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ANIMAL REPRODUCTION

Volume 15 Supplement 1 2018

REPRODUCTION IN DOMESTIC RUMINANTS IX

PROCEEDINGS OF THE 10TH INTERNATIONAL RUMINANT REPRODUCTION SYMPOSIUM

Official journal of the
Brazilian College of Animal Reproduction

10th IRRS 2018
Brazil South America
SEPTEMBER 16-20
Foz do Iguaçu

ISSN 1806-9614

ANIMAL REPRODUCTION

Reproduction in Domestic Ruminants IX – Proceedings of the 10th International Ruminant Reproduction Symposium

Official journal of the Brazilian College of Animal Reproduction

v.15, Supplement, 1 **2018**

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All the correspondence should be sent to the following address:
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Animal Reproduction, v.1- , n.1- , 2004-
Belo Horizonte, MG, Brazil: Colégio Brasileiro de Reprodução Animal, 2004-

Quarterly. ISSN 1806-9614 (printed; 2004-2011); 1984-3143 (online; 2004-)

1. Animal reproduction - Periodicals. I. Colégio Brasileiro de Reprodução Animal. II. Brazilian College of Animal Reproduction.

CDU - 636.082.4 (81) (05) - AGRIS L10

Indexed in:
Biological Abstracts
Biosis Previews
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Current Contents: Agric Biol Environ Sci
Directory of Open Access Journals (DOAJ)
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Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018

From the IRRS Local Organizing Committee

Dear colleagues

It is with great joy that we welcome you to the 10th International Ruminant Reproduction Symposium (IRRS) at the Wish Resort Golf & Convention in Foz do Iguaçu – Brazil. The IRRS is recognized as one of the most prestigious international conferences on reproduction, which we believe is clearly reflected in this year's Scientific Program and correspondent review articles published in these proceedings. The IRRS 2018 organizing committee has selected 32 speakers among the most recognized scientists from around the world to present cutting-edge topics on reproductive biology and technology in a variety of ruminant species, including cattle, sheep, goats, buffaloes, and camelids.

We are extremely honored to organize the IRRS in South America for the first time. As you know, South America has garnered significant international attention during the last 15 years because of the increase in the number of high-impact studies in reproductive biology and the wide application of assisted reproductive technologies in ruminants, particularly IVF and fixed-time AI in cattle. Several productive collaborations between local, North American, European, and Australian groups have been fundamental in promoting this intense scientific and technological progress in South America. The IRRS 2018 scientific program illustrates and celebrates the importance of these partnerships. While providing up to date scientific information and an enjoyable social program, we hope to benefit participants of the 2018 IRRS by offering a fruitful environment for new collaborations and the reinforcement of those already underway

Our special thanks to the IRRS editors and session chairs, who put together a fantastic group of speakers, generating this valuable compilation of reviews. Many thanks also to all speakers for their time and generosity in sharing their precious time and knowledge with us. The quality of our scientific program and social environment certainly required investment. We immensely thank our public and private sponsors for providing funds without which this meeting would not be possible.

We sincerely hope that you enjoy the IRRS 2018 and the papers contained herein.

Very best wishes,

José Buratini, Roberto Sartori, Gabriel Bó and Guilherme Nogueira

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018

Pioneer Award: by J.E.P. Santos, Department of Animal Sciences, University of Florida, Gainesville, USA

The recipient of the 2018 International Ruminant Reproduction Symposium Pioneer Award, granted to distinguished scientists who have trained students and have made major contributions to the understanding of reproductive biology in ruminants, is Dr. William Watters Thatcher, Graduate Research Professor Emeritus in the Department of Animal Sciences at the University of Florida, Gainesville, USA.

Dr. William W. Thatcher ("Bill") grew up in the Baltimore-Perry Hall area, Maryland, USA, where he spent time in his grandfather's farm and developed a passion for livestock and agriculture. When it was time to go to college, he was accepted at the University of Maryland in College Park and received his Bachelor of Science degree in Agriculture and Animal Husbandry in June of 1963. He went on to do the Master of Science program at the University of Maryland and conducted his research on milk proteins and blood group polymorphisms in cattle at the Agricultural Research Service of the United States Department of Agriculture in Beltsville, Maryland under the supervision of Dr. Charles A. Kiddy. Upon completion of his MSc degree in December of 1965, Bill moved to Michigan State University where he was awarded the PhD degree in physiology of lactation and reproduction under Dr. Herbert Allen Tucker ("Tuck") in December of 1968 with the dissertation entitled "Physiological, Biochemical and Hormonal Factors Limiting Lactation".

In January, 1969, Bill joined the Department of Dairy Science (now Animal Sciences) at the University of Florida where he spent his entire professional career with a research focus on dairy cattle reproductive physiology. He published his first scientific abstract while in graduate school entitled "Associations Among Blood and Milk Polymorphisms in Dairy Cattle" at the Eastern Divisional Meeting of the American Dairy Science Association in College Park, Maryland in July of 1965, and his first scientific paper also was published in 1965 in the June issue of the Journal of Dairy Research, and he has not stopped since. Bill has published over 50 book chapters and reached the impressive 400-mark on scientific manuscripts published in the peer-reviewed literature, with his latest contribution appearing recently in the Journal of Dairy Science in 2018. Only a few animal scientists have had the distinction of such a prolific research career. He has over 35,000 citations with an h-index of 105 in Google Scholar. This means that not only he has been an extremely prolific publisher of scientific papers, but his peers recognize the importance of his research and they want to read and cite his papers.

Throughout his career, Bill has been the consummate professor who has always believed in interdisciplinary programs and has pushed his students to be the best scientists with a broad view of science and with tools to apply scientific methods to develop new knowledge. He believes the purpose of his program is to conduct research to create new knowledge and develop solutions to solve problems affecting animal agriculture, at the same time that he imposed an interdisciplinary approach to training graduate students. Bill has directly touched and influenced the lives of hundreds of professionals as a mentor and colleague. He has served as the advisor for 73 graduate students, postdoctoral fellows, sabbatical scientists, and visiting trainees. He has served as graduate committee member to more than 100 graduate students in Florida as well as other Universities. His graduate students originated from all corners of the world, many of which came from countries in South America, including Brazil where this symposium is being held. Graduate students and post-doctorates who worked under Bill have taken positions as faculty members, industry scientists, or industry technical service specialists in 30 countries. A great deal have become well recognized leaders in their area of work. Many of the students from South America returned to their countries and formed a legacy that has perpetuated to this day and has impacted science and teaching of cattle reproduction. As a graduate professor, he taught courses in endocrinology and reproductive physiology, and he has been an active member of the Interdisciplinary Reproductive Biology and the Animal

Molecular Cell Biology programs at the University of Florida to this day. He has served as associate editor or on the editorial boards of Biology of Reproduction, Journal of Dairy Science, Journal of Animal Science, Theriogenology, Animal Reproduction Science, and Reproduction-Nutrition-Development. He has been the president of the Society for Study of Reproduction (2005-2006) and of the International Congress on Animal Reproduction (2004-2008).

Bill arrived at the University of Florida shortly before one of his mentors, Dr. Donald Henry Barron who occupied the J. Wayne Reitz Chair in Reproductive Biology and Medicine, joined the University in 1969. Bill's efforts to foster collaborative and multidisciplinary approaches to basic and applied research, in collaboration with colleagues in Animal Sciences and the Medical College, established a joint weekly seminar in reproductive and perinatal biology in 1969 that is ongoing every Wednesday afternoon still today. Early in his career, Bill developed a strong friendship and close working relationship with Dr. Fuller Bazer in the Department of Animal Sciences at Florida. The combined inquisitive minds, with limitless imagination and complementary biological and quantitative skills resulted in a strong and very productive scientific collaboration over many years. They conducted experiments to understand the mechanisms underlying the conceptus-maternal cross-talk that lead to maintenance of the corpus luteum, critical for survival of pregnancy. Eventually, the two collaborated with Dr. Michael Roberts in the Department of Biochemistry in Florida, and their research led to the initial experiments demonstrating that protein extracts isolated from the uterine flushes from pregnant ewes or proteins purified from cultured conceptuses extended the corpus luteum lifespan for days to weeks. Those findings eventually culminated with the discovery of interferon-tau as the key molecule produced by the trophoblast of the conceptus necessary for maintenance of pregnancy in ruminants and several of the mechanisms regulating prostaglandin $F_{2\alpha}$ secretion from the bovine endometrium.

The hot and humid subtropical climate in the state of Florida urged scientists to focus efforts on understanding the impacts of heat stress and find solutions to mitigate the losses caused by hyperthermia in livestock. That was one of Bill Thatcher's initial tasks when he arrived at the University of Florida because of the need to understand the impacts of heat stress on cattle and improve reproduction under such conditions. He and colleagues (Dr. Peter J. Hansen) characterized many of the early aspects of how heat stress and consequent hyperthermia affects the reproductive physiology and the reproductive performance of dairy cows. They showed that heat stress affects ovarian follicular development, follicle steroidogenesis, and endometrial synthesis of prostaglandins. His work showed that cows under heat stress have reduced expression of estrus, which deters the efficient use of artificial insemination (AI). Bill studied and developed methods that facilitate the implementation of AI in herds, which has been a hallmark of his scientific contributions. He was a member of a team of scientists and allied industry professionals who developed prostaglandin $F_{2\alpha}$ as a reproductive hormone to be used in estrous synchronization protocols to control luteal lifespan. His initial work with colleagues (Dr. Jim W. Lauderdale) on the use of prostaglandin $F_{2\alpha}$ led to the development of commercial products marketed to induce estrus in cattle. Work from his group as well as others characterized the early findings of follicular dominance in the bovine that eventually led to a better understanding of the control of the estrous cycle in cattle. His laboratory in collaboration with Dr. Keith L. MacMillan ("Jock MacMillan") conducted the initial experiments on manipulation of ovarian follicle development with the use of GnRH and then incorporated prostaglandin $F_{2\alpha}$ to control luteal lifespan to better synchronize estrus in dairy cattle. This early groundbreaking work eventually led to development of timed AI protocols that are currently used for reproductive management of lactating dairy cows and dairy heifers all over the world. Their findings on the use of GnRH and prostaglandin $F_{2\alpha}$ became the foundation for therapy of cows with ovarian cystic disease and other types of anovulatory conditions in dairy cows. This same work on control of follicle development and luteal lifespan was eventually applied to embryo transfer programs to allow for fixed timed embryo transfer in cattle. Bill always had strong quantitative skills and he collaborated with Dr. Charlie J. Wilcox, a statistician at Florida to make sure his research was sound and that his students understood the importance of proper design of experiments and data analyses.

Bill not only fostered interdisciplinary training for his students, but he also applied the same concepts to his research program. The progression of his career eventually led to integration of concepts on reproductive management, nutrition, and health to improve reproduction of lactating dairy cows. The collaborations with Dr. Charles R. Staples and Dr. José E. Santos in the area of nutrition and reproduction improved our understanding of the roles of animal health and fatty acids on reproduction in dairy cattle. Polyunsaturated fatty acids are potent regulators of cellular function in mammals and work conducted by Thatcher and colleagues at the University of Florida have characterized some of the cellular and reproductive effects of omega-3 and omega-6 fatty acids on regulating prostaglandin $F_{2\alpha}$ synthesis, embryo development, and maintenance of pregnancy in cattle. Bill has been a pioneer on the understanding of how growth hormone and insulin-like growth factor 1 affect ovarian follicle development and pregnancy in cattle. His work, integrating use of recombinant bovine growth hormone with estrous synchronization protocols for timed AI demonstrated the benefits of bovine somatotropin to improving embryo development and pregnancy in lactating dairy cattle.

It is safe to say that the work developed by Bill Thatcher over many years has greatly impacted animal production and has changed how dairy producers manage reproduction today. His influence on how we think about dairy cattle reproduction is present every day in the scientific setting or on the farm. It is very likely that many of the concepts discussed in this Symposium has, one way or another, something related to Bill's scientific contributions. His impact has extended to all corners of the world in which dairy cattle are raised to produce food for humans. He has been invited to 50 countries to give lectures and seminars to extend knowledge in dairy cattle reproduction and management.

Bill has been officially retired and became Emeritus Professor at the University of Florida since 2004. Nevertheless, he still comes to work and contributes with the mission of the Department and the University by advising faculty and graduate students. He continues to contribute to scientific societies by editing books, writing and reviewing manuscripts. He is an exceptional example of how, through collaboration, scientists can educate one another and create an environment of rich scientific stimulation. It is important to mention that Bill's success in the scientific arena did not preclude him from enjoying his time and creating strong friendships with colleagues. Everyone who knows him is aware of his intensity as a scientist, but also recognize his collegiality and friendship. At home, he has always had a strong pillar and a safe harbor in his wife, Marie-Joelle Thatcher. She has supported him throughout his career and has made sure his health stays strong.

Bill has received more than 20 awards recognizing his scientific contributions and graduate student mentoring ability, among them the Journal Dairy Science 100 Club Award from the American Dairy Science Association (2017), the Carl G. Hartman Award from the Society for Study of Reproduction (2014), the Pioneer Award from the International Embryo Transfer Society (2014), the Hetzel Award for lifetime achievement from Hungarian Society for Animal Production (2008), the Morrison Award from the American Society of Animal Science (2006), honorary member of the American College of Theriogenologists (2003), the Merial Dairy Management Research Award from the American Dairy Science Association (2002), the Research Award from the National Association of Animal Breeders (2000), the Doctoral Dissertation Mentoring Award from the University of Florida (2002), the Graduate Teaching Award from the College of Agriculture and Life Sciences at the University of Florida (2001), and the Lester E. Casida Award for Excellence in Graduate Training from the American Society of Animal Science (1997), among others. He is a fellow of the American Dairy Science Association (2007) and the American Society of Animal Science (2011).

The 2018 Pioneer Award from the International Ruminant Reproduction Symposium is the latest of many international accolades recognizing the distinguished scientific career in reproductive biology of Dr. William W. Thatcher and his contributions to our understanding of reproduction in ruminants.

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018

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DOI: 10.21451/1984-3143-AR2018-0011

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Factors influencing establishment of the ovarian reserve and their effects on fertility

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Abstract

A reserve of primordial follicles is set up in the ovaries of fetuses or neonates, depending on the species, and serves as the source of developing follicles throughout the reproductive lifespan. This review focuses on the cellular and molecular mechanisms currently known to control the establishment of this reserve, and their regulation by environmental factors. Most mutations in genes controlling germ cell proliferation and survival, meiosis or follicle assembly lead to the absence of primordial follicles or a sharp reduction in their number, incompatible with fertility in adults. Inadequate maternal nutrition affects the cellular metabolism, increases the oxidative stress and delays follicle formation in fetal ovaries. Despite the existence of compensation mechanisms of some developmental processes, the early-life nutritional environment imprints the long-term ability of follicles to enter growth and develop in adult ovaries. However, maternal undernutrition, overfeeding or high-fat diet during the establishment of the ovarian reserve does not seem to affect the fertility of the female offspring, unless their metabolism or neuroendocrine status is altered. Exposure of fetal or neonatal ovaries to excess steroids inhibits or stimulates follicle formation in a complex manner depending on the nature of the steroid, the dose and the animal species. Estrogens can control follicle formation through intra-ovarian mechanisms involving members of the TGF-beta family such as activin and BMP2. Early-life exposure to synthetic estrogens or environmental pollutants with estrogen-like activity impairs meiotic progression and follicle assembly, and affects long-term primordial follicle activation in adult ovaries. The effects of compounds with estrogen-like activity on the ovarian reserve can be transmitted to several generations through the female germline. Further investigations are needed to establish the early-life effects of the environmental factors on the female reproductive lifespan and decipher the mechanisms of their epigenetic effects on the size and quality of the ovarian reserve.

Keywords: cyst breakdown, germ cells, oocyte, ovary, primordial follicle.

Introduction

In the ovary, the growing follicles develop from a reserve of primordial follicles constituted early in life and gradually emptied by both follicle growth activation and follicle degeneration. From this first reserve of primordial follicles, a second ovarian reserve is formed, which consists of gonadotropin-responsive small antral growing follicles and is a dynamic reserve for ovulation. The mechanisms regulating the transitions between reserves have been recently reviewed (Monniaux *et al*., 2014).

When established, the reserve of primordial follicles is oversized in all species. For instance, in the fetal bovine ovary at the end of the first trimester of pregnancy, it comprises millions of follicles (Erickson, 1966), of which only some hundreds of them will grow up to the ovulatory stage during the postnatal life. An intriguing observation is that the size of the ovarian reserve in the early postnatal life varies importantly between individuals of the same species or strain (sheep: McNatty *et al*., 1995; pig: Black and Erickson, 1968; humans: Block, 1952; Baker, 1963; Forabosco and Sforza, 2007). Recently, Ireland *et al* working on cattle reproduction proposed that the maternal environment has a critical role in regulation of the inherent high variation in the ovarian reserve. Moreover, they raised the important question: does size matter in females? and argued that young adults with a low ovarian reserve of primordial follicles have low numbers of growing follicles and phenotypic characteristics usually associated with ovarian aging and infertility (Ireland *et al*., 2011). How the establishment of the first reserve during the fetal or the early postnatal life can affect in a sustainable way the quality and growing features of the follicles of the second reserve, and finally the female fertility in adult life, remains a challenging question, however.

This review will outline the different steps of the establishment of the reserve of primordial follicles and our current knowledge of the molecular and environmental control of this set up. From recent data available in different animal models and in humans, we will try to understand how the environmental factors may regulate the size of the ovarian reserve and the developmental capacity of the primordial follicles, and discuss their possible long-term effects on female fertility and longevity of reproduction.

Main steps of ovarian reserve establishment

The setting up of the reserve of primordial follicles occurs through similar mechanisms in all mammals, but the timing of the processes underlying the formation of the reserve is species-specific (Monniaux *et al*., 2014; Fig. 1). Gonad morphogenesis involves the invasion of the genital ridge by mesonephros-derived cells, which associate with a founder population of primordial germ cells (PGC), as demonstrated in sheep and cattle (Zamboni *et al*., 1979; McNatty *et al*., 2000;

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Received: January 30, 2018
Accepted: April 30, 2018

Hummitzsch *et al*., 2013). Upon arrival at the gonad, the germs cells, named oogonia at this stage, enter synchronous mitotic divisions with incomplete cytokinesis, forming clonal cell clusters named germ cell cysts or nests, as shown in mouse (Pepling and Spradling, 1998). While the oogonia are dividing to form cysts, they also interact with somatic cells in the ovary. The germ cells and epithelial pre-granulosa cells become organized into ovigerous or ovarian cords, outlined by a basement membrane that separates them from the mesenchymal cells of the developing ovary. The ovigerous cords are notably well developed in sheep and cattle and they remain until primordial follicles begin to form (Juengel *et al*., 2002; Sawyer *et al*., 2002; Burkhart *et al*., 2010; Garverick *et al*., 2010; Hummitzsch *et al*., 2013). At this stage of intensive mitotic activity of oogonia, the number of germ cells increases exponentially up to about 15 000 per ovary in mouse (Myers *et al*., 2014), 2 700 000 in cow (Erickson, 1966), 500 000 to 1 000 000 in sheep (Smith *et al*., 1993) and more than 5 000 000 in humans (Baker, 1963).

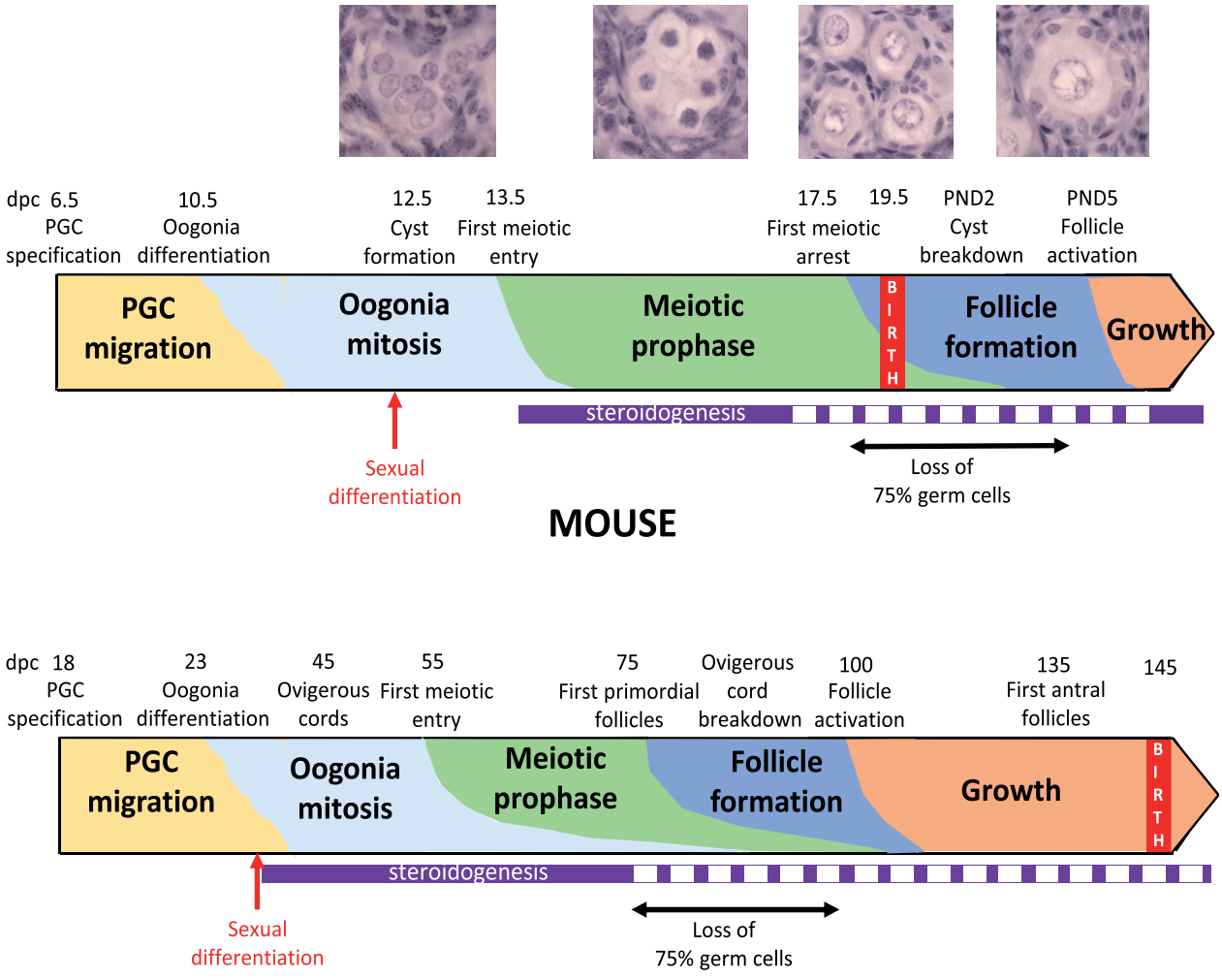

Figure 1. Timeline of the different steps of primordial follicle formation and activation in mouse and sheep ovaries. The periods of ovarian steroidogenesis activity are indicated in purple (full line: high activity, dotted line: low activity). Photographs illustrate the histological appearance of germ and somatic cells from germ cell cyst formation to the primary follicle stage (Monniaux and Brisard, 2018; INRA, Nouzilly, France; unpublished pictures). From Dutta *et al*., 2014; Findlay *et al*., 2015; Grive and Freiman, 2015 for mouse and McNatty *et al*., 1995; Quirke *et al*., 2001; Juengel *et al*., 2002 for sheep. PGC: primordial germ cells; dpc: days post-conception.

After cessation of mitosis, the oogonia enter meiosis and become oocytes, which progress through the stages of meiotic prophase I arresting in the diplotene stage. Afterwards, the germ cell cysts break apart and individual oocytes become surrounded by granulosa cells forming primordial follicles. It is estimated that during cyst breakdown, about 75% of the oocytes are lost through programmed cell death, including apoptosis and autophagy, as demonstrated in mouse (Pepling and Spradling, 2001; Rodrigues *et al*.,

2009).

The functional significance of germ cell development in the mouse cysts was unknown until recently. Using lineage tracing, Lei and Spradling investigated how the mouse germ cells are physically connected to one another and they observed centrosomes, Golgi material, and mitochondria traveling through large gaps in the plasma membrane and accumulating as a Balbiani body in a subset of oocytes during mouse perinatal oocyte development. These Balbiani body-containing oocytes survive and give rise to mature oocytes whereas the oocytes without Balbiani bodies would be only nurse cells as they look smaller, lose most of their cytoplasm, and appear to undergo apoptosis (Lei and Spradling, 2016). Each oocyte is associated with about four nurse cells (Pepling, 2016), that accounts for the dramatic loss of germ cells occurring during cyst breakdown. It appears that this development step determines in a large part the size of the reserve of primordial follicles and their ability to develop.

The formation of follicles proceeds centrifugally from the interface of the cortex and medulla towards the outer region of the cortex, as shown in humans (Konishi *et al*., 1986), rodents (Hirshfield, 1992; Mork *et al*., 2012), sheep (Juengel *et al*., 2002; Sawyer *et al*., 2002) and cattle (Burkhart *et al*., 2010; Hummitzsch *et al*., 2013). Moreover, in the bovine fetal ovary, the ovarian cortex forms lobes appearing to have slightly different developmental ages and giving rise to primordial follicles asynchronously within the same ovary (Burkhart *et al*., 2010; Garverick *et al*., 2010). In mouse, the primordial follicles were shown to consist of two classes that harbor a distinct development path. The medullary primordial follicles are synchronously activated after birth, forming the first wave of activated fast-growing follicles that may aid in the onset of puberty and provide mature oocytes up to 3 months of age. In contrast, the cortical primordial follicles are gradually activated, are slower growing and contribute to ovulation at later stages of the reproductive life (Zheng *et al*., 2014a). The existence of two populations of primordial follicles that exhibit distinct developmental dynamics and contribute differently to ovarian physiology is also suggested in primates (Zheng *et al*., 2014b). Their possible existence in sheep and cattle might explain the bi-phasic pattern of changes in ovarian follicle recruitment and growth observed in these species before puberty (Rawlings *et al*., 2003).

Recent evidence supports the existence of stem cells of a number of the different cell types within the ovary (Hummitzsch *et al*., 2015). Particularly, the isolation of oogonial stem cells from adult mouse and human ovaries has been reported; these cells exhibit both germ and stem cell markers in culture (White *et al*., 2012). When reintroduced into an ovarian somatic environment, the mouse oogonial stem cells have generated follicles capable of producing healthy offspring (Zou *et al*., 2009). However, the oogonial stem cells are unable to sustain by themselves the ovarian function into an advanced age, partly due to age-related changes in the ovarian microenvironment (Truman *et al*., 2017). Moreover, there are no data on their potential physiological role within the ovary, and specifically no evidence that they can contribute to the primordial follicle pool (Grieve *et al*., 2015). The dogma that a fixed pool of primordial follicles formed early in life serves as the only source of developing follicles throughout the reproductive lifespan still holds true (Lei and Spradling, 2013; Zhang *et al*., 2014).

Molecular control

During the last decades, the role of factors controlling the different phases of ovary and follicle formation has been deciphered, thanks to the generation of transgenic mice using gene knock out, knock in, targeted deletion, or over-expression strategies (Edson *et al*., 2009; Baillet and Mandon-Pépin, 2012; Monget *et al*., 2012; Pepling, 2012; Kerr *et al*., 2013; Findlay *et al*., 2015; Grive and Freiman, 2015). Mouse genetic models have identified numerous genes involved in PGC differentiation and migration, oogonia survival and proliferation, as well as in the initiation and execution of meiotic prophase in oocytes (Fig. 2). The mechanisms underlying these processes are generally well conserved from *Drosophila* to mice. The recently established transcriptome and DNA methylome landscapes of human migrating and gonadal PGCs were found in general similar to those of mouse PGCs at comparable stages (Guo *et al*., 2015). Owing to this high degree of conservation, factors shown to affect ovarian reserve establishment in mouse models are all potential candidates for identifying mutations associated with premature ovarian insufficiency in humans (Jagarlamudi *et al*., 2010; Pelosi *et al*., 2015), and with infertility in domestic animal species. However, despite conserved principles between species, some mechanistic differences exist between mice and a range of species including pigs, monkeys and humans, as exemplified for PGC specification at early developmental stages (Tang *et al*., 2016; Kobayashi *et al*., 2017). In this review, in addition to the knowledge acquired from mouse models, data from various species are presented and compared where possible.

As said above, primordial follicles are formed by germ cell cyst breakdown and assembly of individual oocytes with pre-granulosa cells. Intricate regulation of gene expression, including the oocyte-specific Figla (Soyal *et al*., 2000), Nobox (Lechowska *et al*., 2011) and Taf4b (Grive *et al*., 2014) transcription factors, is critical for these processes in mouse. The oocyte-specific secreted factors BMP15 and GDF9, known to drive the growth of small follicles and the maturation of the cumulus-oocyte complex during folliculogenesis (Monniaux, 2016), might participate also in controlling follicle formation. Indeed, the formation of multi-oocyte follicles, indicative of the presence of defects in cyst breakdown and oocyte assembly with pre-granulosa cells, has been observed in Bmp15-/- Gdf9+/- mice (Yan *et al*., 2001). Moreover, it was recently reported that GDF9 and BMP15 can induce the formation of follicles from human embryonic stem cells expressing the germ cell-specific proteins DAZL and BOULE (Jung *et al*., 2017). However, the role of GDF9 and

BMP15 in follicle formation *in vivo* remains speculative in humans and other species, particularly because BMP15 is not expressed until the follicle is actually formed and begins to grow. In the fetal sheep ovary, *GDF9* expression starts at 56 days post-conception (dpc) and is located in oocytes (Mandon-Pépin *et al*., 2003; Juengel *et al*., 2004). However, *BMP15* expression is hardly detectable before 94 dpc, and in sheep homozygous for the *FecX*^{I} inactivating mutation in *BMP15*, follicle formation seems normal (McNatty *et al*., 1995). Some observations suggest that BMPR1B, a BMP receptor known to bind BMP15/GDF9 heterodimers and *BMP15* homodimers, may still participate in follicle formation in sheep. In the fetal sheep ovary, *BMPR1B* expression starts as soon as 25 dpc, is high at the time of germline cyst breakdown (Mandon-Pépin *et al*., 2003) and is located in the mesonephric-derived cell streams and the ovigerous cords (Reader *et al*., 2012). In the ovaries of fetuses and newborn lambs homozygous for the *FecB*^{B} mutation in *BMPR1B*, the oocytes of the primordial follicles are larger and contain a greater volume of mitochondria, smooth endoplasmic reticulum and ribosomes, suggesting that the mutant form of BMPR1B has influenced the process of germline cyst breakdown and led to follicles better equipped for the initiation of follicular growth (Reader *et al*., 2012). The role of BMPR1B in the mechanisms of follicle formation and the BMP ligands able to activate BMPR1B signaling at this stage in the sheep ovary remain to be defined. Nevertheless, it can be speculated that BMP15 secreted by the first growing follicles may activate BMPR1B signaling in the ovigerous cords and influence the subsequent formation of follicles.

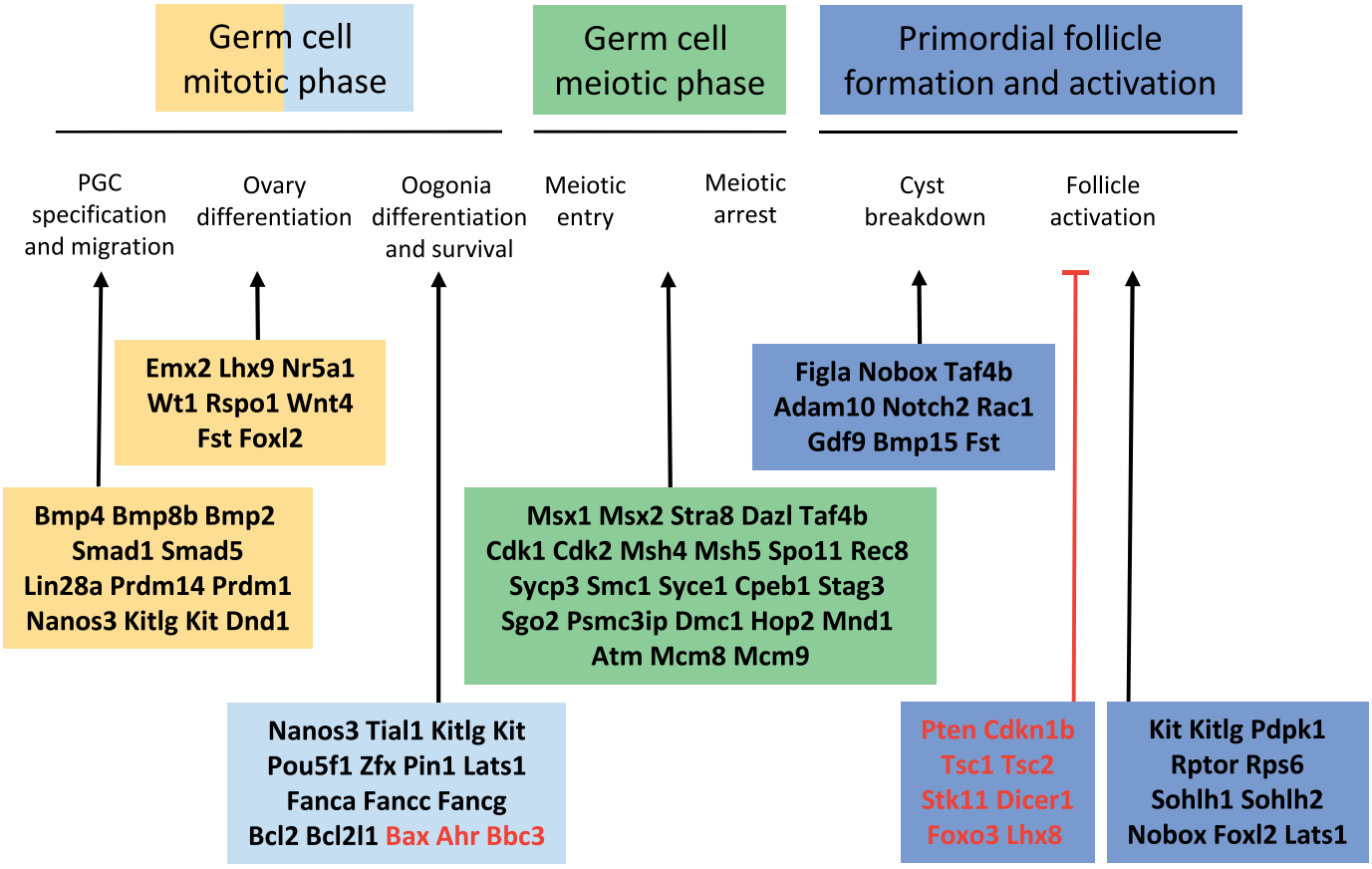

Figure 2. Genes with *in vivo* mutations affecting the different steps of primordial follicle formation and activation in mouse. Genetically modified mouse models exhibit phenotypes similar to those of premature ovarian insufficiency in humans. Black font: positive regulators, red font: negative regulators. From Edson *et al*., 2009; Jagarlamudi *et al*., 2010; Baillet and Mandon-Pépin, 2012; Monget *et al*., 2012; Pelosi *et al*., 2015.

In addition, some somatic cell-derived factors play important roles in cyst breakdown. Indeed, estradiol signaling is critical for inhibiting this process until birth in rodents (Chen *et al*., 2007) and may regulate it in ruminants as well (Fortune *et al*., 2010, 2013). In mouse, growth factors such as activin/follistatin and neurotrophins also influence cyst breakdown and the size of the primordial follicle pool (Bristol-Gould *et al*., 2006; Kerr *et al*., 2009; Kimura *et al*., 2011), and recently the disintegrin Adam10 has been shown to govern the recruitment of the pre-granulosa cells in cysts (Feng *et al*., 2016). From data in mice, the actions of these various factors converge to modulate the Jagged/Notch signaling pathway in germ cell cysts. Particularly, the Notch2 gene encoding a Notch receptor in pre-granulosa cells orchestrates cyst

breakdown, postnatal apoptosis of oocytes and primordial follicles assembly (Xu and Gridley, 2013) and the oocyte-specific secretory proteins Jagged1 (a Notch ligand), Gdf9 and Bmp15 activate the Notch signaling pathway in pre-granulosa cells (Zhao *et al*., 2016). All these observations highlight the importance of crosstalk between germ cells and pre-granulosa cells for the formation of primordial follicles.

The inactivation of genes controlling the processes of PGC specification and migration, ovary differentiation, meiosis initiation and execution of meiotic prophase leads generally to the absence of germ cells in the ovaries of mutant mice. Most inactivation of genes involved in the regulation of oogonia proliferation and survival, or germ cell cyst breakdown lead also to female infertility due to the absence of primordial follicles or a sharp reduction in their numbers at birth. However, in some cases, as in the inactivation of the pro-apoptotic factors *Bax* and *Bbc3*, or the over-expression of the pro-survival factor *Bcl2*, the number of primordial follicles of the initial reserve is increased (Perez *et al*., 1999; Flaws *et al*., 2001; Myers *et al*., 2014). In mice over-expressing *Bcl2* in ovaries, the surfeit of primordial follicles is not maintained in the long term, suggesting that the ovary may contain a sensing mechanism by which excess numbers of primordial follicles at birth are detected and removed from the ovary by adulthood (Flaws *et al*., 2001). The importance of postnatal regulations of the number of primordial follicles has also been shown in ewes carrying the $FecB^B$ mutation in *BMPR1B*. The establishment of the ovarian reserve is delayed in mutant ewes (McNatty *et al*., 1995) and at birth their ovaries contain lower numbers of primordial follicles than wild-type ewes, but at 5 years of age mutant ewes are still fertile and their ovarian reserve is even higher, due to a lower rate of primordial follicle activation (Ruoss *et al*., 2009). These examples in mouse and sheep genetic models demonstrate that, in the presence of a mutation affecting moderately the establishment of the initial reserve of primordial follicles, the fertility and reproductive longevity of the female are not determined at birth. Similar compensatory postnatal mechanisms have been observed following the administration of activin to neonatal mice, since despite an increased number of primordial follicles in the mice ovaries after treatment, the excess follicles containing oocytes of poor quality is eliminated prior to puberty (Bristol-Gould *et al*., 2006).

Environmental and hormonal control

In sheep, as in cattle and humans, primordial follicles are formed before birth (Fig. 1) so that the establishment of the ovarian reserve is under the control of the maternal environment. All metabolic, hormonal or health changes in the maternal compartment and pollutants able to cross the placenta may affect the development of the fetal gonads. These maternal or external environmental factors can act directly on the developing ovary, but they impact also on various organs, particularly the hypothalamo-pituitary complex, the liver and the pancreas (Rhind *et al*., 2001; Padmanabhan and Veiga-Lopez, 2013), as well as the adipose tissue, all able to affect indirectly ovarian function and fertility. Whether the environmental factors acting directly on follicle formation can also affect long-term fertility, and through which mechanisms, is a difficult question. This review will focus on the known effects of nutrition, steroids and some endocrine-disrupting factors on ovarian reserve establishment, and discuss their mechanisms of action and potential consequences for fertility.

Nutritional factors

Maternal nutritional status participates in programming growth, development, and function of the major fetal organ systems. Placental insufficiency impairs fetal development and reduces the number of primordial follicles in the ovaries of fetuses and neonates in humans (de Bruin *et al*., 1998) and sheep (Da Silva *et al*., 2003). Maternal overnutrition reduces also the number of primordial follicles in fetal bovine ovaries at the end of pregnancy, but concomitantly the number of growing follicles is increased, suggesting that the initiation of follicular growth has been activated (Weller *et al*., 2016). There is little information on the effects of maternal nutrition on the initial size of the pool of primordial follicles, but increasing evidence demonstrates that nutrition modulates the dynamics of ovarian reserve establishment in ruminant fetuses and rodent neonates, with functional consequences on follicular growth.

Undernutrition of ewes from the time of mating significantly retards ovarian development in fetal ovaries. Particularly, the ovaries of fetuses from feed-restricted ewes contain more germ cells entering the initial stages of meiosis at a time when a large proportion of them has completed this process (Borwick *et al*., 1997). This delay in meiosis onset is induced by the combination of maternal undernutrition before ovarian differentiation (days 0-30 of pregnancy) and during the phases of germ cell mitosis and meiosis entry (days 31-65 of pregnancy; Rae *et al*., 2001). Interestingly, undernutrition imposed during each of these gestational periods reduces also the incidence of follicle development beyond the primordial stages (Rae *et al*., 2001). These observations indicate the existence of a precocious window of ovarian sensitivity, during which nutritional disorders can affect the dynamics of follicle formation and compromise subsequent follicular development.

The mechanism of action of nutrients on follicle formation remains poorly understood. Female rats born to mothers fed a high-fat diet throughout pregnancy have fewer oocytes in fetal ovaries at embryonic day 20 and it was speculated that increased maternal-fetal inflammation associated with maternal obesity (Aye *et al*., 2014) may have contributed to accelerated fetal oocyte loss (Tsoulis *et al*., 2016). In a murine pharmacological model of maternal diabetes induced by streptozotocin administration, *in utero* exposure of mice to hyperglycemia decreases the expression of genes involved in meiosis initiation

(*Stra8*, *Dmc1* and *Sycp3*) and germ cell cyst breakdown (*Nobox*, *Figla* and *Bmp15*) and impairs the initiation of meiosis and follicle assembly in the fetal offspring ovaries (Qiu *et al*., 2017). The starvation of mouse pups between 1.5 and 3 days of postnatal life, when most primordial follicles are assembling, leads also to a decrease in the expression of *Nobox* and the impairment of germ cell cyst breakdown; in the ovaries of the starved pups, the alteration of metabolic parameters, exemplified by the lower expression of genes encoding proteins of fatty acid synthesis such as *Fabp5*, *Cpt2* and *Acsl3*, could have triggered an oxidative stress responsible for the increased autophagy and apoptosis observed in the oocytes within cysts and in the primordial follicles (Wang *et al*., 2017).

Besides a possible direct effect of glucose and other nutrients on ovarian development, metabolic hormones can regulate follicle formation. Insulin accelerates primordial follicle assembly in rodent ovaries and increases apoptosis in germ cell cysts *in vitro*, as shown in hamster (Yu and Roy, 1999) and mouse (Feng *et al*., 2015). Leptin, a major adipokine able to modulate lipid and glucose metabolism and insulin sensitivity, could also regulate follicle formation. Indeed, in the ovaries of piglets containing a substantial proportion of germ cells not yet enclosed in follicles but grouped into germ cell cysts, leptin receptors are present on oogonia and oocytes (Fig. 3). These germ cells were found to express six different isoforms of leptin receptors, potentially able to activate the MAP kinases and PI3/AKT pathways (Attig *et al*., 2013). Moreover, postnatal leptin treatment of piglets with intra-uterine growth retardation accelerates follicle formation and activation, since the ovaries of leptin-treated piglets contain lower percentages of oogonia and oocytes in meiotic prophase, but higher percentages of oocytes in the dictyate stage within germ cell cysts, primordial and primary follicles (Attig *et al*., 2013).

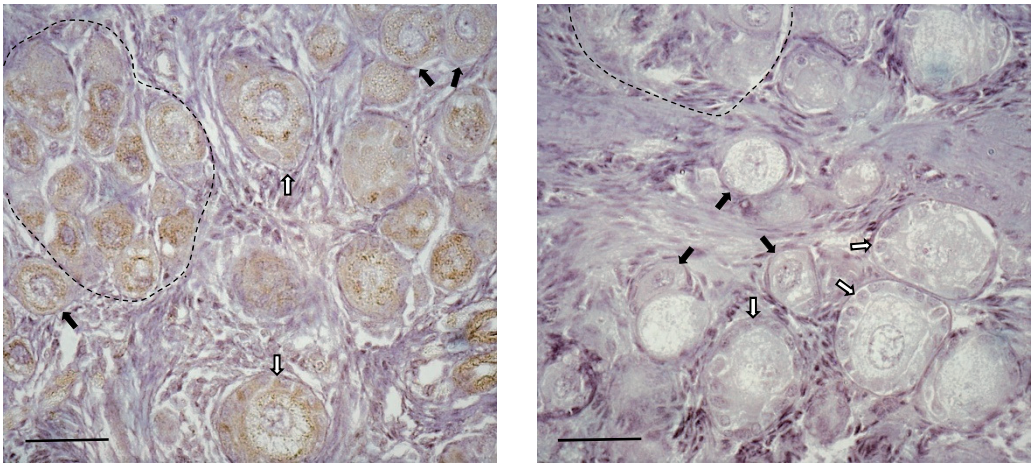

Figure 3. Expression of leptin receptors in the ovaries of 21 days-old piglets. For immunostaining, ovarian sections were incubated with leptin receptor antibody (Ob-R (M-18)-R, sc-1834-R; Santa Cruz Biotechnology Inc, Heidelberg, Germany; dilution 1/250), without (A) and with (B) displacement by the peptide used as immunogen for antibody preparation. Leptin receptors are detectable in all germ cells and some granulosa cells of primary follicles. Dotted lines delineate germ cell cysts. Primordial and primary follicles are indicated by black and open arrows, respectively. Bar = 50µm. (Monniaux and Brisard, 2018; INRA, Nouzilly, France; unpublished pictures).

Whether the early-life nutritional environment affects fertility and reproductive longevity of mammals is a controversial issue (Gardner *et al*., 2009; Sloboda *et al*., 2011). After induction of maternal diabetes by streptozotocin in mouse, the reproductive performance of the offspring is severely decreased in the long term, but their postnatal growth and physical development are also impaired or delayed, indicating the establishment of a sustainable metabolic syndrome (Spadotto *et al*., 2012). In sheep, inadequate maternal nutrition has little effect, if any, on puberty and ovulation rate of the offspring when postnatal growth is normal (Da Silva *et al*., 2001; Rae *et al*., 2002). None of the studies of maternal undernutrition, overfeeding or high-fat diet during the period of establishment of the ovarian reserve of their offspring have demonstrated that the fertility or reproductive longevity of the offspring is affected in the absence of metabolic syndrome.

Some changes in germ cell numbers induced by undernutrition or overfeeding in the early phases of ovarian development are compensated in offspring at birth or after a period of normal feeding. For instance, in rats born to mothers fed a high-fat diet throughout pregnancy and lactation, despite a significant decrease in oocyte number during late fetal life, neonates at day 4 of postnatal life demonstrate higher numbers of primordial follicles than control offspring, suggesting that a recovery mechanism has occurred during follicle formation (Tsoulis *et al*., 2016). Also, following a reduced rate of germ cell cyst breakdown induced by the starvation of mouse pups in early postnatal life, the animals recover a normal number of primordial follicles in their ovaries after 3 weeks from re-feeding (Wang *et al*., 2017). Despite the existence of compensation mechanisms in developmental processes, the early-life nutritional environment before or/and during primordial follicle formation leaves an imprint since it modulates the long-term ability of follicles to enter growth and

develop. Indeed, neonatal overfeeding of rats induces the accelerated activation of primordial follicles before puberty and at adulthood (Sominsky *et al*., 2016). Moreover, maternal high-fat diet induces follicular atresia in the ovaries of rabbit and rat offspring in adulthood (Leveillé *et al*., 2014; Tsoulis *et al*., 2016). Interestingly, in the rat model, antral follicles of adult offspring exhibit reduced FSH responsiveness and express low levels of the estrogen receptor *Esr1* and the oocyte secreted factor *Gdf9* (Tsoulis *et al*., 2016), both factors well known to regulate follicular development and FSH sensitivity. Furthermore, undernutrition of rats during pregnancy decreases the number of antral growing follicles in the ovaries of adult offspring (Bernal *et al*., 2010). In a similar way, offspring from cows nutritionally restricted during their first trimester of gestation (before the full establishment of the ovarian reserve in offspring) exhibit, from birth to the adult age, lower numbers of antral follicles than control offspring from non-restricted mothers (Sullivan *et al*., 2009; Mossa *et al*., 2013). This difference in folliculogenesis activity between offspring from restricted and non-restricted cows cannot be attributed to metabolic differences since offspring have similar birth weights and postnatal growth rates (Mossa *et al*., 2013). Cows with low numbers of antral follicles on their ovaries are known to have numerous phenotypic characteristics usually associated with subfertility (Ireland *et al*., 2011), but whether alterations in folliculogenesis of the offspring after precocious maternal nutritional restriction may, in turn, impact on their fertility and reproductive longevity has not been assessed in this bovine model.

Steroids and endocrine-disrupting factors

In cattle and sheep, fetal ovarian capacity to produce steroids is high before follicle formation begins and decreases around the time follicles first appear (Quirke *et al*., 2001; Yang and Fortune, 2008; Fig. 1). In mice, fetal ovaries have also a significant steroidogenic activity, which drops before cyst breakdown; in addition, they are exposed to high levels of maternal steroids, which fall dramatically after birth (Dutta *et al*., 2014). From these observations, the hypothesis that steroids may inhibit follicle formation was tested *in vitro* on postnatal rodent ovaries and fetal bovine ovaries. In rat (Kezele and Skinner, 2003), mouse (Chen *et al*., 2007) and cattle ovaries (Nilsson and Skinner, 2009), progesterone inhibits follicle formation, whereas the non-aromatizable androgen 5alpha-dihydrotestosterone does not in cattle ovaries (Fortune *et al*., 2010). Estradiol at high concentrations impairs primordial follicle formation in mice and cattle (Chen *et al*., 2007; Fortune *et al*., 2010) but not in rats, whereas conversely low concentrations of estradiol stimulate primordial follicle formation *in vivo* and *in vitro* in hamster ovaries (Wang and Roy, 2007). *In vivo*, the number of primordial follicles is reduced by 50% in the ovaries of near-term fetal baboons deprived of estrogen by administration of an aromatase inhibitor *in utero*, and restored to normal in animals supplemented with estrogen (Zachos *et al*., 2002). Moreover, fetuses of sheep androgenized *in utero* from day 30 of pregnancy have, at day 90, nearly double the proportion of germ cells enclosed in follicles compared with control animals (Comim *et al*., 2015), indicating that follicle formation is accelerated by testosterone or by estrogenic action stemming from aromatization of testosterone to estradiol. Altogether, these observations indicate that each steroid acts upon follicle formation in a complex species-specific and dose-dependent fashion.

In sheep, the receptors of estrogens (ESR1 and ESR2), androgens (AR), as well as progesterone (PGR), are all expressed in the surface epithelium and ovarian stroma of the fetal ovaries (Juengel *et al*., 2006). In cattle, ESR1 is mostly found in the surface epithelium whereas ESR2 is expressed in the medulla, germ cells and pre-granulosa cells during early fetal life, then both receptors seem to be expressed in all cell types after 110 dpc (Burkhart *et al*., 2010; Garverick *et al*., 2010). In humans, ESR2 is localized primarily to germ cells, but AR expression is confined to somatic cells between clusters of germ cells (Fowler *et al*., 2011). The potential exists therefore that all steroids could interfere with somatic-to-germ cell signaling during follicle formation, but it needs exploring. Interestingly, estrogen receptors, particularly ESR2, are expressed in all types of germ cells and in pre-granulosa cells in sheep, cattle and humans, suggesting that both direct and indirect actions of estrogens are possible on germ cells during follicle formation. From the data available, estrogens could control follicle formation through intra-ovarian mechanisms involving members of the TGF-beta family. Indeed, estrogens regulate the formation of primordial follicles in baboons by controlling the intra-ovarian inhibin/activin ratio (Billiar *et al*., 2003) and recently, BMP2 has been shown to mediate the effect of estrogens on follicle formation in the hamster ovary, since the interference of BMP2 production or its receptor function disrupts estradiol-stimulated primordial follicle formation (Chakraborty and Roy, 2017). However, the mechanism by which low and high concentrations of estrogens exert opposite effects on follicle formation are not yet understood.

Increasing evidence indicate that environmental factors with estrogen-like activity modulate importantly follicle formation. Mice treated neonatally with genistein, the primary soy phytoestrogen, have multi-oocyte follicles in ovaries; genistein was found to inhibit germ cell cyst breakdown and attenuate oocyte cell death, and these effects are mediated by the estrogen receptor Esr2 (Jefferson *et al*., 2006). Similar effects were observed after exposure of neonatal mice to synthetic estrogens, such as diethylstilbestrol, ethinylestradiol and bisphenol A (Karavan and Pepling, 2012). Oral administration of zearalenone (a mycoestrogen produced by *Fusarium graminearum*) to pregnant mice impairs germ cell meiotic progression, decreases the expression of the meiosis-specific genes *Dazl*, *Stra8*, *Scp1* and *Scp3*, increases DNA double-strand breaks at the diplotene stage and affects primordial follicle assembly in the fetal ovaries (Liu *et al*., 2017). In a similar way,

following exposure of pregnant mice to diethylhexylphthalate (DEHP), a widespread plasticizer with estrogen-like activity, the first meiotic progression of female fetal germ cells is delayed, associated with an increase in DNA methylation level of *Stra8* and a decrease in its expression levels (Zhang *et al*., 2015). In neonatal mice, DEHP impairs also primordial follicle assembly, while decreasing the gene and protein expression of Esr2 and components of Notch signaling in mouse ovaries (Mu *et al*., 2015). In sheep, exposure (by grazing pastures fertilized with sewage sludge) of pregnant ewes to a real-life mixture of environmental chemicals with pro-estrogenic actions disrupts fetal ovarian development and alters the fetal ovarian transcriptome and proteome at day 110 of pregnancy (Fowler *et al*., 2008).

Estradiol is not required for the initiation of follicle growth in mice (Britt *et al*., 2000) but aromatase knockout mice have reduced numbers of primordial and primary follicles compared with wild-type mice at 10 weeks of age (Britt *et al*., 2004). The primordial follicles that form in ovaries of estrogen-deprived baboon fetuses contain oocytes with a marked reduction in microvilli, structures essential for uptake of substrates from surrounding granulosa cells and presumably long-term follicle survival (Zachos *et al*., 2004). However, lowering estrogen levels during the period of follicle formation does not impair folliculogenesis and ovulation at adulthood in baboons (Pepe *et al*., 2013).

Steroid excess during follicle formation can be deleterious for subsequent follicular development. The exposure of ewes between 60 and 80 days of pregnancy to a mixture of environmental chemicals with estrogen-like activity decreases primordial follicle activation in fetal ovaries near term and increases atresia rate in activated follicles (Bellingham *et al*., 2013; Lea *et al*., 2016). In contrast, prenatal testosterone excess between days 30 and 90 of pregnancy enhances the activation of primordial follicles and early follicular development in fetal and postnatal sheep ovaries (Steckler *et al*., 2005; Smith *et al*., 2009). In sheep, fetal exposure to excess testosterone was shown to disrupt the ovarian proliferation/apoptosis balance, with a decrease in BAX expression in the primordial and primary follicles of fetuses appearing to be programmed by androgenic actions, and changes in PCNA, BCL2, and CASP3 expression in the growing follicles of adults programmed by estrogenic actions of testosterone (Salvetti *et al*., 2012). This prenatal treatment by testosterone leads to early reproductive failure in adult ewes (Clarke *et al*., 1977; Birch *et al*., 2003), resulting from the combination of neuroendocrine, metabolic and ovarian defects (Padmanabhan and Veiga-Lopez, 2013) and similar conclusions were drawn from rodent models of early exposure to steroids (Zambrano *et al*., 2014).

Early exposure to environmental factors with estrogen-like activity affects long-term primordial follicle activation and the observed effects are transgenerational. For instance, maternal DEHP exposure significantly accelerates the recruitment of primordial follicles in the F1 and F2 generations of mice (Zhang *et al*., 2015). The modification of the DNA methylation of imprinted genes in F1 mouse oocytes induced by maternal DEHP exposure is heritable to F2 offspring (Li *et al*., 2014). Recently, it was shown that the reduction of the ovarian follicular reserve and of the oocyte developmental capacity induced by maternal DEHP exposure is transmitted through the female germline up to the third generation (Pocar *et al*., 2017).

Conclusions

During the last decades, our knowledge of the cellular mechanisms and the molecular and environmental control of the establishment of the ovarian reserve of the primordial follicles has made significant progress. Some of the intra-ovarian mechanisms currently known to control germ cell cyst breakdown and primordial follicle assembly are illustrated in Fig. 4. Inadequate maternal nutrition, exposure to steroid excess or to environmental pollutants with estrogen-like activity at the time of follicle formation can delay or modulate the mechanisms controlling the establishment of the ovarian reserve, leading to the formation of a decreased number of primordial follicles in the fetal or neonatal ovaries.

The size of the initial follicular reserve is primarily dependent on genetic determinants, and mutations affecting factors controlling the different steps of ovarian reserve establishment can lead to the absence of primordial follicles, or a sharp reduction in their numbers, incompatible with fertility in the adult. However, having two- or three-fold more or less follicles than the mean number characteristic of the species at birth is not a determinant for fertility and reproductive longevity in adulthood, as demonstrated in mice (Flaws *et al*., 2001) and sheep (Ruoss *et al*., 2009). Rather, the dynamics of follicle consumption from the initial primordial follicle pool determine the female reproductive lifespan (Monniaux *et al*., 2014).

From the data available, the ovarian changes induced by environmental factors present during the fetal or neonatal life do not seem to alter the fertility of young females, unless their metabolism or neuroendocrine status is affected, but their consequences on the reproductive lifespan have not yet been established. To understand the impact of the early-life environment on fertility of both young and older adults, there is now a significant need to consider the different types of populations of primordial follicles, which have distinct developmental dynamics and contribute differently to ovarian functionality (Zheng *et al*., 2014b).

The early-life ovarian environment modulates in the long term the rate of primordial activation and the dynamics of the small growing follicles in adult ovaries. As these processes are little or not influenced by the gonadotropins, the observed changes result likely from direct imprinting effects of environmental factors on the germ and/or somatic ovarian cells, occurring before and during follicle formation. Increasing evidence indicate that environmental chemicals with estrogen-like activity leave epigenetic marks on the germ cells, and their effects are transmitted through the female germline up to the third generation in mice (Pocar *et al*., 2017).

Further investigations are needed now to decipher the underlying mechanisms of these epigenetic effects and understand the long-term consequences of gene imprinting on the size and quality of the ovarian reserve.

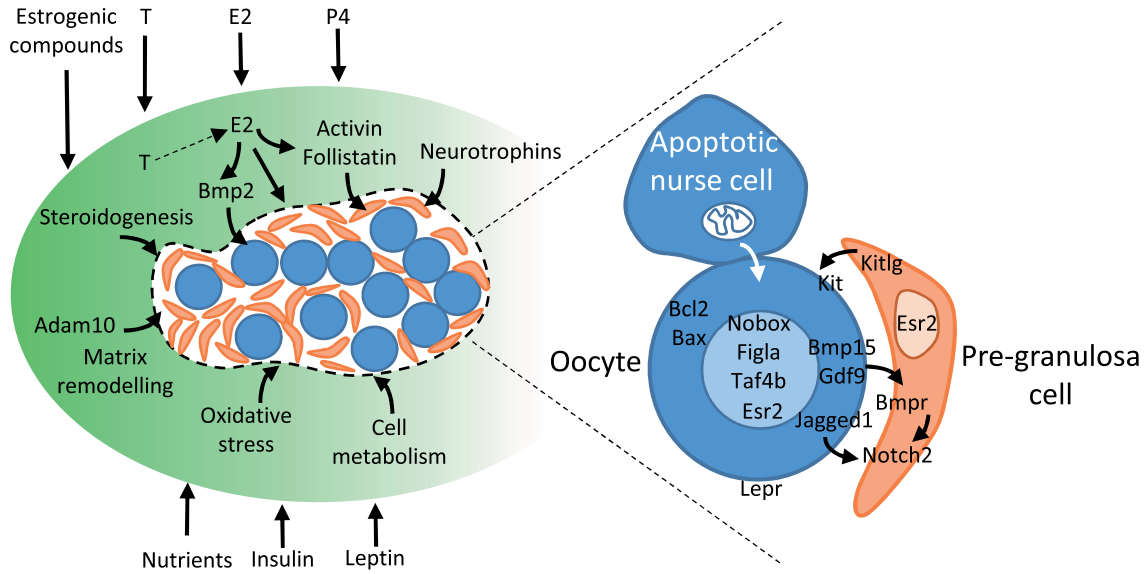

Figure 4. Schematic representation of some mechanisms currently known to regulate germ cell cyst breakdown and follicle assembly. A germ cell cyst is represented (delineated by a dotted line), containing oocytes (blue round cells) and pre-granulosa cells (small pink cells). The intra-ovarian mechanisms regulating germ cell cyst breakdown and the environmental factors currently known to modulate them are indicated in the left part of the figure. Some known interactions between germ and somatic cells, and between germ cells themselves, are zoomed in the right part of the figure.

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DOI: 10.21451/1984-3143-AR2018-0019

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Control of growth and development of preantral follicle: insights from *in vitro* culture

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Abstract

The regulation of folliculogenesis involves a complex interaction among endocrine, paracrine and autocrine factors. The mechanisms involved in the initiation of the growth of the primordial follicle, i.e., follicular activation and the further growth of primary follicles up to the pre-ovulatory stage, are not well understood at this time. The present review focuses on the regulation and development of early stage (primordial, primary, and secondary) folliculogenesis highlighting the mechanisms of primordial follicle activation, growth of primary and secondary follicles and finally transition from secondary to tertiary follicles. We also discuss the importance of *in vitro* follicle culture for the understanding of folliculogenesis during the preantral phase. Studies suggest that follicular development from primordial to early antral stages is primarily controlled by intra-ovarian ligands but it can also be influenced by many extra-ovarian factors. The control of early folliculogenesis is, therefore, extremely complex because several ligands act through distinct signaling pathways that form sophisticated information networks responding to multiple, often opposing, stimuli. The balance among different stimuli determines follicular survival or death as well as quiescence or activation (growth). The distribution of the ligands and their corresponding receptors varies among follicular compartments and species, and significant changes in gene expression pattern among follicular categories have been reported. Knowing that follicular requirements during early folliculogenesis can be stage-specific and species-specific, *in vitro* culture studies offer an alternative to evaluate single and combined factors during a specific period of follicular development. Herewith we summarize the main findings obtained *in vitro* together with the mechanisms regulating folliculogenesis.

Keywords: folliculogenesis, *in vitro* development, ovary, preantral follicle.

Introduction

Folliculogenesis is the physiological process of formation, activation, growth and maturation of ovarian follicles. It describes the progression of some small primordial follicles into large preovulatory follicles. The regulation of folliculogenesis involves a complex interaction among endocrine, paracrine and autocrine factors, which in turn affects the steroidogenesis, angiogenesis, basement membrane turnover, oocyte growth and maturation as well as follicular atresia (reviewed by Atwood and Meethala, 2016). It is well known that mammalian ovaries contain from thousands to millions of follicles, whereby about 90% of them are represented by preantral follicles (PFs). The mechanisms involved in the initiation of growth of the primordial follicles, i.e., follicular activation and the further growth of primary follicles up to the pre-ovulatory stage, are not well understood at this time. It is important to emphasize that despite the large number of follicles in the ovary, the vast majority (approximately 99.9%) of them become atretic during their growth and development stages (reviewed by Figueiredo *et al*., 2011).

The *in vitro* follicle culture (IVFC) technology represents a valuable tool to preserve the fertility in individuals subjected to cancer treatment as well as sub- or infertility treatment, to create gamete banks from endangered species and breeds, to complement other reproductive technologies (e.g., *in vitro* embryo production), and to be used as models for studies in reproductive toxicology (reviewed by Figueiredo *et al*., 2011). Furthermore, IVFC provides insights on the control of growth and development of preantral and antral follicles (Cadenas *et al*., 2017). The present review focuses on the regulation and development of early stage (primordial, primary, and secondary) of folliculogenesis highlighting the mechanisms of primordial follicle activation, growth of primary and secondary follicles and, finally, transition from secondary to tertiary follicles. We also discuss the importance of IVFC for the understanding of folliculogenesis during the preantral phase.

Basic aspects of follicle structure and populations

The ovarian follicle is the functional unit of the ovary composed of an oocyte surrounded by companion somatic cells (granulosa and theca cells). To facilitate the understanding of this review the following follicular classification was adopted: quiescent or dormant follicles represented by the primordial follicles (one layer of flattened granulosa cells around the oocyte) and growing follicles (intermediate: one layer of flattened and cuboidal granulosa cells; primary: one layer of cuboidal granulosa cells, and secondary: two or more

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Received: February 1, 2018
Accepted: June 1, 2018

layers of cuboidal granulosa cells around the oocyte; Silva *et al*., 2004). However, some authors named dormant follicles the ones containing either one layer of flattened granulosa cells or with flattened and/or cuboidal (Jimenez *et al*., 2016). All preantral follicle categories contain an immature oocyte at the germinal vesicle stage.

Regulation of folliculogenesis during the preantral follicle phase

Folliculogenesis is a highly regulated developmental sequence resulting in the growth and differentiation of the oocyte and associated somatic cells. Capacity of the oocyte to resume meiosis, complete maturation (oocyte maturational competence), undergo successful fertilization, support normal embryo and fetal development and produce healthy offspring (oocyte developmental competence) is gradually acquired as the oocyte develops as the follicles pass through the primordial to the preovulatory stages (reviewed by Figueiredo *et al*., 2011). The production of a good quality oocyte (developmental competence) depends on a fine crosstalk between the oocyte and its surrounding follicular cells that begins during the preantral follicle phase of folliculogenesis. The development of PFs is primarily controlled by intraovarian (autocrine/paracrine regulation) ligands (e.g., growth factors, cytokines, and gonadal steroids) even though it can be influenced by many extraovarian ligands (endocrine regulation) from different tissues including the endocrine glands (reviewed by Atwood and Meethala, 2016; Fig. 1-2). The control of folliculogenesis is, therefore, extremely complex because the aforementioned ligands act through distinct signaling pathways. These cell-signaling pathways do not act in isolation, but interact in various ways forming sophisticated information networks that respond to multiple, often opposing, stimuli. This connection may involve components that are common between pathways, as well as positive and negative feedback loops (Hunter, 2000). The main pathways currently studied are adenylate cyclase, MAPK / Erk, PI3K / Akt, phospholipase C, JAKS / STATS, SMADS and nuclear receptors. The ligands that regulate folliculogenesis act by binding to different types of receptors that activate one or more of these pathways leading to responses related to activation, survival, proliferation and follicular maturation (reviewed by Atwood and Meethala, 2016). The following sections describe the proposed mechanisms involved in the regulation of follicle growth from primordial to antral stage.

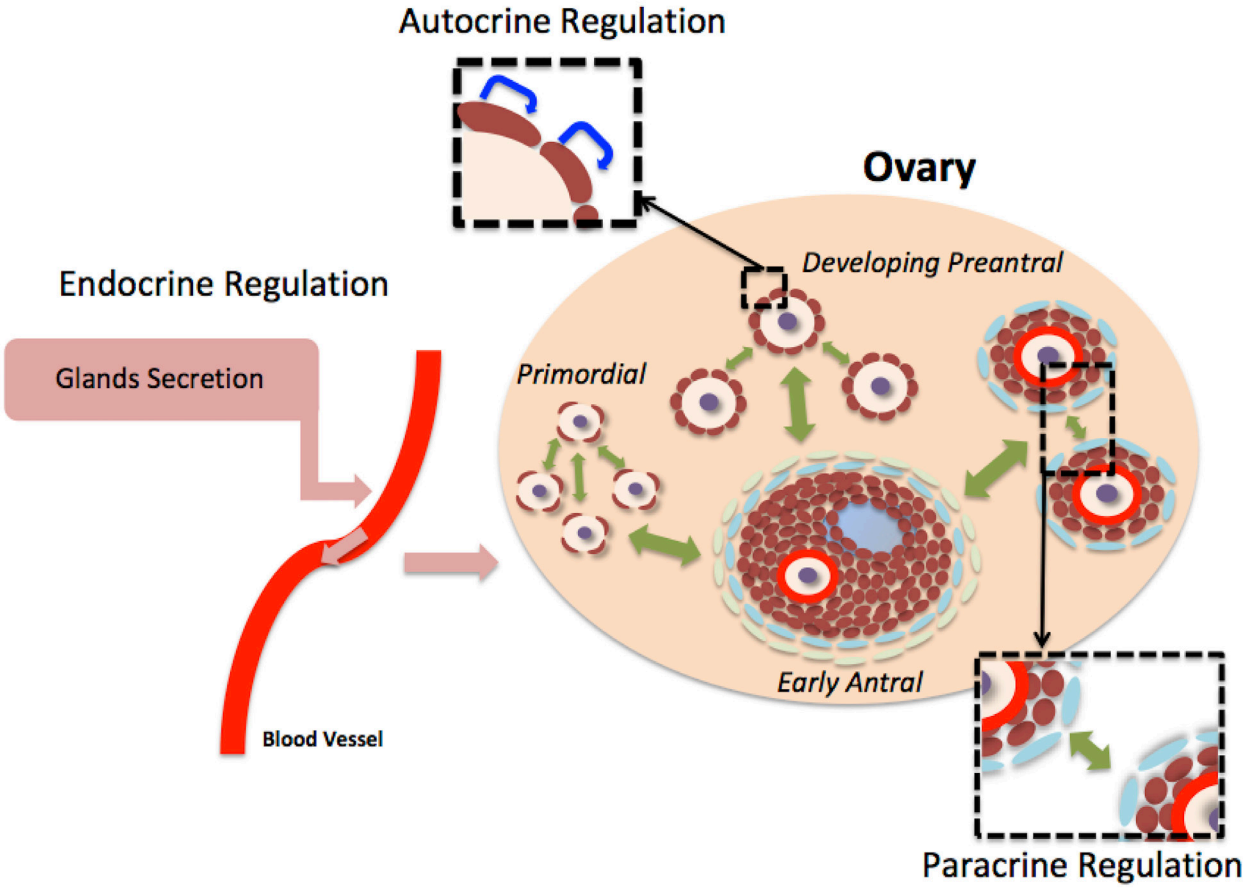

Figure 1. Control of the development of preantral follicle. Follicular development from primordial to early antral stages is primarily controlled by intra-ovarian ligands, but it can also be influenced by many extra-ovarian factors. This process is complex and involves autocrine (blue arrows), paracrine (green arrows) and endocrine (pink arrows) regulations.

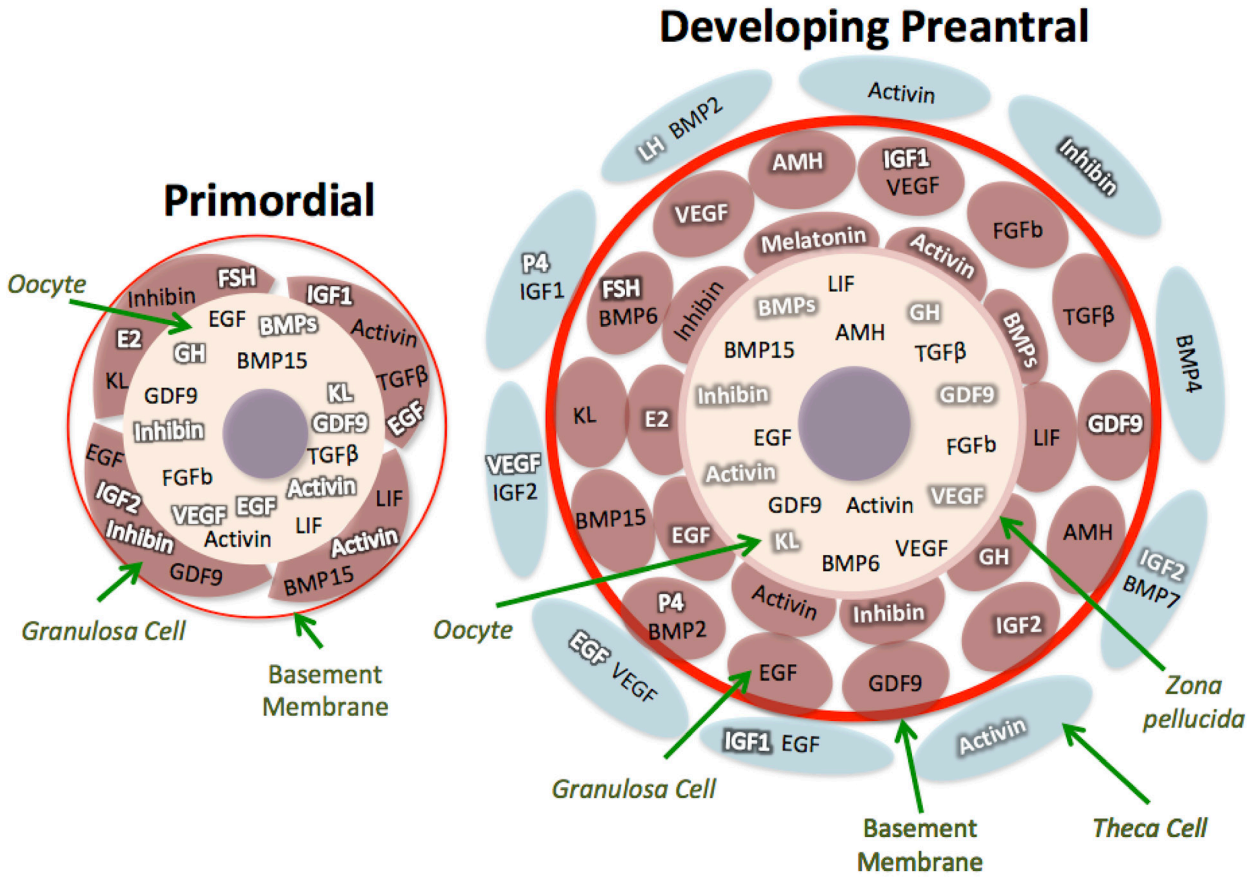

Figure 2- Distribution of some key growth factors and hormones within primordial and developing follicular compartments (oocyte, granulosa and theca cells) in ruminants. Ligands (bold black letter) and receptors (bold white letter). KL – Kit ligand; E2 - 17b-estradiol; FGFb – basic fibroblast growth factor; VEGF – vascular endothelial growth factor; GDF-9 – Growth differentiation factor-9; LIF – leukemia inhibitory factor; GH – growth hormone; EGF – Epidermal growth factor; AMH – Antimullerian hormone; BMP15 – Bone morphogenetic protein 15; BMP6 - Bone morphogenetic protein 6; BMP2 - Bone morphogenetic protein 2; BMP4 - Bone morphogenetic protein 4; IGF1 and IGF2 – Insulin growth factors 1 and 2; TGFβ- tumor growth factor beta; BMPs - Bone morphogenetic proteins superfamily; FSH – Follicle stimulating hormone; LH – Luteinizing hormone.

Activation of primordial follicle

Follicular activation or recruitment is defined as the transition from primordial (quiescent follicle) to primary follicle (growing follicle). This process is marked by a rapid increase in oocyte volume that is accompanied by differentiation and proliferation of the surrounding pregranulosa cells into more cuboidal granulosa cells (reviewed by McLaughlin and McIver, 2009). The first visible sign that a primordial follicle is being activated is that some granulosa cells begin to change from a squamous to a cuboidal shape (reviewed by Figueiredo *et al*., 2011). The shape change is followed by the onset of DNA synthesis and mitosis in the granulosa cells. Activation is pituitary independent, and it probably is controlled by autocrine/paracrine mechanisms (reviewed by McLaughlin and McIver, 2009).

Culture of ovarian cortical tissue has been used to study primordial follicle activation *in vitro*. Kawamura *et al*. (2013) reported in human and mice that ovarian fragmentation increases actin polymerization and disrupts the Hippo signaling pathway, leading to an increase in expression of CCN growth factors. The name CCN is derived from major family members including cysteine-rich angiogenic protein (CYR61 or CCN1), connective tissue growth factor (CTGF or CCN2), and overexpression of nephroblastoma (NOV or CCN3). Secreted CCN2 and related factors promote primordial follicle growth *in vitro* (reviewed by Hsueh *et al*., 2015). Also, various growth factors (GDF9, BMP15, IGF1, kit ligand, BMP7, LIF, FGF2, FGF10 and TGFβ) and hormones (FSH, melatonin, growth hormone, oestradiol, and progesterone) are involved in the activation of primordial follicles *in vitro* (reviewed by Silva *et al*., 2016).

The mechanisms that regulate the initiation of growth of primordial follicle have been studied *in vivo* in murine species. It has been reported that growth factors, like KL, activate the phosphatidylinositol 3 kinase (PI3K) pathway in oocytes. Liu *et al*. (2007) showed that forkhead transcription factor 3 (FOXO3), serine/threonine kinase (Akt), phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and glycogen synthase kinase 3A and 3B (GSK) are

components involved in the PI3K pathway. FOXO3a is a downstream effector of the PTEN/PI3K/AKT pathway (Tran *et al*., 2003). In mouse ovaries, FOXO3a causes suppression of follicular activation, preserving the follicular reserve *pool* (Castrillon *et al*., 2003). A considerable proportion of the signaling mediated by PI3Ks converges at 3-phosphoinositide-dependent protein kinase-1 (PDK1). The PI3K–PDK1 cascade in mice oocytes regulates ovarian aging by regulating the survival of primordial follicles (Reddy *et al*., 2008). PTEN may act as a phosphoinositide-3 (PIP3)-phosphatase that antagonizes the activity of PI3K by dephosphorylating PIP3 to PIP2 (Maehama and Dixon, 1998). Oocyte-specific deletion of PTEN causes premature activation of mice primordial follicle pool, suggesting that the mammalian oocyte is the initiator of follicle activation and that the oocyte PTEN-PI3K pathway governs follicle activation through control of initiation of oocyte growth (Reddy *et al*., 2008). In bovine species, primordial follicle activation *in vitro* is associated with loss of the primordial follicle PTEN and cytoplasmic translocation of FOXO3 (Bromfield and Sheldon, 2013). Oocyte-specific loss of PTEN promotes in FOXO3 hyperphosphorylation, and FOXO3 nuclear export, which triggers the initiation of growth of primordial follicle, thus indicating that FOXO3 controls this step of follicular development (mice: John *et al*., 2008).

In vivo studies have shown that serine/threonine kinase mammalian target of rapamycin (mTORC) is another factor that controls primordial follicle activation since a suppression of its activity maintains the quiescence of mice primordial follicles (Reddy *et al*., 2008). These authors also reported that elevation of mTORC activity in the oocyte activates primordial follicles. In addition, the tumor suppressor tuberous sclerosis complex (TSC), which negatively regulates mTORC, functions in oocytes to maintain the quiescence of primordial follicles (Adhikari *et al*., 2010), Thus, the activity of both TSC and PTEN suppress mice follicular activation, but in distinct ways. They play an essential role in oocytes of mice primordial follicles to preserve the female reproductive lifespan (Adhikari *et al*., 2009).

In vitro studies have shown that various growth factors (GDF9, BMP15, IGF1, kit ligand, BMP7 LIF, FGF2, FGF10 and TGFβ) and hormones (FSH, melatonin, growth hormone, estradiol and progesterone) promote the activation of primordial follicles *in vitro* (reviewed by Silva *et al*., 2016). *In vitro* studies have shown that BMP15 increases follicle and oocyte diameters during culture of cortical tissues rich in primordial follicles (goat: Celestino *et al*., 2011). However, *in vivo* overexpression of *BMP15* gene in mice has no effect on the rate of primordial to primary follicle transition (McMahon *et al*., 2008). In addition, sheep containing homozygous mutations in this gene have normal primordial follicle activation, but folliculogenesis is arrested at the primary stage (Galloway *et al*., 2000). Recently, Zhao *et al*. (2018) showed that the growth factor activates phosphorylated mitogen-activated protein kinase3/1 (MAPK3/1) signaling in pregranulosa cells from mice to elevate mTORC1 signaling, leading to enhanced expression of Kit Ligand and subsequent activation of PI3K signaling in oocytes *in vitro*. Activation of this signaling pathway results in primordial follicle activation. Bisphenol, a chemical widely used in mineral water bottles and food-can linings, can initiate excessive premature activation of primordial follicles in mature mouse ovaries via the PTEN/PI3K/AKT signaling pathway by down regulating PTEN expression *in vivo* (Hu *et al*., 2017). Novella-Maestre *et al*. (2015) also showed that ovary-specific treatment with PTEN inhibitor enhances the activation mechanisms of primordial follicles, and also augments estradiol secretion in rat ovaries. In addition, Sun *et al*. (2005) treated mice and human ovaries with mTORC stimulators, i.e., phosphatidic acid (PA) and propranolol, and demonstrated that the stimulators increased activation of primordial follicles. On the other hand, gremlin-2 maintains the store of primordial follicles by suppressing Smad 1/5/8 signaling in the human ovary (Ikeda *et al*., 2016). However, the mechanisms of primordial follicle activation seem to be very complex, since it was reported that expression of 223 genes are down-regulated, while expression of 268 other genes are up-regulated in the oocytes during the human primordial-to-primary follicle transition (Ernst *et al*., 2017). The tyrosine kinase inhibitor imatinib mesylate blocks the activity of tyrosine kinase c-Kit, and is used as treatment for multiple cancers. It has also been proposed as an agent to prevent primordial follicle loss during chemotherapy based on its role as a c-Abl kinase inhibitor via PI3K/PTEN/Akt signaling pathways (Roness *et al*., 2014). However, it was shown recently that this compound down-regulates Kit ligand and GDF9 expression, with a delayed activation of rat primordial follicles *in vitro*. It is not clear if such a delay will affect the further follicular development (Asadi-Azarbaijani *et al*., 2017).

Development of primary and secondary follicles

Once primary follicles are formed, the cuboidal granulosa cells begin to express FSH receptors probably through autocrine/paracrine mechanisms induced by granulosa-derived activin (reviewed by Silva *et al*., 2016). From the primary stage onwards, the oocyte begins to grow and differentiate as a result of a progressive increase in the level of oocyte RNA synthesis. Some oocyte genes including those encoding the zona pellucida (ZP) proteins (i.e. ZP1, ZP2 and ZP3) are transcribed and translated. During primary follicle development, gap junctions (intercellular channels composed of proteins called connexins-Cx that directly couple adjacent cells) are observed. Connexin 37 (C×37) is an oocyte-derived connexin that forms gap junctions between the oocyte and surrounding granulosa cells and has an obligatory role for folliculogenesis while C×43 is a major gap junction protein expressed in the granulosa cell layer (reviewed by Brito *et al*., 2014a). The communication between the granulosa cells and oocyte remains throughout folliculogenesis and is responsible for the synchronous expression of important

activities. The acquisition of a theca layer (inner theca interna and outer theca externa) is an important event observed during the development of a secondary follicle. Some stromal cells in the inner layer express LH receptors (Young and McNeilly, 2010).

During the primary to secondary follicle transition, the granulosa cells proliferate to form multiple layers and thereby support oocyte growth (Richards and Pangas, 2010). During this early stage of folliculogenesis, oocytes begin to express abundant cell-cell communication proteins, including Cx37, N- and E-cadherin, and G-protein coupled receptors (Dharma *et al*., 2009). E- and N-cadherin are localized in the oocyte membrane and establish oocyte-granulosa cell contacts (Wang and Roy, 2010; Mora *et al*., 2012). In Cx37-deficient mice, folliculogenesis is arrested at the early antral stage, while in mice lacking Cx43, the follicles do not develop beyond the primary follicle stage, and oocyte growth is disrupted (Simon *et al*., 1997, Ackert *et al*., 2001). The mechanism to keep this bidirectional communication as well as to stimulate the primary to secondary follicles transition was recently reported by Jiang *et al*. (2017) in mice. These authors showed that geranylgeranyl diphosphate (GGPP), a metabolic intermediate involved in protein geranylgeranylation, is required to establish the oocyte-granulosa cell communication. In mice ovaries, the levels of GGPP and geranylgeranyl diphosphate synthase (GGPPS) in oocytes are increased during early folliculogenesis (Jiang *et al*., 2017). The depletion of GGPP in mouse oocytes impairs the proliferation of granulosa cells and disturbs the primary to secondary follicle transition. Regarding other mechanisms, GGPP depletion inhibits Rho GTPase geranylgeranylation and its GTPase activity, which is responsible for the accumulation of cell junction proteins in the oocyte cytoplasm. As a consequence, the physical connection between oocyte and granulosa cells is lost and the secretion of oocyte growth factors, like GDF9 is impaired (Jiang *et al*., 2017). It is important to consider that GDF9 knockout mice have follicular development arrested at the primary follicle stage (Dong *et al*., 1996). However, sheep with a homozygous mutation in GDF9 gene have normal follicular development up to the antral stages (McNatty *et al*., 2005). Additionally, GDF9 and BMP15 immunization experiments in sheep species caused a reduction in ovarian volume and abnormal follicular development beyond the primary follicle stage (Juengel *et al*., 2002).

Figure 3 shows the mechanisms by which GGPP-mediated protein geranylgeranylation regulates oocyte-granulosa cell communication and promotes the growth of primary follicles. Farnesyl diphosphate (FPP) is a metabolic intermediate of the mevalonate pathway that is catalyzed into geranylgeranyl diphosphate (GGPP) by geranylgeranyl diphosphate synthase (GGPPS). Then, GGPP activates Rho GTPase and Rab GTPase that stimulates the expression of cell junction proteins that are localized in the oocyte membrane to maintain the connection between granulosa cells and oocyte. This mechanism may be important for the secretion of growth factors that are synthesized exclusively by the oocyte, like GDF9 (mice: Jiang *et al*., 2017). These events stimulate proliferation of granulosa cells and primary to secondary follicle transition. Pires *et al*. (2013) also reported in mice and non-human primates that sperm acrosomal SLLP1 binding (ASTL) protein (ovastacin) starts expression during the primary to secondary transition, but not in primordial follicles, which suggest this zinc metalloprotease deserves consideration as a candidate to control primary to secondary follicles transition.

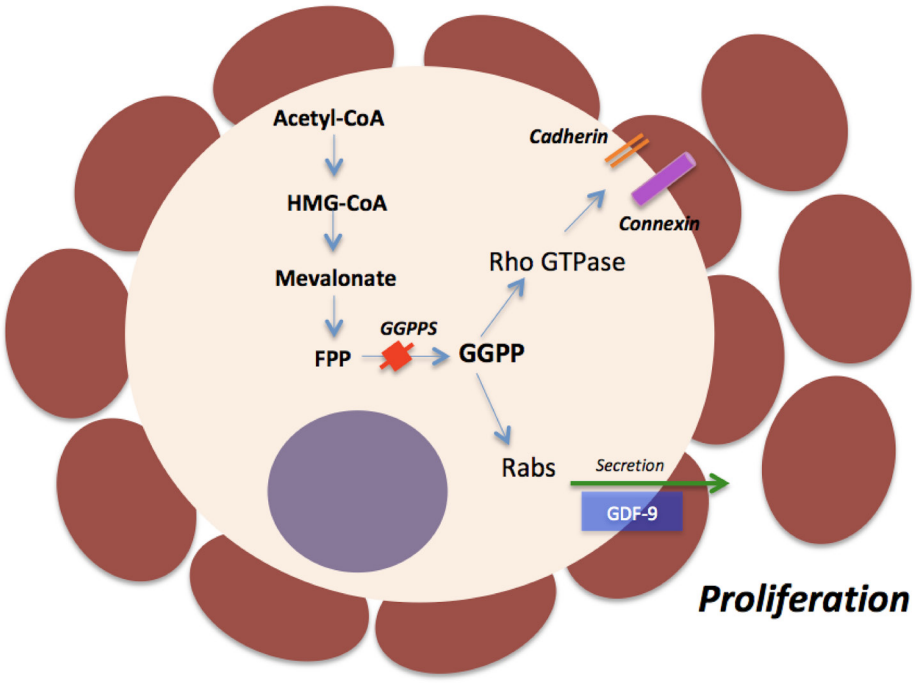

Figure 3. Mechanisms involving GGPP-mediated protein geranylgeranylation in the regulation of primary to secondary follicle transition.

Transition from preantral to antral follicle

The transition from secondary to tertiary follicle is marked by the appearance of a cavity in the granulosa cells named cavitation and/or beginning antrum formation. Early antrum formation is more likely to be controlled by autocrine/paracrine mechanisms rather than extra-ovarian factors like pituitary hormones such as FSH. As a matter of fact, cavitation occurs in hypophysectomized animals (Erickson, 1983) as well as in FSH-β-deficient mice (Kumar *et al*., 1998). In addition to gap junctions (Simon *et al*., 1997) two growth factors expressed in the follicle itself have been implicated in cavitation: activin and KIT ligand. Treating cultured granulosa cells with activin induced the formation of antrum-like cavity in rat follicles (Li *et al*., 1995). Blocking the action of the KIT ligand in the ovary prevents the formation of mouse antral follicles (Yoshida *et al*., 1997).

Significant changes in gene expression pattern occur in the transition from late secondary to early tertiary follicles. To date, a study performed by our team investigated the temporal changes in transcriptional profiles of secondary and early antral (tertiary) follicles in caprine ovaries using microarray analysis. A total of 14,323 genes were hybridized with goat mRNAs while 9,664 genes were not. Of all the hybridized genes, 2,466 were stage-specific up- and down-regulated in the transition from secondary to early tertiary follicles (Fig. 4). Gene expression profiles showed that three major metabolic pathways (lipid metabolism, cell death, and hematological system) were significantly differentiated between the two follicle stages. (Magalhaes-Padilha *et al*., 2013).

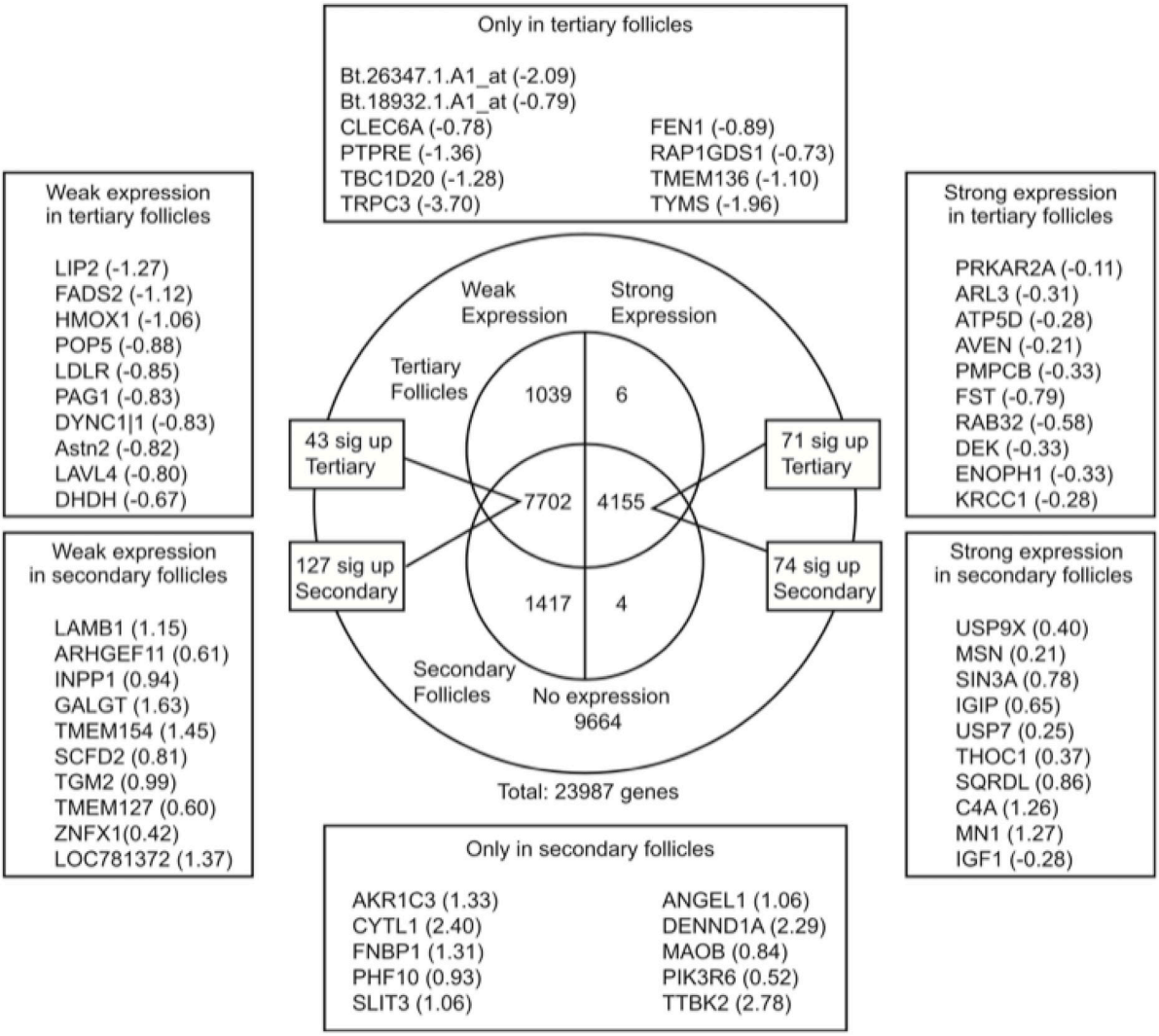

Figure 4. Overview of differential gene expression between secondary and tertiary ovarian follicles in a Venn diagram. Expressed genes were grouped into two categories weakly (left half) and strongly (right half) expressed. Strong expression means the expression level is above the genome average and weak expression means it is below the genome average. Top 10 representative genes from six categories were listed in boxes with their M value. M value was the log2 ratio of secondary follicle/tertiary follicle expression (Maglhães-Padilha et al., 2013).

Some studies have shown that the production of hyaluronan and proteoglycan by granulosa cells generates an osmotic gradient that enhances the formation of follicular fluid during transition from secondary to early tertiary follicles (Rodgers and Irving-Rodgers, 2010). These substances are osmotic solutes that act to increase the osmotic pressure inside the bovine follicle, resulting in fluid accumulation (Clarke *et al*., 2006). Versican and versican proteoglycans have been identified in the follicular fluid of various species, including bovine (McArthur *et al*., 2000; Clarke *et al*.; 2006) and human (Eriksen *et al*.; 1999), whereas perlecan protein have been identified in granulosa cells of bovine antral follicles (McArthur *et al*.; 2000; Princivalle *et al*.; 2001; Irving-Rodgers *et al*.; 2004). Familiari *et al*. (1987) reported that hyaluronan and proteoglycans, located on the apical side of granulosa cells, are then secreted into follicular fluid. Among the substances that promote the formation of antrum in bovine follicles, GDF9 increases the expression of versican and perlecan, as a consequence of a positive interaction with FSH. Both GDF9 and FSH also increase the *in vitro* expression of other antral fluid proteins, like versican and HAS2 (Vasconcelos *et al*., 2013).

Contribution of *in vitro* follicle culture for the understanding of folliculogenesis

Immature oocytes are usually cultured *in vitro* enclosed in preantral (primordial, intermediate, primary and secondary) or antral (tertiary or early antral) follicles. This biotechnology is known as IVFC, and is mainly carried out in two forms: in situ, which means that ovarian follicles are cultured within the ovarian tissue, or in the isolated form (reviewed by Figueiredo *et al*., 2011). As mentioned before, the IVFC is an important tool to enhance the knowledge of the mechanisms involved in the control of ovarian folliculogenesis, including the development of PFs. The following sections discuss the major contribution of IVFC for the understanding of preantral follicle development focusing on domestic animals, especially ruminant species.

Shared signaling pathways and similar effects of ligands on preantral follicle activation and growth in vitro

As in other tissues, when a ligand binds to its receptor in the ovary it will eventually affect gene expression, which in turn controls cell survival, proliferation and differentiation. As shown in Fig. 5 many intra and extraovarian ligands share the same signaling pathway. The addition of different ligands to a given control medium can result in similar effects. Studies have shown that the individual addition of many ligands to a determined culture medium improved in a concentration-depend manner the survival, activation and follicle and oocyte diameters after IVC of caprine ovarian tissue (reviewed by Figueiredo *et al*., 2011). However, the action of an individual ligand can be affected by the type of basic medium (TCM 199, MEM, etc.), the type of supplements present in this medium, as well as the volume of the medium and interval of its replenishment during IVFC (reviewed by Figueiredo *et al*., 2011). Consequently, care must be taken in the interpretation of the data and their possible translation to *in vivo* conditions.

Gene expression in vivo *and specific follicle requirements* in vitro

Previously, temporal changes in transcriptional profiles of caprine secondary and early antral follicles have been reported. Such difference has a great impact on follicular requirements during IVC. Cadenas *et al*. (2017) investigated the effect of GH and VEGF added alone, sequentially, or in combination, in a medium supplemented with insulin at physiological concentration (10 ng/ml) on the IVC of large secondary preantral and early antral follicles from goats. Contrarily to PFs, in the IVC of early antral follicles, GH addition improved oocyte growth and maturation. It was previously shown that caprine secondary and early antral follicles present distinct gene expression (Magalhães-Padilha *et al*., 2013), which may result in different responses to some compounds. Based on RNA-seq technology, Bonnet *et al*. (2015) determined that the changes in gene expression that occur in both oocytes and granulosa cells respect a spatio-temporal process in sheep. In summary, depending on the follicular preantral phase the involved pathways are related to the acquisition of meiotic competence, migration and cellular organization, whereas in granulosa cells they are related to adhesion, formation of cytoplasmic projections and steroid synthesis. Also, changes in gene expression are more abundant in oocytes than in granulosa cells, with the transition between the primary to secondary follicles being the most active period. Knowledge of the important biomarkers of preantral follicular phase will allow the improvement and development of specific step-wise IVFC protocols.

Heat stress and in vitro *follicle development*

Even though the impact of heat stress (HS) on antral follicles is well documented with *in vivo* studies, little is known about the influence of HS on the preantral phase of folliculogenesis. It was shown in bovine ovarian tissue *in vitro* that HS (41ºC) for 12 h induces early activation of primordial follicles with increased production of reactive oxygen species after 7 days IVFC (Paes *et al*., 2016). Furthermore, these authors showed that secondary PFs appear to be less sensitive to such stress, and that HS disrupts E2 and P4 secretion and reduces oocyte nuclear maturation of COCs from antral follicles grown *in vivo*.

Follicle dominance (antral vs. *preantral and influence of follicular fluid*)

The importance of grouping PFs retrieved from domestic animals to increase growth rates in comparison with the culture of single follicles during

IVFC obtained from pigs (Wu *et al*., 2001) and buffaloes (Gupta *et al*., 2002) was demonstrated. Duarte *et al*. (2010) observed that caprine secondary PFs cultured in groups (3 follicles/group) demonstrated enhanced viability, growth and antrum formation rates when compared to individually cultured follicles. However, co-culture of PFs with an early antral follicle had a detrimental effect on viability, antrum formation and production of oocytes for IVM. During *in vitro* culture of mouse follicles, Spears *et al*. (1996) demonstrated the occurrence of dominance in pairs of preantral follicles cultured in contact with each other until the antral stage of development. Probably, there is a specific interaction between adjacent follicles, which determines the success of dominant follicle development. Besides this, Duarte *et al*. (2012) showed that IVFC medium with follicular fluid from a dominant follicle enhanced caprine follicular survival, the maintenance of ultrastructure, as well as promoting follicular growth, meiosis resumption and early antrum formation. Follicular fluid is not only a source of hormones and growth factors. It was shown previously that follicular fluid increases the antioxidant capacity in *in vitro* cultured porcine granulosa cells, being also a source of anti-apoptotic factors (Santos *et al*., 2015). These authors also observed that such a protective effect is more robust when using follicular fluid from sows than from gilts.

Extracellular matrix stiffness and primordial follicle activation

Usually, immature follicles gradually move from the rigid collagen-dense cortex zone to the less dense peri medullary region as they grow. To maintain the spherical structure of the follicle *in vitro*, 3D culture systems were developed in which the follicle floats in rotating tubes or inverted micro drops, or is encapsulated in a culture matrix, such as alginate. Although major advances in our understanding of follicle biology have been achieved with 2D attachment culture systems, recent studies have shown that 3D culture systems more closely mimic the physiological environment of the ovary, preserving follicular architecture and the interaction between somatic and germ cells (reviewed by Brito *et al*., 2014a). The use of IVC of isolated follicles allowed the evaluation of the importance of extracellular matrix rigidity (3D culture system) and of the contact between follicular cells in the activation of the primordial follicles and in the development of secondary follicles. Hornick *et al*. (2012) confirmed this fact using primate primordial follicles cultured in groups embedded in different alginate concentrations. They found that only the highest alginate tested concentration (2%) maintained the three-dimensional shape of the follicle and consequently an intimate contact between the follicular cells. This fact favored the activation and the *in vitro* growth that ends up in the production of secondary follicles. On the other hand, Brito *et al*. (2014b) increased oocyte maturation rates (59.52%) after culturing caprine secondary follicles in a less rigid alginate matrix (0.25%). These *in vitro* studies correlate with *in vivo* observation that primordial follicles are found primarily in the rigid and dense ovarian cortex rich in collagen, whereas the developing follicles are located closer to the medulla, in a less rigid environment.

Role of oocyte and theca cells on in vitro *follicle development*

Interaction between the vascular theca and the avascular granulosa cells also play an important role in follicle development, not only because theca cells produce androgen that is aromatized in granulosa cells. Granulosa cells also regulate Theca cell steroidogenesis by providing steroids, growth factors, cytokines, and extracellular matrix, as shown in bovine (Parrott and Skinner, 2000). Liu *et al*. (2015) showed that co-culture of mouse theca cells with granulosa cells derived from PFs increased steroidogenesis in theca cells, together with the increase of mRNA expression of steroidogenic enzymes. Signaling between theca cells and oocyte also deserves attention when developing *in vitro* protocols. It is possible to observe migration of theca cells between co-cultured follicles (Campbell *et al*., 2013), which may contribute to the formation of mouse multiple oocyte follicles *in vitro* (Christensen *et al*., 2017) if these cells are not properly active.

Antrum formation and the role of extra-ovarian factors

As the follicle diameter increases rapidly during late folliculogenesis, it was suggested that antrum is formed to overcome transport limitations (Gosden and Byattsmith, 1986). Both granulosa and thecal cells contribute to antrum formation by the synthesis and action of steroids and growth factors. However, extraovarian factors are necessary to allow PFs to form antrum *in vitro*, even though it is known that PFs are not gonadotropin dependent. Gutierrez *et al*. (2000) showed the importance of adding FSH to the culture medium to obtain an antrum in bovine cultured PFs. Araujo *et al*. (2014), however, showed also in bovine that the influence of extra-ovarian factors depends on the culture system. For instance, growth hormone addition in the culture medium was effective to stimulate estradiol synthesis only under 3D conditions.

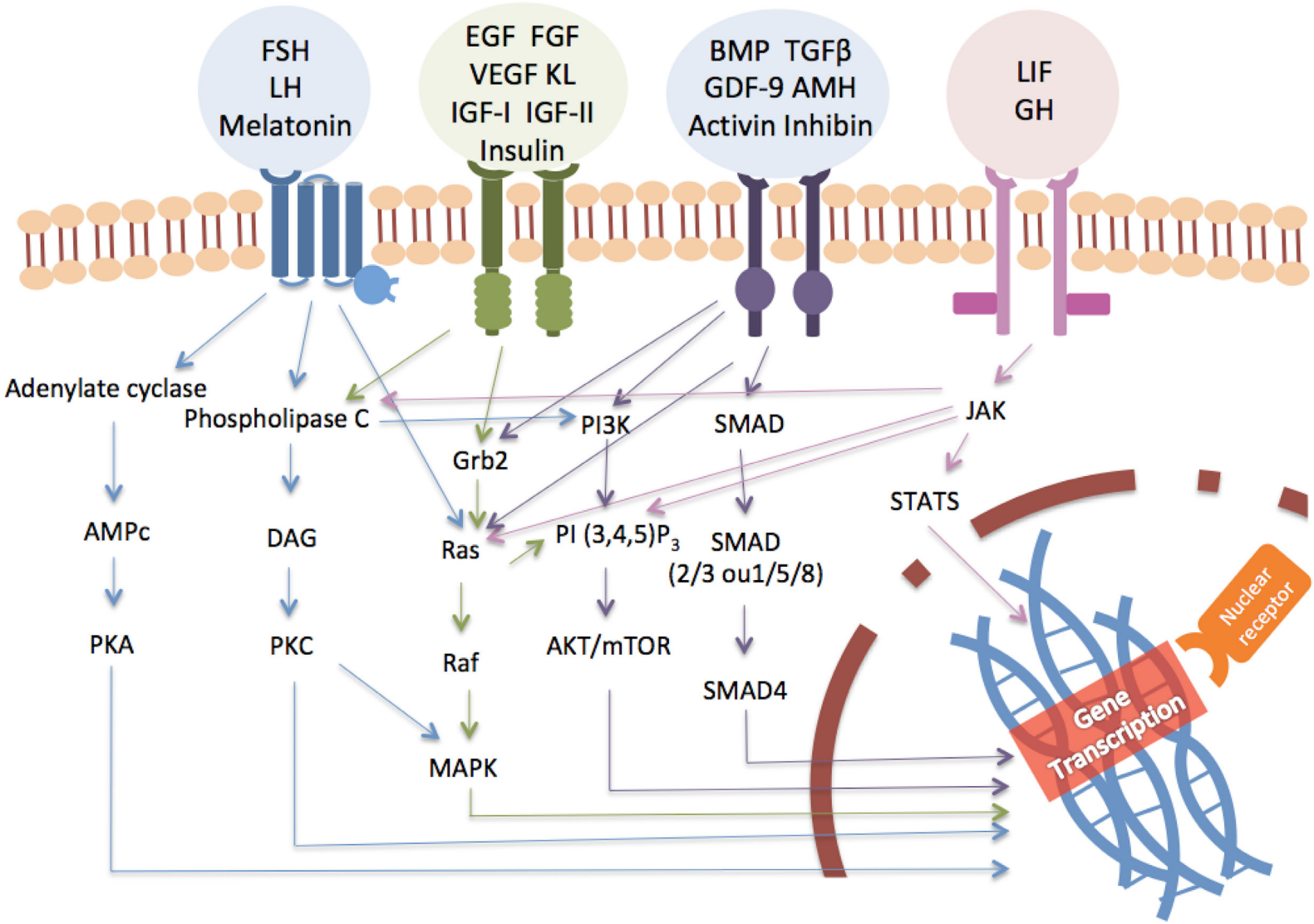

Figure 5. Signaling pathway of intra and extraovarian ligands in follicular development.

Final considerations

Follicular development from primordial to early antral stages is directly controlled by intra-ovarian ligands, and is influenced by many extra-ovarian ligands from different tissues including the endocrine glands. The control of early folliculogenesis is, therefore, extremely complex because several ligands act through distinct and interactive signalling pathways (for instance. adenylate cyclase, MAPK/Erk, PI3K/Akt, phospholipase C, JAKS/STATS, SMADS and nuclear receptors), forming sophisticated information networks that respond to multiple, often opposing, stimuli. The balance among different stimuli can lead to responses related to follicular survival or death as well as quiescence or activation (growth). It is important to emphasize that the distribution of the ligands and their corresponding receptors varies among follicular compartments (oocyte, granulosa and theca cells) and species (for instance between sheep and rodents) and significant changes in gene expression pattern among follicular categories have been reported. As a result, follicular requirements during early folliculogenesis seem to be stage-specific and species-specific. *In vivo* assessment of the mechanisms regulating follicle development is dependent on the animal species, and it can be affected by multiple factors including nutrition, breed, age, as well as individual variations. Therefore, especially for large domestic animals, interactions of factors and their effect on follicular development can be analyzed via IVFC studies. Hence, the IVFC technique enhances our understanding of the control of folliculogenesis. It also enhances the future use of a large number of immature oocytes enclosed in PFs in assisted reproductive technologies in humans as well as in others mammalian species. Last but not the least, it is important to highlight that the IVFC technique is a helpful tool to replace and reduce animal experimentation.

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DOI: 10.21451/1984-3143-AR2018-0027

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Mechanisms regulating follicle selection in ruminants: lessons learned from multiple ovulation models

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Abstract

Selection of a single dominant follicle from a cohort of growing follicles is a unique biological process, a key step in female reproductive function in monovular species, and lies at the core of reproductive technologies in cattle. Follicle growth and the number of follicles that ovulate are regulated by precise endocrine, paracrine, and autocrine mechanisms. Most of our current understanding about follicle selection focuses on the role of FSH, LH, and the IGF family in follicle growth and selection of the dominant follicle. However, more recently the role of members of the TGF-ß family has been highlighted, particularly in high fecundity genotypes in sheep. Intercellular signaling between the oocyte and granulosa cells (GC) regulates proliferation and differentiation due to actions of bone morphogenetic protein 15 (BMP15) and growth and differentiation factor 9 (GDF9) within the follicle. Mutations that either knockout or reduce the activity of BMP15 or GDF9 have been found to increase ovulation rate in heterozygotes and generally cause severe follicle abnormalities in homozygotes. A mutation in the intracellular kinase domain of the BMPR1B receptor (Booroola fecundity gene) increases ovulation rate in heterozygotes with further increases in ovulation in homozygotes. The physiological mechanisms linking these mutations to increased ovulation rates are still not well defined. A recently identified high fecundity bovine genotype, Trio, causes increased expression of SMAD6, an intracellular inhibitor of the BMP15/GDF9 signalling pathways. This bovine model has provided insights into the mechanisms associated with selection of multiple dominant follicles and multiple ovulations in carriers of fecundity alleles. The present review focuses on the mechanisms involved in follicle selection in ruminants with a special emphasis on the contribution made by multiple ovulation models in both cattle and sheep. The evaluation of multiple ovulation models in ruminants has allowed us to construct a new physiological model that relates changes in the BMP15/GDF9 signalling pathways to the physiological changes that result in selection of multiple dominant follicles. This model is characterized by acquisition of dominance at a smaller follicle size but at a similar time in the follicular wave with multiple follicles acquiring dominance in a hierarchal sequence, delaying FSH suppression and, thus allowing additional follicles to continue to grow and acquire dominance.

Keywords: follicle selection, high fecundity, ruminants.

Introduction

Selection of a single dominant follicle from a cohort of growing follicles is a unique biological process and a key step in female reproductive function. Disturbances in this process can lead to anovulation and infertility or, alternatively, multiple ovulations and multiple births. The improvement in our understanding of follicle development and selection has fueled the development of synchronization protocols for fixed time artificial insemination as well as the development of other reproductive biotechnologies such as superovulation and embryo transfer. As a result, these advances have highlighted the importance of optimal follicle growth and selection as a critical step towards achieving reproductive efficiency in livestock species in order to feed a growing world population.

Alterations in follicle selection can lead to the occurrence of codominant follicles and multiple ovulations which are the basis for dizygotic twinning in cattle and sheep. A particularly useful approach, suitable for study of many biological processes, is the examination of abnormal phenotypes. In this regard the occurrence of multiple ovulations in otherwise monovular species provides a physiological model in which the follicle selection mechanism has been altered in such a way to allow multiple follicles to be selected and ovulate. The available multiple ovulation models in sheep (Juengel *et al*., 2013) and, more recently, in cattle (Kamalludin *et al*., 2018) have contributed to the identification of members of the transforming growth factor-ß (TGF-ß) superfamily as key regulators of ovulation rate and prolificacy unveiling a new set of genes, pathways, and autocrine mechanisms with critical roles in the ovarian physiology of ruminants.

The term follicle selection has been widely used in the literature, however there is a lack of consensus on its scope and implications for reproductive function. Selection has been used in relation to terminal growth of antral follicles, however the loss of preantral follicles by atresia during the early stages of folliculogenesis could arguably be included as part of the selection mechanism. Ginther *et al*. (2001) provided a precise definition for the term in relation to monovular species: is the process wherein one follicle develops from a wave of growing follicles and becomes the only follicle with ovulatory capacity. However, this definition does not account for the possibility of

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Received: February 13, 2018
Accepted: April 12, 2018

multiple follicles being selected. Conversely, the definition of the word selection is: a collection of things chosen from a group of similar things. Therefore, in order to determine whether follicle selection is occurring, two related events need to be observed: 1) a cohort of growing follicles, and 2) the ability of one (or multiple) follicles to continue to grow and acquire the capacity to ovulate while the remaining follicles do not. In the present review we will use the latter definition which provides the opportunity for more than one follicle to be selected, and thus the occurrence of multiple ovulations can be studied in relation to the follicle selection mechanism.

The purpose of this review is to briefly explore the follicular, endocrine and paracrine factors associated with selection of the dominant follicle and more importantly to explore the contribution of multiple ovulation models in ruminants for understanding the follicle selection mechanism. After introducing the genetic models that have been found to increase ovulation rate in ruminants, the review will explore the potential physiology that underlies the increased ovulation rate in both ovine and bovine models.

Selection of a single dominant follicle

Follicle development is an essential aspect of female reproduction with intricate mechanisms driving all stages of this process. Initial follicle development involves gradual activation of primordial follicles, which occurs throughout the reproductive lifespan of the female. The complex mechanisms involved in primordial follicle activation are slowly becoming clearer with a key role for KIT ligand from the granulosa cells (GC) activating PI3K/Akt/mTOR pathways in the oocyte and eventually blocking the transcriptional machinery that tonically inhibits primordial follicle activation, such as Foxo3a and p27 (Zhang and Liu, 2015; Bertoldo *et al*., 2018; Kallen *et al*., 2018). Subsequent growth of the primary, secondary, and early antral follicles involves FSH and a series of paracrine factors, such as C-type natriuretic peptide, that stimulate follicle growth allowing the growing follicles to overcome inhibitory pathways, such as the Hippo pathway, ultimately allowing follicles to enter the antral stages of follicle development (Hsueh *et al*., 2015). The final development stages of antral follicle growth occurs in a wave-like pattern in ruminants, a model first proposed by Rajakoski (1960). The introduction of ultrasonography and the resulting ability to image the bovine ovary allowed for the concept of follicular waves to be revisited and investigated (Pierson and Ginther, 1987; Sirois and Fortune, 1988). Ultrasonography has now provided substantial information on the dynamics of follicle development and has provided convincing data supporting the follicular wave concept. Moreover, the ability to monitor follicle growth patterns has provided the ability to study follicle selection from a dynamic perspective and allow for the evaluation of temporal associations between follicle events and endocrine changes.

A follicular wave is defined as the synchronous growth of a group of small antral follicles, from which a single follicle is selected becoming the dominant follicle, whereas the remainder of the follicles (subordinate follicles) undergo regression (Ginther *et al*., 1989; Adams, 1994). The follicular wave pattern in ruminants is characterized by the development of typically 2 or 3 follicular waves in cattle (Knopf *et al*., 1989), and 3 to 6 waves in sheep (Ginther *et al*., 1995), during each cycle. Traditionally the emergence of the follicular wave has been defined, in cattle, as the day or examination at which the retrospectively identified dominant follicle is first detected at a diameter of 4-5 mm (Ginther *et al*., 1989).

The pivotal event leading to the occurrence of a single ovulation in monovular species, such as cattle, is referred to as follicle selection. The morphological visualization of the follicle selection process has been termed, diameter deviation, and consists of the continuous growth of the future dominant follicle while the subordinate follicles slow their growth rate or completely cease to grow (Ginther *et al*., 1996, 1997a, 2001). In cattle, diameter deviation occurs, on average, between 2 and 3 days after emergence of the follicular wave, and, although there is individual variability, this range appears to be very representative as it has been observed in multiple studies using both *Bos taurus* and *Bos indicus* breeds (Ginther *et al*., 1996; Sartori *et al*., 2001; Gimenes *et al*., 2008). The future dominant follicle is on average 8 to 9 mm at deviation while the largest subordinate follicle is 7 to 8 mm in *Bos taurus* (Ginther *et al*., 1996, 1997a). Conversely, in *Bos indicus* the future dominant follicle and largest subordinate follicle at deviation onset are 5.9 to 7 mm and 5.4 to 5.9 mm, respectively (Sartorelli *et al*., 2005; Gimenes *et al*., 2008; Sartori *et al*., 2016). These data support the idea that the future dominant follicle generally has a size advantage over the largest subordinate follicle. In this regard, a study in *Bos taurus* found that in 76% of 33 waves the future dominant follicle was larger at deviation than the largest subordinate follicle, in 21% they had the same diameter, while in only 3% of the waves the future dominant follicle was smaller (Ginther *et al*., 1997a). Interestingly, despite diameter deviation occurring at similar times after wave emergence in *Bos taurus* and *Bos indicus*, the size of both the future dominant follicle and the largest subordinate are significantly smaller in *Bos indicus*. The precise mechanism that causes *Bos indicus* to have a smaller follicle size at the time of deviation has not been fully elucidated.

Activation of diameter deviation occurs rapidly (<8 h), as shown by the inhibition of follicle growth less than 8 h after FSH suppression and the growth cessation of the largest subordinate follicle within 8 h after the future dominant follicle reaches 8.5 mm in diameter (Ginther *et al*., 1999). As the follicular wave develops in response to the FSH surge, the developing follicles themselves secrete FSH suppressors, mainly inhibin, that causes the circulating FSH to decline as the wave progresses (Gibbons *et al*., 1999b). The role of FSH in the selection process can be summarized through what

has been termed the two-way functional coupling hypothesis between the follicles and FSH. This hypothesis states that during the common growth phase all follicles within the wave contribute (i.e. inhibin) to suppress FSH while depending on FSH for continued growth. Once diameter deviation occurs, the future dominant follicle assumes the coupling role by providing the final suppression of FSH, causing it to reach its nadir while acquiring the ability to survive in the face of basal FSH concentrations (Ginther *et al*., 1996, 1997a, 2000b; Ginther, 2000). Evidence for this hypothesis has been provided by the following results: 1) deviation occurs in association with FSH concentration reaching its nadir (Ginther *et al*., 1996, 1997a; Ginther, 2000); 2) initially all the follicles within the wave contribute to the suppression of FSH as seen by the increase in FSH when a portion or all the follicles are ablated (Gibbons *et al*., 1997); 3) depression of FSH concentrations during the common growth phase by treatment with estradiol (E2) inhibits the growth of all follicles in the wave (Ginther *et al*., 2000b); 4) administration of exogenous FSH allows for continuous growth of follicles and rescue of those destined to become subordinate allowing for multiple ovulations (Adams *et al*., 1993); 5) administration of an inhibin antiserum early in the follicular wave results in development of more than one dominant follicle (Kaneko *et al*., 1993; Takedomi *et al*., 1997); 6) removal of the dominant follicle after deviation is followed by an increase in FSH within 1 h and subsequent increase in the diameter of the largest subordinate follicle occurring 3 h after the increase in FSH (Ginther *et al*., 2016); 7) reduction of FSH concentrations at the time of deviation, by either treatment with a steroid-free fraction of follicular fluid or E2, led to a reduction in the growth of the dominant follicle but not the subordinate follicles (Bergfelt *et al*., 2000; Ginther *et al*., 2000b); and 8) treatment with E2 antiserum leads to an increase in circulating FSH and a delay in deviation (Beg *et al*., 2003).

Circulating luteinizing hormone (LH) has also been implicated in follicle selection. Perhaps the most compelling piece of evidence for the role of LH in follicle selection is the lack of continuous growth of the dominant follicle beyond deviation in the absence of LH pulses (Fike *et al*., 1997; Haughian *et al*., 2013). For example, treatment with acyline (GnRH antagonist) during the first follicular wave reduced circulating LH concentrations and prevented diameter deviation and growth of the largest follicle past 9 mm. Interestingly, the reduction of circulating LH did not affect follicle growth up to the onset of diameter deviation (Haughian *et al*., 2013).

Circulating E2 concentrations begin to increase at the time of deviation as a result of increased E2 production by GC of the future dominant follicle (Ginther *et al*., 1997b; Kulick *et al*., 1999). As previously discussed, the increase in E2 contributes to the final suppression of FSH, such that ablation of the dominant follicle at deviation (~8.5 mm) causes an increase in FSH and, if exogenous E2 is administered, the increase in FSH is delayed for a period of time associated with the increase in E2 (Ginther *et al*., 2000a).

Follicle selection and acquisition of dominance not only involves morphological and endocrine changes but also profound changes in follicular fluid, GC and theca cells (TC). Among the intra-follicular components, E2 concentration has been one of the best characterized changes associated with diameter deviation and acquisition of dominance. Intrafollicular E2 has been shown to increase in the future dominant follicle at or immediately after deviation, and this is associated with increased mRNA expression for CYP19A1 (aromatase) in GC (Beg *et al*., 2000; Luo *et al*., 2011).

Induction of LH receptors (LHCGR) in GC has been proposed as one of the initial signatures of the dominant follicle phenotype (Beg *et al*., 2001; Luo *et al*., 2011). The induction of LHCGR in GC appears to be stimulated, at least in part, by FSH and is mediated by increases in cAMP (Luo *et al*., 2003; Nogueira *et al*., 2007). First detection of a difference in LHCGR between the future dominant follicle and the largest subordinate was at 8 to 8.4 mm, immediately prior to diameter deviation at ~8.5 mm (Beg *et al*., 2001). More interestingly, it appears that LH pulses are required for induction of LHCGR in GC, as demonstrated by the lack of LHCGR after treatment with acyline 24 h prior to expected deviation (Luo *et al*., 2011). Follicles that have acquired dominance are the only follicles that ovulate after an LH surge, termed ovulatory capacity, and this capacity is preceded by the acquisition of LH receptors in GC (Sartori *et al*., 2001). Acquisition of ovulatory capacity occurs just after follicle selection in *Bos taurus* and *Bos indicus*, even though the size of the selected dominant follicle is quite different (Sartori *et al*., 2001; Gimenes *et al*., 2008; Simões *et al*., 2012). Two other factors that are involved in acquisition of follicle dominance are free IGF1, which decreases in the subordinate follicle but remains elevated in the dominant follicle (Beg *et al*., 2000, 2001) due to breakdown of IGF binding proteins (IGFBP) by the IGFBP protease, PAPPA (Rivera and Fortune, 2003) and FGF10 or FGF18 which increase in the subordinate follicles (Gasperin *et al*., 2012; Portela *et al*., 2015).

Multiple ovulation models in ruminants

Genetic models of multiple ovulation

The Booroola Merino ewe was the first high prolificacy line described. It originated from a flock of the Commonwealth Scientific and Industrial Research Organization (CSIRO) using triplet, quadruplet, and quintuplet born ewes and a quintuplet born ram obtained from a commercial operation, Booroola, owned by the Seears Brothers in Cooma, NSW, Australia in 1958 (Bindon, 1984). Since then, at least 19 different mutations affecting ovulation rate related to 6 different genes (Table 1) have been described in sheep and more recently a new mutation has been identified in cattle (Kirkpatrick and Morris, 2015).

Table 1. High fecundity genotypes in sheep showing affected gene, allele, line and breed, reported ovulation rate and percentage increase over wild type allele in heterozygous and homozygous carriers and proposed functional modifications in the proteins.

			Number of ovulations (% increase relative to controls)		
Gene	Mutated allele	Name/Breed	Heterozygous	Homozygous	Ref.
BMPR1B	$FecB^{B}$	Booroola	2.8 (+85%)	4.6-9.7 (+204% to 439%)	Davis *et al*., 1982; Mulsant *et al*., 2001; Wilson *et al*., 2001; McNatty *et al*., 2017
	-	Mehraban	1.3† (+25%)	NR	Abdoli *et al*., 2013
BMP15	$FecX^{I}$	Inverdale (Rommney)	2.5-3.2 (+35% to 64%)	POF (primary stage)	Braw-Tal *et al*., 1993; Shackell *et al*., 1993; Galloway *et al*., 2000; Davis *et al*., 2001a
	$FecX^{H}$	Hanna (Romney)	2.6-3.2 (+46% to 72%)	POF (primary stage)	Galloway *et al*., 2000; Davis *et al*., 2001a; Hanrahan *et al*., 2004
	$FecX^{B}$	Belclare (Belclare)	3.3 (+70%)	POF	Hanrahan *et al*., 2004
	$FecX^{G}$	Galway (Belclare, Cambridge)	2.7-3.1 (+37 to 42%)	POF	Hanrahan *et al*., 2004; McNatty *et al*., 2004
	$FecX^{L}$	Lac X-mutated (Lacaune)	3.3-7.2 (+69% to +269%)	POF (primary stage)	Bodin *et al*., 2007; Drouilhet *et al*., 2009
	$FecX^{R}$	Raza Aragonesa	2.0 (+46%)	POF	Martinez-Royo *et al*., 2008; Lahoz *et al*., 2011
	$FecX^{Gr}$	Grivette	2.9 (+16%)*	4.6 (+81%)	Demars *et al*., 2013
	$FecX^{O}$	Olkuska	2.0 (+32%)	3.3 (+142%)	Demars *et al*., 2013
	$FecX^{Bar}$	Tunisian Barbarine	1.8 (+64%)	POF	Lassoued *et al*., 2017
GDF9	$FecG^{H}$	High Fertility (Belclare, Cambridge)	4.3 (+88%)	POF	Hanrahan *et al*., 2004
	$FecG^{T}$	Thoka (Icelandic)	+32% (lambing rate)	POF (primary/secondary)	Nicol *et al*., 2009; Juengel *et al*., 2013
	$FecG^{E}$	Embrapa (Santa Ines)	1.3 (+10%)*	2.2 (+82%)	McNatty *et al*., 2004; Silva *et al*., 2011
	$FecG^{V}$	Vacaria (Ile de France)	2.4-2.5 (+94%)	POF (small antral follicles abnormal)	Souza *et al*., 2014
	$FecG^{F}$	Finnsheep	2.48 (+6%)	2.98 (+28%)	Vage *et al*., 2013; Mullen and Hanrahan, 2014
$B4GALNT2^{\dagger}$	$FecL^{L}$	Lacaune	3.1 (+114%)	4.6 (+214%)	Drouilhet *et al*., 2009
Unknown	$FecX^{2W}$	Woodlands (Coopworth)	2.7 (+25%)	NR - fertile	Davis *et al*., 2001b
Unknown	FecW	Wishart (Romney)	+0.8-1.0 ovulations	NR- fertile	Davis *et al*., 2006
Unknown	FecD	Davisdale (Border Leicester X Romney)	+0.4-0.8 ova	NR - fertile	Juengel *et al*., 2011

*Non-significant increases from wild-type controls. POF = Primary ovarian failure. NR = not reported.

Ovine models - Role of TGF-β family members

The evaluation of the aforementioned high ovulation rate phenotypes in sheep has led to the identification of specific genes and pathways with previously unknown effects on ovulation rate. Single gene mutations such as Booroola/FecB, Inverdale/FecX^{I}, Hannah/FecX^{H} and FecG^{H} led to the discovery of the role of TGF-β signaling in folliculogenesis and ovulation rate. The lines listed above resulted in the identification of two oocyte-secreted factors (OSF), namely bone morphogenetic protein 15 (BMP15), and growth and differentiation factor-9 (GDF9), as key regulators of GC function (Juengel *et al*., 2013). The original Booroola mutation had a non-conservative substitution in the intracellular kinase domain of the BMPR1B receptor (Mulsant *et al*., 2001; Souza *et al*., 2001; Wilson *et al*., 2001), affecting subsequent signaling pathways. Other mutations identified in sheep result in absent, non-functional, or modified forms of BMP15 or GDF9 (Galloway *et al*., 2000; Hanrahan *et al*., 2004).

BMP15 and GDF9 are primarily OSF (Juengel *et al*., 2002), translated as larger precursor proteins, with a pro-region required for proper folding. Upon cleavage by furin-like proteases the mature dimeric form of the protein is produced (McNatty *et al*., 2004; Weiss and Attisano, 2013). Interestingly, GDF9 and BMP15 have been shown *in vitro* to form homodimers and heterodimers, however, unlike most TGF-β members these are not covalently linked by disulfide bonds (Liao *et al*., 2003). As TGF-β members, both BMP15 and GDF9 signal through an assembly of type-I and type-II receptors into heterotetrameric complex receptors (Fig. 1). The type II receptor BMPRII is common to both (Moore *et al*., 2003), however, GDF9 and BMP15 differ in the type I receptor utilized, with GDF9 signaling through ALK5/TGFβRI or ACVR1B/ALK4 (Li *et al*., 2011; Peng *et al*., 2013) and BMP15 signaling through ALK6/BMPRIB (Moore *et al*., 2003). Upon BMP15 binding to its receptor, phosphorylation of the type-I receptor (ALK6/BMPR1B) occurs by means of the kinase domain of the type II receptor, which is constitutively active, ultimately leading to phosphorylation of receptor-regulated SMAD1/5/8 (R-SMAD) and formation of heteromeric complexes of activated R-SMADs with SMAD4 which accumulate in the nucleus and affect gene expression in a cell-type dependent manner (ten Dijke and Hill, 2004; Weiss and Attisano, 2013). Conversely, GDF9 receptor type I (TGFβRI/ALK5) is phosphorylated upon ligand binding and results in the activation of a different set of R-SMADs (SMAD2/3) with subsequent formation of heteromeric complexes with SMAD4 (Gilchrist *et al*., 2006). Two inhibitory SMAD proteins have been described, SMAD6 and SMAD7, which act as negative modulators of BMP15 and GDF9 signalling (Li, 2015). Knockdown of SMAD7 significantly enhanced expression of GDF9-stimulated genes indicating that SMAD7 appears to preferentially inhibit the SMAD2/3 pathway. Conversely, SMAD6 preferentially inhibits BMP signaling by several proposed mechanisms: 1) acting as a SMAD4 decoy thus reducing the formation of SMAD1-SMAD4 heteromers (Hata *et al*., 1998); 2) interaction with type 1 receptors (e.g BMPR1B) preventing phosphorylation of SMAD1/5/8 (Imamura *et al*., 1997; Goto *et al*., 2007); 3) acting as an adaptor protein for Smad ubiquitin regulatory factor 1 (Smurf1) which leads to ubiquitination and degradation of type 1 receptors and R-SMADs (Murakami *et al*., 2003). The high fecundity alleles that act through the GDF9/BMP15 pathways are shown in Fig. 1 (n = 16).

Recently, GDF9 and BMP15 have been shown to act synergistically to regulate GC function, through activation of their receptor complexes as homodimers or heterodimers (Liao *et al*., 2003; McNatty *et al*., 2004). A series of studies have shown that addition of both, recombinant BMP15 (murine, human or ovine) and GDF9 (murine, human or ovine) to murine or rat GC produced a greater effect in GC proliferation than either one alone (Reader *et al*., 2011, 2016; Mottershead *et al*., 2012). Moreover, this effect appears to be mediated by SMAD2/3 activation rather than SAMD1/5/8. Another study also evaluated the effect of recombinant non-purified murine and ovine GDF9 and BMP15 on rat GC (McNatty *et al*., 2005a). Thymidine incorporation indicated that murine GDF9 was the only factor that stimulated proliferation on its own, while ovine GDF9 or BMP15 alone had no effect. However, the combination of murine GDF9 with ovine BMP15 or ovine BMP15 with ovine GDF9 had a dose-dependent synergistic effect on thymidine incorporation, much greater than murine GDF9 alone. In addition, these authors demonstrated that either factor alone had no effect on progesterone (P4) production, but mGDF9+oBMP15 or oGDF9+oBMP15 significantly reduced P4 production in a dose-dependent manner.

The individual and cooperative role of BMP15 and GDF9 has also been investigated in ruminant GC (McNatty *et al*., 2005b). Granulosa cell proliferation was stimulated by oBMP15 and oBMP15+oGDF9 to similar levels in bovine GC, while oGDF9 had no effect and intriguingly mGDF9 inhibited bovine GC proliferation. In ovine GC, addition of the aforementioned factors yielded similar results as those observed in bovine GC; however, the addition of both GDF9 and BMP15 regardless of species of origin resulted in a significantly greater effect on proliferation than with either factor alone. Progesterone production by bovine GC was inhibited by oBMP15 and oGDF9 alone at the highest dose, while combination of both greatly inhibited P4 secretion. In sheep GC, oBMP15 was without effect on P4 secretion, however oGDF9 inhibited while mGDF9 stimulated secretion, and addition of oBMP15 to GDF9 of either species did not modify the effects observed with GDF9 alone (McNatty *et al*., 2005b).

The results previously described emphasize that: 1) both GDF9 and BMP15 regulate GC proliferation and differentiation measured through P4 production *in vitro*; 2) the effect of each factor depends on the species of origin for BMP15 and GDF9, as well as the species of origin of the GC; and 3) a cooperative effect between BMP15 and GDF9 has been observed in

all species evaluated, albeit differences appear to exist among species regarding which particular GC function is affected by the cooperation. In addition, recent results indicate that BMP15 and GDF9 proteins can occur as dimers, monomers, and multimers and that components of the immature protein such as the pro-region may have important roles in the biological effects of these OSF (Reader *et al*., 2011; Heath *et al*., 2017). Further, it has been suggested that the dimerization of BMP15 and GDF9 may be occurring at the time of receptor binding with minimal dimerization prior to secretion from GC or within the follicular fluid (Heath *et al*., 2017). It seems clear that further research is needed in this important area in order to establish the precise roles of BMP15 and GDF9 and their dimers in regulation of GC function, particularly with respect to species differences and the possibility that these differences may underlie differences in the number of follicles selected.

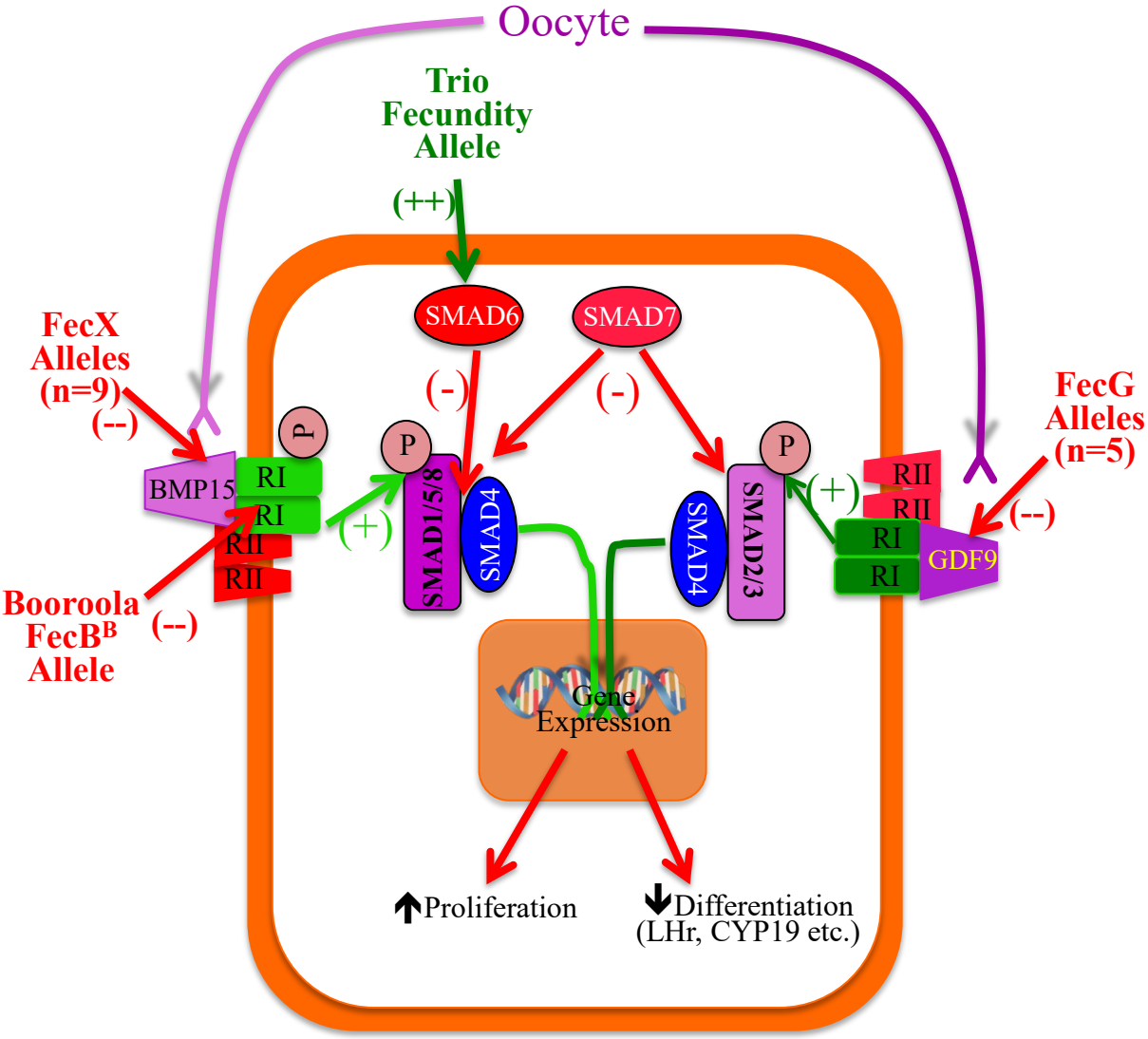

Figure 1. Our current working model of mechanisms in granulosa cells (GC) that lead to reduced follicle growth rate and earlier follicular dominance in carriers of some (n = 16) high fecundity alleles that have been identified in ruminants. The ovine FecX alleles (n = 9) alter the activity or knockout the BMP15 protein; whereas, ovine FecG alleles (n = 5) alter the activity or knockout the GDF9 protein. Both of these proteins are members of the TGF-ß family that are secreted by the oocyte as homodimers or heterodimers and regulate independently or, more likely, cooperatively the proliferation and function of the GC. The ovine Booroola FecB allele causes an alteration in the kinase region of the BMPR1B receptor and thus reduces the activity of the BMP15 pathway. Carriers of the novel bovine Trio allele have a mutation in a regulatory region for SMAD6 causing a dramatic increase in expression of SMAD6 in GC with subsequent inhibition of BMP-15 action by inhibiting Smad-1/5/8 and thus reduces proliferation and allows differentiation.

Bovine models

Multiple ovulation models in cattle can be divided into three main types, namely: the USDA-MARC twinner population (Echternkamp *et al*., 2004), the high producing lactating dairy cow (Lopez *et al*., 2005), and the recently identified Trio high fecundity allele (Kirkpatrick and Morris, 2015). Genomic contributions to twinning rate in cattle have been studied by QTL mapping within paternal half-sib

families and genome-wide association analyses (GWAS). QTL mapping analyses typically allowed identification of genomic regions potentially associated with twinning rate with broad confidence intervals, making identification of causative genes and polymorphisms extremely challenging. GWAS, by exploiting association across a population rather than within family, potentially narrows localization of underlying genes, though validation is critical. Multiple positional candidate gene regions have been identified by QTL and GWAS analyses (Table 2), though only a few have been replicated.

One of the most replicated regions is the bovine chromosome 5 segment containing IGF1. QTL mapping work in both the Norwegian dairy cattle population and the US Holstein population has provided strong support for a contribution of this genomic region to variation in twinning rate. In a follow-up study, polymorphisms in the IGF1 gene were identified and tested for association in two samples of US Holstein sires representing different timeframes. IGF1 polymorphisms were identified with repeatable associations across these two data sets, one of which was subsequently further validated in the MARC twinner population (Kirkpatrick, 2018: University of Wisconsin-Madison, Madison, WI, USA; unpublished). Two other regions with some degree of replication across studies are the region of bovine chromosome 23 containing the steroid 21-hydroxylase (CYP21) gene and chromosome 7 near the anti-Müllerian hormone (AMH) gene. In all three cases, contribution of these positional candidate genes to genetic variation is uncertain, and further work is needed to establish any causal relationship between these and variation in ovulation or twinning rate.

The search for major genes affecting ovulation in cattle led to the identification of the cow Treble with an exceptional record of prolificacy (Morris *et al*., 2010). This cow produced three sets of triplets in her lifetime among which was a son (Trio) who sired several daughters that had either twin or triplet births, suggesting transmission of a genetic factor across generations. Semen from Trio was used in artificial insemination at the University of Wisconsin-Madison and daughters born to these matings were evaluated for ovulation rate and genotypes for an initial within-family linkage analysis. The linkage analysis provided strong evidence ($P < 1x10^{-28}$) of segregation of a single gene, located on chromosome 10, with a large effect on ovulation rate (1.02 ± 0.08 additional CL per cycle) (Kirkpatrick and Morris, 2015). Examination of this narrowed bovine genomic region suggested multiple candidate genes for the high ovulation rate phenotype. SMAD3 and SMAD6 reside in this region and are part of the TGF-ß signaling system (ten Dijke and Hill, 2004). Considering the mediating roles of SMAD3 and SMAD6 in GDF9 and BMP15 signaling, respectively, and the high fecundity genotypes in sheep associated with GDF9 and BMP15, these two Smads were considered strong candidates for the causative mutation. However, screening for polymorphisms within the coding and flanking regions failed to identify the likely causative polymorphism (Kirkpatrick and Morris, 2015), suggesting that the functional polymorphism was either in a regulatory element located at a greater distance from the gene or is in a different gene in the region. Subsequent gene expression analyses in GC identified and confirmed significant overexpression of SMAD6 in Trio carrier cattle vs non-carrier, strongly implicating SMAD6 as the gene responsible for the high ovulation rate phenotype, though the actual mutation remains unknown (García-Guerra *et al*., 2018a; Kamalludin *et al*., 2018).

Table 2. Chromosomal locations of quantitative trait loci and single nucleotide polymorphisms associated with twinning rate and ovulation rate in cattle.

Trait	Chromosome and approximate location within chromosome (Mb)	Population	Positional candidate genes (chromosome)	Ref.
Ovulation rate	7 (40) and 23 (27)	MARC twinner	CYP21 (23)	Blattman *et al*., 1996
Twinning rate	5 (64), 7 (108), 12 (10) and 23 (26)	Norwegian cattle	IGF1 (5), CYP21 (23)	Lien *et al*., 2000; Meuwissen *et al*., 2002
Ovulation rate	5 (46)	MARC twinner		Kappes *et al*., 2000; Allan *et al*., 2009
Twinning rate	5 (68)	US Holstein	IGF1 (5)	Cruickshank *et al*., 2004; Kim *et al*., 2009b
Twinning rate	8 (108), 10 (26) and 14 (51)	US Holstein		Cobanoglu *et al*., 2005
Ovulation rate	14 (61)	MARC twinner		Gonda *et al*., 2004
Ovulation rate	7 (22), 10 (75) and 19 (42)	MARC twinner	AMH (7), ESR2 (10), IGFBP4(19)	Arias and Kirkpatrick, 2004
Twinning rate	4 (44), 5 (67), 6 (8, 44), 7 (68, 76), 8 (58), 9 (34), 11 (47), 14 (21, 38), 15 (23), 23 (51), and 28 (9)	US Holstein	IGF1 (5)	Kim *et al*., 2009a; Bierman *et al*., 2010
Twinning rate	6 (51), 7 (19), 23 (27)		AMH (7), CYP21 (23)	Weller *et al*., 2008
Twinning rate	24 (40)	Italian Maremmana		Moioli *et al*., 2017

Physiology underlying multiple ovulation models

Ovine high fecundity

Mutations in high fecundity ovine genotypes, in general, result in reduced signaling of the GDF9 and/or BMP15 pathways leading to ovulation of multiple smaller follicles as compared to wild type ewes (McNatty *et al*., 1986b). However, the overall ovarian phenotype depends on the precise mutation and the carrier status of the animal (heterozygous or homozygous). Table 1 shows a comprehensive list of the mutations identified in sheep and the associated ovarian phenotype (ovulation rate, percent increase, etc.) according to their carrier status. Homozygous carriers of high fecundity alleles show two distinct ovarian phenotypes. Approximately 60% of the mutations result in primary ovarian failure (POF) and an infertile phenotype in the homozygous state, due to block of follicle development at the primary or secondary stage. Similarly, immunization of ewes against GDF9 and BMP15 results, in most cases, in an anovulatory state and the presence of normal follicles up to the primary stage but only a few abnormal follicles developing past the primary stage (Juengel *et al*., 2002; McNatty *et al*., 2007). Conversely, immunization with some antigens, resulted initially in increased ovulation rate in some ewes. Taken together, these results indicate that a partial decrease in the availability of BMP15 and GDF9 underlies the occurrence of multiple ovulations.

On the other hand, some of the high fecundity ovine genotypes are fertile when present in the homozygous state. The Booroola/FecB results in a further increase in ovulation rate when present in the homozygous state, with ovulation rates that can reach up to 14, with homozygous ewes being fully fertile (Bindon and Piper, 1986; McNatty *et al*., 2017). This mutation does not result in reduction in BMPR1B mRNA or protein concentrations, but there is diminished receptor activity (Fabre *et al*., 2006). More recently, mutations in BMP15 and GDF9 that do not result in POF and sterility in the homozygous state have been described (Table 1). These mutations have a further increase in ovulation rate when present in the homozygous state, although the increase is not as substantial as the one observed in Booroola (Silva *et al*., 2011; Demars *et al*., 2013). Furthermore, ewes with multiple fecundity mutations have substantial increases in ovulation rate indicating that they are independent and do not cancel each other out or lead to anovulatory phenotypes (Hanrahan *et al*., 2004; Drouilhet *et al*., 2009; McNatty *et al*., 2017).

Among the models proposed to explain the occurrence of multiple ovulations in high fecundity ovine genotypes, one states that multiple ovulations could be the result of increasing the number of follicles available for selection (Baird and Campbell, 1998; Scaramuzzi *et al*., 2011; Monniaux, 2016). Data on antral follicle counts (AFC) from ewes with or without high fecundity genes show considerable variation, with some studies indicating no differences in AFC while others indicate greater AFC in ewes carrying a high fecundity allele. Booroola carrier ewes exhibit similar AFC (≥1 mm) as non-carriers as shown by multiple studies (McNatty *et al*., 1985, 1986b; Henderson *et al*., 1987; Gibbons *et al*., 1999a). In agreement, no correlation between AFC and ovulation rate in the previous cycle was found in Booroola ewes (Driancourt *et al*., 1985). However, a recent study showed lower circulating and intrafollicular AMH levels in Booroola/FecB homozygous ewes than wild type ewes but greater number of follicles≥ 1 mm (Estienne *et al*., 2015). In addition, the authors showed that BMP4-induced AMH production by GC was impaired in Booroola/FecB carriers. The authors postulated a model in which greater number of follicles coupled with reduced AMH production, could increase FSH and LH sensitivity and this would allow follicles to mature at a smaller size and in greater number (Estienne *et al*., 2015).

Follicle populations in BMP15/FecX mutations have been the subject of fewer studies and there appears to be differences between specific lines. Heterozygous ewes for the Inverdale/FecX^{I} mutations, were found to have greater AFC (≥1 and ≥2.5 mm) compared to wild types ewes (Shackell *et al*., 1993; McNatty *et al*., 2009). Conversely, in heterozygous Raza Aragonesa/FecX^{R}, another BMP15 mutation, no differences were found in AFC (≥3 mm) or circulating AMH when compared to controls (Lahoz *et al*., 2013, 2014). Reports for other mutations are scarce in the literature, however, reports available on Ile de france/FecG^{V} and Lacaune/FecL^{L} ewes showed no differences in AFC between carrier and wild type ewes (Drouilhet *et al*., 2010; Souza *et al*., 2014). The role of antral follicle numbers on selection of multiple follicles has yet to be elucidated and further research is needed.

Several studies have conclusively demonstrated that ewes carrying high fecundity alleles such as Booroola/Fec^{B} (McNatty *et al*., 1985, 1986b; Souza *et al*., 1997), Inverdale/FecX^{I} (Shackell *et al*., 1993), Ile de france/FecG^{V} (Souza *et al*., 2014), and Lacaune/FecL^{L} (Drouilhet *et al*., 2010) have preovulatory follicles that are significantly smaller than those observed in non-carrier controls. Ewes carrying high fecundity alleles have fewer GC per follicle, compared to wild type control ewes even when comparing similar-sized follicles (McNatty *et al*., 1985, 1986b, 2017). However, when number of GC in all presumptive preovulatory follicles were taken together, there were no differences in total GC number between wild types, Inverdale/FecX^{I}, homozygous or heterozygous Booroola/FecB, or even ewes carrying combinations of these mutations and Woodlands/FecW2^{W} (McNatty *et al*., 1979, 1985, 1986b, 2017). Follicle dynamics have not been evaluated to a great extent between ewes carrying high fecundity alleles and wild type controls with the exception of two studies (Souza *et al*., 1997; Gibbons *et al*., 1999a). Both of these studies found smaller follicle sizes in high fecundity ewes starting 48 h after wave emergence.

In agreement with the smaller preovulatory follicle size, the resulting corpora lutea (CL) of ewes carrying high fecundity alleles (i.e Booroola/FecB or

Inverdale FecX^{I}) are smaller on an individual basis and have fewer cells than those observed in non-carrier control ewes (McNatty *et al*., 1985, 1986b, 2017; Niswender *et al*., 1990; Shackell *et al*., 1993). However, circulating P4, total luteal weight, luteal cell volume, number of cells/gr of tissue, and average cell dimensions were similar between genotypes (Niswender *et al*., 1990; Souza *et al*., 1997). Overall, high fecundity genotypes ovulate smaller follicles, leading to smaller individual CL, however due to the higher ovulation rate total luteal tissue and circulating P4 is similar.

Two independent studies showed that GC from Booroola/FecB carrier ewes have reduced proliferation compared to wild type ewes (Monniaux *et al*., 2000; Mulsant *et al*., 2001). Furthermore, treatment of GC with BMP4 increased thymidine uptake in wild-types while the same treatment applied to homozygous Booroola/FecB carriers was without effect (Mulsant *et al*., 2001; Fabre *et al*., 2003). Interestingly, the sera of ewes immunized against BMP15 or GDF9 reduced GC proliferation by 70% when sera originated from ewes that were anovular after immunization against BMP15 or GDF9. On the other hand, sera from animals with more than 3 CL inhibited thymidine incorporation by 24-27% (McNatty *et al*., 2007). These results, taken together, provide some evidence that there is impaired signaling of the mutated BMPR1B receptor in Booroola/FecB GC and that in order for multiple ovulations to occur GC proliferation needs to be only partially reduced.

Follicles of ewes carrying the Booroola mutation reached peak aromatase activity and follicular fluid E2 concentrations at progressively smaller diameters related to whether they were heterozygous or homozygous for the mutated allele (McNatty *et al*., 1985, 1986b). Similar to E2, follicles from ewes carrying high fecundity alleles have GC that are responsive to LH stimulation at a smaller follicle size (McNatty *et al*., 1986a, 2009, 2017), are capable of producing levels of cAMP, in response to LH, at smaller sizes (Henderson *et al*., 1987), and have greater mRNA for LHCGR in GC of small (1-3 mm) and medium (3-4.5 mm) follicles than similar follicles from wild type controls (Juengel *et al*., 2017). Thus, acquisition of LHCGR in GC, increased E2 production, and decreased IGFBP (Monniaux *et al*., 2000) at smaller follicle sizes indicate that acquisition of dominance at a smaller follicle size is a hallmark of the physiology of ewes carrying high fecundity alleles.

It is well-known that exogenous FSH can override the selection mechanisms allowing for codominance and multiple ovulations in both sheep and cattle. Initial studies on circulating FSH in Booroola/FecB ewes found elevated mean concentrations in homozygous carriers, while heterozygous carriers were intermediate but not different from controls (McNatty *et al*., 1987; Boulton *et al*., 1995). Similarly, a recent study in ewes containing both the Booroola/FecB and Inverdale FecX^{I}, had greater circulating FSH on day 5-6 of the estrous cycle compared to controls (Juengel *et al*., 2017). The differences found in circulating FSH however do not appear to be associated with differences in circulating inhibin (McNatty *et al*., 1992; Shackell *et al*., 1993; Souza *et al*., 1997). Conversely, other studies found no differences in circulating FSH between Booroola ewes (homozygous and heterozygous combined) and wild type controls (Souza *et al*., 1997; Gibbons *et al*., 1999a). The conflicting results in circulating FSH concentrations between carriers of the Booroola mutation and wild type ewes could potentially result from the use of different breed backgrounds, differences in assays, and finally different strategies to normalize FSH patterns between ewes. In this regard, only two studies evaluated circulating FSH normalized to emergence of the follicular wave and both failed to identify differences in FSH (Souza *et al*., 1997; Gibbons *et al*., 1999a). However, small follicle size and less pronounced diameter deviation in ewes make it reasonable to expect that subtle differences in FSH near follicle selection would go unnoticed.

One study that provided evidence against the role of changes in circulating FSH driving the occurrence of multiple ovulations in ovine, high-fecundity genotypes was done in carriers and non-carriers of Booroola FecB ewes using autotransplanted ovaries, a GnRH antagonist and controlled patterns of FSH and LH in both genotypes (Campbell *et al*., 2003). Interestingly, the differences in ovulation rate between carriers and wild types were maintained despite similar FSH/LH patterns being delivered. The authors stated that these results did not support the idea that the FecB gene acts simply through increasing gonadotropin stimulation but that sensitivity of follicular cells to gonadotropins must also be critical for increased ovulation rate produced by the FecB allele. Thus, descriptive studies on FSH patterns support increases/alterations in circulating FSH in carriers of fecundity genotypes but definitive evidence has not yet been provided, whereas this manipulative study supports the idea that differences in circulating FSH may be secondary to ovarian gonadotropin sensitivity in determining the ovulation rate in carriers and non-carriers of fecundity genotypes.

USDA MARC twinner cattle

The twinner cattle population at USDA-MARC has been the result of selection over multiple generations for twinning and ovulation rate (Echternkamp *et al*., 1990a; Gregory *et al*., 1990). As a result, twinner cattle have on average 2.1 ovulations per cycle, with ~60% of the cycles having 2 ovulations and rarely exceeding 4 ovulations (Echternkamp *et al*., 2009).

Follicle numbers have been investigated in MARC twinner cattle as a potential component of the mechanism underlying multiple ovulations, as previously described for high fecundity alleles in sheep. Total antral surface follicles are greater in twinner cows than in control cows from an unselected population (Echternkamp *et al*., 1990b, 2004). Furthermore, differences have been found in the preantral follicle population, with twinner cows having significantly

more (~2-fold) secondary follicles than controls, while no differences were found in primordial, primary, or tertiary follicles (Cushman *et al*., 2000). However, the control population used for comparison is of paramount importance as follicle numbers have been shown to be highly variable among individuals. The control population utilized in MARC twinner studies may have a different genetic makeup and this could potentially confound reported associations. Twinner cattle preovulatory follicle size (12 h after estrus) was also evaluated in relation to ovulation rate, indicating that individual follicle diameter decreased as ovulation rate increased from 1 to 3, but no further decrease was seen thereafter although sample size of cows with ovulation rates greater than 3 was low (Echternkamp *et al*., 2009). Likewise, individual CL volume decreased with increased ovulation rate, however total CL volume, weight and circulating P4 increased with increasing ovulation rate (Echternkamp *et al*., 2004, 2009). Circulating FSH in the USDA-MARC twinner population did not indicate differences when compared with unselected controls, although interpretation of the results is hampered by lack of normalization to wave emergence or deviation (Echternkamp, 2000; Echternkamp *et al*., 2004). Thus, precise follicle dynamics and associated hormonal patterns of MARC twinner cattle, in relation to diameter deviation, have not yet been conclusively investigated.

Several aspects of the follicular microenvironment have been evaluated in relation to selection of multiple dominant follicles in the MARC twinner cattle population. The IGF1 system appears to be a key component of the altered selection mechanism in MARC twinner cows as evidenced by: 1) greater plasma and intrafollicular IGF1 (Echternkamp *et al*., 1990b, 2004); 2) greater binding activity of IGFBP-3 and one form of IGFBP-5, but lower binding activity of one form of IGBP-4 (Echternkamp *et al*., 2004); and 3) decreased IGF2R mRNA in GC, an IGF2 receptor lacking kinase activity (Echternkamp *et al*., 2012; Aad *et al*., 2013). Thus, lower IGF2R and IGFBP-4 and greater IGF1 have been implicated in selection of additional follicles, although the precise sequence of events has not been resolved.

Trio, a novel high fecundity allele

The recent discovery of Trio provides an outstanding opportunity to investigate the physiologic mechanisms associated with selection of a single follicle (non-carriers) or multiple follicles (Trio carriers) during different experimental conditions (Kirkpatrick and Morris, 2015). The cow is a particularly useful research model for studies of follicular selection due to ease and accuracy of ultrasound evaluation of bovine follicular growth, ability to evaluate concurrent endocrine profiles, and methods to manipulate or evaluate follicular fluid (Ginther *et al*., 1997b, 2004; Beg *et al*., 2002; Beg and Ginther, 2006).

Evaluation of more than 243 estrous cycles in Trio carrier cattle (heterozygote) and half-sib non-carriers, revealed that Trio carriers had a consistent increase in ovulation rate with a mean ovulation rate of 3.5 ± 0.2 while non-carriers had a mean ovulation rate of 1.1 ± 0.1 (García-Guerra *et al*., 2017b). In Trio carriers, most (70.4%; 95/134) cycles had 3 or 4 ovulations, and few cycles had >5 ovulations (4.4%) or only a single ovulation (5.2%), while ~89% of the cycles of non-carrier controls had single ovulations, and no cycles were observed having 3 ovulations or more. Thus, heterozygous carriers of Trio have a dramatic and consistent increase in ovulation rate. More recently, ovulation rate was obtained from 3 homozygous Trio allele carriers during a minimum of 4 estrous cycles (Garcia-Guerra *et al*., 2018; University of Wisconsin-Madison, Madison, WI, USA; unpublished). Mean ovulation rate per cycle was 4.3 ± 0.5 for homozygous Trio allele carrier heifers, indicating that the Trio allele does not result in primary ovarian failure in the homozygous state, unlike many of the high fecundity genotypes found in sheep. However, due to low number of homozygote animals, it is currently unclear if homozygous Trio carriers have similar or increased ovulations compared to heterozygotes.

We recently investigated differences in antral follicle populations in carriers *vs*. non-carriers of Trio (García-Guerra *et al*., 2017b). Trio carriers had AFC (≥2 mm) at the time of wave emergence that were similar to non-carriers. Furthermore, no association was observed between AFC and mean ovulation rate for the preceding four estrous cycles in Trio carriers or non-carriers despite large variation in AFC. Production of AMH in females is from GC of growing preantral and small antral follicles, thus providing a good representation of the dynamic follicle reserve (Rico *et al*., 2011). In cattle, AMH and AFC have been found to have relatively high correlations ranging from 0.59 to 0.88 (Ireland *et al*., 2008). As expected, our results indicate that circulating AMH concentrations were positively associated with AFC in both Trio carrier and non-carrier cattle (García-Guerra *et al*., 2017b). However, there was no difference between genotypes in circulating AMH. Thus, differences in size of the antral follicle population is not causing selection of multiple follicles in cattle carrying the high fecundity allele, Trio.

Trio carriers exhibit predominantly (>70%) 3 follicular waves during the estrous cycle, similar to non-carriers (García-Guerra *et al*., 2017a). In agreement with increased ovulation rate, each follicular wave contained greater number of dominant follicles in Trio carriers (3 to 4 per wave) compared to non-carriers (1 per wave). In addition, Trio carriers ovulate multiple smaller-sized follicles, however, the study of follicle dynamics in Trio carriers also indicates that follicles are smaller during the entire follicular wave (García-Guerra *et al*., 2017a, 2018b). Evidence for smaller follicle size in Trio carriers arises from the following observations: 1) largest, smallest and mean preovulatory follicle diameter is greatly reduced; 2) future dominant follicles are smaller at wave emergence, at deviation, and at time of maximum diameter; 3) future first subordinate follicle is smaller at wave emergence and deviation; 4) reduced follicle growth rate; and 5) resulting CL are significantly smaller on an individual basis.

Nevertheless, there is clear evidence of subordinate follicles in Trio carriers, supporting the idea that selection still occurs but at a smaller size and with a greater number of selected follicles in Trio carriers. Thus, diameter deviation between future dominant follicles and subordinate follicles occurred at similar times (~3 days) in Trio carriers and non-carriers albeit at significantly smaller follicle sizes (6 mm *vs*. 8.6 mm, respectively).

Evaluation of CL volume and circulating P4 indicates that despite individual CL being smaller in Trio carriers, total luteal volume and resulting P4 concentrations are not different than those observed in non-carriers (García-Guerra *et al*., 2017b, 2018b). As a result, it is reasonable to evaluate follicle size and growth patterns on a volume basis rather than diameter. As shown in Fig. 2, the diameter and volume of the largest or mean dominant follicle (average of all dominant follicles) normalized to onset of diameter deviation is significantly smaller in Trio carriers compared to non-carrier controls (García-Guerra *et al*., 2018b). However, total dominant follicle volume, calculated as the sum of each individual dominant follicle, is similar between genotypes. Interestingly, there is a close association between ovulation rate and individual follicle size such that Trio carrier cattle have ~4-fold greater number of ovulations than single-ovulating controls but their preovulatory follicles are individually ~4-fold smaller on a volume basis than in controls, thus the total preovulatory follicle volume is similar. In agreement, circulating E2, normalized to day of ovulation, is similar between genotypes (García-Guerra *et al*., 2017a), indicating that combined hormonal output is similar regardless of whether it originates from one large follicle or four smaller follicles.

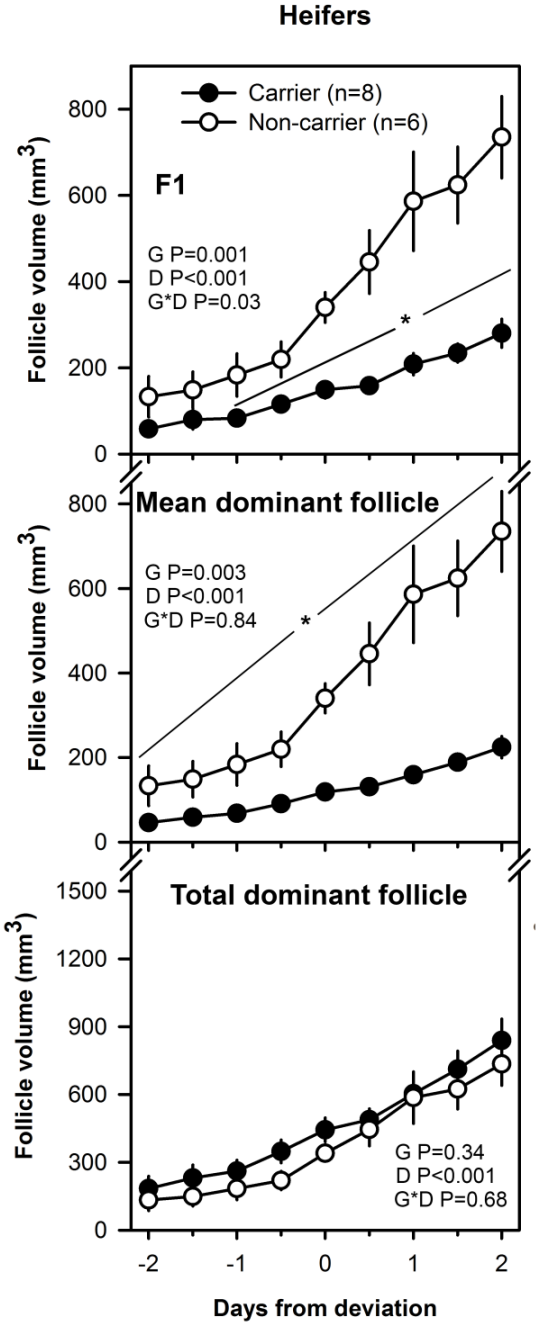

Figure 2. Growth profile of largest follicle volume (F1), mean dominant follicle volume, and total dominant follicle volume in Trio carrier and age-matched, half-sib non-carrier control heifers (adapted from García-Guerra *et al*., 2017b). Data were normalized to deviation and each point represents mean (± SEM). *Indicates significant differences between genotype for a given time point ($P < 0.05$). G, genotype; D, day; G*D, genotype by day interaction.

The known role of FSH in stimulating follicle growth and the ability to induce multiple ovulations by administration of exogenous FSH (Adams *et al*., 1993), leads naturally to the evaluation of FSH levels in selection of multiple dominant follicles in high fecundity genotypes. In this regard, FSH surges of similar magnitude were found to precede emergence of each follicular wave during the estrous cycle of Trio carriers and non-carriers; although circulating FSH was greater near follicular deviation (García-Guerra *et al*., 2017a). In a second experiment, we synchronized the emergence of the follicular wave and evaluated circulating FSH at frequent intervals (García-Guerra *et al*., 2018b). Overall, average circulating FSH was greater in Trio carriers than non-carriers. However, precise analysis focusing on the time encompassing observed deviation found that FSH was greater only in a narrow window encompassing deviation (Fig. 3). This elevation in circulating FSH concentrations near the time of follicular deviation is consistent with FSH being a key component of the process that allows selection of multiple dominant follicles in this high fecundity genotype. Nevertheless, provision of large doses of FSH produced a similar superovulatory response in either carriers or non-carriers of the Trio allele (Garcia-Guerra *et al*., 2018; University of Wisconsin-Madison, Madison, WI, USA; unpublished).

Based on the observation that diameter deviation occurs at a similar time but smaller follicle size in Trio carriers, we hypothesized that the dominant phenotype and ovulatory capacity also occurred at a smaller follicle size. We found that follicles of Trio carriers acquired a dominant phenotype, as determined by intrafollicular E2 concentrations, CYP19A1, LHCGR, and PAPPA mRNA abundance in GC, at a significantly smaller size than in non-carrier controls (García-Guerra *et al*., 2018a). As expected, non-carrier single-ovulating cattle acquired a dominant phenotype when follicles were ~8.5 mm. Conversely, Trio carriers acquired a similar phenotype when follicles were ~6 mm, thus supporting the idea that dominance is acquired at a smaller size and in agreement with observed diameter deviation. Moreover, in another experiment, challenge with an exogenous ovulatory stimulus revealed that ovulatory capacity (50% probability of ovulation) was achieved at a reduced follicle size in Trio carriers (5.5 mm) than non-carriers (8.3 mm). Moreover, when each factor pertaining to the dominant phenotype (i.e. E2, LHCGR) were analyzed individually, as a function of follicle size, the 50% probability of dominance was acquired at 5-5.5 mm and 8-8.5 mm for Trio carriers and non-carriers, respectively (García-Guerra *et al*., 2018a). Consideration of follicle size on a volume basis further confirmed our previous findings indicating that acquisition of dominance occurred at a similar time but follicles of Trio carriers were approximately 1/3 to 1/4 the size, on a volume basis, compared to non-carriers.

Finally, based on timing of acquisition of the dominant phenotype, it appears follicles in Trio carriers acquire dominance in a hierarchal manner, as indicated by the increasing number of dominant follicles, based on intrafollicular E2, between days 2 and 4 after wave emergence (García-Guerra *et al*., 2018a). Thus in Trio carriers, the first follicle to acquire dominance would be unable to provide sufficient FSH inhibitor (E2 and/or inhibin), due to its smaller size, to inhibit FSH thus the window for acquiring dominance remains open. As a result, the next follicle(s) in the hierarchy is (are) able to acquire a dominant phenotype and contribute to increasing circulating E2. The metaphorical gate of selection then, would remain open, until a sufficient number of dominant follicles (3 to 4) are present that equate to the same total follicle volume as that observed in a single dominant follicle in non-carrier cattle. This will produce sufficient FSH inhibitor (circulating E2) necessary to provide the final suppression of FSH and thus prevent selection of additional follicles.

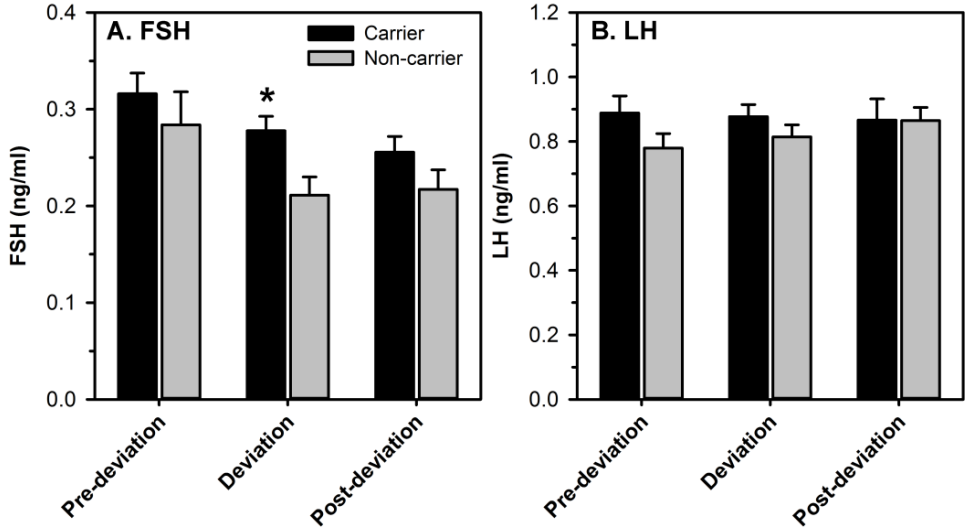

Figure 3. Mean FSH (A) and LH (B) concentrations at 3 distinct time points: pre-deviation (36 ± 12 h before the onset of deviation), at deviation (24 h encompassing the onset of deviation), and post-deviation (36 ± 12 h after the onset of deviation) in cattle with (Trio; n = 13) or without (Non-carriers; n = 9) the high fecundity allele (adapted from García-Guerra *et al*., 2017b). Data represent mean (± SEM). *Indicates significant differences between genotypes ($P < 0.05$).

Conclusions - A model for selection of multiple follicles

Several models have been proposed to explain the occurrence of multiple ovulations in high fecundity genotypes in sheep, primarily based on studies of Booroola/FecB ewes (Baird 1987; Scaramuzzi *et al*., 2011; Juengel *et al*., 2013; Monniaux, 2016). Four potential, not mutually-exclusive, mechanisms have been proposed: 1) widening the selection gate by increasing FSH, which translates into a longer period of time during which FSH is above the threshold; 2) increasing the number of follicles, thus increasing the probability that more than one follicle is allowed through the gate; 3) decreasing GC proliferation and size at which follicles acquire LH receptors; and 4) increased sensitivity of follicles to FSH, thus allowing follicles to continue to grow in a lower FSH environment. Our recent data obtained from cattle carrying the high fecundity allele, Trio, are consistent with the first (slight FSH increase) and third (smaller follicles at dominance due to decreased GC proliferation) mechanisms but provide clear evidence against the second mechanism, increased number of follicles, as a mechanism to increase ovulation rate in Trio carriers. Although certain high fecundity ovine genotypes and the MARC-twinner bovine model have been reported to have greater numbers of small follicles, the mere increase in follicle numbers alone does not seem sufficient to explain the occurrence of increased multiple ovulations, since there is still no explanation for why only a portion of available follicles are selected. In addition, substantial variation in follicle numbers has been shown in single ovulating cattle, and no evidence has been provided so far to link this variation with the occurrence of multiple ovulations. Moreover, *Bos indicus* are known to have much greater numbers of antral follicles than *Bos taurus*, however the occurrence of multiple ovulations and births appears to be rare (Sartori and Barros, 2011). In a recent review (Monniaux, 2016), the increase in follicle numbers in regulation of multiple ovulations has been integrated with the fourth proposed mechanism (greater FSH sensitivity). The increase in FSH sensitivity has been hypothesized to arise from changes in number of FSH receptors, decreased AMH, increased response of the receptor upon stimulation, or increase in other factors that modulate or act in conjunction with FSH to stimulate GC (i.e. IGF1). Recent results from Trio carriers did not show an increased expression of FSH receptor or increased intrafollicular IGF1 but found decreased expression of both factors in dominant follicles in Trio carriers as compared to controls (García-Guerra *et al*., 2018a).

The most consistent finding for all high fecundity genotypes is smaller-sized dominant and ovulatory follicles in Trio carriers or ovine fecundity genotypes. In addition, our evidence in Trio, as well as evidence in high fecundity ovine genotypes support another aspect of the third mechanism, related to acquisition of LH receptors in smaller-sized follicles. Thus, development of smaller follicles that acquire dominance and ovulate at smaller sizes appears to be a key component for selection of multiple follicles. Thus, multiple smaller follicles function as a cohesive unit that has the same hormonal output (i.e. E2) and upon ovulation results in multiple CL of smaller individual size but the same total number of luteal cells with corresponding similar P4 output.

An important aspect that stems from the development of smaller follicles in Trio carriers is their reduced growth rate. The reduced follicle growth is likely mediated by a reduction in the rate at which GC proliferate, resulting from attenuation of BMP15 signaling pathways in response elevated SMAD6 (García-Guerra *et al*., 2018a; Kamalludin *et al*., 2018). Thus, overexpression of SMAD6 in GC of Trio carriers would be similar, functionally, to high-fecundity ovine mutations that affect BMP15 and GDF9. However, the precise effects of these factors in bovine GC along with the regulation of such pathways have not been investigated and should be the focus of future research.

The potential for FSH being the major driver for occurrence of multiple ovulations is extremely appealing. Our findings of elevated FSH concentrations, particularly around the time of expected deviation provides clear support for this mechanism, and this would provide a logical physiologic explanation for increased ovulation rate. Thus, our current model (Fig. 4) for Trio carriers is: 1) at wave emergence follicles are smaller (~50% of volume); 2) follicles develop at a reduced rate resulting in follicles ~25-30% of the volume of non-carriers at deviation; 3) upon reaching the decisive period (2.5-3 days) the largest follicle acquires dominance (i.e. LH receptors) with a corresponding increase in E2 production, however because of much smaller follicle size, the resulting E2 production is not sufficient to completely suppress FSH; 4) as a result the next follicle is allowed to acquire dominance (following a hierarchal order) until sufficient dominant follicle volume is reached, resulting in sufficient GC numbers to induce final suppression of FSH. Future studies are needed to definitely evaluate this model in other high fecundity genotypes.

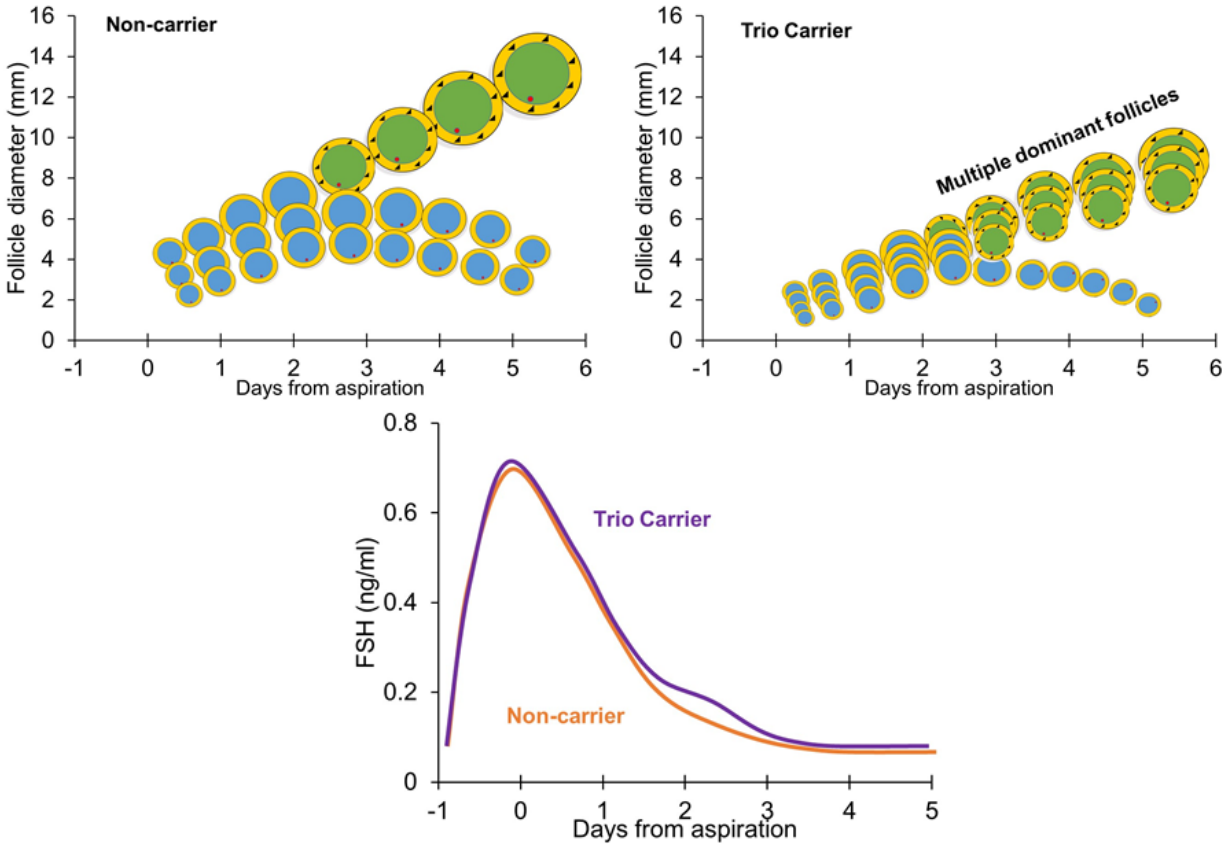

Figure 4. Physiological model for follicle selection in single ovulating cattle (left) and Trio carriers with multiple ovulations (right). The inset below shows the FSH concentrations during the period after follicle aspiration, emphasizing the comparison between carriers and non-carriers of the Trio allele.

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DOI: 10.21451/1984-3143-AR2018-0030

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

The life and death of the dominant follicle

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Abstract

Much work has been conducted over the years to determine the major factors that control follicle growth, including the role of FSH, LH and IGF1. These factors permit the dominant follicle to grow while subordinate follicles regress. The dominant follicle enters a phase of growth, and then that growth slows as the follicle reaches maximum size. The dominant follicle remains morphologically larger for a few days in the static phase, before starting to regress with the loss of functional dominance. Few studies have addressed the factors that determine follicle fate during the static phase. In this review, we summarize the differences in gene expression between growing and non-growing (static or early regressing) dominant follicles, highlighting areas that require further study. Potential factors that may help survival of the dominant follicle include IGF1, estradiol and BMP4/BMP7, and intrafollicular factors that likely initiate regression and apoptosis include FGF18 and AMH acting through FASLG. It is also very likely that the influence of microRNAs, especially miR-21, play a role in determining the fate of the dominant follicle.

Keywords: apoptosis, atresia, follicle, granulose.

Introduction

The bovine dominant follicle, once established, continues to grow from about 9 to 15 mm diameter over the course of about 4 days, then enters a 4 to 5-day plateau or static phase with little further growth, after which it starts to regress concomitantly with the recruitment of a new follicle wave. The dynamics of dominant follicle growth and gonadotropin control of the establishment of follicular dominance in ruminants have been extensively reviewed (Ireland *et al*., 2000; Ginther, 2016; Shimizu, 2016; Webb *et al*., 2016). The growing dominant follicle is highly estrogenic and the granulosa cells proliferate as the follicle increases in diameter, initially under the influence of FSH. As plasma FSH concentrations decline, the continued growth of the follicle is supported by LH and IGF1 (Shimizu, 2016). As the follicle reaches the end of the growth phase, intrafollicular concentrations of estradiol decrease (Ireland and Roche, 1983; Badinga *et al*., 1992; Price *et al*., 1995) and the follicle enters the static phase.

It is well known that the dominant follicle is functionally as well as morphologically dominant, as it suppresses the development of smaller follicles; ablation of the dominant follicle allows immediate recruitment of a new follicle wave or can rescue the regressing subordinate follicle if performed early during the growth of the dominant follicle (Ko *et al*., 1991; Siddiqui *et al*., 2015). The static phase, despite its name, is a period of change for the dominant follicle and the fate of the follicle is decided during this time. Early static dominant follicles are estrogen-active and approximately half of static dominant follicles are morphologically healthy by light microscopy (Price *et al*., 1995; Irving-Rodgers *et al*., 2001), and they frequently respond to induced luteolysis by ovulating (Ali *et al*., 2001). In contrast, the late static dominant follicle is estrogen-inactive, mostly atretic and fails to ovulate after luteolysis (Ali *et al*., 2001; Irving-Rodgers *et al*., 2001). Therefore, the static phase is a plastic period of the dominant follicle lifespan during which the fate of the follicle is determined.

To determine the mechanisms of follicle growth and regression, many studies have been performed comparing growing dominant with regressing subordinate follicles of the same wave. Although these studies are of great value and have identified many characteristics of growth and regression, they do not address the 'static' phase of the dominant follicle lifespan. The purpose of this review is to summarize our understanding of the physiology of late growing, static and early regressing dominant follicles in cattle (*Bos taurus*), with reference to other species where appropriate, and to explore potential intrafollicular mechanisms that may determine follicle fate during the static phase. Several factors involved in early dominant follicle growth are described out of necessity when data for the late growing/static phase are unavailable.

Gene expression patterns in late growing, static & early regressing dominant follicles

Numerous studies of the expression of candidate genes and of global transcriptomics have been performed comparing granulosa cells from dominant follicles and from subordinate follicles during and after selection in cattle (reviewed in Zielak-Steciwko and Evans, 2016), but less attention has been paid to the different phases of dominant follicle growth. An early series of studies measured steroidogenic enzyme and gonadotropin receptor mRNA levels by in-situ hybridization (reviewed in Bao and Garverick, 1998), and as follicle growth slowed in healthy dominant follicles (from day 4 to 6 after wave emergence), there were decreases in *CYP11A1* mRNA levels in theca and granulosa cells, and of *CYP17A1* and *LHCGR* in theca cells. These same studies demonstrated that atretic dominant follicles contained less *CYP19A1* and *LHCGR* mRNA in the granulosa cell layer compared to healthy dominant follicles of the same size. In a later study, a comparison between dominant follicles in the early and late growing phases, from day 2 to day 5.5 after wave

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Received: February 15, 2018
Accepted: April 30, 2018

emergence, demonstrated that as the dominant follicle reaches maximum diameter, granulosa *FSHR* mRNA levels decrease and *LHCGR* mRNA levels increase (Mihm *et al*., 2006), however this study included a number of smaller follicles that were no larger than the next subordinate follicle.

Other candidate gene studies have revealed that some fibroblast growth factors have been shown to differ between healthy and atretic dominant follicles; FGF18 is expressed in theca cells and mRNA levels are higher in atretic compared with healthy large follicles and in subordinate vs dominant follicles (Portela *et al*., 2010). FGF9 is predominantly expressed in granulosa cells and mRNA levels are higher in atretic compared with healthy large follicles (Schütz *et al*., 2016). In contrast, thecal *FGF2* and *FGF10* mRNA levels have been reported to be higher in healthy compared to atretic bovine follicles of abattoir origin (Berisha *et al*., 2004; Buratini *et al*., 2007).

Some members of the bone morphogenetic protein family also change with follicle health: Glister *et al*. demonstrated that granulosa cell *BMP2* mRNA levels decreased as follicle size increased (from 7 to 18 mm, abattoir ovaries; Glister *et al*., 2010) whereas Selvaraju at al. showed that BMP2 mRNA levels increase as dominant follicles progressed from pre- to mid-dominance (8 - 16 mm; timed collection) and remained high in static phase follicles (Selvaraju *et al*., 2013). Comparing estrogen-active and inactive large follicles, granulosa cell *BMP2* mRNA levels were higher in large atretic follicles compared to large healthy follicles (Glister *et al*., 2010). This latter study also showed that *BMP4*, *BMP6* and *BMP7* mRNA levels did not differ between healthy and unhealthy large follicles.

Cocaine- and amphetamine-regulated transcript (CARTPT) mRNA levels are very low in granulosa cells of dominant follicles in cattle compared with pre-selection follicles and do not change during dominant follicle growth (Lv et al., 2009). Unfortunately, data are not available for these genes in growing, static and early regressing dominant follicles.

Two studies have investigated the follicular transcriptome during the late growing/static phase of the dominant follicle lifespan. In one study, the static phase was mimicked in cows by stimulating with FSH followed by a 'coasting' period; in this model, the abundance of multiple mRNA species changed as the follicle coasts, with most changes reflecting an increase in genes encoding anti-proliferative and pro-apoptotic proteins as the static phase progresses (Nivet *et al*., 2013). In a study of follicles >9 mm diameter grouped as growing, static and regressing by flow cytometry, microarray analysis identified a number of genes that were differentially expressed between the three groups, suggesting that the follicles undergo distinct changes rather than a gradual slide from healthy to atretic (Girard *et al*., 2015). In this latter study, *BMP4* mRNA levels were not different between growing, static and regressing dominant follicles, in agreement with Glister *et al*. (2010).

MicroRNAs have also been the target of profiling during follicle growth. Using large bovine follicles of abattoir origin, 57 miRNA were differently expressed in estrogen-active compared to estrogen-inactive follicles (Sontakke *et al*., 2014). In a study comparing the dominant and subordinate follicles on day 3 and day 7 of the cycle, Salilew-Wondim and colleagues found few (16) differentially expressed miRNA between dominant and subordinate follicles on day 3, and a larger number (108) differentially expressed on day 7 (Salilew-Wondim *et al*., 2014). A direct comparison of dominant follicles on day 3 (growing) and day 7 (static or regressing) identified 131 differentially expressed miRNA in granulosa cells (Salilew-Wondim *et al*., 2014). The number of miRNA that were differentially expressed in both these datasets is small (Table 1), which might be a reflection of the different biological models used.

A study comparing preovulatory dominant follicles with subordinate follicles identified 34 miRNA enriched and 30 miRNA reduced in granulosa cells of preovulatory follicles compared to subordinate follicles; interestingly, PCR analysis indicated that selected miRNA differentially expressed in granulosa cells were also differentially expressed in theca cells (Gebremedhn *et al*., 2015). Preovulatory follicles would be expected to be different from non-ovulatory dominant follicles owing to the considerable increase in oestradiol levels and LH pulse frequency as well as a decrease in peripheral progesterone levels, so this particular comparison is not so relevant for the current discussion.

No global profiling has yet been reported for theca cells during this stage of follicle growth. A microarray study compared the theca cells of healthy and atretic follicles 3-5 mm diameter of abattoir origin, and concluded that most differentially expressed genes were related to inflammation and vascularization rather than apoptosis (Hatzirodos *et al*., 2014).

A glycoproteomic study was conducted with bovine granulosa and theca samples and atresia was associated with increased levels of certain sulphated chondroitin polysaccharides in granulosa cells and of sulphated heparan polysaccharides in theca cells (Hatzirodos *et al*., 2012). It is not known whether such changes occur during the growing-static-regressing phases of the dominant follicle.

Table 1. MicroRNA identified in both Sontakke et al. (2014) and Salilew-Wondim et al. (2014) as differentially expressed between growing and non-growing large follicles.

Upregulated in growing follicles	Upregulated in non-growing follicles
bta-miR-202	bta-miR-149-3p
bta-miR-31	bta-miR-21
bta-miR-873	bta-miR-150
bta-miR-652	bta-miR-204-3p
bta-miR-450b	bta-miR-409a
bta-miR-15b	bta-miR-142
bta-miR-424-p5	

Prolonging the life of the dominant follicle

FSH, IGF1 and estradiol

FSH is a major folliculogenic factor and administration of exogenous FSH induces the growth of multiple dominant follicles in a superovulatory setting; lower levels of FSH are also able to maintain the growth of 2 dominant follicles and/or delay regression of subordinate follicles (Adams *et al*., 1993; Mihm *et al*., 1997; Rivera and Fortune, 2001). This is likely to be caused by stimulation of the early growth phase of the dominant follicle which permits the selection of multiple dominant follicles. Once the dominant follicle is established it can remain viable in the face of low circulating FSH concentrations, and continue to grow past its normal lifespan if pulsatile LH secretion is increased (Stock and Fortune, 1993; Bigelow and Fortune, 1998). A regressing dominant follicle (as well as subordinate follicles) can also be rescued if FSH levels are increased (Ginther *et al*., 2016).

Intrafollicular estradiol is another major player involved in follicle health. It is well known that estradiol increases proliferation of granulosa cells of numerous species (Drummond and Findlay, 1999; Rosenfeld *et al*., 2001), enhances cell cycle progression from G1 to S phase (Quirk *et al*., 2006), and protects cells against FASLG- and FGF18-induced apoptosis (Quirk *et al*., 2006; Portela *et al*., 2015). It should be noted here that exogenously administered systemic estradiol induces atresia of the dominant follicle (Burke *et al*., 2005) by reducing LH pulse amplitude as well as FSH secretion (Price and Webb, 1988).

Although IGF1 is probably best known for its role in follicle deviation and the establishment of dominance, it also likely plays a role in supporting dominant follicle survival through regulation of IGF binding proteins (IGFBP) and hence IGF1 bioactivity (Mazerbourg and Monget, 2018). Large estrogen-active sheep and cattle follicles contain lower levels of IGFBPs - and thus higher IGF bioavailability - than do smaller growing or atretic follicles (Besnard *et al*., 1996; Roberts and Echternkamp, 2003), and addition of IGF1 to granulosa cells increased cell proliferation and estradiol secretion in a follicle-size specific manner (Monniaux and Pisselet, 1992; Gong *et al*., 1993; Spicer *et al*., 1993).

The transforming growth factor-β (TGFβ) family

The TGFβ superfamily roles in ovarian function are critical and complex, and it seems that the disappearance of one of them or a disturbance of the equilibrium formed by these factors will strongly influence folliculogenesis and then ovulation. The role of TGFβ superfamily members in preantral follicle development and follicle selection has been reviewed (Knight and Glister, 2006).

Two BMPs, BMP4 and BMP7, are generally considered to be theca-derived proteins that act on granulosa cells. In cattle, both BMP4 and BMP7 mRNA are detected predominantly in theca cells (Fatehi *et al*., 2005; Glister *et al*., 2010), whereas in sheep neither BMP4 nor BMP7 mRNA were detected in follicles by in-situ hybridization (Juengel *et al*., 2006). Neither BMP4 nor BMP7 mRNA levels appear to be regulated by LH in bovine theca cells (Glister *et al*., 2011).

In vitro studies have shown that these BMPs affect granulosa cell steroidogenesis and proliferation. Theca-derived BMP4 and BMP7 increased granulosa proliferation and estrogen secretion, and inhibited progesterone synthesis in some studies with ruminants (Glister *et al*., 2004), but did not alter granulosa proliferation in another study (Yamashita *et al*., 2011). The effect of BMP7 on progesterone synthesis is due to a reduction of *STAR* mRNA levels (Yamashita *et al*., 2011). These BMPs may also promote follicle development/survival by increasing granulosa cell VEGF secretion and angiogenesis (Shimizu *et al*., 2012). Paradoxically, BMP4 and BMP7 potently inhibit androgen secretion from bovine theca cells (Glister *et al*., 2005) and as levels of neither appear to change with follicle health (Glister *et al*., 2010), the physiological role of these proteins remains to be established.

Levels of granulosa cell *BMP2* mRNA were lower in estrogen active dominant follicles compared with smaller growing follicles, and tended to increase in atretic follicles in cattle (Glister *et al*., 2010), and in sheep *BMP2* mRNA was only detected by in-situ hybridization in atretic follicles (Juengel *et al*., 2006). In contrast, *BMP2* mRNA levels were higher in large estrogen active dominant follicles compared with smaller growing follicles in water buffalo (Rajesh *et al*., 2018). Addition of recombinant BMP2 increased estradiol secretion but decreased progesterone secretion from ovine and bovine granulosa cells in vitro without altering cell proliferation (Souza *et al*., 2002; Juengel *et al*., 2006; Selvaraju *et al*., 2013).

Two other BMPs of interest are BMP15 and GDF9, which are expressed in the oocyte. GDF9 is critical for primary follicle growth and knock-out of *Gdf9* in the mouse results in arrest of folliculogenesis at the primary stage (Dong *et al*., 1996). In sheep, the role of GDF9 appears similar because in the case of a natural loss of function mutation of GDF9 in several breeds of ewes show abnormal folliculogenesis with arrest of follicle development at the primary stage (Nicol *et al*., 2009). Loss of *Bmp15* in mice results in reduced litter size owing to ovulation defects (Yan *et al*., 2001). In sheep there are several natural mutations that alter antral follicle growth, including $FecX^{I}$, $FecX^{R}$ and $FecX^{L}$, for which homozygous ewes are sterile with follicle arrest at the primary stage whereas ewes heterozygous for this same mutation have increased ovulation rate (Galloway *et al*., 2000; Bodin *et al*., 2007; Martinez-Royo *et al*., 2008). Another mutation in the BMP subfamily, called *FecB*, is in the coding sequence of the *BMPR1B* gene and induces a partial loss of function of this BMP receptor which leads to increased ovulation rate (Souza *et al*., 2001) and influences the proliferation and steroidogenesis of granulosa cells (Mulsant *et al*., 2001; Campbell *et al*., 2006). Recently, a mutation in a BMP signalling pathway termed 'Trio' has and been described in cattle, which results in increased *SMAD6* mRNA

levels and, similar to the situation in sheep, in the growth and survival of two or more dominant follicles (Garcia-Guerra *et al*., 2018).

Both BMP15 and GDF9 affect granulosa cell proliferation and steroidogenesis, but in complex species-specific patterns. Recombinant BMP15 stimulated granulosa cell proliferation in ruminants (McNatty *et al*., 2005) and protects granulosa and cumulus cells against apoptosis in ruminants (Hussein *et al*., 2005). In sheep and cattle, BMP15 and GDF9 have been reported to inhibit FSH-induced progesterone synthesis by granulosa cells (McNatty *et al*., 2005; Fabre *et al*., 2006), although species of origin of the protein has been reported to alter its effect: ovine GDF9 inhibited progesterone secretion from sheep granulosa cells whereas mouse GDF9 increased progesterone secretion (McNatty *et al*., 2005). There is also a species difference in the amounts of BMP15/GDF9 secreted by the oocyte of polyovular vs monovular species, as sheep secrete both whereas rats secrete primarily GDF9 (Lin *et al*., 2012). BMP15 and GDF9 synergize, and this may be in the form of secreted heterodimers (cumulin) or secreted monomeric proteins that form dimers at the receptor of the target cell (Mottershead *et al*., 2015; Heath *et al*., 2017).

There is also evidence that GDF9 can alter theca cell function in pre-selection follicles, as it decreased proliferation and steroidogenesis of bovine theca cells from follicles <6 mm diamater, but had no effect on theca cells from follicles >8 mm diameter (Spicer *et al*., 2008).

Induction of atresia in the dominant follicle

The fate of the dominant follicle is determined during the static phase of development, and the follicle can regress and become atretic 'passively' if the survival signals described above are reduced/absent. However, it is not clear what endocrine changes occur between the end of the growing period and the end of the static period. Alternatively, intrafollicular events may predispose a follicle toward atresia; the following section describes some potential pro-apoptotic factors that may play a role in determining the fate of the dominant follicle.

Fas antigen and Fas ligand

Fas antigen is a transmembrane receptor which induces apoptosis when activated by the protein Fas ligand (FASLG). In cattle, granulosa cell *FAS* mRNA levels were not different between growing and atretic dominant follicles, but were significantly higher in the theca layer of atretic compared with healthy dominant follicles (Porter *et al*., 2000). *FASLG* mRNA levels are also higher in atretic vs healthy follicles in non-ruminants, and can be increased in ruminant granulosa and theca cells in vitro by treatments that increase apoptosis including serum withdrawal (Hu *et al*., 2001), FGF18 (Portela *et al*., 2015) and toxins (Guerrero-Netro *et al*., 2015, 2017). Alone, FASLG does not cause apoptosis in granulosa cell cultures with serum but requires the presence of IFN gamma - however, in serum-free culture, bovine GC are susceptible to FASL-induced apoptosis (Quirk *et al*., 2000), although this was prevented in the presence of IGF, FGF2 and EGF, but not FGF7, TGF, PDGF or gonadotropins (Quirk *et al*., 2000).

In rodents, Faslg induces granulosa cell death and decreased levels of inducible nitric oxide synthase (Nos2) mRNA levels, and this can be prevented by nitric oxide (Chen *et al*., 2005). In cattle, inhibition of endogenous NO production increased FASLG expression and granulosa cell apoptosis (Zamberlam *et al*., 2011). Estradiol stimulated *NOS2* mRNA levels in bovine granulosa cells (Zamberlam *et al*., 2011) and also attenuates FASLG-induced apoptosis (Quirk *et al*., 2006). It seems likely that FASLG is a mediator of apoptosis induced by various effectors.

Fibroblast growth factors

FGF18 has been clearly demonstrated as a pro-apoptotic factor. This member of the fibroblast growth factor family is produced in vivo by the theca layer in cattle, and protein and mRNA levels are higher in atretic compared with healthy follicles. Moreover, recombinant FGF18 inhibits granulosa cell estradiol secretion and abundance of *CYP19A1*, *CYP11A1*, *HSD17β1*, *STAR*, *HSD3β1* and *FSHR* mRNA (Portela *et al*., 2010), and increases DNA fragmentation and abundance of cleaved caspase-3 in granulosa cells (Portela *et al*., 2010, 2015; Fig. 1). Injecting FGF18 into the growing dominant follicle *in vivo* caused follicle regression (Portela *et al*., 2015). It is interesting to note here that some growth factors promote granulosa cell proliferation but decrease estradiol secretion in vitro, FGF9 for example (Schreiber and Spicer, 2012); this apparent paradox has been referred to a dedifferentiating effect, but proliferation of cells may be caused by growth factor activation of MAPK pathways that drive proliferation irrespective of lower estradiol levels.

In support of this notion, FGF18 appears not to activate the typical FGF signalling pathways in granulosa cells; specifically, FGF18 does not increase MAPK3/1 phosphorylation or abundance of typical FGF response genes including SPRY2 and EGR3 (Jiang *et al*., 2013; Han *et al*., 2017), although it does increase MAPK14 phosphorylation (Portela *et al.,* 2015). The mechanism of action of FGF18 remains obscure.

The transforming growth factor-β (TGFβ) family

Anti-Müllerian Hormone (AMH), another member of the TGFβ super-family, is secreted by granulosa cells of small follicles and is known to inhibit the recruitment of primordial follicles in rodents (Durlinger *et al*., 1999) but not in sheep (Campbell *et al*., 2012). In ruminants, as in non-ruminant species, AMH levels decrease with increasing size of antral follicles, and appears to be inversely correlated with CYP19A1 expression (Monniaux *et al*., 2008; Campbell *et al*., 2012; Liang *et al*., 2016). Recombinant AMH decreases granulosa and theca cell steroidogenesis in vitro (Campbell *et al*., 2012), and has been shown to

increase apoptosis in human granulosa tumour cells (Anttonen *et al*., 2011). AMH mRNA levels and protein secretion from granulosa cells in vitro are stimulated by BMP2, BMP4 and BMP6 in sheep and cattle (Rico *et al*., 2011; Estienne *et al*., 2015), and *AMH* mRNA levels are increased by BMP15 in sheep granulosa cells, and GDF9 enhanced this effect (Pierre *et al*., 2016). At least part of the ability of the *FecB* mutation to decrease granulosa apoptosis may be the reduced expression of AMH mRNA and protein in this genotype (Fig. 2).

It is worthy of mention that certain BMP family members appear to have both pro-survival and pro-apoptotic actions, as they have been shown to stimulate estradiol secretion, which is a pro-survival factor, or stimulate AMH secretion which promotes apoptosis (Fig. 3). It is most likely that the predominant activity depends on stage of follicle growth and endocrine/paracrine milieu of the follicle at the time in question. Studies in which multiple endocrine/paracrine factors are studied in combination are needed to resolve this enigma.

Fig x

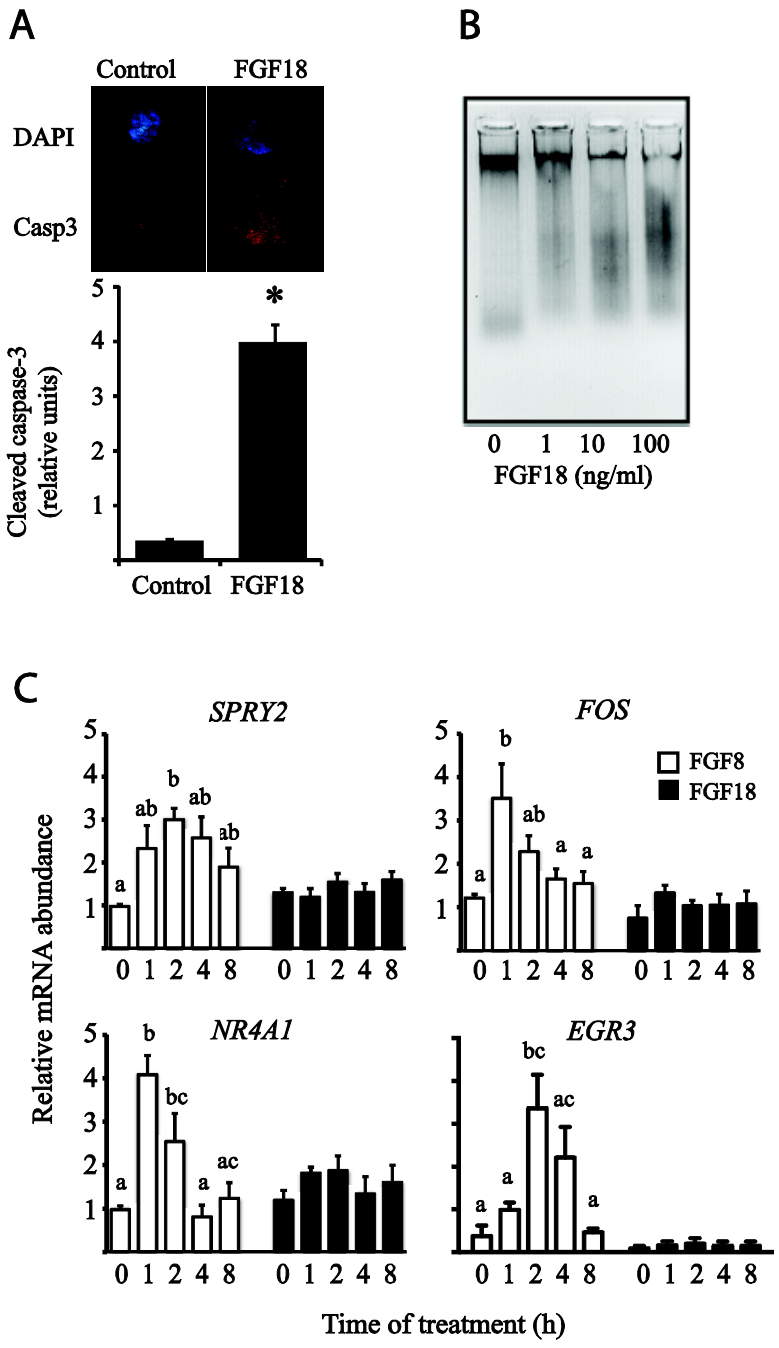

Figure 1. FGF18 increases apoptosis in granulosa cells and does not activate typical FGF signaling pathways. Culture of bovine granulosa cells with recombinant human FGF18 increases cleaved caspase-3 protein levels (A) and DNA fragmentation (B), and addition of FGF18 (10 ng/ml, filled bars) does not increase levels of mRNA of typical response genes (compare with FGF8; 10 ng/ml, hollow bars). Bars with common letters are not statistically different. Data from (Portela *et al*., 2010, 2015; Jiang *et al*., 2013; Han *et al*., 2017).

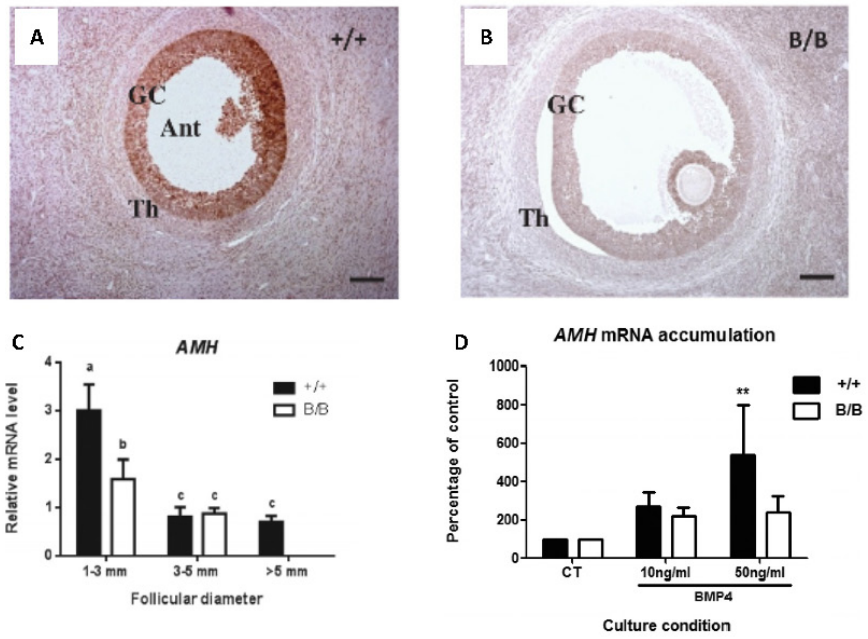

Figure 2. Regulation of AMH by BMPs in sheep follicles. Anti-Mullerian hormone protein (A,B) and mRNA (C) levels are markedly reduced in sheep carrying the hyperprolificacy Booroola mutation in BMPR1 compared to non-carriers, and this mutation leads to reduced granulosa cell responsiveness to BMP4 (D). Bars with common letters are not statistically different, and asterisks denote a significant stimulation of *AMH* mRNA abundance by BMP4. Data from Estienne *et al*. (2015).

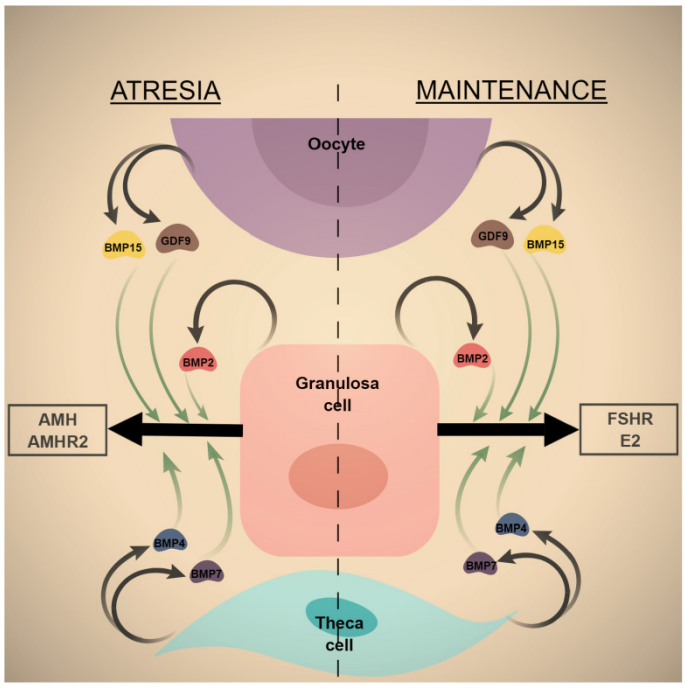

Figure 3. The duality of BMP action in the ovarian follicle. BMPs of theca, oocyte and granulosa cell origin have been shown to stimulate FSHR expression and estradiol secretion from granulosa cells, thus supporting granulosa cell survival and growth or maintenance of the dominant follicle. However, these same factors have also been shown to increase AMHR2 expression and AMH secretion, which is a pro-apoptotic signal. The net impact on the follicle is likely determined by other endocrine/paracrine factors present during the static phase of the dominant follicle lifespan.

miRNA

The roles of miRNA in dominant follicle development remain obscure. Some miRNA upregulated in atretic follicles (Table 1) have been shown to block apoptosis, including miR-21 in mouse granulosa cells (Carletti *et al*., 2010), miR-149 in lymphoma cells (Fan *et al*.,2016) and miR-142 in cancer cells (Li *et al*., 2016). However, miR-150 promotes cell growth in ovarian cancer (Li *et al*., 2015) but causes apoptosis in lymphocytes (Sang *et al*., 2016) as well as endothelial cells. It is possible that these miRNAs are upregulated in atretic follicles as a defence against apoptosis, and are thus an effect of atresia rather than a cause.

Conclusions

As growth of the dominant follicle slows, the follicle faces two possible fates: maintenance of growth/survival, and atresia. Increases in gonadotropin concentrations will drive survival, likely through increased intrafollicular estradiol, IGF1 and mRNA levels, and evidence is accumulating that other intrafollicular factors may either predispose the follicle to or provide protection against atresia. The pro-apoptotic factors likely include increased secretion of AMH by granulosa cells and of FGF18 by theca cells, which increase FASLG-mediated apoptosis of granulosa cells and thus initiate an irreversible process of follicle atresia. The BMPs have been implicated, and they may help or hinder follicle survival depending perhaps on other endocrine or paracrine factors present. The potential role of each is summarized in Fig 4. Exactly when and how this fate determination occurs is unknown, and future research is required to determine the paracrine and autocrine events that occur within the follicle during the static phase of its lifespan.

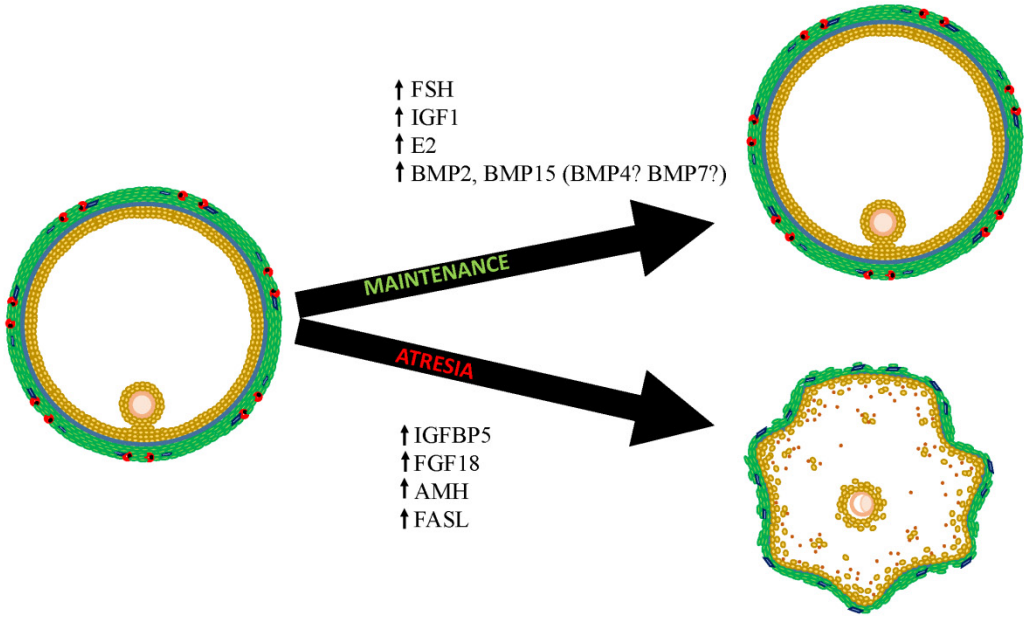

Figure 4. Schematic representation of the fate of the dominant follicle as it enters the static phase of its lifespan. The follicle may survive and go on to the preovulatory stage if appropriate gonadotrophic stimuli are provided. Alternatively, lack of gonadotropin support in combination with the secretion of local proapoptotic factors including but not limited to FGF18 and AMH may initiate FASLG-mediated granulosa cell apoptosis and irrevocably drive the follicle into regression.

Acknowledgments

We thank NSERC and FRQ-NT for funding the work from our laboratory, and apologize to all those whose work we were not able to cite in this short review.

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DOI: 10.21451/1984-3143-AR2018-0029

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Seminal plasma proteins and metabolites: effects on sperm function and potential as fertility markers

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Abstract

Molecular components of sperm and in the media surrounding them influence male fertility. In this regard, seminal plasma proteins and metabolites modulate various reproductive events, including sperm motility and capacitation, cell protection, acrosome reaction, fertilization and embryonic development. Empirical associations between seminal proteins and metabolites and fertility indicate that these molecules are potential molecular markers of male reproductive status in cattle and other species.

Keywords: fertility, metabolites, proteins, seminal plasma, sperm.

Introduction

Pregnancy after artificial insemination (AI) is the best indicator of reproductive potential of sires. However, this information usually becomes available only after bulls are mature and have been selected for commercial use in the AI industry. Moreover, criteria such as sperm motility and morphology have limited associations with sire fertility, particularly in bulls selected by the AI industry (Killian *et al*., 1993; Moura, 2005; Moura *et al*., 2006; Oliveira *et al*., 2013; Dogan *et al*., 2015; Kaya and Memili, 2016). There can be substantial differences in fertility among bulls with normal semen parameters and those with non-compensable sperm defects may never achieve adequate fecundity (Oliveira *et al*., 2013; Dogan *et al*., 2015; Kaya and Memili, 2016). Therefore, mechanisms by which sperm molecular profiles influence bull fertility are not fully understood. In this context, there are efforts to identify molecular markers of gamete function in farm animals and humans. Candidate makers include sperm RNA, proteins and various molecules in reproductive fluids. These studies are based on the hypothesis that molecular components of sperm and/or from the surrounding media influence fertilizing capacity. In this regard, analysis of seminal plasma proteome and metabolome will provide information about mechanisms regulating sperm fertilizing potential and reproductive performance. Thus, the present review discusses the roles of selected seminal plasma proteins and metabolites and how their expression relates to fertility, especially in cattle.

Seminal plasma proteins

Proteins involved in sperm protection

Seminal plasma contains proteins that protect sperm in the epididymis (Hinton *et al*., 1995; Kraus *et al*., 2005), after ejaculation and in the female reproductive tract. Production of reactive oxygen species (ROS) is a component of sperm physiology (MacLeod, 1943); however, excessive ROS disturbs sperm homeostasis through formation of lipid peroxidation, reduction of enzymes that regulate calcium influx, and loss of ATP (Ohta *et al*., 1989; Aitken *et al*., 1993). To mitigate deleterious effects of excessive ROS, the epididymis secretes antioxidant enzymes (Hinton *et al*., 1996), including glutathione S-transferase, tioredoxin peroxidase, superoxide dismutase, glutathione peroxidase (GSHPx) and catalase (Alvarez and Storey, 1983; Jeulin *et al*., 1989; Fouchécourt *et al*., 2000; Dacheux *et al*., 2006). Of these, GSHPx catalyzes the reduction of hydrogen peroxide (Halliwell and Gutteridge, 1990), protecting sperm against excessive ROS (Perry *et al*., 1992; Dacheux *et al*., 2005). For example, increased GSPHx activity in ram semen is linked to maintenance of sperm viability (Casao *et al*., 2010). Another seminal plasma molecule, acidic seminal fluid protein (aSFP), also controls oxidative stress in the bovine reproductive tract (Einspanier *et al*., 1993; Schöneck *et al*., 1996). This protein shares identity with molecules of the spermadhesin family (Romão *et al*., 1997) and, in the bull, is secreted by the epididymis and accessory sex glands (Moura *et al*., 2007a, 2010). Although aSFP binds to ejaculated sperm, it is lost after capacitation (Dostàlovà *et al*., 1994). Therefore, unlike porcine spermadhesins (Caballero *et al*., 2004, 2005), it appears that bovine aSFP does not participate in sperm-oocyte interactions. However, aSFP has been associated with survival of cryopreserved bull sperm (Jobim *et al*., 2004).

Ion chelators in seminal plasma, such as lactoferrin (LF), also protect sperm against effects of lipid peroxidation (Ochsendorf, 1999). Lactoferrin sequesters ionic iron (Nozaki *et al*., 2003) and adsorption to sperm during epididymal transit (Jin *et al*., 1997) and ejaculation (Thaler *et al*., 1990). In stallions, LF represents 41.2% of all proteins secreted by the epididymis (Fouchecourt *et al*., 2000) and high

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Received: February 15, 2018
Accepted: May 17, 2018

concentrations of LF in horse and dog seminal plasma relate to total number of sperm (Kikuchi *et al*., 2003a, b). Seminal albumin, in turn, binds to lipid peroxides, contributing to sperm protection (Alvarez and Storey, 1983) and is positively correlated with percentage of morphologically normal sperm in bovine semen (Elzanaty *et al*., 2007).

Clusterin, another seminal plasma molecule with protective roles, acts as a chaperone (Humphreys *et al*., 1999) and inhibits cell lysis by complement-mediated mechanisms present in female secretions (Ibrahim *et al*., 1999; Meri and Jarva, 2001). In the epididymis, clusterin affects maturation, lipid transport (Tenniswood *et al*., 1992) and sperm membrane remodeling (Humphreys *et al*., 1999). Clusterin chaperone activity is consistent with its ability to interact with various types of proteins *in vivo* (Carver *et al*.; 2003). *In silico* analysis of clusterin networking indicates potential interactions with proteases and protease inhibitors, such as plasminogen, alpha-2-macroglobulin, TIMP-1, alpha-2-antiplasmin precursor and plasminogen activator inhibitor 1. Clusterin also has putative links to fibronectins, which participate in cell adhesion, wound healing and maintenance of cell structure, including GTP protein-coupled receptors and modulators of cell growth. Seminal plasma clusterin is inversely related to percentage of sperm with intact membrane in peccaries (*Peccari tajacu* L.; Santos *et al*., 2014) and with percentage of morphologically normal sperm in semen from Brahman bulls (Boe-Hansen *et al*., 2015). In contrast, bull and ram sperm with morphologic defects have extensive clusterin binding (Ibrahim *et al*., 2001a, b). This association probably occurs as a result of clusterin´s ability to bind to damaged portions of hydrophobic regions of sperm membranes (Bailey and Griswold, 1999). Sertoli cell-secreted clusterin prevented apoptosis in rat testes subjected to hyperthermia (Matsushita *et al*., 2016) and in humans, clusterin secreted in the fluid of the seminiferous epithelium has positive associations with fertility (Salehi *et al*., 2013). In addition, seminal clusterin promotes immune tolerance to male antigens in humans, mitigating female immune reactions to male factors (Merlotti *et al*., 2015). High levels of clusterin are associated with advanced physiopathological states, such as kidney diseases, neurodegenerative disorders, artheriosclerosis, heart attack and cancer (Wehrli *et al*., 2001; Trougakos *et al*., 2002; Pucci *et al*., 2004; Calero *et al*., 2005). We have characterized the seminal plasma proteome of several domestic and wild species, including bulls (Moura *et al*., 2007a; Rego *et al*., 2014, 2016; Menezes *et al*., 2017), rams (Souza *et al*., 2012), boars (González-Cadavid *et al*., 2014), peccaries (Santos *et al*., 2014), dogs (Aquino-Cortez *et al*., 2017) and coatis (Silva *et al*., 2018), among others. Clusterin is present in the semen of all these species, in moderate to high concentrations. Thus, many animals have a clusterin-based, conserved mechanism for sperm protection and regulation of immune reactions initiated by male gametes in the female reproductive tract.

Proteins associated with sperm motility

Seminal plasma contains various proteins associated with sperm motility (Baas *et al*., 1983), e.g., kallikrein-cinins. In this case, kininogenin seminal plasma is a specific substrate for kalikrein (Fink *et al*., 1989), an important stimulator of post-ejaculation sperm motility (Schill *et al*., 1989). There is a positive correlation between seminal plasma kallikrein activity and sperm motility, with exogenous kallikrein enhancing bovine sperm motility (Somlev *et al*., 1996). Angiontensin converting enzyme (ACE) is another seminal plasma component related to the kalikrein system (Hohlbrugger*et al*., 1984). This enzyme catalyzes formation of angiontensin II and binds to receptors on sperm, enhancing motility (Vinson *et al*., 1996). Furthermore, ACE activity in ram seminal plasma is positively correlated with sperm concentration and fertility (Métayer *et al*., 2001; Gatti *et al*., 2004). In contrast, inhibition of ACE activity in bovine seminal plasma decreases progressive motility and inhibits acrosome reaction after *in vitro* capacitation (Costa and Thundathil, 2012).

Proteins involved in sperm capacitation, acrosome reaction and fertilization

Phospholipid binding proteins belonging to the family of BSPs (Binder of Sperm Proteins) are present in seminal plasma of several species, including bulls, bucks (male goats and rabbits), rams, rodents, stallions and men (Moura *et al*., 2007a; Manjunath *et al*., 2009; Souza *et al*., 2012; Plante *et al*., 2016). BSPs comprise ~60% of all proteins of the accessory sex gland fluid (Moura *et al*., 2007a) and seminal plasma (Manjunath and Sairam, 1987) of *Bos taurus* bulls and nearly the same amount in seminal plasma of *Bos indicus* bulls (Rego *et al*., 2014). In cattle, BSP proteins are secreted as isoforms with 14-15 kDa (BSP1 and BSP3) and 30 kDa (BSP5). Both BSP1 and BSP5 have two fibronectin type II domains arranged in tandem and amino terminal extensions that are O-glycosylated at threonine residues. Such biochemical attributes allow BSP1 and BSP5 to interact with sperm and to modulate ligand-binding activities by similar mechanisms (Calvete *et al*., 1996; Manjunath *et al*., 2009), with functional similarities (Manjunath and Thérien, 2002). Bovine BSPs are typical accessory sex gland proteins (Manjunath and Thérien, 2002; Moura *et al*., 2007a, 2010). BSPs bind to bull sperm at ejaculation (Manjunath and Thérien, 2002) and remain there after sperm contact oviductal secretions *in vitro* (Souza *et al*., 2008), as well as in acrosome-reacted or frozen-thawed sperm (Rodriguez-Villamil *et al*., 2016). BSPs also mediate the interaction between sperm and the oviduct epithelium (Gwathmey *et al*., 2006; Suarez, 2016). The most studied role of BSPs is their ability to bind and remove phospholipids and cholesterol from the sperm membrane, an initial event of capacitation (Thérien *et al*., 1999). Capacitating

effects of BSPs have been reported in other species, including mice (Plante and Manjunath, 2015) and humans (Plante *et al*., 2014). However, while ruminant BSPs originate mainly from the accessory sex glands (Manjunath *et al*., 2009; Souza *et al*., 2012; van Tilburg *et al*., 2014), they are expressed in the epididymis of mice and humans.

In addition to sperm capacitation, BSP1 affects *in vitro* fertilization and embryonic development. The study conducted by Rodriguez-Villamil *et al*. (2016) evaluated cumulus-oocyte complexes (COCs) incubated with frozen-thawed ejaculated sperm (18 h) in Fert-TALP medium containing: heparin and BSP1. With ejaculated sperm, cleavage rates were similar when Fert-TALP medium was incubated with heparin, 10 or 20 µg/ml BSP1. Day-7 blastocyst rates were equivalent after incubations with heparin or 10 µg/ml BSP1, but there were marked reductions in blastocyst formation after IVF media were supplemented with 20 or 40 µg/ml. Therefore, BSP1 is as efficient as heparin for inducing capacitation and fertilizing capacity of frozen-thawed ejaculated sperm. However, damage caused to embryo development may have been caused by BSP1 itself. High concentrations of and/or prolonged exposure to BSP proteins are harmful to cryopreserved sper due to membrane destabilization and excessive phospholipid and cholesterol efflux (Thérien *et al*., 1995, 1998; Manjunath and Thérien, 2002). Furthermore, content of BSP5 in accessory sex gland fluid has a quadratic association with bull fertility (Moura *et al*., 2006), suggesting that excessive BSP was detrimental to sperm physiology and/or embryo development.

In the same study (Rodriguez-Villamil *et al*., 2016), cleavage rates were higher after IVF using cauda epididymal sperm and any BSP1 concentration (10, 20 or 40 µg/ml) embryo development (day 8) was greater after inclusion of 20 or 40 µg/ml BSP1 in the IVF media, with or without heparin. Thus, we concluded that: 1) heparin has limited effect on cauda epididymal sperm *in vitro* (based on fertilization rates and blastocyst formation; 2) BSP1 has better effects on embryo growth than heparin; and 3) a combination of BSP1 with heparin does not enhance cleavage rates and embryo development beyond those obtained with BSP1. We also verified that SP1 did not cause reductions in bovine blastocyst growth after IVF with epididymal sperm, in contrast to results obtained with ejaculated sperm. Therefore, previous exposure of sperm or not to seminal plasma determines how sperm will respond to BSP in vitro. Additionally, combining heparin and BSP1 did not increase capacitation rates of ejaculated sperm. And both cleavage rates and blastocyst growth were similar after ejaculated sperm were incubated with heparin, BSP-1+heparin or BSP-1. With epididymal sperm, the best results or capacitation and blastocyst growth were obtained with BSP-1, when compared to heparin and heparin+BSP-1 (Rodriguez-Villamil *et al*., 2018; Federal University of Ceara, Fortaleza, Brazil; unpublished data). Thus, BSP-1 is a potent capacitating factor for bovine sperm and it increases fertilization rates, with no dependence on heparin.

Despite multiple beneficial roles of BSPs, these molecules can damage sperm during cryostorage, as they extract phospholipids and cholesterol from the membrane in a concentration- and time-dependent manner (Manjunath *et al*., 2002; Plante *et al*., 2016). Such deleterious effects occur when sperm are exposed for prolonged periods-and/or to excessive concentrations of BSPs. In this regard, extenders used for sperm preservation, such as egg-yolk (EY) and milk, contain components that associate with BSPs (Manjunath *et al*., 2002). There are interactions between low-density lipoproteins in EY (Bergeron and Manjunath, 2006) or milk proteins; the latter can prevent excessive BSP binding to sperm and excessive phospholipid removal from the membrane, thereby protecting sperm during cryopreservation (Plante *et al*., 2015). In goats, milk proteins (casein and β-lactoglobulin) bind to BSPs and reduces BSP interactions with sperm (Menezes *et al*., 2016). Currently, BSPs are one of the most studied mammalian seminal plasma proteins and effects on ejaculated sperm, including capacitation, interaction with the oviduct epithelium and fertilization. That BSPs interact with components of semen extenders suggest that these proteins are potential targets for development of biomolecules that could enhance assisted reproductive technologies.

Seminal plasma phospholipase A2 (PLA2) participates in capacitation, acrosome reaction and sperm-oocyte membrane fusion (Soubeyrand *et al*., 1997; Pietrobon *et al*., 2005; Roldan and Shi, 2007), promotes release of fatty acids and phospholipids involved in final stages of gamete fusion (Roldan, 1998) and has antimicrobial effects. Furthermore, its expression in bovine seminal plasma is associated with fertility (Moura *et al*., 2006). Osteopontin (OPN) concentrations in bovine seminal plasma were related to *in vivo* fertility of Holstein bulls (Killian *et al*., 1993; Moura *et al*., 2006) and to fertilizing capacity of cauda epididymal sperm treated with accessory sex gland fluid in IVF trials (Henault *et al*., 1995; Moura *et al*., 2007b). OPN is mainly secreted by the accessory sex glands and binds to sperm after ejaculation and after they contact secretions of the oviduct and are capacitated (Souza *et al*., 2008). Also, OPN has a calcium binding site and a domain to link with heparin, consistent with its effects on sperm capacitation (Monaco *et al*., 2009; Boccia *et al*., 2013).

Alterations in the OPN gene reduce seminal plasma OPN concentration (Rorie *et al*, 2016). Furthermore, in IVF studies, percentage of fertilized bovine oocytes was significantly reduced by addition of OPN antibodies to fertilization media and exposure of sperm or oocytes to antibodies against alpha V and alpha5 integrins before fertilization (Gonçalves *et al*., 2007). Also, pre-treatment of bovine sperm and oocytes with OPN enhances both *in vitro* fertilization and early embryo development (Gonçalves *et al*., 2008a, b). The

RGD amino acid sequence of osteopontin mediates its link with α5 and αv integrins (Denhardt, 2002; Wai and Kuo, 2004) and the ability of osteopontin to support cell adhesion is prevented when the RGD sequence is mutated (Liaw *et al*., 1995; Xuan *et al*., 1995). Treatment of sperm or oocytes with an RGD peptide, but not with an RGE sequence, reduced both the number of sperm bound to the zona pellucida and fertilization rates, similar to effects of anti-osteopontin antibodies. It appears that OPN interacts with sperm through integrins (Gonçalves *et al*., 2007). Incubation of oocytes with osteopontin purified from bovine milk increased cleavage rates on day 4, blastocyst development on day 8 and hatched blastocysts on day 11 (Gonçalves *et al*., 2007). Furthermore, OPN purified from milk improved sperm capacitation and addition of OPN to IVF media enhanced bovine blastocyst formation (Monaco *et al*., 2009). Moreover, in an IVF system, using frozen-thawed bull semen, OPN improved fertilization rates and blastocyst development on day 8 (Gonçalves *et al*., 2008a). In swine, supplementation of fertilization media with recombinant rat OPN enhanced fertilization rates by 41% and reduced polyspermy (Hao *et al*., 2006). Exogenous OPN added to IVF media improved cleavage rates and swine embryo development, and inhibited apoptosis and DNA fragmentation (Hao *et al*., 2008). Moreover, anti-OPN antibodies decreased rates of *in vitro* fertilization and blastocyst growth in mice (Liu *et al*., 2015). Clearly, OPN affects fertilization and post-fertilization events.

Osteopontin is typically involved in cell adhesion, tissue and extracellular remodeling, inflammation and immune-mediated events (Denhardt, 2002; Wai and Kuo, 2004; Rittling and Singh, 2015; Bouleftour *et al*., 2016). Despite substantial knowledge regarding actions of osteopontin in several tissues, an understanding of its function in male reproduction is far from complete. There is general consensus that OPN secreted by the accessory sex glands binds to sperm during ejaculation through integrins and that the integrin-OPN complex interacts with the zona pellucida (D'Cruz, 1996). This model is supported by the presence of OPN in bovine oviductal fluid (Gabler *et al*., 2003). Additionally, OPN binds to the CD44 receptor, which usually participates in cell adhesion (Cichy and Puré, 2003), and has been expressed in sperm (Bains *et al*., 2002) and oocyte membranes (Schoenfelder and Einspanier, 2003). In the bull, OPN binds to the acrosome at ejaculation (Cancel *et al*., 1999) and this sperm-OPN link is preserved after sperm contacts with oviductal fluid and undergoes an acrosome reaction *in vitro* (Souza *et al*., 2008). In addition to sperm binding, OPN interacts with the zona pellucida and oolemma of bovine oocytes (Souza *et al*., 2008). Consequently, we propose that OPN adheres to sperm and this complex connects to the zona pellucida or to OPN-zona pellucida, as OPN can form high-affinity bonds with other OPN molecules (Kaartinen *et al*., 1999; Goldsmith *et al*., 2002). When entering the periviteline space, OPN attached to the post-equatorial segment would mediate the interaction of sperm and oolema, also through integrins and/or CD44. Integrins (αv and α5) are present in bovine (Erikson *et al*., 2008) and human sperm (Fusi *et al*., 1996; Reddy *et al*., 2003), as well as on human oolema (D'Cruz, 1996) and CD44 transmembrane glycoproteins are present in bovine sperm and oocytes. Interactions of sperm OPN with oocyte integrins and CD44 receptors could trigger intracellular signaling, as reported for other cell types (Wai and Kuo, 2004; Rangaswami *et al*., 2006), and affect fertilization and early embryo development.

Metabolomics

Metabolites are the result of metabolic reactions associated with various biochemical pathways (Dunn *et al*. 2011). Many of these molecules have important roles in biological processes and represent potential biomarkers for predicting or detecting developmental states, physiological events, diseases or specific phenotypes. Therefore, metabolomics is used to understand networks of metabolites and have provided comprehensive identification and quantification of small molecules, including amino acids, peptides, vitamins, minerals, lipids, and carbohydrates in diverse cells, tissues, fluids, organs and organisms (Oliver *et al*., 1998; Fiehn 2001, 2002; Dunn *et al*., 2005).

Substantial progress has been made in the study and development of methodological strategies for using metabolomics, metabonomics, metabolic-fingerprinting, metabolite targeting, and metabolic profiling. Metabonomics is used to measure differences in levels of metabolites resulting from such factors as pathological or genetic changes, toxins, or use of drugs. Metabolic fingerprinting is a rapid method to evaluate and classify biologic samples or biopsies. Furthermore, metabolite target analysis is used to identify specific metabolic pathways of a limited number of metabolites, whereas metabolic profiling evaluates a cluster of metabolites that participate in a specific metabolic pathway (Dunn and Ellis, 2005; Hollywood *et al*., 2006; Holmes *et al*., 2008; Dunn *et al*., 2011; Patti *et al*., 2010). Metabolomics can be performed associated with other omics approaches, e.g. genomics, transcriptomics, and proteomics. For example, an Integrative Personal Omics Profile (iPOP) to identify markers for possible diseases affecting an individual could lead to an early diagnosis and perhaps prevention of certain diseases (Chen *et al*., 2012). Although complementary to other omics, metabolomics provides identification and quantification of products from metabolism and its pathways, analyses of modifications in metabolic reactions, and characterization of phenotypes and identification of potential biomarkers for such phenotypes (Fiehn, 2001, 2002; Dunn *et al*., 2005; Goodacre *et al*., 2004; Hollywood *et al*., 2006; Patti *et al*., 2010).

Metabolomics and reproductive biology

Advanced and more sensitive methods are vital for addressing major questions in biology and biotechnology, including those related to assisted reproductive technologies (ART). It is well known that metabolites have critical roles in specific pathways related to fertilization, implantation and embryonic development. Some techniques used in metabolome analysis for studies of reproductive biology include proton nuclear magnetic resonance (1H NMR), mass spectrometry (MS), fourier transform infrared spectroscopy (FTIR), near infrared (NIR) and Raman (Singh and Sinclair, 2007, Seli *et al*., 2010a; Kovac *et al*., 2013; Muñoz *et al*., 2014a; b). Metabolomics methods have been used as noninvasive approaches to improve assessment of embryo quality (Singh and Sinclair, 2007; Bromer and Seli, 2008; Nagy *et al*., 2008; Seli *et al*., 2010a; Montag *et al*., 2013). For example,1H NMR scans compared metabolomes in the culture media for human embryos before transfer. In that study, glutamate was associated with subsequent developmental potential (Seli *et al*., 2008). In addition, NIR, Raman and 1H NMR used for metabolome analysis of human embryo culture media were valuable for predicting successful implantation and pregnancy after IVF (Seli *et al*., 2007, 2008, 2010b). FTIR metabolomics were used to determine gender of bovine embryos (Muñoz *et al*., 2014a, b). This is also an effective and non-invasive method to determine embryo viability and the metabolic profile of blood plasma from recipient cows. Further, FTIR can be used to identify superior embryos and recipient females for optimum pregnancy outcome (Muñoz *et al*., 2014b).

As indicated above, methods of conventional semen evaluation most often give only descriptive information and have limitations to predict in fertility. However, various molecular approaches, such as metabolomics, have provided more in depth understanding of mechanisms causing male infertility (Deepinder *et al*. 2007; Aitken, 2010). Metabolomics has promise in identifying potential biomarkers of male fertility and infertility (Gilany *et al*., 2014; Goodacre *et al*., 2004; Deepinder *et al*., 2007; Kovac *et al*., 2013). The presence or changes in specific metabolites could be related to male gamete functions, perhaps enabling evidence-based techniques to prevent or mitigate infertility (Aitken, 2010). Metabolomics approach using Raman spectroscopy to analyze human seminal plasma facilitated diagnosis of normospermic and asthenozoospermic men (Gilany *et al*., 2014). Furthermore, 1H NMR identified fertility-associated biomarkers in seminal plasma and serum of high- and low-fertility bulls. Metabolites, such as citrate, tryptamine, taurine, and leucine were identified in seminal plasma, whereas asparagine, glycogen, citrulline, and isoleucine were present in serum (Kumar *et al*., 2015). Using 1HNMR, Hamamah *et al*. (1998) detected increased choline/citrate, choline/lactate, and glycerophosphorylcholine/choline ratios in seminal plasma of men afflicted with spermatogenic failure versus those with obstructive azoospermia. Several small molecular markers were identified in the urine of men with normozoospermic infertility using liquid chromatography-mass spectrometry (LC-MS) in combination with bioinformatics and multivariate analyses. In this research, leukotriene E4,3-hydroxypalmitoylcarnitine, aspartate, xanthosine, and methoxytryptophan were biomarkers of infertility (Zhang *et al*., 2014). Clearly, metabolomics can be used to identify molecular markers of male fertility.

Conclusions

In recent decades, methods in proteomics and metabolomics have enabled detection of unprecedented numbers of molecules in the seminal plasma of farm animals, wild species and humans. This broadens our knowledge regarding roles of these molecules and their contributions to male fertility. Metabolomics can identify numerous classes of substances associated with metabolic pathways, leading to challenges in interpretation. Empirical associations exist between specific seminal proteins (Table 1), metabolites and fertility indexes. Experiments also confirm cause and effect relations between seminal plasma proteins (e.g.osteopontin and BSPs) and IVF and early embryo development, suggesting that seminal proteins have potential in animal biotechnology.

Studies to describe components of the seminal plasma are vital to construct comprehensive libraries of seminal plasma compounds. As many as 4,000 proteins have already been identified in human seminal plasma, although there may be up to 10,000 present (Gilany *et al*., 2014). A human proteome atlas (https://www.proteinatlas.org/) of human tissues and organs is under development (Omenn *et al*., 2017; Uhlén *et al*., 2015; Thul *et al*., 2017). Fundamental research sets the foundation of science and technology. However, investigations that use omics approaches and reproduction need to be focused on finding markers of traits that are important for livestock industry in different regions of the world. Lessons could be learned from translational research, where research is carried out by multidisciplinary teams, joining efforts from basic science, applied investigators and professionals in the front of technology.

Table 1. Functional groups and mechanism of actions of major seminal plasma proteins.

Major functional group	Protein	Mechanism of action	Major references
Proteins involved in sperm protection	Glutathione peroxidase	Catalyzes reduction of H_2O_2, protects sperm against excessive ROS.	(Halliwell and Gutteridge, 1990; rams); (Perry *et al*., 1992; Dacheux *et al*., 2005; bulls)
	Acidic seminal fluid protein	Sperm decapacitation, oxidative stress control, survival of cryopreserved sperm	(Einspanier *et al*., 1993; Schöneck *et al*., 1996; bull); (Dostàlovà *et al*., 1994; bull); (Jobim et al., 2004; bull)
	Lactoferrin	Ion chelators, protects sperm against effects of lipid peroxidation	(Ochsendorf, 1999; men)
	Albumin	Binds to lipid peroxides in sperm membrane, sperm protection	(Alvarez and Storey, 1983; rabbit)
	Clusterin	Chaperone and sperm membrane remodeling, protects against female reproductive tract immune response, binds to damage sperm membrane	(Humphreys *et al*., 1999; men); (Ibrahim *et al*., 1999; bull; Merlotti *et al*., 2015; men); (Bailey and Griswold, 1999; rat)
Proteins associated with sperm motility	Kallikrein-cinins	Substrate for kallikrein, which enhance sperm motility	(Somlev *et al*., 1996; bull)
	Angiotensin converting enzyme	Catalyzes angiotensin II formation, Enhance motility.	(Vinson *et al*., 1996; rat and men; Costa and Thundathil, 2012; bull)
Proteins involved in sperm capacitation, acrosome reaction and fertilization	Binder of sperm proteins	Bind to sperm at ejaculation; mediate gametes interaction, phospholipids and cholesterol efflux from sperm membrane; enhance in vitro fertilization and embryonic development.	(Manjunath and Thérien, 2002; bull); (Gwathmey *et al*., 2006; bull); (Thérien *et al*., 1999; bull) (Rodriguez-Villamil *et al*., 2016; bull); Manjunath *et al*., 2002; bull; Plante *et al*., 2016; bull)
	Phospholipase A2	Release of fatty acids and phospholipids involved in final stages of gamete fusion; antimicrobial effects.	(Soubeyrand *et al*., 1997; bull; Pietrobon *et al*., 2005; mouse; Roldan and Shi, 2007); Moura *et al*., 2006; bull)
	Osteopontin	Binds to sperm acrosome after ejaculation; sperm capacitation; interacts with sperm through integrins, interacts with zona pellucida and oolemma; binds to sperm CD44 receptor; enhances *in vitro* fertilization and embryo development; reduces polyspermy and inhibits apoptosis and DNA fragmentation.	(Souza *et al*., 2008; bull; Cancel *et al*., 1999; bull); (Boccia *et al*., 2013; buffalo; Monaco *et al*., 2009; bull); (Gonçalves *et al*., 2007; bull; D'Cruz, 1996; men); (Souza *et al*., 2008; bull); (Cichy and Puré, 2003; men); (Gonçalves *et al*., 2008a; bull); (Hao *et al*., 2006; swine; Hao *et al*., 2008; swine)

Acknowledgments

The present work has been supported by the Brazilian Federal Reseach Councils (CAPES and CNPq) and the Ceara State Foundation for Scientific Development and Research (FUNCAP).

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DOI: 10.21451/1984-3143-AR2018-0041

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Impacts of oxidative stress on bovine sperm function and subsequent *in vitro* embryo development

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Abstract

Low levels of reactive oxygen species (ROS) in sperm are essential for various sperm functions such as capacitation, hyperactivation and acrosome reaction. However, increased synthesis of ROS or a disruption of antioxidative status (e.g. in cryopreserved sperm) can induce oxidative stress (OS). Sperm are particularly vulnerable to OS, as their plasma membrane contains large amounts of polyunsaturated fatty acids and they have limited antioxidative capacity (due to low cytoplasmic volume). Oxidative stress disturbs sperm function by damaging sperm proteins, lipids and DNA. Under relatively low OS sperm may retain their fertilizing ability, which might result in transfer of impaired paternal molecules (e.g. damaged DNA) to the fertilized oozyte. Oocytes can repair damaged paternal DNA, but only to a certain extent. Most embryos are either repaired (based on limited DNA damage in blastocysts) or eliminated (based on low percentage of blastocyst formation when sperm with damaged DNA is used for fertilization). However, some blastocysts had increases in both DNA damage and apoptosis, which could have important implications for subsequent development. In several studies, exogenous antioxidants improved quality of sperm exposed to oxidative stress and subsequent embryo development. However, there is still a knowledge gap regarding whether these alterations affect embryonic survival and further development to a live fetus and healthy offspring.

Keywords: embryo development, Oxidative stress, sperm function.

Introduction

Oxidative stress (OS) has a major role in pathophysiology of nearly all biological systems. MacLeod (1943) was apparently the first to report toxic effects of O_2 on sperm. He noted in experiments carried out *in vitro* that increased O_2 concentrations in seminal fluid hastened reductions in sperm motility and suggested that H_2O_2, generated by cells from O_2, was the actual toxic agent. Three years later, Tosic and Walton (1946) described deleterious effects of H_2O_2 on bovine sperm motility and viability. Currently, there is no doubt that OS decreases sperm quality and male fertility *in vivo* (Agarwal and Saleh, 2002; Aitken and Baker, 2006).

Nature of reactive oxygen species and oxidative stress

Reactive oxygen species (ROS) is a collective term that includes oxygen radicals (e.g. superoxide radical and hydroxyl radical) as well as highly reactive derivates of O_2 that do not contain unpaired electrons (non-radicals), including hydrogen peroxide (H_2O_2), singlet oxygen (O_2) and hypochlorous acid (HOCl) (Halliwell and Gutteridge, 1989).

ROS can be generated both outside and inside cells. Intracellularly, ROS can be produced by enzymes and non-enzymatically. NADPH oxidases in the cell membrane and cytochrome P450-dependent oxygenases in mitochondria and endoplasmatic reticulum are the main enzymatic sources. In addition to membrane-associated oxidases, soluble enzymes, including amino oxidases, xanthine oxidase, aldehyde oxidase, flavoprotein dehydrogenase and tryptophan dioxygenase, can also generate ROS (Freeman and Capro, 1982).

Non-enzymatic production of ROS occurs when an electron is directly transferred to oxygen. This occurs, for example, in the mitochondrial electron transport chain, where redox centers leak electrons that are accepted by oxygen to form superoxide, which is then quickly dismutated to H_2O_2 (Loschen *et al*., 1974; Liu *et al*., 2002). It is now widely accepted that mitochondria are the main source of intracellular ROS. A sophisticated antioxidative system counteracts and regulates ROS homeostasis. Antioxidative enzymes, including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), are primary compounds providing antioxidative defense. In addition, a number of low-molecular-weight antioxidants such as zinc, ascorbate, tocopherol, pyruvate, flavonoids, carotenoids, and glutathione also contribute to total antioxidative capacity (Finkel *et al*., 2000). Oxidative stress denotes a condition where ROS production overwhelms antioxidative capacity (Sies, 1993).

Physiological roles of reactive oxygen in semen

After ejaculation, sperm undergo a series of physiological changes such as capacitation and acrosome reaction in the female genital tract (Austin and Bishop 1958; Hunter and Rodriguez Martinez, 2004). *In vitro* studies demonstrated that exogenous O_2^- and H_2O_2 promote capacitation and the acrosome reaction, whereas appropriate antioxidants prevent them

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Received: 27 February, 2018
Accepted: May 24, 2018

(De Lamirande *et al*., 1997). Furthermore, low levels of $NO^{\cdot}$ promote capacitation of human sperm (Zini *et al*., 1995) as well as hyperactivation and zona pellucida binding of sperm (De Lamirande *et al*., 1997; Sengoku *et al*., 1998). Therefore, it has been demonstrated that capacitation and the acrosome reaction are redox-regulated (free radical regulated) processes enabling sperm to fertilize an ovum (reviewed by Aitken, 2017).

Oxidation sources in semen

The major reason for incidence of OS in semen (Fig. 1) is depletion of seminal antioxidants and excess generation of free radicals by sperm (Wathes *et al*., 2007). In cattle semen cryopreservation is well known to cause excessive production of ROS (Bilodeau *et al*., 2000) and to decrease antioxidative activity (Gürler *et al*., 2016). Free radicals appear to have important roles in cell damage after freezing and thawing, as antioxidant supplementation improved the quality of cryopreserved sperm (reviewed by Agarwal and Majzoub, 2017). However, mechanisms that increase oxidative stress in frozen-thawed sperm are not yet clear. While some authors attributed them to depletion of antioxidative enzymes (Bilodeau *et al*., 2000; Stradaioli *et al*., 2007) others propose that osmotic stress during sperm freezing and thawing induces oxidative stress (McCarthy *et al*., 2010). In somatic cells, hyperosmotic-induced cell swelling may activate membrane-associated phospholipase A2, which causes formation of free polyunsaturated fatty acids as arachnoic acid, which subsequently activates NADPH oxidase, thereby increasing O_2 production (Lambert *et al*., 2006).

Immature sperm may produce substantial amounts of ROS that are negatively correlated with sperm quality (Ollero *et al*., 2001; Agarwal and Saleh, 2002).

There are also age-related differences in oxidative stress in bulls. For example, compared to older Simmental bulls, young bulls of the same breed were more sensitive to a decrease in sperm motility during summer, when the enzymatic antioxidative protection in seminal plasma and spermatozoa were insufficient to counteract the intensive oxidative processes in spermatozoa (Vince *et al*., 2018). Furthermore, there an effect of breed concerning the effect of heat stress on sperm quality under tropical conditions has been described. Nichi et al. (2006) noticed a higher oxidative sperm damage in Simmental and Nelore bulls during summer compared to winter, but lipid peroxidation was higher in Simmental bulls than in Nelore bulls. The authors mentioned that the GPx activity in semen of Simmental bulls might have been insufficient to avoid sperm damage that occurred concurrent with increased ROS production in summer. Our own group (Gürler *et al*. 2015) has shown that there are also inter-individual differences in OS within the same breed. We examined frozen thawed semen in nine Holstein-Friesian bulls and detected differences of the total antioxidant capacity (TAC) in seminal plasma between the bulls. The TAC values were negatively related to lipid peroxidation of sperm.

Peroxidase-positive leukocytes (mainly polymorphonuclear leukocytes and macrophages) are other sources of ROS in semen (Ochsendorf and Fuchs, 1997). Additionaly, various exogenous factors are described, which either directly induce oxidative stress in sperm or stimulate endogenous production of ROS in the ejaculate. Many chemical toxins have a negative impact on sperm function. For example, lead and cadmium are heavy metals that reduce antioxidative activity of seminal plasma and sperm motility (Tvrda *et al*., 2012).

Impacts of oxidative stress on sperm

Harmful effects of OS on sperm function can occur in many ways, as excess ROS damages a variety of important molecules, including lipids (Gutteridge and Halliwell, 1990), proteins (Stadtman and Levine, 2000) and DNA (Richter *et al*., 1988).

Lipid peroxidation

Sperm have high levels of polyunsaturated fatty acids in their plasma membrane, which are vulnerable to oxidative attack. Lipid peroxidation generates a variety of lipid metabolites, including lipid peroxyl radicals, alkoxyl radicals, malonaldehyde, 4-hydroxynonenal, and acrolein (Jones *et al*., 1979). Lipid peroxyl radicals destabilize the plasma membrane by abstracting hydrogen atoms from adjacent polyunsaturated fatty acids. This process generates lipid radicals that react with oxygen to produce lipid hydro-peroxide and hydroxides, which in turn propagate a lipid peroxidation chain reaction. Lipid hydroperoxide is cleaved out of the membrane by phospholipase 2 and is further reduced by glutathione-peroxidase to truncated phospholipids. These molecules stop the chain reaction but concurrently destabilize the membrane (Storey, 1997). This affects functionality of integral membrane proteins as ion channels, with consequences for membrane integrity and fluidity (Aitken *et al*., 1989; Niki *et al*., 2005). Both membrane fluidity and integrity are important for a sperm to fuse with an oocyte. High concentrations of lipid peroxides in sperm were negatively correlated with their ability to fuse with the oocyte (Aitken *et al*., 1989). In addition, lipid peroxidation is also known to reduce sperm motility. Mechanisms involve modulation of ion channel function by membrane structure alterations (Nishikawa *et al*., 1989; Lundbaek and Andersen, 1994) and adduct formation of lipid metabolites with flagellar axonemal proteins (Baker *et al*. 2015; Moazamian *et al*., 2015) and mitochondrial electron transport proteins. Modulation of mitochondrial proteins disrupts mitochondrial electron transport, resulting in an efflux of electrons. These in turn combine with oxygen in a vicious cycle to form additional ROS (Aitken *et al*., 2012).

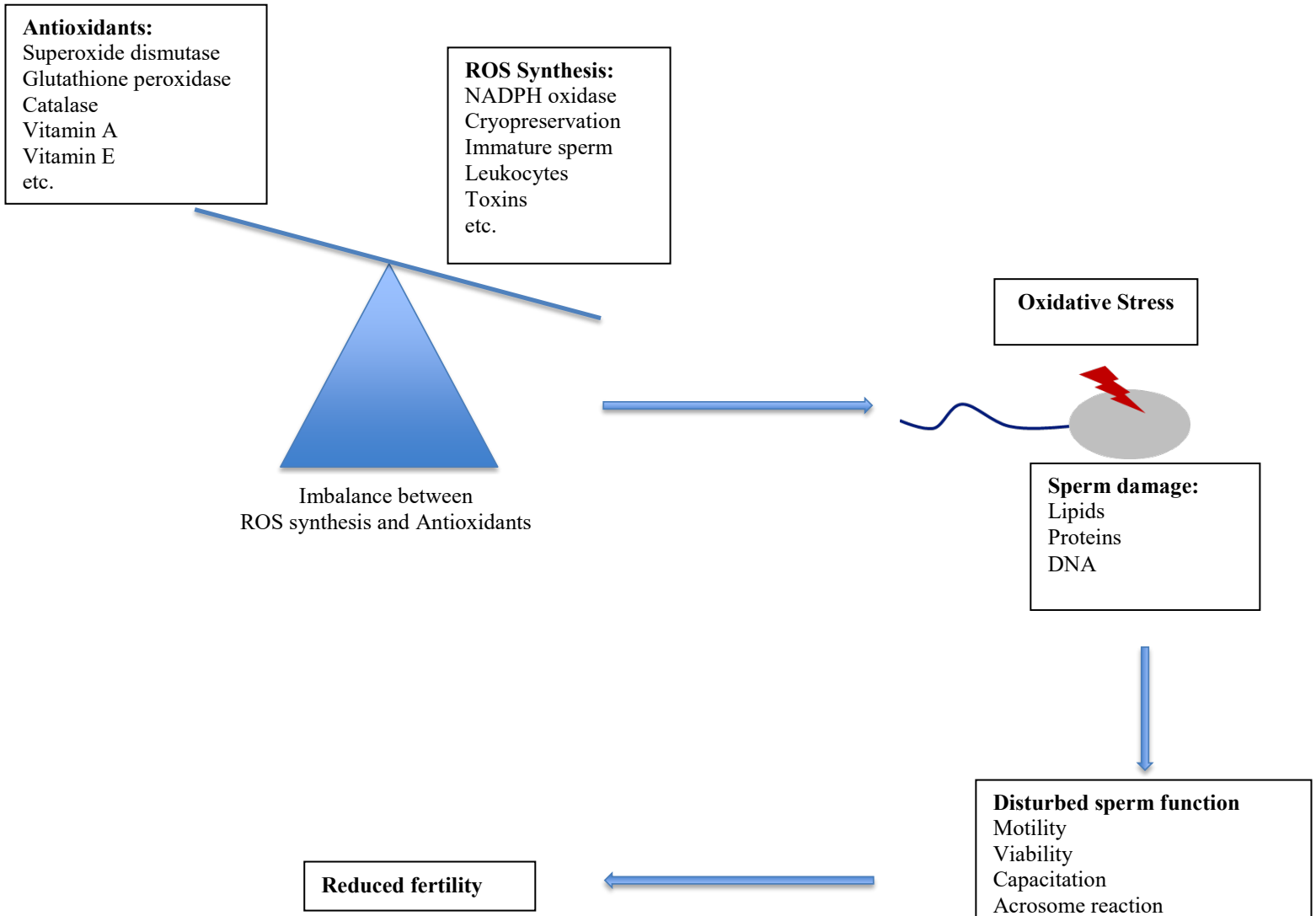

Figure 1. Oxidation sources in semen and their role in disturbances of sperm quality and male fertility.

Protein modifications

Reactive oxygen species are also postulated to directly modify proteins by oxidizing amino acid residue side chains, cleavage of peptide bonds, and formation of covalent protein-protein cross links (Garrison *et al*., 1962; Schuessler and Schilling, 1984; Davies, 1987). Conformation or activity of proteins can be influenced by oxidation of, for example, thiol groups. The amino acid cysteine contains a thiol group, and consequently, many proteins can be affected. In sperm, the enzyme tyrosine phosphatase, which has an important role in sperm capacitation, has a thiol group and is hence vulnerable to oxidation by ROS. Tyrosine phosphatase activity is inhibited by oxidation of the sulfhydryl group, initiating a cascade and finally inducing capacitation and the acrosome reaction in the sperm cell. Prolonged oxidative stress can also lead to over-oxidation of thiol groups of protamines and thus induce hyper-condensation of DNA, which adversely affects function (De Lamirande *et al*., 1997).

DNA damage

Free radicals damage DNA in numerous ways. Hydroxyl radicals bind to the double-bonds of DNA bases and abstract hydrogen from the deoxyribose sugar (Breen and Murphy, 1995). The abstraction of hydrogen from deoxyribose carbon causes strand breaks and base releases. The ROS attack on bases leads to numerous base alterations (Henle and Linn, 1997). One of the most abundant modifications is oxidation of guanine. Hydroxyl radical addition to aqueous solutions induces formation of 8-oxo-7,8-dihydro-20-deoxyguanosine and 2,6-diamino-5-formamido-4-hydroxypyrimidine in cells (Burrows and Muller, 1998; Cadet *et al*., 2003). The former is one of the most mutagenic lesions, since it causes transversion mutations due to its ability to pair with adenine as well as cytosine bases (Wood *et al*., 1990).

Protamines cover most of the DNA, but depending on the species, up to 50% of histones are retained. In bulls, 13% of the paternal genome is still bound to histones (Samans *et al*., 2014). The exact biological function of this retention is unknown, but it is hypothesised that retained histones are marking sets of genes preferentially activated during early embryo development (Gardiner-Garden *et al*., 1998; Hammoud *et al*., 2009; Samans *et al*., 2014). These regions may be prone to DNA damage since they are less condensed (Noblanc *et al*., 2013).

Repair mechanisms of DNA damage

Cells have diverse means of repairing DNA damage, including nucleotide excision repair, base excision repair, mismatch repair, and DNA double strand break repair (Evans and Cooke, 2009). In mature sperm, capacities for repairing DNA damage are limited. They only express one enzyme of the base excision repair pathway, 8-oxoguanine DNA glycosylase. Downstream components of the base excision repair pathway, apurinic endonuclease 1, and XRCC-1 (X-ray repair complementing defective repair in Chinese hamster cells 1) were not detectable (Smith *et al*., 2013). Consequently, mature sperm were reported to lack the ability to repair paternal DNA damage.

Impacts of sperm damaged by oxidative stress on embryonic development

There is no doubt that damage in the sperm can impair fertility and disrupt embryo development (Fig. 2). High levels of oxidative stress induce sperm plasma membrane alterations and hamper motility with the consequence of failure of fertilization. However, at lower levels of ROS, sperm may retain their ability to fertilize oocytes (Aitken and Baker, 2006). Through fertilization, not only the haploid paternal genome is transferred into the oocyte, but the entire content of the sperm, which may contain damaged molecules or toxic metabolites. Much of the content is disassembled, as it is not required for development (Cummins, 2001; Krawetz, 2005). Sperm mitochondria, for example, are degraded by the ubiquitination system (St John *et al*., 2000). However, other components of paternal origin are stable and have been followed until much later in embryonic development, including DNA, centrioles, some transcription factors, signalling molecules, and even ribonucleic acid (RNA) (Shalgi *et al*., 1994). Consequences of oxidative damage of paternal-derived molecules are extensively described for DNA. Effects of oxidative modifications in paternally derived centrioles or cytoplasmic factors on embryonic development have not been investigated. However, errors in microtubule assembly resulted in human fertilization failure and may contribute to a form of male infertility (Asch *et al*., 1995; Cummins, 2001).

Several studies investigated developmental consequences of sperm DNA damage induced by oxidative stress. Aitken and Baker (2006) demonstrated that exposure of sperm to low levels of hydrogen peroxide only marginally affected oocyte-sperm fusion, despite causing substantial DNA damage in sperm (Aitken and Baker, 2006). Thus using oxidatively damaged sperm for embryo production can lead to numerous developmental abnormalities. Low levels of pro-oxidants in bovine sperm cells had negative effects on blastocyst formation, but not on cleavage. Exposure of sperm to more severe oxidative stress reduced the blastocyst rate, cleavage rate and embryo quality (Silva, 2007; de Assis *et al*., 2015; De Castro *et al*., 2016). Simões *et al*. (2013) classified semen samples according to their sensitivity to OS and reported that increased susceptibility of sperm to OS compromised sperm DNA integrity and consequently reduced embryo quality.

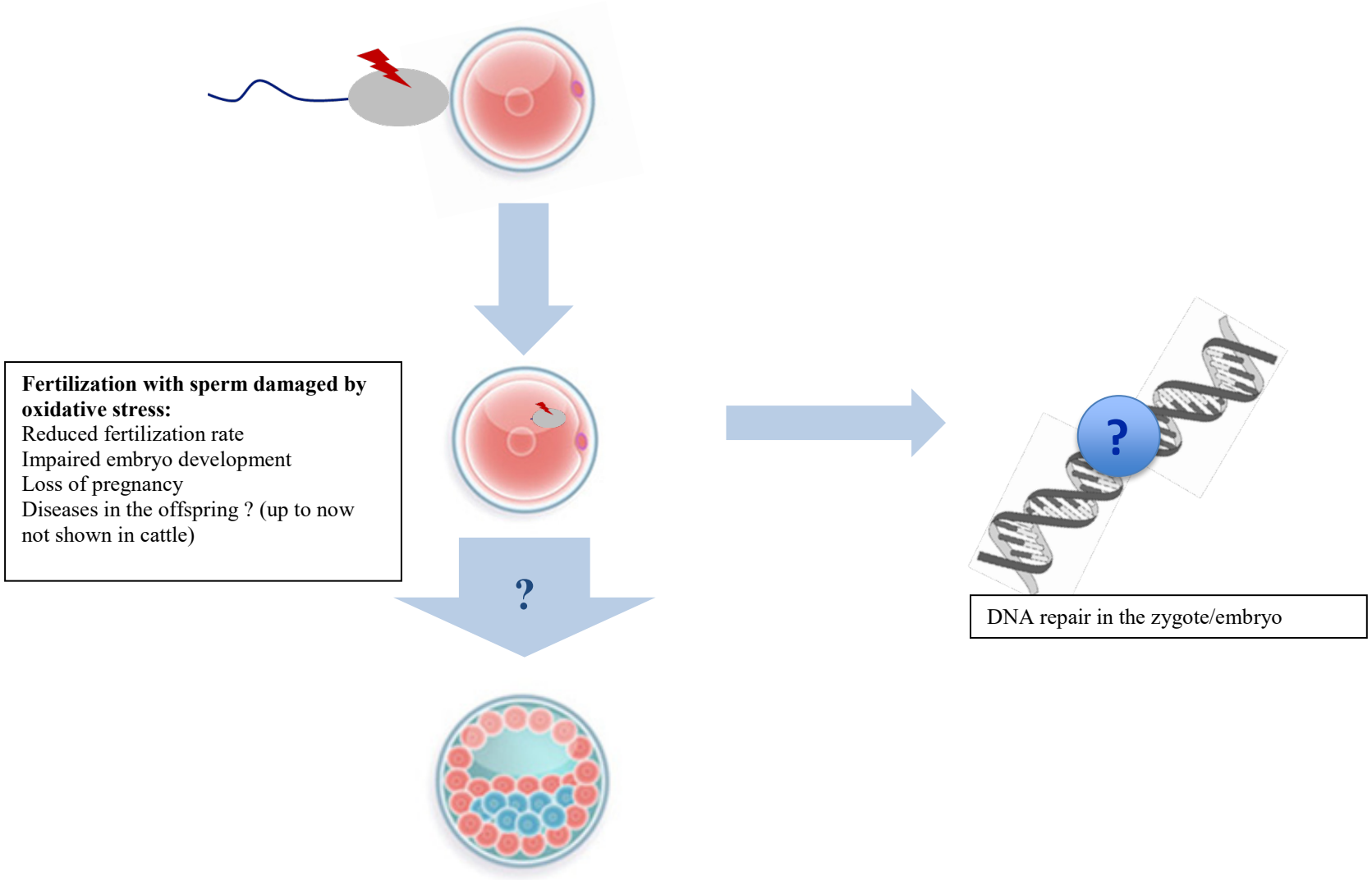

Figure 2. Effects of fertilization with sperm damaged by oxidative stress on embryo development.

In addition to negative effects on pre-implantation development, it is widely accepted that damaged sperm can support embryo development, implantation, and even pregnancy up to term, although development may be severely impaired. To the best of our knowledge, there are no reports in cattle on this topic. In mice, following IVF of mice using hydrogen peroxide damaged sperm, embryos developed, but were less likely to implant, were lighter, had a smaller crown-rump length, and female fetuses had metabolic abnormalities (Lane *et al*., 2014). In human assisted reproduction, fertilization with oxidatively damaged sperm, especially in regard to DNA damage, has been associated with loss of pregnancy or diseases in the offspring (Gavriliouk and Aitken, 2015).

As sperm cannot repair their genome before fertilization, DNA repair in newly fertilized embryos relies entirely on messenger ribonucleic acid (mRNA) and proteins stored in the oocyte (Ashwood-Smith and Edwards, 1996). It is suggested that a newly fertilized oocyte can cope with at least 10% of sperm DNA damage. This was derived from a study on trout, in which sperm with more than 10% tail DNA (based on a COMET assay) produced embryos, suggesting damage was either repaired or tolerated (Pérez-Cerezales *et al*., 2010).

Over 150 DNA repair genes have been identified in humans (reviewed by Jaroudi and SenGupta, 2007). Most belong to one of the four main DNA damage signaling and repair pathways: nucleotide excision repair, base excision repair, mismatch repair, and DNA double strand break repair. One of the earliest steps for DNA double-strand break repair is phosphorylation of the histone H2AX, referred to as gammaH2AX, which recruits DNA repair proteins (Celeste *et al*., 2003). Many DNA repair pathways are already active in early developmental stages. DNA damage repair pathways during early development interact with cell cycle progression. A cell with a damaged genome has three choices; remove the lesion, survive despite the lesion (with potential functional consequences), or undergo cell death. The importance of these repair mechanisms has been demonstrated by Barton (2007), who has used damaged sperm for fertilization or blocked DNA repair pathways in the zygote. When male rats were treated with cyclophosphamide, known to induce DNA damage, zygotes had enhanced gammaH2AX staining in the male pronucleus, compared to the control. In addition, Poly (ADP-Ribose) polymerase 1 (an enzyme which mediates DNA single-strand break repair in the base excision repair pathway) was upregulated in both pronuclei after fertilization with damaged sperm (Barton, 2007).

Rahman *et al*. (2012) studied the influence of oocyte quality on embryo production with damaged sperm in cattle and reported that bovine oocytes with a larger diameter were able to support embryo development after fertilization of sperm incubated in media with hydrogen peroxide better than their smaller counterparts. However, factors other than oocyte size can also influence oocytes repair capacity.

Conclusions

Bovine sperm are exposed to OS under different conditions, causing damage to various cell structures. In particular, damage to sperm DNA can affect embryo development and increase embryo mortality. Whether disturbances of embryo development affect postnatal health of cattle, documented for other species, has apparently not been reported. It is well established that exogenous antioxidants can reduce negative effects of oxidative stress on sperm function and embryo development. However, there is still a knowledge gap regarding how oxidative stress can be avoided without inhibiting essential physiological effects of reactive species on fertilization.

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Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Na/K-ATPase and Regulation of Sperm Function

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Abstract

A standard bull breeding soundness evaluation (BBSE) identifies bulls with semen that is grossly abnormal. Nonetheless, semen samples classified as satisfactory based on these traditional approaches differ in fertility; perhaps there are submicroscopic differences in sperm characteristics affecting fertility. Therefore, a better understanding of molecular regulation of sperm function could promote development of novel, evidence-based approaches to predict male fertility. Recently the α4 isoform of Na/K-ATPase (ATP1A4) has received considerable attention, due to its testis-specific expression in post-meiotic germ cells and mature sperm, in addition to its regulation of sperm motility and capacitation. Using fresh bull sperm, we determined that ATP1A4 resided in specialized microdomains (raft and non-raft) of the sperm plasma membrane and activated specific signaling (caveolin-1, EGFR, Src, ERK1/2) molecules during sperm capacitation. Furthermore, ATP1A4 was the predominant isoform responsible for total Na/K-ATPase activity in capacitated sperm. Despite the widely accepted dogma of transcriptional/translational quiescence, bovine sperm translated ATP1A4 mRNA on mitochondrial or mitochondrial-type ribosomes, increasing their content and activity during capacitation. Proteomic analysis of raft and non-raft fractions revealed a significant interaction between ATP1A4 and plakoglobin, a member of the β-catenin family of proteins involved in cell adhesion, in the equatorial segment of capacitated sperm, suggesting a potential role in sperm-oolemma fusion. In frozen-thawed sperm, ATP1A4 content and activity was greater in high- versus low-fertility bulls. Additionally, ATP1A4-induced increases in ROS, calcium, actin polymerization and tyrosine phosphorylation were also involved in regulating post-thaw sperm function in these bulls. Overall, results demonstrated that ATP1A4 had unique roles in controlling several aspects of sperm physiology, acting through well-established enzyme activity and signaling functions. Consequently, isoforms of Na/K-ATPase are potential biomarkers for male fertility.

Keywords: bull sperm, Na/K-ATPase, ouabain.

Introduction to bull fertility evaluation

Dairy and beef industries strive to achieve high pregnancy rates from genetically superior bulls. Therefore, fertility is more important than production traits. Based on the assessment from the American Gelbvieh Association's Alliance marketing program on 110,000 feedlot cattle, estimated relative importance of reproductive traits to growth and carcass traits are in the ratio of 4:2:1, respectively (Schiefelbein, 1998). Bulls with reduced sperm fertility can cause substantial economic losses due to delayed conception, prolonged calving seasons, reduced calf weaning weights, and increased number of breeding females that are culled due to either failure to become pregnant or to delayed pregnancy establishment. Although subfertility of beef bulls may not be evident when used in a multiple-sire or low breeding pressure system, such bulls typically have reduced fertility when they are used for single-sire mating or AI (Kastelic and Thundathil, 2008; Kasimanickam *et al*., 2012). Therefore, bull effects are of utmost importance. For example, a modest 1% increase in the reproductive rate would generate up to three times more return on investments (Hansen, 2006). Due to the subjective nature of conventional semen analysis, acceptable semen may be erroneously rejected, and concurrently, semen of unacceptable quality may be used for inseminations (Christensen *et al*., 2005). Therefore, in addition to frozen semen evaluation, bull or semen selection could be complemented by laboratory assays focused on molecular sperm function.

Role of biomarkers in bull fertility evaluation

High-throughput technologies such as mass spectrometry, along with appropriate bioinformatics tools, may provide better cues for annotation of proteins in the context of gamete biology. As proteins define a cell phenotype, changes at the proteome level could lead to differences in phenotypes influencing economically important traits and could enable identification of biomarkers of fertility. For example, content of P25b, a bovine sperm membrane antigen, was lower in semen from subfertile bulls than in high-fertility bulls (Parent *et al*., 1999). A 30-kDa heparin-binding protein (fertility-associated antigen, FAA), was differentially expressed in sperm membranes of beef bulls with varying fertility (Bellin *et al*., 1998). Furthermore, Sutovsky *et al*. (2015) described positive and negative protein biomarkers of fertility. Negative fertility markers include proteins that are exclusively associated with certain types of sperm defects, whereas positive biomarkers include proteins that are present in normal sperm, although they may either be upregulated or downregulated. One of the negative protein biomarkers of sperm quality is ubiquitin, which has been assessed in

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Received: February 6, 2018
Accepted: July 17, 2018

numerous species, including humans (Sutovsky *et al*., 2001) horses (Sutovsky *et al*., 2003), cattle (Sutovsky *et al*., 2002), and pigs (Kuster *et al*., 2004), and is correlated with infertility and indications of poor sperm quality, namely primary and total morphological defects (Purdy, 2008). Comparing normal versus abnormal sperm induced by elevated testicular temperature, Newton *et al*. (2009) demonstrated differential expression of several sperm proteins in morphologically abnormal sperm, including the testis-specific isoform of Na/K-ATPase (ATP1A4), as the molecular basis for impaired function.

Na/K-ATPase structure and isoforms

Na/K-ATPase is a plasma membrane protein with two fundamental roles in regulation of cell function. First, it is responsible for maintenance of Na^+ and K^+ gradients across the plasma membrane of most mammalian cells. In that regard, this enzyme is responsible for cell volume and pH, resting membrane potential, osmotic balance, and generation of a Na^+ gradient essential for secondary transmembrane ion transport (Skou and Esmann, 1992). Secondly, it acts as a receptor for cardiotonic steroids such as ouabain (specific inhibitor of Na/K-ATPase) and its interaction initiates signaling critical for regulation of various cell functions. The functional Na/K-ATPase consists of two subunits, the α subunit (110 kDa) and the β subunit (35-60 kDa, depending on glycosylation; (Blanco and Mercer, 1998). The α polypeptide is the catalytic unit responsible for ionic translocation as well as ouabain-dependent signaling events (Jorgensen *et al*., 2003), whereas the β subunit is essential for the enzyme's activity, as well as folding and localisation in the membrane (Geering, 1991). There are four α isoforms (α1, α2, α3, and α4) and three β isoforms (β1, β2, and β3) that are expressed in mammalian tissues (Blanco and Mercer, 1998; Mobasheri *et al*., 2000). The α1 and β1 isoforms are expressed in almost every cell (function as housekeeping Na/K-ATPase), whereas other α polypeptides have more restricted expressions, with specific roles (Mobasheri *et al*., 2000).

Ouabain in secreted from the adrenal gland (Doris *et al*., 1984) and is also identified in the bovine vaginal fluid (Daniel *et al*., 2010). Therefore, the presence of Na/K-ATPase in sperm and ouabain in the female reproductive tract suggests that this protein has a specific role in sperm physiology during capacitation or in other steps leading to fertilization.

Expression and localization of Na/K-ATPase isoforms in testis and sperm

The Na/K-ATPase α4 isoform (ATP1A4) has received considerable attention in recent years due to its sperm-specific expression (Blanco *et al*., 2000). The Na/K-ATPase α1 (ATP1A1; ubiquitous isoform) and α4 subunits are co-expressed in sperm with the β1 and β3 isoforms (Arystarkhova and Sweadner, 1997). In addition to α1, α4, β1, and β3 isoforms, the α3 and β2 subunits are also present in bovine sperm (Hickey and Buhr, 2011). In sperm two-thirds of total Na/K-ATPase activity is attributed to ATP1A4 (Wagoner *et al*., 2005). ATP1A4 expression peaked in mature testes in rats (Woo *et al*., 2000; Wagoner *et al*., 2005), whereas ATP1A1 expression was constant throughout spermatogenesis. In terms of localization, ATP1A4 was restricted to the mid-piece (rat) and principal piece (human; (Woo *et al*., 2000; Hlivko *et al*., 2006) of the flagellum, whereas ATP1A1 was present throughout the flagellum (Wagoner *et al*., 2005). In total contrast, in our studies with fresh bovine sperm, ATP1A4 was restricted to the sperm head (Thundathil *et al*., 2006).

Ion transport-dependent functions of Na/K-ATPase in sperm

An isoform of Na/K-ATPase (ATP1A4) specifically expressed in sperm suggests that this protein has a specific role in sperm physiology. Consequently, it was no surprise that sperm from ATP1A4 KO mice demonstrated severe reduction in total motility due to a characteristic bend in the sperm tail and cell membrane depolarization (Jimenez *et al*., 2011a). Simultaneously, over-expression of ATP1A4 resulted in plasma membrane hyperpolarization, higher progressive motility and enhanced hyperactivation, implicating ATP1A4 in sperm motility under both noncapacitating and capacitating conditions (Jimenez *et al*., 2011b). In addition, Jimenez *et al*. (2012) also reported that ATP1A4 activity was upregulated at the plasma membrane during sperm capacitation. ATP1A4 indirectly influences sperm motility by regulating intracellular pH, membrane potential and intracellular calcium release. ATP1A4 is linked to other K^+ channels which are involved in depolarization and hyperpolarization. Consequently, ouabain inhibition of ATP1A4 caused sperm membrane depolarization (Jimenez *et al*., 2012). ATP1A4 is also coupled to Na/H-exchanger (NHE), a flagellar protein, that uses the Na^+ gradient established by the Na/K-ATPase to remove H^+ from the cell in exchange for Na^+ (Counillon and Pouyssegur, 2000), thereby regulating the intracellular pH. Therefore, inhibition of ATP1A4 by ouabain eliminates the Na^+ gradient used by NHE to move H^+ out of the cell. Loss of NHE activity may lead to acidification of the intracellular compartment, which abolishes dynein activity and reduces flagellar movement (Woo *et al*., 2002). Furthermore, ATP1A4 is also functionally linked to sperm calcium regulation via a Na/Ca-exchanger (NCX), which uses the secondary Na^+ influx generated by Na/K-ATPase for calcium efflux. Inhibition of Na/K-ATPase increases intracellular Na^+ concentration which disrupts calcium efflux by NCX, thereby increasing intracellular calcium concentrations (Jimenez *et al*., 2010).

Na/K-ATPase as a signaling molecule during bovine sperm capacitation

Based on known signaling roles of Na/K-ATPase in somatic cells, ouabain (Na/K-ATPase inhibitor) induces ERK1/2 activation via transactivation of EGFR and activation of PLC and PKC. In addition, Na/K-ATPase-ouabain interaction generates ROS from mitochondria through the Src/EGFR/Ras pathway (Liu *et al*. 2000; Xie *et al*., 1999). Therefore, the sequalae of Na/K-ATPase signaling events in somatic cells resemble some events associated with sperm capacitation, namely increases in intracellular Na^+ and Ca^{2+} concentrations, generation of ROS, and activation of ERK1/2. Protein kinase A (PKA) mediated activation of protein tyrosine kinase (PTK) induces tyrosine phosphorylation (a hallmark of capacitation) in sperm (de Lamirande and O'Flaherty, 2008). There is also cross-talk between ROS and elements of ERK1/2, PKA and PTK (O'Flaherty *et al*., 2006) to promote tyrosine phosphorylation during capacitation. Therefore, we tested involvement of these signaling molecules in bovine sperm and demonstrated that PKA, RTK and Src kinases are involved in this process (Newton *et al*., 2010). Another study in bovine sperm also confirmed involvement of the ERK pathway in this process (Anpalakan, 2010).

Our previous study (Newton *et al*., 2010) demonstrated that incubation of bovine sperm with a PKA inhibitor (H89) inhibited ouabain-induced tyrosine phosphorylation. In addition, activated PKA interacts with PKC and activates phospholipase D (PLD), which subsequently hydrolyses phosphatidyl choline (PC) to phosphatidic acid (PA), mediating polymerization of globular (G)-actin to filamentous (F)-actin. Actin polymerization is involved in capacitation and the acrosome reaction in bovine sperm (Yagi and Paranko, 1995; Cohen *et al*., 2004). However, mechanisms by which ATP1A4 regulates these multiple signaling pathways during capacitation remain unknown. We proposed that raft and non-raft pools of ATP1A4 exist in sperm membrane and activate specific signaling molecules/pathways (investigated in studies reported below).

Role of lipid rafts and non-rafts in ATP1A4 signaling during bovine sperm capacitation

In somatic cells, the majority of Na/K-ATPase resides in specialized microdomains of the plasma membrane called lipid rafts, which facilitates its signaling functions, due to proximity to other signaling molecules within these microdomains (Liu *et al*, 2003; Liang *et al*, 2007). Domains similar to somatic cell lipid rafts have been demonstrated in mammalian sperm (Cross, 2004; Shadan *et al*., 2004; Sleight *et al*., 2005; Bou Khalil *et al*., 2006; Nixon and Aitken, 2009) and several raft subtypes have also been identified (Asano *et al*, 2009). Although previous studies demonstrated a role for lipid raft proteins in sperm-oocyte interactions (van Gestel *et al*., 2005; van Gestel *et al*., 2007), involvement of raft and non-raft proteins in signaling events leading to sperm capacitation has apparently not been reported. In our recent study (Rajamanickam *et al*., 2017a), content of ATP1A4 was increased in both raft and non-raft fractions of capacitated sperm.

In addition, ATP1A4 was the predominant isoform contributing to total Na/K-ATPase activity in both rafts and non-rafts of capacitated sperm. With reference to signaling functions of ATP1A4, there were comparative increases in phosphorylation of signaling molecules in both raft (Cav1) and non-raft (EGFR, Src, and ERK1/2) membrane fractions during capacitation. We also inferred that ATP1A4 interacted with Cav1 and EGFR in the raft fraction, whereas interactions of ATP1A4 with Src, EGFR, and ERK1/2 occurred in the non-raft fraction of ouabain-capacitated sperm. Therefore, we concluded that both raft and non-raft cohorts of ATP1A4 contributed to enzyme activity and phosphorylation of signaling molecules during bovine sperm capacitation.

We proposed a model regarding potential raft- and non-raft-mediated ATP1A4 signaling pathways (Fig. 1) in the bovine sperm plasma membrane during capacitation. Upstream events in rafts may involve interactions between Na/K-ATPase isoforms and CAV1, which can bind and activate Phospholipase C (PLC), thereby increasing hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), generating inositol 1,4,5-triphosphate (IP_3) and diacyl glycerol (DAG), which in turn activate protein kinase C (PKC). Co-localization of ATP1A4 and PLC isoform (PLCζ) was performed to determine involvement of PLCζ in our raft signaling model. PLCζ was localized to the equatorial segment (ES) and ATP1A4 co-localised with PLCζ at the ES and postacrosome region in ouabain-capacitated sperm (Fig. 2). IP_3 binds to its cognate receptor (IP_3R), leading to an increase in intracellular calcium, whereas PKC through other mediator proteins promotes polymerization of globular actin (G-actin) to filamentous-actin (F-actin). Within non-rafts, ATP1A4-ouabain interaction involves Src and EGFR leading to activation of ERK1/2 and protein tyrosine kinase (PTK)-mediated tyrosine phosphorylation of proteins. Increases in F-actin, intracellular calcium, and protein tyrosine phosphorylation contribute to capacitation-associated changes in sperm. The above-mentioned model assumes that activation/phosphorylation of signaling molecules occurs within the respective domains (raft or non-raft) of the plasma membrane. However, it is likely that a subset of the non-raft pool of ATP1A4 merges with the raft pool of ATP1A4 (or vice-versa), due to cholesterol efflux, either amplifying the existing signaling process or initiating a new downstream pathway during capacitation. All these possibilities open exciting areas for future investigation and could unravel the dynamic organization of signaling complexes mediated by ATP1A4 in sperm.

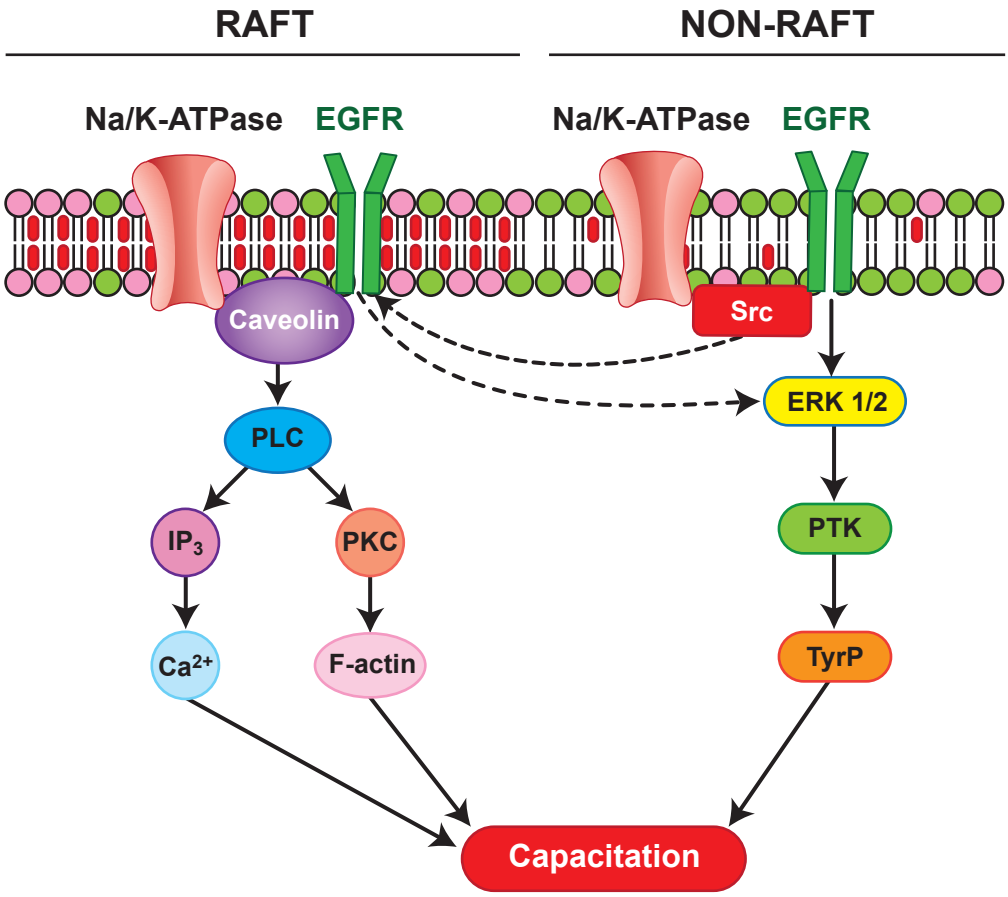

Figure 1. A hypothetical model for ATP1A4-mediated raft and non-raft signaling pathways during bovine sperm capacitation. Interactions between Na/K-ATPase isoform and Cav1, can activate phospholipase C (PLC), thereby generating inositol 1,4,5-triphosphate (IP_3) and diacyl glycerol (DAG), via hydrolysis of phosphatidylinositol 4,5-bisphosphate which in turn activates protein kinase C (PKC). IP_3 binds to its cognate receptor (IP_3R), leading to an increase in intracellular calcium, whereas PKC mediates conversion of globular actin (G-actin) to filamentous-actin (F-actin) through other mediator proteins. Within non-rafts, Na/K-ATPase-ouabain interaction involves Src and EGFR. Signals from non-raft interactions are relayed downstream, leading to activation of ERK1/2 and protein tyrosine kinase (PTK)-mediated tyrosine phosphorylation of proteins. Increase in F-actin, intracellular calcium, and protein tyrosine phosphorylation contribute to capacitation-associated changes in sperm

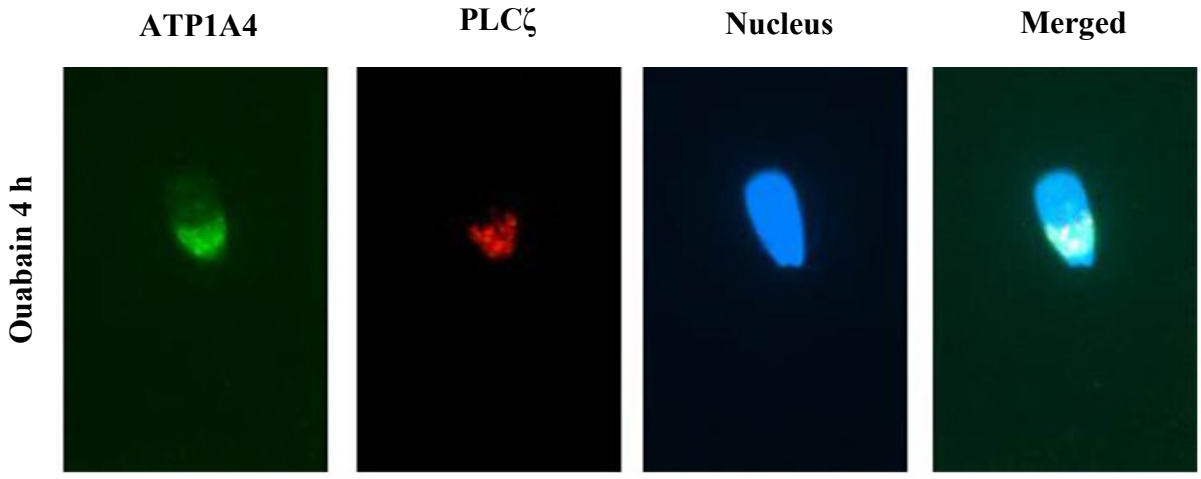

Figure 2. Co-localisation of ATP1A4-PLCζ during sperm capacitation. Representative images of ATP1A4 (green), PLCζ (red), nuclei (blue) and merged ATP1A4, PLCζ and DAPI staining in ouabain-capacitated sperm.

Characterization of the ATP1A4 interactome in lipid rafts and non-rafts during bovine sperm capacitation

In the aforementioned study, we highlighted two distinct pools (raft and non-raft) of ATP1A4 that trigger specific downstream signaling pathways. However, during spermatogenesis, sperm acquire several unique proteins or their isoforms to meet its functional demands, including lactate dehydrogenase (LDH-C4), sperm adhesion molecule 1 (PH-20), and testis-specific isoform of Angiotensin Converting Enzyme (t-ACE). Therefore, it is likely that ATP1A4 interacts with other sperm proteins during capacitation. To test this, we used an immunoprecipitation-mass spectrometry approach to compare the interactome profile of ATP1A4 between raft- and non-raft membrane fractions from capacitated sperm.

Proteomic analysis using a gel-based LC-MS/MS approach identified that the non-raft interactome was comprised of hexokinase, plakophilin-1, 14-3-3 protein, cathepsin D, and heat shock protein. A disintegrin and metalloprotease (ADAM) and annexin A2 were exclusive to the raft fraction, whereas actin and plakoglobin were identified in both raft and non-raft fractions of ouabain-capacitated sperm. These differentially interacted proteins are putatively involved in sperm-oocyte interactions, metabolism, and protease activity and they also act as adaptor and cytoskeletal proteins, based on gene ontological information. We validated plakoglobin, among other proteins, due to its significant interaction with ATP1A4. Immunocytochemical staining demonstrated that plakoglobin was localized to the equatorial segment of uncapacitated and capacitated sperm. The ATP1A4 signal was predominantly localized to the anterior acrosome in uncapacitated sperm, became pronounced in the equatorial segment, and co-localized with plakoglobin in capacitated sperm (Fig. 3).

In epithelial cells, E-cadherin, catenins and the actin cytoskeleton mediate cell-cell adhesion (Yamada *et al*., 2005). In addition, E-cadherin and catenin proteins are also expressed in sperm and oocytes (Rufas *et al*., 2000; DeVries *et al*., 2004) where they are specifically localized to the equatorial segment of the sperm head and microvillar region on oolemma (Takezawa *et al*., 2011). If gamete interaction involves mechanisms that are like epithelial cell-cell adhesion, it is likely that E-cadherin and catenin are involved in events leading to sperm-oocyte adhesion and fusion, considering their strategic locations in sperm and in oocytes. Since plakoglobin belongs to the catenin family, interaction between ATP1A4 and plakoglobin could potentially mediate gamete fusion, leading to fertilization. However, the physiological relevance of Na/K-ATPase-plakoglobin interactions remains to be investigated.

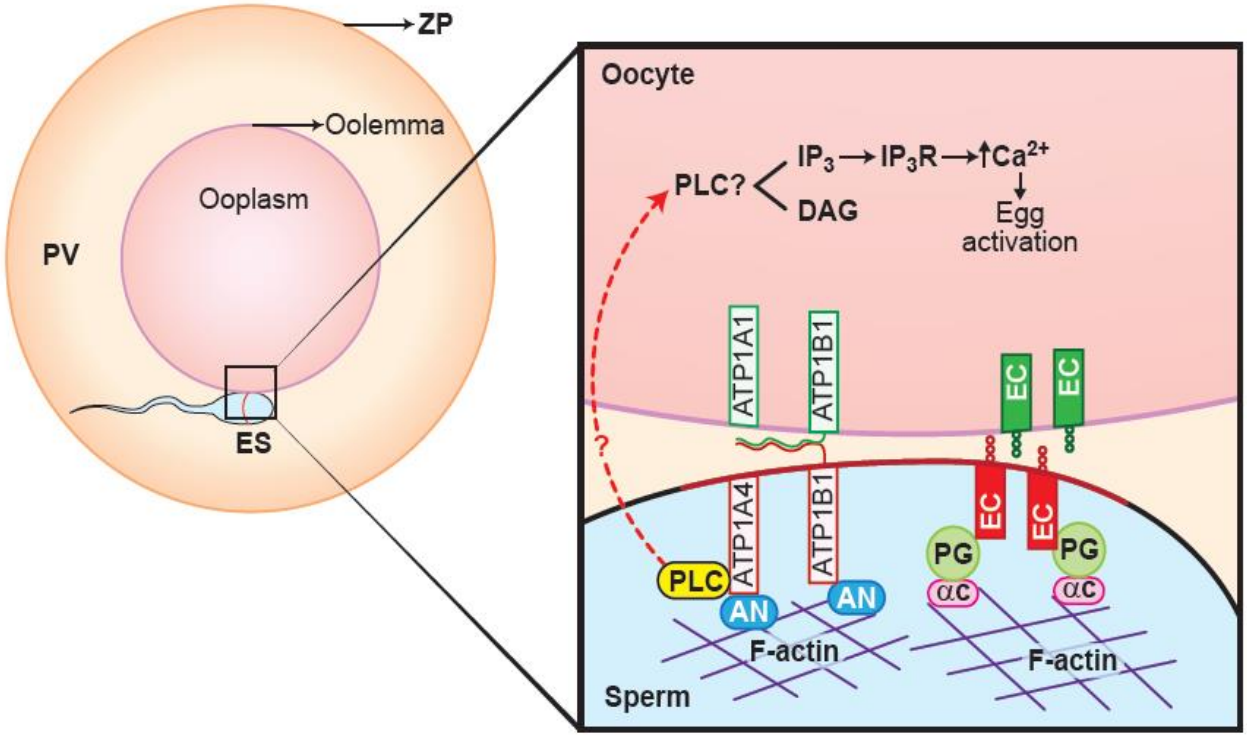

Figure 3. A hypothetical model depicting the involvement of plakoglobin, α and β subunits of ATP1A4, E-cadherin and PLCζ during sperm-oocyte fusion. Complementary E-cadherin molecules on the sperm and oocyte could bind to each other; these interactions could be further strengthened by binding of the cytoplasmic domain of E-cadherin (EC) to plakoglobin (PG) –α-catenin (αC) –actin network. ATP1A4 could bind to an anchor protein, ankyrin (AN), which in turn interacts with the actin cytoskeleton and indirect links ATP1A4 to plakoglobin-E-cadherin complex. This interaction could facilitate entry of sperm-derived PLCζ (indicated by broken arrows) which results in increase of intracellular calcium and resumption of meiosis in the oocyte. In addition, ATP1A4 could be involved in sperm-oocyte interactions by N-glycan motifs-mediated binding of β-subunits of Na/K-ATPase (ATP1B1/ATP1B3) present in gametes.

Capacitation increases the content of ATP1A4 in bovine sperm via mitochondrial translation

Despite the widely accepted dogma of transcriptional/translational quiescence in sperm, we observed that increased ATP1A4 content in raft and non-raft membrane fractions during capacitation with either ouabain or heparin. This increase in ATP1A4 content in the plasma membrane was further confirmed with immunoblotting using total sperm protein extracts, flow cytometry in fixed capacitated sperm, and a simultaneous increase in the enzyme activity of the protein. Furthermore, the increase in content and activity were not due to relocation of this protein to plasma membrane from other subcellular components. Upon further investigation, we also identified that this capacitation-associated increase was partially sensitive to the mitochondrial translation inhibitor chloramphenicol but insensitive to actinomycin D (a transcription inhibitor), suggesting mitochondrial translation of ATP1A4 during capacitation.

Ejaculated sperm contain a complex repertoire of transcripts and intact mRNAs (Sendler *et al*., 2013; Das *et al*., 2013). Furthermore, subcellular localization of mRNA in sperm has been documented (Kumar *et al*., 1993). It was suggested that *de novo* protein translation from sperm mRNA is essential to supplement degraded proteins or to support functional changes during capacitation. This *de novo* protein production was also demonstrated in other mammals, suggesting widespread existence of this mechanism across species (Gur and Breitbart 2006). Mitochondrial ribosomes seem to be responsible for *de novo* protein synthesis during capacitation as sperm lacks classical cytoplasmic machinery (Ostermeier *et al*., 2002; Gilbert *et al*., 2007). Accordingly, several publications have highlighted that cycloheximide (cytoplasmic ribosome inhibitor) did not affect sperm protein synthesis (Bragg and Handel 1979; Ahmed *et al*., 1984; Naz 1998; Gur and Breitbart 2006).

A standard assay to demonstrate protein synthesis in any cell type is incorporation of labeled amino acids into newly synthesized peptides. In our study, bodipy-tagged lysine was incorporated into several proteins at ~110, 75, 50 and 37 kDa; however, in the presence of chloramphenicol (mitochondrial translation inhibitor), band intensities at ~110 and 37 kDa were partially inhibited, whereas bands at 75 and 50 kDa were completely abolished, suggesting that protein synthesis occurred during bovine sperm capacitation. Mitochondrial or mitochondrial-type ribosomes may perform a similar function in sperm, as cytoplasmic machinery is lost during spermatogenesis (Toshimori, 2009). Interestingly, extra-mitochondrial localization of mitochondrial ribosomes, especially the 16S rRNA, has been demonstrated in mouse oocytes and zygotes (Ninomiya and Ichinose, 2007) and sperm nucleus (Villegas *et al*., 2002) and functional competence of such mitochondrial ribosomes has been demonstrated in *Drosophila* embryos (Amikura *et al*., 2001; Amikura *et al*., 2005). In the current model (Fig. 4), we hypothesize that ATP1A4 mRNA, mitochondrial or mitochondrial-type ribosomes and translation initiation and elongation factors are present outside mitochondria (perinuclear theca and post-acrosome regions in the head) in ejaculated sperm. However, ATP1A4 mRNA is prevented from being translated due to the inhibitory action of RBPs (RNA binding proteins) and the unavailability of a functional 55 S mitochondrial ribosome in ejaculated sperm. Capacitation associated changes remove the inhibitory effect of RBPs and the binding of the mRNA and $tRNA^{Met}$ to the small ribosome subunit (28 S). The latter step is markedly enhanced in the presence of GTP. These initial steps recruit the large ribosome subunit (39 S) to join the small ribosome subunit (28 S) and thereby a fully functional mitochondrial ribsome is assembled. The initiation phase is completed, and translation can proceed with elongation and termination phases leading to production of a functional protein (Rajamanickam *et al*., 2017c).

Perhaps post-translational modifications of ATP1A4 are carried out through hydrophobic and electrostatic interactions of membrane-associated molecular chaperones ultimately guiding them to appropriate folding and transportation pathways. Finally, insertion and anchoring of the protein to the plasma membrane could be facilitated by hydrophobic amino acids in the transmembrane domains of ATP1A4 (Homareda *et al*., 1989). Our study indicates the ability of sperm to synthesize protein suggests the importance of atypical, yet functional translation pathways to meet physiological demands during capacitation and opens interesting areas of investigation in mammalian sperm biology.

ATP1A4 as a fertility marker in dairy bulls

In the Canadian Dairy Network (CDN), bull fertility is assessed by 56-day non-return rates (NRR), based on a linear mixed model adjusted for several factors, such as month of insemination, age of the cow or heifer at insemination, breed of service sire, price of inseminating dose, AI technician, and an overall herd management effect, all combined into one index (FERTSOL value). The bovine artificial insemination (AI) industry in Canada uses high-fertility (HF) and low-fertility (LF) Holstein bulls for breeding purposes. Based on this FERTSOL assessment, bulls were classified as either high-fertile (HF; FERTSOL > +1; range: 3.6–6.7) or low-fertile (LF; FERTSOL < -1; range: - 4 to -19).

Since mature sperm DNA is generally transcriptionally quiescent, sperm functions are carried out by existing proteins without additional protein synthesis (with few exceptions). Therefore, sperm proteins may serve as molecular markers for variations in bull fertility (Peddinti *et al*., 2008; D'Amours *et al*., 2010). We proposed that comparing sperm from bulls with varying levels of fertility may identify molecular differences (e.g. expression of specific proteins) in sperm and determine markers of fertility. Using frozen semen from HF and LF bulls as a research model, we investigated the role of ATP1A4 in regulation of fertility in dairy bulls.

Result of this study indicated that frozen-thawed sperm from HF bulls had increased ATP1A4 content and activity compared to LF bulls. A possible explanation for the difference in content between HF and LF bull sperm could be attributed either to loss of ATP1A4 from the sperm membrane of LF bulls during the process of freeze-thawing, or these bulls inherently might have had a lower content of ATP1A4. Unfortunately, it was not possible to investigate the latter possibility, due to a lack of access to fresh semen from these bulls.

Furthermore, post-thaw sperm from HF bulls had increased tyrosine phosphorylation, ROS, F-actin content, and low intracellular calcium concentrations compared to LF bulls. Subsequent incubation of HF sperm with ouabain further augmented post-thaw increases in tyrosine phosphorylation, ROS production, and F-actin content, whereas the increase in intracellular calcium was still low compared to LF sperm. Even though it is widely understood that increased ROS was related to cryo-capacitation (Bailey *et al*., 2000), results from our study indicate otherwise as HF sperm had low ROS content (Del Olmo *et al*., 2015). Perhaps there is a higher subpopulation of normal sperm (with a viable plasma membrane) compared to the cryo-capacitated sperm in HF and LF groups that is responsible for controlled ROS production after thawing. Furthermore, the higher level of ROS in HF sperm may correspond to the minimum threshold needed for successful capacitation (de Lamirande and Gagnon, 1995).

Calcium is another intracellular second messenger, with dynamic roles in hyperactivation and sperm capacitation. The interaction between Na/K-ATPase and inositol 1,4,5-triphosphate receptor (IP_3R), an intracellular calcium store receptor, is regulated by ouabain, thereby increasing calcium concentrations. The presence of IP_3 ligand and its cognate receptor, IP_3R has already been confirmed in sperm (Ho and Suarez, 2003; Wennemuth *et al*., 2003). It is noteworthy that calcium increased in both fertility groups in response to ouabain, albeit at a higher magnitude in LF sperm. Like calcium, regulation of actin dynamics is pivotal for many sperm processes, including capacitation. Controlled production of ROS modulates reorganization of actin cytoskeletal components through a process that involves GTP binding protein Rho and actin binding protein cofilin (Carlier *et al*., 1999; Moldovan *et al*., 2000) or through involvement of gelsolin (Shahar *et al*., 2014). Therefore, it is likely that increased ROS could promote increased F-actin content in HF bull sperm.

Collectively, quantification of ATP1A4 and its associated downstream effectors (ROS, calcium or F-actin) may aid in development of improved laboratory assays for better prediction of fertility of bulls (Rajamanickam *et al.,* 2017b). This could prevent sub-fertile semen from entering the market and thereby improve efficiency of cattle reproduction.

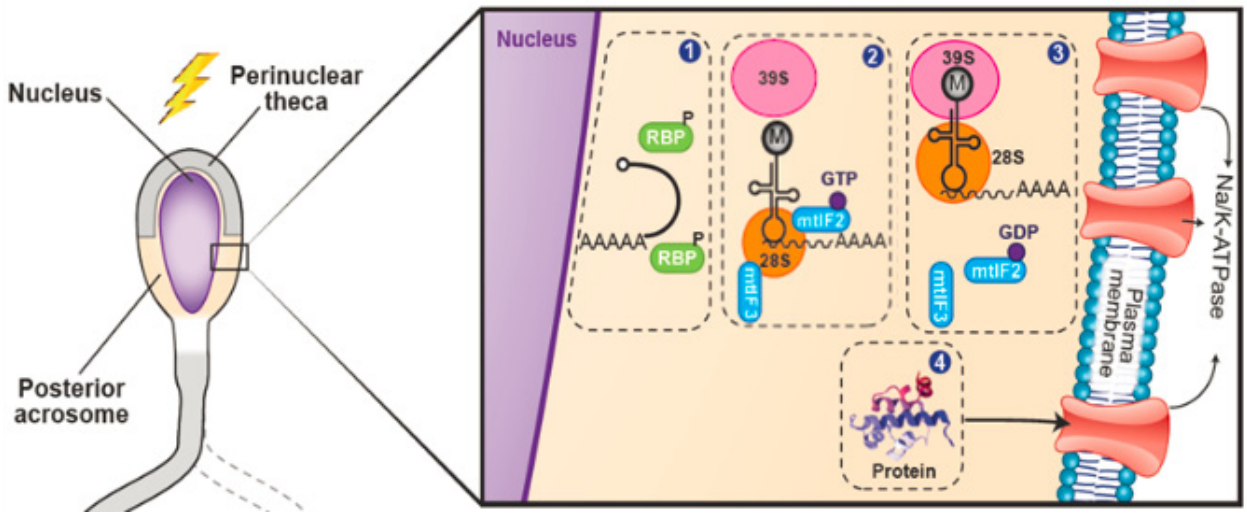

Figure 4. Hypothetical schematic view of the possible mechanisms involved in translation of sperm mRNA during capacitation. During spermatogenesis, RBPs bind with pre-mRNA in the nucleus and are subsequently exported together to the cytoplasm of spermatocytes and early round spermatids. The perinuclear theca (PT) and postacrosomal sheath (PS) in the sperm could serve as translation sites in the sperm head. (1) During capacitation, post-translational protein modifications such as phosphorylation, release the mRNA from the inhibitory effect of RBPs. (2) Mitochondrial translation initiation factors, mtIF3 and mtIF2 facilitate binding of the mRNA and $tRNA^{Met}$, respectively, to 28S (small mitochondrial ribosome subunit) in the presence of GTP hydrolysis. (3) Molecular events described in Step 2 initiate binding of 39S (large mitochondrial ribosome subunit) to 28S-mRNA-tRNA complex and formation of a functional 55S mitochondrial or mitochondrial-type ribosome. (4) Synthesis of ATP1A4 protein and its translocation to the plasma membrane.

Conclusions and future directions

Using fresh and frozen-thawed bull semen, we demonstrated the physiological relevance of ATP1A4 in regulation of sperm function and its potential as a fertility marker. In the raft and non-raft study, we determined that raft and non-raft pools of ATP1A4 stimulated independent signaling pathways, leading to capacitation. Deducing downstream pathways mediated by ATP1A4 could help understand the molecular basis by which this protein functions in normal sperm, which in turn could help develop diagnostic approaches to identify male infertility. Mass spectrometry revealed interesting candidate proteins such as plakoglobin that interacted with ATP1A4 during capacitation. The functional significance of PLCζ as a sperm oocyte activating factor and the role of plakoglobin has been well studied in sperm and testis, respectively. However, the relevance of both these proteins interacting with ATP1A4 during capacitation and fertilization remains to be investigated.

Our results also indicated that there is ATP1A4 mRNA in capacitated sperm. Functionally intact and stable sperm mRNAs can be delivered to the oocyte during fertilization and are important during the critical window of activation of the embryonic genome and its epigenetic regulation (Sendler *et al*., 2013). Perhaps this sperm-specific mRNA persists in the oocyte until activation of the embryonic genome and has critical roles in embryo development. Further studies are expected to advance our current knowledge in the role of this protein in sperm function and fertilization.

Acknowledgements

This study received funding from Natural Sciences and Engineering Research Council (NSERC) of Canada (Grant # RGPN-2014-04850) and Alberta Livestock and Meat Agency (Grant # 2010F027R) awarded to JT.

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DOI: 10.21451/1984-3143-AR2018-0035

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Follicular guidance for oocyte developmental competence

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Abstract

The advancement of folliculogenesis is coincident with the sequential acquisition of oocyte developmental competence. In practical bovine/porcine ART, cumulus-oocyte complexes (COCs) aspirated from small antral follicles have low developmental competence relative to COCs from medium/large antral follicles, as evidenced by a poor capacity to support embryogenesis up to the blastocyst stage. This is in part because of incomplete differentiation of cumulus cells in small antral follicles, in particular under-developed functionality of EGF signalling. Gonadotrophins and oocyte-secreted paracrine factors cooperate to establish EGF receptor functionality in cumulus cells, which appears to be involved in the acquisition of oocyte developmental competence. Here we review the modification of follicular cumulus cells during antral folliculogenesis involved in oocyte developmental competence.

Keywords: cumulus cells, oocyte**,** oocyte secreted factors.

Introduction

The developmental competence of an oocyte, referring to its capacity to support fertilization, preimplantation embryo and subsequent full-term development, is inherently linked to environmental cues it receives from the follicular somatic cell compartment (Gilchrist and Thompson, 2007). The follicular soma is regulated principally by gonadotropic cues (FSH and LH) during the course of folliculogenesis (Scaramuzzi *et al*., 2011).

Oocytes acquire increasing developmental competence sequentially with advancing folliculogenesis and as such oocyte and follicle development are inter-dependent processes (Thibault, 1977). Hence, as the obligatory driver of folliculogenesis, FSH is also the central driver of the acquisition of oocyte developmental competence, facilitating a myriad of follicular somatic cell functions that nurture the growth and development of the oocyte. During the terminal phases of folliculogenesis and oogenesis, FSH promotes expression of the LH and epidermal growth factor (EGF) receptors on granulosa cells (Erickson *et al*., 1979; El-Hayek *et al*., 2014). FSH also facilitates the bilateral communication between granulosa cells and between the oocyte and granulosa/cumulus cells (CCs) by promoting gap junctional communication (GJC; El-Hayek and Clarke, 2015), likely via cyclic adenosine monophosphate (cAMP)-phosphate kinase A (PKA) pathway activation. Oocyte-CC GJC is responsible for the transfer of small molecules such as cAMP, cyclic guanosine monophosphate (cGMP) and metabolites from the CCs to the oocyte (Sugimura *et al*., 2014; Li *et al*., 2016). In addition, vesicles and potentially RNA may traffic to the oocyte from the CC's transzonal projections (Macaulay *et al*., 2014) which, together with GJC, regulate oocyte meiotic maturation and developmental competence (Sugimura *et al*., 2014; Li *et al*., 2016; Russell *et al*., 2016).

Key propagators of the ovulatory cascade within the follicle are the EGF-like peptides; amphiregulin (AREG), epiregulin, and betacellulin, which are produced by mural granulosa cells in response to LH, and signal via the EGF receptor (EGFR) that is expressed on mural granulosa and CCs (Park *et al*., 2004; Shimada *et al*., 2006; Hsieh *et al*., 2007). EGFR activation, in cooperation with the oocyte-secreted factors (OSFs), stimulates gene expression that enables cumulus expansion and ovulation. Two OSFs that facilitate this and are essential for ovulation and oocyte capture by the infundibulum are bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9), which are structurally similar members of the transforming growth factor β family that can also form a heterodimer termed cumulin (Mottershead *et al*., 2015). Hence, the final steps of oocyte development prior to ovulation are dictated by endocrine cues mediated by the gonadotrophins and by paracrine cues from the oocyte, intersecting in the CCs as the key facilitators of oocyte developmental competence (Russell *et al*., 2016; Richani and Gilchrist, 2018).

This review will focus on the recent advances in our knowledge of the contribution of endocrine and paracrine cues in the differentiation of follicular granulosa and CCs during antral folliculogenesis, and their contribution to oocyte developmental competence. This knowledge is important for the application of advanced reproductive technologies in domestic animal breeding and in humans. The successful clinical application of the key reproductive technologies of superovulation (combined with artificial insemination or IVF) and oocyte *in vitro* maturation (IVM) are critically dependent on this knowledge.

EGFR signaling and oocyte developmental competence

It is now well established that the EGF network is an essential propagator of the ovulatory signal in the

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Received: February 22, 2018
Accepted: May 22, 2018

Graafian follicle to the cumulus-oocyte complex (COC; Park et al., 2004; Conti et al., 2012). The concept that the oocyte sequentially acquires developmental competence to support fetal development throughout antral follicle development has long been clear (Eppig *et al*., 1992; Lonergan *et al*., 1994). Hence, oocytes from small antral follicles have a significantly poorer capacity for cumulus expansion and for the support of early embryogenesis, compared with those from large antral follicles. Indeed this concept serves as the basis for the need for superovulation of women undergoing IVF and of cattle undergoing multiple ovulation and embryo transfer (MOET).

Prochazka *et al*. (2003) hypothesized that porcine COCs from small antral follicles (3-4 mm) have an under-developed EGFR. Indeed, there is a growing body of evidence showing that the development of a functional EGF network in follicular granulosa cells is suppressed until the peri-ovular stage as a mechanism of limiting ovulation to the dominant follicle(s), and thus to the developmentally competent oocyte(s); Richani and Gilchrist, 2018). The exact timing of acquisition of functional EGFR signalling in COCs and the associated molecular changes in the oocyte are still unclear, but for experimental purposes such studies commonly divide the antral phase of folliculogenesis in half into small versus large antral follicles. In pigs for example, <4mm (small follicles) vs. >4 mm (medium-large follicles) are typically compared (Prochazka *et al*., 2003; Ritter *et al*., 2015), whereas in cattle which ovulate larger follicles, a medium-large antral follicle is 6-10 mm (Lonergan *et al*., 1994). Using such models, porcine COCs derived from small antral follicles (<4 mm) are unresponsive to all EGF family ligands and COCs exhibit progressive acquisition of EGF responsiveness with follicle growth (Prochazka *et al*., 2000, 2003; Diaz *et al*., 2007; Ritter *et al*., 2015). The acquisition of EGF signalling capability by CCs with advancing follicle growth coincides with oocyte acquisition of developmental competence, and the two are likely to be related (Ritter *et al*., 2015; Sugimura *et al*., 2015). These latter recent findings suggest that the EGF network may contribute to oocyte quality by providing regulatory cues from the cumulus cells to the oocyte which regulate oocyte integrity (Gilchrist and Richani, 2013; Richani and Gilchrist, 2018). The EGF network also mediates mRNA translation in the transcriptionally silent oocyte during the course of meiotic maturation to the metaphase II stage (Chen *et al*., 2013). This directly impacts oocyte quality as genetic perturbation of *Areg* expression in mouse cumulus cells leads to reduced fecundity (Chen *et al*., 2013).

An understanding of the cellular and molecular mechanisms leading to the development of EGF signalling capabilities with follicle development can provide new approaches for improving the success of oocyte IVM, a reproductive technique that involves the maturation of COCs derived from small antral follicles of unstimulated ovaries (Gilchrist, 2011). Granulosa-type cells (preantral, mural, and cumulus) express EGFR mRNA and protein throughout folliculogenesis, and is activated by a range of EGF family members to promote follicle growth (Roy 1993; Garnett *et al*., 2002). Several studies have shown that EGFR mRNA expression is lower in small antral follicle CCs than their large antral follicle counterparts (Singh *et al*., 1995; Prochazka *et al*., 2003; Caixeta *et al*., 2009; El-Hayek *et al*., 2014). However, in a porcine model, we observed (Ritter *et al*., 2015) CCs from small antral follicles (<4 mm) exhibit equal expression of EGFR transcripts as those from larger antral follicles (>4 mm), however EGFR protein production and phosphorylation and subsequent downstream ERK1/2 activity were perturbed. Hence, COCs from small antral follicles are unresponsive to EGF peptides and cannot undergo expansion. There is now good evidence that the EGF signalling network is under-developed in COCs from small antral follicles (Richani and Gilchrist, 2018; Fig. 1).

Establishing cumulus EGFR signaling requires orchestration by endocrine cues and oocyte-secreted factors

Details of the mechanisms responsible for the maturation of the EGF network in CCs remain unclear, however recent evidence suggests that cooperation between endocrine (FSH) signaling and paracrine signals from the oocyte is essential in this process. FSH plays a major and essential role in follicle development. Follicles of $Fshb^{-/-}$ null mice exhibit perturbations in CC differentiation and oocyte meiotic maturation, as well as a deficiency in follicle Egfr mRNA expression (El-Hayek *et al*., 2014). FSH sensitivity is long understood to be a determining factor in follicle selection; recent evidence suggests that this may in part be due to FSH promoting development of a functional EGF network throughout antral follicle growth. Prochazka *et al*. (2003) demonstrated that *in vitro* treatment of porcine small antral COCs with FSH promotes EGF responsiveness and subsequent EGFR signaling. EGFR signaling is essential for cumulus expansion, and requires activity of the FSH downstream effector protein kinase A (PKA; Prochazka *et al*., 2012). Hence, evidence from mice and domestic animal models suggests that endocrine cues are needed for the maturation of the EGF signaling network in CCs, and that the FSH-cAMP-PKA signalling axis mediates this.

Recent evidence has shown that the oocyte itself also significantly contributes to the development of the EGF network throughout the antral phase of folliculogenesis by mediating the effects of FSH. Alone, FSH is insufficient in inducing EGF responsiveness in granulosa and cumulus cells (Fig. 1). Prior to differentiation into cumulus cells, preantral granulosa cells are unable to undergo expansion. Diaz *et al*. (2006, 2007) showed that this is due to the immaturity of the pre-antral oocyte; murine preantral granulosa acquire the capacity to undergo EGF-induced expansion if cultured with oocytes from large antral follicles as well as treated with FSH, suggesting that the oocyte modulates its secretome to regulate the follicle's response to external endocrine cues. In addition, Sugimura *et al*. 2015) recently showed that small antral porcine COCs can be induced to respond to AREG following exposure

to the FSH effector cAMP. However, this required the exogenous addition of GDF9 or BMP15, since cAMP alone had little effect on AREG-induced meiotic maturation, cumulus expansion, ERK1/2 phosphorylation, or blastocyst development in small antral oocytes in the absence of GDF9 and BMP15 (Sugimura *et al*., 2015). This is consistent with the findings that GDF9 and BMP15 facilitate CC EGFR expression via SMAD2/3 (Su *et al*., 2010), and that for full GDF9 signalling to occur, cooperation between the EGFR-ERK1/2 and SMAD2/3 pathways in granulosa and cumulus cells is required (Sasseville *et al*., 2010). EGF responsiveness can be induced in CCs from small antral follicles (<4 mm) by co-culture with oocytes from larger antral follicles (>4 mm) (Ritter *et al*., 2015). In contrast, EGF responsiveness in CCs from small antral follicles cannot be induced by co-culturing with low competence oocytes of small antral follicles, demonstrating that native factors secreted from developmentally competent oocytes mediate this process, and that the oocyte alters its secretome throughout folliculogenesis to regulate CC acquisition of EGFR signaling. This suggests that EGF responsiveness is a milestone in the growth and development of the COC throughout folliculogenesis (Ritter *et al*., 2015). This mechanism is likely in place to perturb meiotic resumption and ovulation of oocytes that have not completed folliculogenesis which are growing in the presence of continual surges of endocrine LH and FSH during menstrual cycles. Hence, in the same way that FSH promotion of LH receptor signalling marks the development of mural granulosa cells, dual oocyte- and endocrine FSH-induction of EGF responsiveness in CCs represents a developmental milestone in folliculogenesis (Fig. 1; Ritter *et al*., 2015; Richani and Gilchrist, 2018).

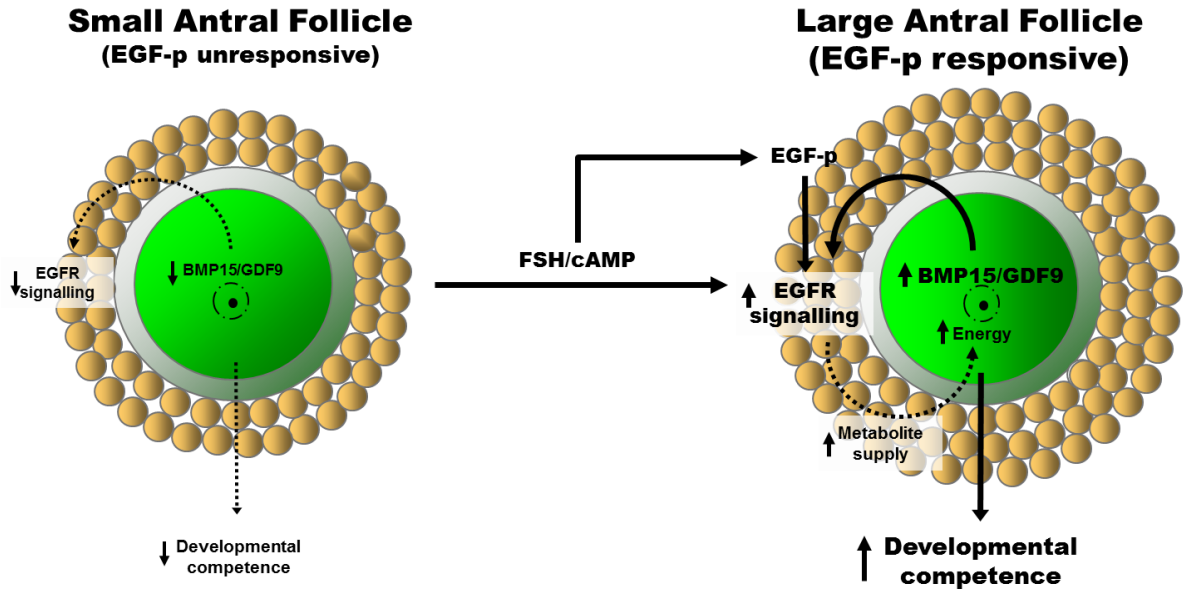

Figure 1. Hypothetical mechanism of cooperation between endocrine FSH priming and oocyte paracrine signals to promote EGFR signaling in cumulus cells. The acquisition of EGFR signaling capability by cumulus cells is a developmental hallmark for the COC. Cumulus-oocyte complexes derived from small antral follicles, which have low developmental competence, exhibit under-developed EGFR signaling as they are unresponsive to EGF-peptides (EGF-p). As folliculogenesis progresses, EGFR functionality is induced in COCs by the concerted actions of FSH/cAMP and oocyte-secreted factors (of which BMP15 and GDF9/cumulin have been identified), and is associated with increased oocyte developmental competence. Improved oocyte developmental competence may be facilitated by EGF-peptide stimulation of cumulus cell glycolysis and provision of metabolites (e.g. NAD(P)H) to the oocyte facilitating oocyte mitochondrial activity and energy production needed for development. Figure from Richani and Gilchrist (2018) adapted from Sugimura *et al*. (2015) with permission.

Modification of cumulus cells by FSH-priming

As mentioned above, promotion of EGFR signalling in CCs might be a key component in the acquisition of oocyte developmental competence (Ritter *et al*., 2015; Richani and Gilchrist, 2018). However, this is not the full story since the CCs of mouse COCs derived from small antral follicles which have received no gonadotropin priming are able to undergo a degree of expansion *in vitro* (Vanderhyden *et al*., 1990; Wigglesworth *et al*., 2015). CCs from small antral follicles exhibit a more immature capacity for metabolism, inter-cell communication and cell differentiation at the transcriptome level (Wigglesworth *et al*., 2015), suggesting that the EGFR network is likely one of several signaling pathways which develop with follicle growth to participate in the acquisition of oocyte developmental competence.

In vivo priming of follicles prior to final oocyte maturation enhances oocyte developmental competence (Hendriksen *et al*., 2000). This priming can be driven by exogenous FSH administration; the oocytes derived from

cows subjected to FSH treatment prior to oocyte collection are more developmentally advanced than those from unstimulated cows (Sirard *et al*., 1999; Sugimura *et al*., 2012), likely due in large part to the drastically altered transcriptome it induces in CCs (Fig. 2; Sugimura *et al*., 2017). Our recent RNA-seq analysis showed substantial alteration to CC gene transcription following *in vivo* FSH-priming, with the majority of altered transcriptomic genes being downregulated (Sugimura *et al*., 2017). Surprisingly, the matrix-formation genes HAS2, TNFIP6, and PTX3, which are typically up-regulated in CCs during oocyte maturation and are associated with oocyte developmental capacity (Gebhardt *et al*., 2011; Wathlet *et al*., 2011), were downregulated, however this may be attributed to the timing of cell collection, whereby CCs were collected from immature unexpanded COCs (Sugimura *et al*., 2017).

FSH-priming also enhances CC transcripts associated with interferon signaling and interferon regulatory factor (IRF) activation, including interferon-stimulated genes (Sugimura *et al*., 2017). IRF7, a regulator of the viral IFNα/β immune response (Honda *et al*., 2005), ranked first in the list of activated upstream regulators in CCs from FSH-primed cows (Sugimura *et al*., 2017). TGFB1 regulates IRF7 expression whereby prolonged exposure to TGFB1 promotes downregulation of IRF7 (Cohen *et al*., 2014). In granulosa cells, increased TGFB1 is a hallmark of the activated inflammatory process, which may stimulate follicular atresia (Hatzirodos *et al*., 2014a). Pathway analysis predicted TGFB1 as an inhibited upstream regulator and the expression of TGFB1 and the receptor TGFR2 were lower in CCs from animals primed with FSH (Sugimura *et al*., 2017). In addition, CC expression of transcripts associated with follicle atresia, including TSP1 downregulation and TGFB and TP53 upregulation, (Thomas *et al*., 2008), is suppressed by FSH-priming. FSH-priming also upregulated cumulus expression of CYP19A1, an indicator of healthy large follicles (Irving-Rodgers *et al*., 2009; Hatzirodos *et al*., 2014b). In the natural oestrus cycle of mono-ovulatory animals, a dominant follicle is ultimately selected for ovulation, whilst other growing follicles undergo atresia induced by granulosa cell signalling; as a result of the decrease in survival factors, predominantly FSH (Scaramuzzi *et al*., 2011). Hence, most COCs collected from antral follicles from a natural oestrus cycle ovary come from follicles at varying stages of atresia. Artificial control of follicular development through the administration of exogenous FSH rescues many antral follicles from atresia via promotion of survival by promoting anti-inflammatory mechanisms such as IRF7 (Sugimura *et al*., 2017). Whilst LH-induced ovulation induces an inflammatory cascade across the follicle, prior to the LH-surge, FSH is responsible for maintaining an anti-inflammatory state to maintain CC integrity. FSH causes suppression of STAT3 signaling (Ilha *et al*., 2015), and genetic perturbation of STAT3 expression has been shown to increase expression of IFNα/β response genes, including OAS and IRF7 (Wang *et al*., 2011). Hence, the net effect on COCs of FSH-priming of animals is decreased inflammatory signals and atresia, contributing to increased oocyte developmental competence (Fig. 2).

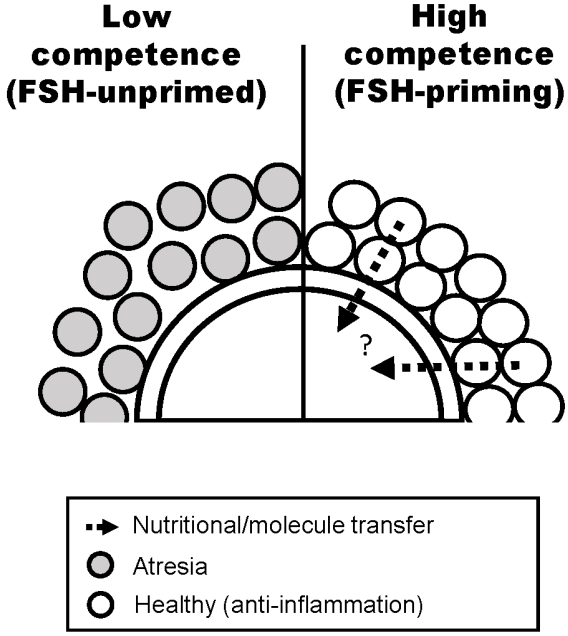

Figure 2. Hypothetical model of the effect of FSH-priming on bovine cumulus cells based on RNA-seq expression signature. Cumulus cells in a low competence model without FSH-priming exhibit a transcriptomic signature suggestive of decreased inter-cell communication, increased atresia, and driving a spontaneous ovulation-like cascade. Conversely, cumulus cells in a high competence model following FSH-priming are in a state of increased inter-cell communication which promotes the transport of molecules from cumulus cells to oocytes, and exhibit increased anti-inflammatory signals at the time of final oocyte maturation. Figure adapted from Sugimura *et al*. (2017).

Conclusion

An important function of follicular somatic cells in follicles approaching ovulation is to prepare the oocyte for final maturation, in part by promotion of cumulus EGFR signaling, as well as prevention of inflammation and promotion of cell–to-cell communication. The latter facilitates the efficient transfer of molecules from the somatic compartment of the follicle to the oocyte. These processes are likely to constitute important components of oocyte developmental competence in humans and ruminants.

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DOI: 10.21451/1984-3143-AR2018-0021

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

The variable success of in vitro maturation: can we do better?

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Abstract

The efficiency of *in vitro* assisted reproductive technologies, consisting of the transfer of embryos obtained *in vitro* through *in vitro* maturation, *in vitro* fertilization and early embryo culture is still limited. The quality of the oocytes is pivotal for assisted reproductive efficiency and the maturation of the oocyte represents the first key limiting step of the *in vitro* embryo production system. At the time of removal from the antral follicles, the oocyte is still completing the final growth and differentiation steps, needed to provide the so-called developmental competence, i.e. the machinery required to sustain fertilization and embryo development. In mono-ovular species only one oocyte per cycle is available for procreation, therefore the current assisted reproduction techniques strive to overcome this natural boundary. However, the success is still limited and overall the effectiveness does not exceed the efficiency achieved in millions of years of mammalian evolution. One of the problems lies in the intrinsic heterogeneity of the oocytes that are subjected to *in vitro* maturation and in the lack of dedicated *in vitro* approaches to finalize the differentiation process. In this review we will try to overview some of the salient aspects of current practices by emphasizing the most critical and fundamental features in oocyte differentiation that should be carefully considered for improving current techniques.

Keywords: cAMP, cGMP, chromatin, gap junction, meiotic arrest, oocyte, pre-IVM.

Introduction: the math of the *in vitro* embryo production (IVP) system

In cattle the efficiency of *in vitro* assisted reproductive technologies (ART), entailing of the transfer of embryos obtained through *in vitro* maturation (IVM), in vitro fertilization (IVF) and *in vitro* early embryo culture (IVC) is still limited.

Despite the potential advances offered by *in vitro* embryo production (IVP) systems, the percentage of success in cow remained stunning stable over the last 30 years and is limited to one third of the oocytes isolated from the ovary reaching the blastocyst stage of embryonic development (Lonergan and Fair, 2008; Galli, 2017). In bovine IVP, on a percentage basis, starting from 100 oocytes only about one third become a blastocyst after IVM, IVF and IVC, and only about one third of these embryos are able to produce a born calf (Fig. 1). Considering that, on average, 5 healthy cumulus-oocyte complexes (COCs) per ovary are collected and subjected to IVP procedures (Merton *et al.,* 2003), each ovary produces 1.75 blastocyst that finally gives approximately 0.6 calves born (Fig. 1). On a per animal basis, the IVP system brings a modest improvement respect to the physiological condition of a mono-ovular species and very close to mother's nature results after 29 million years of natural selection and evolution in the bovine lineage.

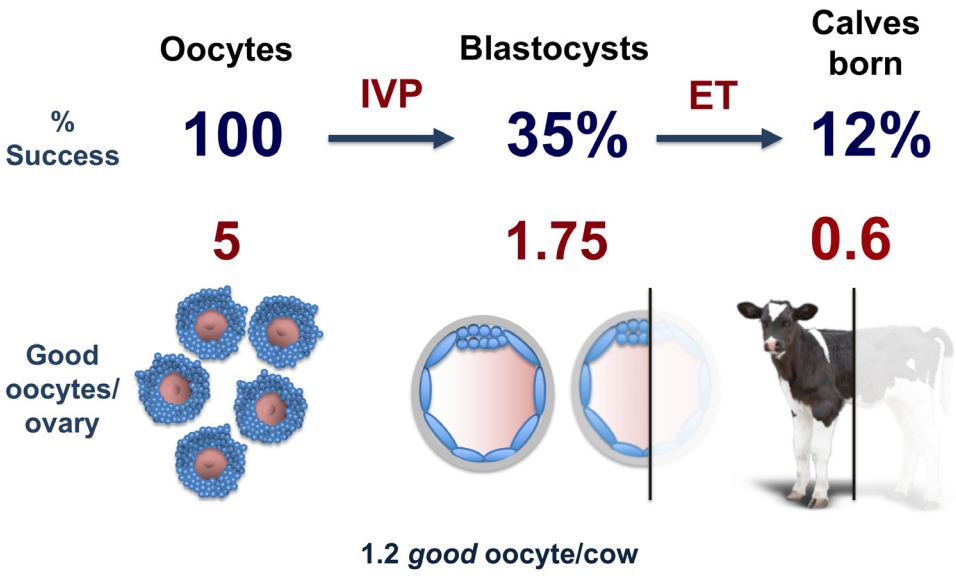

Figure 1. The current efficiency of standard IVP system in the bovine species.

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Received: February 4, 2018
Accepted: May 4, 2018

The physiological issue

The quality of the oocytes is pivotal for assisted reproductive technologies outcome and the maturation of the oocyte represents the first key limiting step of the IVP system. Researches are trying to deal with the improvement of IVP systems and in particular with the IVM step.

Development of IVM techniques was made possible starting from 1935' Pincus and Enzmann observation that oocytes removed from antral follicles before natural ovulation spontaneously resume meiosis (Pincus and Enzmann, 1935). Thus, in standard IVM techniques, oocytes are collected from antral follicles and cultured *in vitro* up to the metaphase II (MII) stage with the emission of the first polar body. However, when oocytes are collected in pools from antral follicles, the processes necessary to confer full meiotic and developmental competence must be completed in a considerably high proportion of them. As a result, the oocytes ability to be fertilized or develop into embryos or to term might be compromised.

In the cyclic cow, the oocyte reaches its final size of around 120 µm when the follicle ranges a diameter of 2-3 mm (Fair *et al.,* 1995). The selection for dominance occurs when the follicles develop from 3 to 8 mm (Dieleman *et al.,* 2002; Adams *et al.,* 2008). Moreover, additional and essential processes occur *in vivo* during the following follicular growth and dominance phase until ovulation (Blondin *et al.,* 1997), when follicles reach a diameter of about 15 mm (Lussier *et al.,* 1987; Fortune, 1994). These processes are referred to as prematuration or capacitation and occur when a follicle is selected to become dominant and are accomplished shortly before the LH surge triggers the final maturation, as depicted in Fig. 2 (Hyttel *et al.,* 1997; Mermillod and Marchal, 1999; Dieleman *et al.,* 2002). Therefore, in the IVP practice, only few oocytes collected from 3-8 mm antral follicles develop *in vitro* into blastocysts and result in viable offspring after transfer presumably because they did not complete the dominant and preovulatory follicular development, which are crucial for the achievement of the full competence proper of the ovulatory oocyte (Hyttel *et al.,* 1997; Dieleman *et al.,* 2002).

From the above observations, it is clear that even if an oocyte collected from a 3 mm follicle has an intrinsic capacity to develop into an embryo after IVM-IVF-IVC, it still requires an additional time to acquire all of the capabilities leading to successful implantation in the uterus and maintenance of gestation to term and to yield healthy offspring, after IVF and IVC. These studies have naturally led to the design of *in vitro* approaches that take into account the need for the oocyte to complete its differentiation, namely a prematuration or capacitation step before *in vitro* maturation.

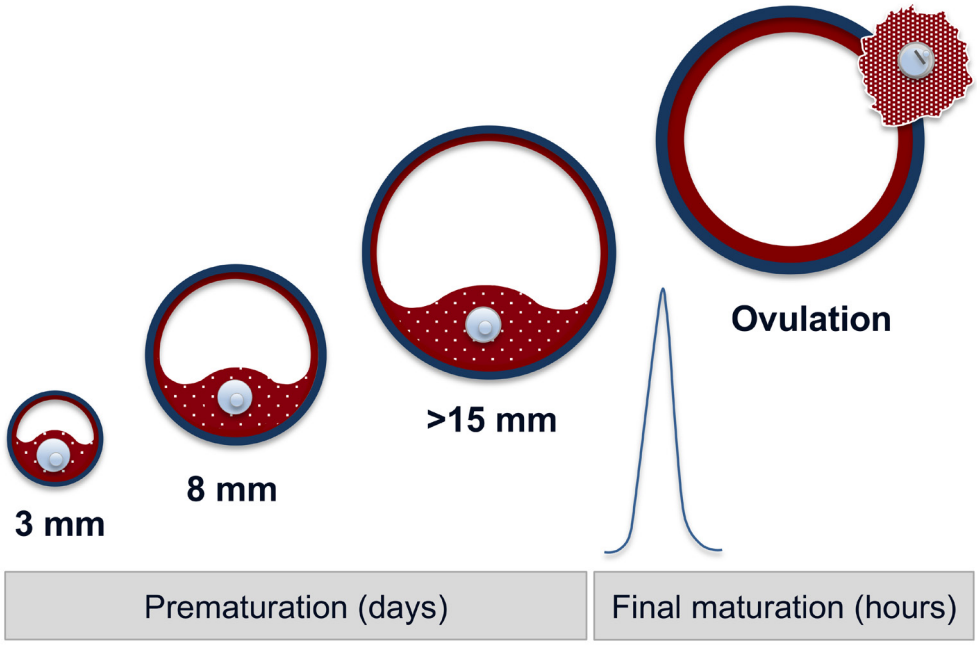

Figure 2. A schematic representation of the prematuration concept. Prematuration refers to the processes occurring *in vivo* during the phase of selection for dominance, when the follicles develop from 3 to 8 mm, and successively when a follicle is selected to become dominant. Finally, prematuration is accomplished shortly before the LH surge triggers the final maturation.

The *in vitro* prematuration step

Controlling cAMP

The identification of the mechanisms that control oocyte meiotic arrest and resumption (reviewed in Conti *et al.,* 2012) has provided the molecular tools to block or delay spontaneous meiotic resumption after removal from the follicle in *in vitro* prematuration studies (reviewed in Bilodeau-Goeseels, 2012). A firmly established concept is that meiotic arrest of the oocyte relies on high concentrations of the second messenger cyclic AMP (cAMP; Cho *et al.,* 1974; Dekel and Beers 1978; reviewed in Conti *et al.,* 2012), which

is known to regulate oocyte and granulosa cell functions (Luciano and Peluso 1995; Conti, 2002). Oocyte cAMP level is sustained by endogenous adenylate cyclase and constitutively active G-protein-coupled receptors (Mehlmann *et al.,* 2002). cAMP is generated also by cumulus cells and then transported into the oocyte through gap junctions (Anderson and Albertini, 1976; Bornslaeger and Schultz, 1985; Luciano *et al.,* 2005). At the same time, intra-oocyte cAMP concentration is regulated by the activity of the oocyte specific phosphodiesterase 3A (PDE3A) enzyme that degrades cAMP into 5'AMP (Tsafriri *et al.,* 1996), whose activity is inhibited by cyclic guanosine 3',5'-monophosphate (cGMP; Norris *et al.,* 2009; Vaccari *et al.,* 2009). cGMP is produced in the cumulus cells upon the activation of the guanilyl-cyclase coupled natriuretic peptide receptor type-2 (NPR2; Robinson *et al.,* 2012) whose activity is induced by its ligand natriuretic peptide precursor C (CNP), which is mainly synthesized by mural granulosa cells (Zhang *et al.,* 2010, 2011). cGMP is then transferred via gap junctions (Richard and Baltz, 2014) to the oocyte where it inhibits PDE3A thus contributing to the maintenance of meiotic arrest (Norris *et al.,* 2009; Vaccari *et al.,* 2009).

Several physiological methods for artificially maintaining meiotic arrest in bovine oocytes have been developed since 1980' with variable success (Leibfried and First, 1980; Sirard and Bilodeau, 1990; Sirard and Coenen, 1993; De Loos *et al.,* 1994; Richard and Sirard, 1996; Fouladi Nashta *et al.,* 1998). Starting from the modulation of cAMP content, numerous IVM systems have been suggested for stimulating oocyte maturation and embryonic developmental competence acquisition (Gilchrist *et al.,* 2016).

Numerous pharmacological and physiological agents have been used to modulate the oocyte cAMP concentration in order to temporally control the oocyte's meiotic arrest and resumption (Gilchrist *et al.,* 2016). For instance, cAMP concentration has been manipulated through the use of broad-spectrum such as IBMX or specific inhibitors of phosphodiesterases (PDEs) such as cilostamide, milrinone or Org9935, by activators of adenylate cyclase (forskolin, iAC), through cAMP analogs (dbcAMP) or by a combination of these agents. Several laboratories have shown that a delay of meiotic resumption has a beneficial effect on embryonic developmental competence. Specifically, the manipulation of intracellular cAMP concentration affects the functional coupling between oocyte and cumulus cells so that a decrease in cAMP determines a drop in gap junction-mediated intercellular communications (Luciano *et al.,* 2004; Thomas *et al.,* 2004). On the other hand, treatments that sustained the intracellular cAMP level prevented the loss of cumulus-oocyte communications and increased oocyte developmental competence (Luciano *et al.,* 1999, 2011; Guixue *et al.,* 2001; Modina *et al.,* 2001; Atef *et al.,* 2005; Nogueira *et al.,* 2006; Ozawa *et al.,* 2008; Shu *et al.,* 2008; Nogueira and Vanhoutte, 2009; Albuz *et al.,* 2010; Dieci *et al.,* 2013; Lodde *et al.,* 2013; Rose *et al.,* 2013; Zeng *et al.,* 2013; Richani *et al.,* 2014). In different systems the preservation of the cAMP concentration during several hours of prematuration culture seems to be the main requirement to promote regular chromatin transition thus endorsing oocyte differentiation (Nogueira *et al.,* 2006; Vanhoutte *et al.,* 2007; Luciano et al., 2011; Dieci *et al.,* 2013; Lodde *et al.,* 2013; Sanchez *et al.,* 2015) and an increase in embryonic developmental competence and/or quality (Luciano *et al.,* 2011; Dieci *et al.,* 2013; Lodde *et al.,* 2013; Franciosi *et al.,* 2014; Azari-Dolatabad *et al.,* 2016; Li *et al.,* 2016; Park *et al.,* 2016; Soares *et al.,* 2017).

However, in most of the above cited cases the reported results showed only a slight improvement in developmental competence depending on the approach used. Often, rather than increasing the number of blastocysts obtained, an improvement in the parameters related to embryo quality was observed (Luciano *et al.,* 2011; Dieci *et al.,* 2013, 2016; Lodde *et al.,* 2013; Rose *et al.,* 2013; Franciosi *et al.,* 2014; Zeng *et al.,* 2014; Azari-Dolatabad *et al.,* 2016; Li *et al.,* 2016; Park *et al.,* 2016; Sanchez *et al.,* 2017; Soares *et al.,* 2017). For instance, blastocysts with higher number of cells or embryo with better developmental kinetics. In other cases, results showed no significant improvement over the current standard *in vitro* embryo production system (Jee *et al.,* 2009; Gharibi *et al.,* 2013; Guimaraes *et al.,* 2015; Diogenes *et al.,* 2017).

Controlling MPF

In addition to cyclic nucleotide manipulation-based methods, other approaches have been used to prevent meiotic resumption. Downstream to cAMP transduction pathway is M-phase promoting factor (MPF), a heterodimer consisting of a kinase, cdk1 and its regulatory partner, cyclin B (cdk1-cyclin B), which is involved in the regulation of G2/M cell cycle transition of all eukaryotic cells. Cyclic AMP-mediated Protein Kinase A (PKA) activity inhibits Cdk1 hence contributing to oocyte meiotic arrest (Mehlmann, 2005). The activation of MPF is also a key point of meiotic resumption in oocytes that corresponds to a G2/M transition (Jones, 2004; Mehlmann, 2005).

Several pharmacological approaches aimed to interfere with MPF activity have been used to artificially maintain mammalian oocyte in meiotic arrest. Cell permeable and selective inhibitors of the CDK1/cyclin B kinase, butyrolactone-I (Kitagawa *et al.,* 1993) and roscovitine (Meijer *et al.,* 1997) have received more attention. Butyrolactone-I has been shown to reversibly inhibit meiotic resumption in bovine (Kubelka *et al.,* 2000; Lonergan *et al.,* 2000; Ponderato *et al.,* 2001, 2002; Imai *et al.,* 2002; Adona *et al.,* 2008; Ferreira *et al.,* 2009; De Bem *et al.,* 2011) and pig (Wu *et al.,* 2002) oocytes for 24 h without negatively affecting the subsequent development to the blastocyst stage. Similarly, roscovitine was effective in reversibly maintain oocyte meiotic arrest in both cow (Mermillod *et al.,* 2000; Sa Barretto *et al.,* 2011) and pig (Marchal *et al.,* 2001). Moreover, the combination of both substances did not cause detrimental effects on development to the blastocyst stage (Ponderato *et al.,* 2001), and subsequent early stages of organogenesis

(Ponderato *et al.,* 2002). However, when compared to the standard IVP system, the majority of the studies reported no significant improvements in embryonic developmental competence when oocytes were arrested with MPF inhibitors and subsequently matured and fertilized *in vitro*. Nonetheless, the use of butyrolactone and roscovitine have been reported to induce some modifications in the oocytes at ultrastructural level (Lonergan *et al.,* 2003), and whether or not these modifications are compatible with normal gestation and live births is still debated.

What's the matter with *in vitro* prematuration?

Notwithstanding the meiotic arrest method used, little to no improvement has been observed in the embryonic developmental competence when oocytes were cultured in bulk, regardless their follicular origin. We believe that the high heterogeneity of the population of COCs subjected to *in vitro* prematuration protocols is responsible for its limited success rate. In most of the cases, indeed, studies on *in vitro* prematuration efficiency have been conducted on ovaries obtained from slaughtered animals, thus contain all types and sizes of antral follicles representing different stages of oocyte development. Considering that in the absence of hormonal synchronization treatments, the follicle population in the ovary is heterogeneous, the stage of differentiation of the oocyte should be taken into account (Luciano and Sirard, 2018). In the following paragraphs we will describe the studies that sustain this hypothesis and, at the same time, have led to define morphological and molecular markers to identify the most suitable prematuration conditions.

A pre-maturation approach thoughtful of oocyte physiology

The oocyte acquires developmental competence just prior to ovulation. Oocyte developmental competence is usually defined as the ability of a female gamete to mature into an egg with its capability to be fertilized and sustain embryo development to the blastocyst stage (Conti and Franciosi, 2018). Nevertheless, identifying oocytes that have achieved the final competence is extremely arduous. One morphological indicator is large-scale chromatin configuration, which changes while the oocyte grows and differentiates during follicular antral development (De La Fuente, 2006; Luciano and Lodde, 2013). This has led to the identification of distinct stages in which the chromatin becomes progressively more compact and occupies a smaller area of the oocyte nucleus or germinal vesicle (GV) in all mammals studied so far (reviewed in Luciano and Lodde 2013; Luciano *et al.,* 2014).

In cows, the four chromatin configurations described correspond to different stages of developmental competence (Lodde *et al.,* 2007). In the GV0 configuration the chromatin appears mostly uncondensed and dispersed throughout the nucleoplasm, while the appearance of few foci of condensation marks the transition to the GV1 configuration. Further compaction into distinct aggregates characterizes the GV2 configuration while the highest level of compaction occurs in GV3, where the chromatin appears as a single clump in a restricted area of the nucleus (Lodde *et al.,* 2007). These stages accompany follicle development. Nearly 90% of the oocytes isolated from early antral follicles (0.5 to 2 mm in diameter) show a GV0 configuration, while medium antral follicles (2-8 mm), which are the follicles most commonly used for IVP, contain nearly no GV0-stage oocytes but GV1, GV2 and GV3 stages in similar proportions (Lodde *et al.,* 2007).

Chromatin configuration is not simply morphology, but a marker of gamete differentiation associated with various functional features (Luciano *et al.,* 2014). In bovine oocytes, the transition from GV0 to GV3 corresponds to progressive transcription silencing (Lodde *et al.,* 2008), changes in epigenetic signatures such as overall methylation (Lodde *et al.,* 2009) and histone modification (Labrecque *et al.,* 2015; Lodde *et al.,* 2017) and changes in nuclear architecture and cytoplasmic organelle redistribution (Lodde *et al.,* 2008). More importantly, the transition from dispersed to compacted chromatin is accompanied by gradual acquisition of meiotic and developmental competence (Lodde *et al.,* 2007, 2008; Luciano *et al.,* 2011). Similar correlations have been described in mice and humans (Zuccotti *et al.,* 1995; Bouniol-Baly *et al.,* 1999; Combelles *et al.,* 2003; Miyara *et al.,* 2003; Sanchez *et al.,* 2015). It is noteworthy that the changes in chromatin configuration also accompany significant changes in the transcriptome signature in the oocyte (Labrecque *et al.,* 2015) and in the corresponding cumulus cells (Dieci *et al.,* 2016) suggesting that chromatin configuration also reflects phases of follicle development (Luciano and Sirard, 2018). Moreover, large-scale changes in chromatin configuration are related to gap-junction functional status through cAMP dependent mechanisms (Luciano *et al.,* 2011; Lodde *et al.,* 2013;Franciosi *et al.,* 2014). In cumulus-oocyte complexes isolated from early antral follicles, characterized by a GV0 chromatin configuration, the maintenance of functional gap-junction communications promotes oocyte growth, gradual transcriptional silencing, large-scale chromatin remodeling and competence acquisition, all of which are controlled via cAMP mediated mechanism (Luciano *et al.,* 2011).

A clear indication that the success of *in vitro* prematuration using cAMP modulators is affected by oocyte heterogeneity, comes from our recent study in cows showing that prematuration may be beneficial for the developmental competence of GV1 oocytes but detrimental for that of GV3 oocytes (Dieci *et al.,* 2016). Previous studies have shown that oocytes within COCs with compact cumulus and homogeneous ooplasm are less competent than those in which the ooplasm appears granulated and the outer layers of cumulus cells exhibit the slight expansion often seen in early atretic follicles (Blondin and Sirard, 1995). In our study, we have shown that oocytes within COCs with compact cumulus and homogeneous ooplasm, tend to be in the GV1 chromatin configuration (loosely condensed). On the contrary in COCs with slight expansion and/or

granulated cytoplasm the oocyte chromatin is in either the GV2 or the GV3 configuration, while GV1 representation is negligible (Fig. 3 A, B; Dieci *et al.,* 2016). In our experiments the GV1-enriched oocyte population benefited of a 6 h of prematuration treatment with cilostamide and physiological concentration of FSH, whereas standard IVM without pretreatment leads to poor pre-implantation development. Strikingly, the same prematuration protocol decreases the blastocyst rate of GV2-GV3 enriched oocyte population (Dieci *et al.,* 2016), which performed better if directly processed for IVM (Fig. 3 D).

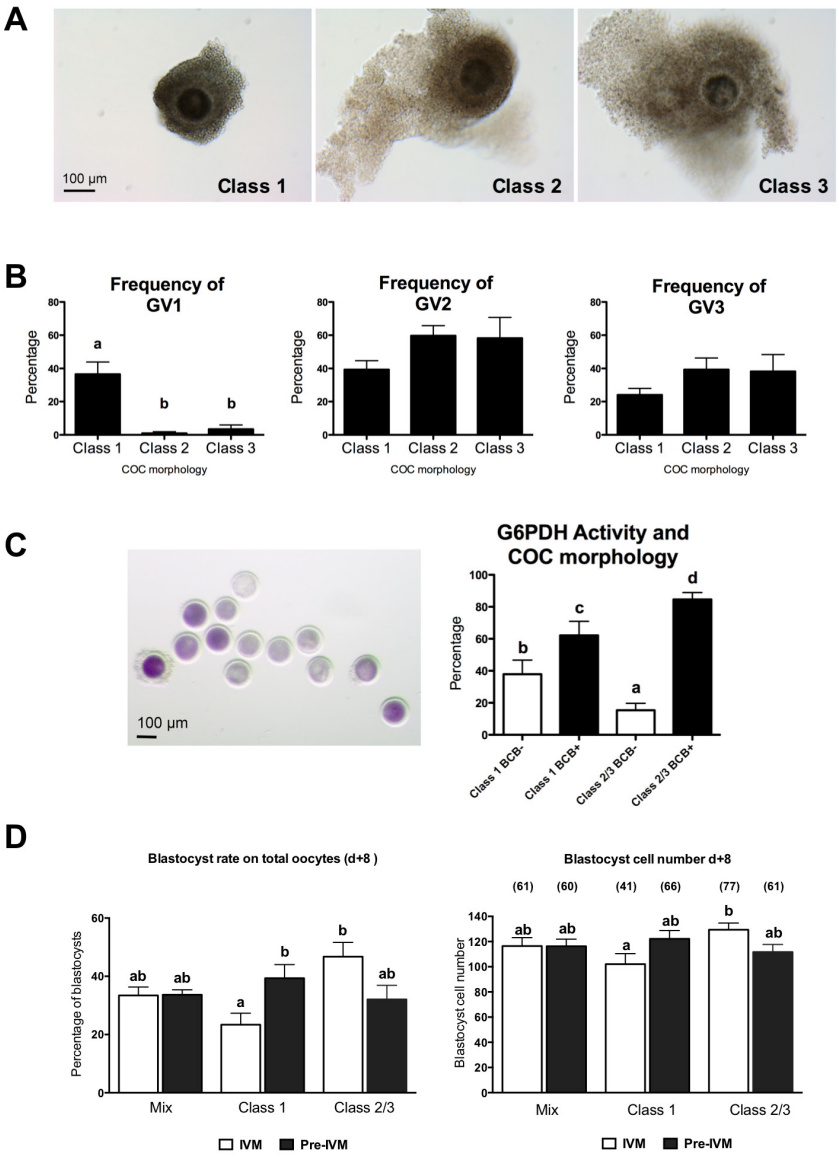

Figure 3. A. Representative images of class 1, class 2 and class 3 COCs. (Class 1: homogeneous ooplasm and absence of expansion of outer layer CC; Class 2: minor granulation of the ooplasm and/or beginning of expansion of outer layer CC; Class 3: highly granulated ooplasm and few CC layers showing expansion). B. Graphs show the frequency of GV1, GV2 and GV3 chromatin configurations in each class. C. After collection, COC were separated into Class 1 and Class 2/3 on the basis of their morphology and subjected to Brilliant Cresyl Blue staining (BCB). After removal of CC, oocytes were classified as BCB+ or BCB− as shown in the representative picture. Graph shows the percentage of BCB+ and BCB− oocytes in Class 1 and 2/3 COC. A total of 337 COC were analyzed (126 Class 1 and 211 of Class 2/3) in nine independent experiments. D. Effect of pre-maturation treatment on COC with different morphology. After collection, COC were separated into Class 1 and Class 2/3 on the basis of their morphology and *in vitro* matured with or without the pre-IVM treatment. Then, oocytes were in-vitro fertilized and in-vitro cultured for 8 days. Groups of unsorted COC (mix of Class 1/2/3) were subjected to the same experimental procedure and were used as controls. Graphs show the effect of the pre-IVM treatment on the blastocyst rate (left) and mean cell number per blastocyst (right). A total of 947 oocytes were analyzed in this study (292 mixed oocytes, 321 Class 1 and 334 Class 2/3) in six independent experiments.
Data were analyzed by one-way ANOVA followed by Newman–Keuls multiple comparison test; data are expressed as means ± SEM; a, b: different letters indicates significant differences ($P < 0.05$). From Dieci *et al*., 2016.

Noteworthy, in animal models characterized by a more homogeneous population of oocytes, prematuration protocols substantially improved the developmental competence. Significant effects were obtained in oocytes with an inherent low embryonic developmental competence such as prepubertal calves, with the use of butyrolactone-I and roscovitine (Donnay *et al.,* 2004) and juvenile mice, with the use of CNP and low concentration of FSH (Romero *et al.,* 2016). Oocytes isolated from early antral follicles (mostly GV0), growth in the presence of cilostamide and a physiologic concentration of FSH were successfully brought to meiotic and embryonic developmental competence (Luciano *et al.,* 2011). In the same line, human oocytes derived from small antral follicles, which have an intrinsically low developmental potential (Sanchez *et al.,* 2017), or compact COCs that are less meiotically competent and characterized by uncondensed chromatin (Nogueira *et al.,* 2006; Sanchez *et al.,* 2017), progressed to a condensed chromatin configuration when prematuration was applied (Vanhoutte *et al.,* 2007).

Moreover, when COCs were selected as homogeneous populations of growing and fully grown oocytes by brilliant cresyl blue staining (Fig. 2 C), the population of growing oocytes was greatly enhanced in embryonic developmental capability while the effect of prematuration was detrimental on the population of fully grown oocytes (Azari-Dolatabad *et al.,* 2016; Dieci *et al.,* 2016; Wang *et al.,* 2016).

Conclusions

The goal of IVP technologies is to improve oocyte quality through a physiological approach in order to obtain a higher number of oocytes with elevated developmental competence. Oocytes coming from non-ovulatory follicles, although can spontaneously resume meiosis are still far to be fully competent. Regardless of the meiotic arrest method used, the rationale of most of the studies aimed at reproducing *in vitro* the final stages of prematuration that normally occur *in vivo*. Several studies have served as proof of concept that prematuration can improve the developmental competence of the oocyte. Nevertheless, little advance has been observed in the embryonic developmental competence when oocytes were cultured in bulk regardless their follicular origin.

The heterogeneity of oocyte population at the start of the procedure greatly affects the outcome of prematuration systems and the specific metabolic needs of the oocyte at the time of isolation should be taken into account. The identification of specific non-invasive biomarker(s) of oocyte health status and final differentiation can provide useful tools for the selection of good quality oocyte, time of prematuration (culture length) as well as the specific environment (hormones, growth factors, molecules, etc.) for optimizing prematuration culture systems. At the same time, the definition of customized culture system can be associated with stimulation strategies to synchronize the growth of ovarian follicles in the donor in order to obtain oocytes specifically suitable for tailored prematuration protocols.

Competing interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality their presentation of the research findings mentioned in this work.

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DOI: 10.21451/1984-3143-AR2018-0063

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Cumulus cells protect the oocyte against saturated free fatty acids

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Abstract

In the cow a major characteristic of metabolic stress is an elevated level of plasma free fatty acid, due to increased lipid mobilization from adipose tissue. Elevated levels of free fatty acids in blood (complexed to albumin) are associated with increased lipotoxicity in non-adipose tissue. An overview is provided on the negative impact of free fatty acids and the metabolic stress imposed on the oocyte and early embryo and thus on bovine fertility. There is increasing evidence that *in vitro* as well as *in vivo* the elevated levels of free fatty acids in blood during metabolic stress can severely hamper oocyte and embryo development. However, fatty acids do also form an essential nutrient source for the oocyte and embryo, which indicates that these good and bad effects of fatty acids should be in subtle balance to optimize the developmental competence of the oocyte and embryo.

Keywords: cumulus, fatty acid, oocyte.

Introduction

This manuscript presents an overview of the current knowledge of fatty acid transfer from blood to the follicle, from the follicular fluid towards the cumulus-oocyte-complex and distribution and use of fatty acids in the oocyte. Cumulus cells appear to play an important role in both fatty acid transfer towards the oocyte and protection of the oocyte against elevated levels of free fatty acids during metabolic stress conditions (Aardema *et al*., 2013; Lolicato *et al*., 2015; Del Collado *et al*., 2017). In particular, saturated free fatty acids appear to have a detrimental impact on oocyte developmental competence (Leroy *et al*., 2005; Wu *et al*., 2010; Aardema *et al*., 2011).

Previously, it has been shown that the negative impact of saturated free fatty acids on oocytes can be compensated by mono-unsaturated oleic acid, which is present in the follicular fluid at high concentration (Aardema *et al*., 2013, 2015). Interestingly, cumulus cells appear to protect the oocyte by converting the potentially toxic saturated fatty acid into mono-unsaturated fatty acid, which is due to Stearoyl-CoA desaturase (SCD) activity and is followed by a safe storage of fatty acids by esterifying them into triacylglycerides (TAG) in lipid droplets (Aardema *et al*., 2017).

Fatty acids are an important nutrient source and building block for the developing oocyte and embryo (Sturmey *et al*., 2009; McKeegan and Sturmey, 2012). A tight fatty acid regulation via the surrounding cumulus cells appears to be a prerequisite for good oocyte developmental competence. The protective properties of the cumulus cells surrounding the oocyte appear to be particularly important during metabolic stress conditions when elevated levels of free fatty acids occur in the follicular fluid.

Fatty acids in metabolic disorders

Metabolic disorders pose one of the major recent health concerns in society, with a high percentage and still growing number of obese and diabetes II patients. Metabolic stress conditions are associated with various medical implications, including impaired fertility in both human and animal. Currently, in the USA nearly half of the women in the fertile age are overweight, with an obesity incidence of more than 20% (Vahratian, 2009; Broughton and Moley, 2017). Since elevated levels of free fatty acids (FFA) are a major characteristic of metabolic stress conditions, which are due to elevated mobilization of body fat reserves, there has been a growing attention for the impact of FFA on oocyte quality and hence early embryonic development, both *in vivo* and *in vitro*. Therefore, the involvement of fatty acids in affecting the developmental competence of the bovine oocyte and embryo is the central topic of this review.

The fatty acid environment of the oocyte

Already from the secondary follicular growth stage onwards, oocytes contain neutral lipids, TAG and cholesteryl-esters, stored in lipid droplets. The number of lipid droplets present in the ooplasm of the oocyte progressively increases during oocyte growth (Fair *et al*., 1997). The origin of the stored neutral lipid in the oocyte is not completely known: neutral lipids may be exogenously derived after uptake from the environment as the receptor CD36 for fatty acid uptake is present in both cumulus cells and oocytes, but moreover oocytes contain the metabolic machinery for fatty acid synthesis, thus lipids may also be of endogenous origin (Cetica *et al*., 2002; Auclair *et al*., 2013; Uzbekova *et al*., 2015). Potential exogenous sources of fatty acids for the oocyte are present in the form of lipoproteins (mainly high-density lipoprotein; HDL), and FFA (fatty acids complexed to albumin) in follicular fluid (Fig. 1). FFA are mobilized from adipose tissue and transfer fatty acids to cells. Follicular fluid reflects the FFA levels of blood in human and bovine, but the free fatty acid compositions differ (Leroy *et al*., 2005; Jungheim *et al*.,

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Received: May 2, 2018
Accepted: June 21, 2018

2011; Yang *et al*., 2012; Aardema *et al*., 2013, 2015; Valckx *et al*., 2014). Metabolites in follicular fluid that originate from blood need to pass the blood-follicle barrier, formed by theca cells, the basal membrane and granulosa cells; a successful passage depends on both size and charge of the metabolite (Gosden *et al*., 1988; Fortune, 1994; Jaspard *et al*., 1997). Plasma lipoproteins are not able to pass the blood-follicle barrier, except for the high-density lipoprotein (HDL), the smallest lipoprotein subclass in particle diameter. Nevertheless, follicular fluid contains low amounts of very low-density lipoprotein (VLDL), which are significantly larger than HDL. Interestingly, VLDL amounts do not correlate with the levels in blood and appear to be secreted by granulosa cells (Gautier *et al*., 2010), possibly executed via selective VLDL transcytosis by these cells. HDL lipoproteins exchange cholesterol and fatty acids with target cells. HDLs are rich in cholesteryl-esters, cholesterol is an important precursor for steroid production in the ovary, but also contain a relatively small amount of TAG. The total fatty acid concentration in follicular fluid is approximately 1.8-2.0 mM and is mostly present in lipoproteins esterified to cholesterol (cholesteryl-ester; 0.7 mM) and in fatty acids of the phospholipid layer of lipoproteins (1.1 mM; Jaspard *et al*., 1997; Valckx *et al*., 2014). During normal physiological conditions, (i.e. no metabolic stress) around 10% of the fatty acids present in follicular fluid is complexed to albumin as FFA (0.23 mM). During periods of metabolic stress, like energy scarcity, obesity or diabetes-type-II, the release of fatty acids from body fat is increased and results in elevated levels of FFA in blood and follicular fluid (Leroy *et al*., 2005; Aardema *et al*., 2013). Aberrant metabolic conditions do not appear to affect the HDL levels in blood and follicular fluid, as demonstrated in women with an increased body-mass-index (BMI) and in dairy cows that suffer from a metabolic stress condition compared with a control condition (Valckx *et al*., 2012; Aardema *et al*., 2013). In contrast, FFA levels massively increase in both blood and follicular fluid during metabolic stress conditions (Leroy *et al*., 2005; Aardema *et al*., 2013; Valckx *et al*., 2014). Whether the fatty acid composition of lipoprotein particles that reside in the follicular fluid is stable or changes during periods of metabolic stress is, to our knowledge, so far not known. It is also not clear whether the cumulus-oocyte-complex (COC) actively incorporates fatty acids that are present in lipoproteins. In this respect, the presence of CD36, a fatty acid translocase, on the cell membrane of cumulus cells and on the oocyte suggests that these cells are able to obtain fatty acids directly from follicular fluid (Uzbekova *et al*., 2015). The type of fatty acids taken up via CD36 depends on the length of the fatty acid; for instance, CD36 facilitates the uptake of long chain fatty acids when present in lipoproteins and of FFA. In both human and cow, the amount and molecular composition of FFA in follicular fluid varies dynamically according to the metabolic status of the individual (Aardema *et al*., 2013; Valckx *et al*., 2014). Although FFA forms only a small proportion of the total fatty acid pool in the follicular fluid, it is the most variable and hence metabolism dependent pool. Furthermore, the CD36 driven uptake of fatty acids by the COC is believed to be predominantly from FFA complexed to albumin (Hughes *et al*., 2011). Therefore, in this review we will focus on the impact of FFA on the oocyte.

Neutral lipids are the major energy storage pool

Fatty acids can serve as an efficient energy source for cells. The complete aerobic catabolism of 1 mole of stearic acid versus 3 moles of glucose (for comparing in both cases 18 C atoms) yields 5-6 x more energy in the form of ATP. Fatty acids also form the main building blocks of cell membranes (phospholipids; the phosphatidyl-backbone of these lipids contains two fatty acids esterified to the sn-1 and sn-2 C atoms of glycerol) and lipid breakdown products can function as cell signalling molecules. In the oocyte, fatty acids can be packed as neutral lipid in the form of TAG (3 fatty acids esterified to glycerol) in lipid droplets. TAG, followed by phospholipids, form the most abundant class of lipid stored in the oocyte, furthermore, in contrast to most other cell types, TAG stored in the oocyte mostly contains saturated fatty acids (Homa *et al*., 1986; McEvoy *et al*., 2000; Kim *et al*., 2001; Aardema *et al*., 2013). The fatty acid composition of oocytes from Israeli Holstein cows shows seasonal variations, with an increased amount of unsaturated fatty acids during the winter period that causes a ΔT of 6°C in the melting temperature of membranes between summer and winter (Zeron *et al*., 2001). An increased level of unsaturated fatty acids lowers the melting temperature of membranes, phenomenon in cells to compensate for a reduced temperature, and improves membrane plasticity (Schumann, 2016). The fatty acid composition of cell membranes can be affected by fatty acids in the diet and during wintertime cows are mostly fed indoors on a ration of grass silage, corn and concentrates. During summer time, there is often supplemental outside grazing next to the ration indoors. Since grass feeding increases the uptake of unsaturated fatty acids, this option does not explain the higher level of unsaturated fatty acids in cell membranes during winter. Therefore, the effect of the dietary fatty acid change and effects on the oocyte during winter remains a yet unexplored but interesting area for future studies.

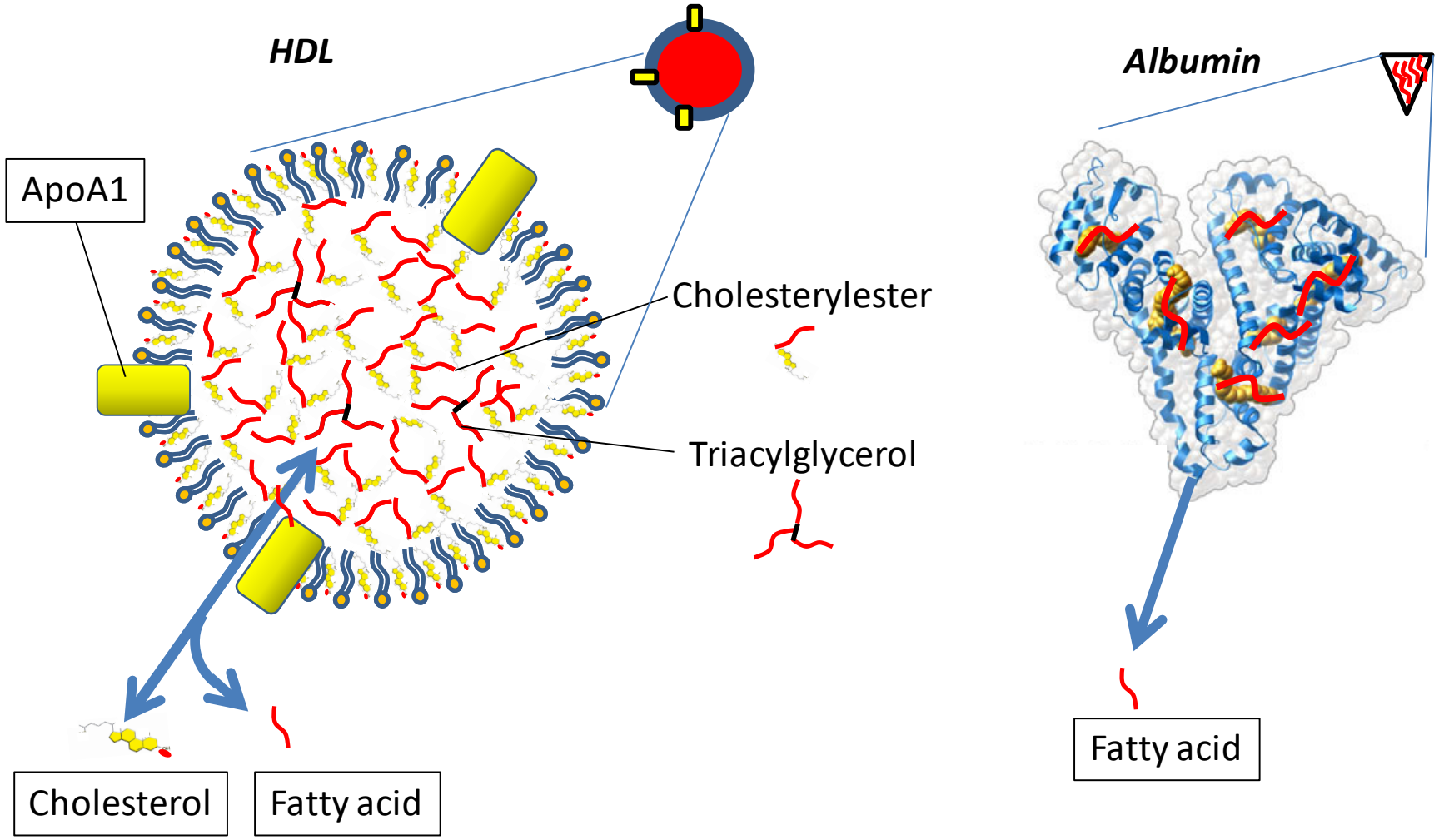

Figure 1. Lipid structure of HDL and the fatty acid albumin complex. HDL is assembled in the liver and secreted in blood cholesterol and fatty acids are exchanged on the surface of cells. Albumin is synthesized in the liver and is secreted in blood, it obtains fatty acids that originate from TAG from adipose tissues and are released after lipolysis and complexed to circulatory albumin. The fatty acid albumin complex can deliver these fatty acids to target cells. Albumin and HDL can both pass the blood-follicle membrane, which likely occurs via transcytosis. The shorthand depictions (diagonal to the right) are used in Fig. 2 to explain lipid delivery from these lipoprotein complexes to the cumulus oocyte complex.

Cumulus cells regulate the transfer of fatty acids to oocytes

Cumulus cells surrounding the oocyte form a natural barrier between follicular fluid and the oocyte. Before fatty acids can reach the oocyte, passage via cumulus cells appears to be unavoidable (Fig. 2). The question is whether and how the transfer of FFA from follicular fluid towards the cytoplasm of the oocyte takes place? It has been indicated that fatty acids are actively transferred from cumulus cells, which do express CD36 for the uptake of long chain fatty acids, to the oocyte (Uzbekova *et al*., 2015; Del Collado *et al*., 2017). The gap junctions, that via the zona pellucida connect cumulus cells and the oocyte and that are able to transfer small metabolites, do also contain fatty acid binding proteins (Dumesic *et al*., 2015; Russell *et al*., 2016; Del Collado *et al*., 2017). Interestingly, blockage of these transzonal projections with cytochalasin B resulted in a reduction of the lipid amount in the oocyte (Del Collado *et al*., 2017). A possible explanation by the authors was that oocytes showed decreased lipid amounts due to the reduction of fatty acid transport via the transzonal projections. This finding also shows that it is likely that diffusion of HDL of albumin FFA complexes through the intercellular matrix between cumulus cells of intact COCs is not sufficient for feeding the oocytes with fatty acids. Unfortunately, the fatty acid breakdown machinery in cytochalasin B treated oocytes was not studied. This could have excluded the possibility that the reduced lipid levels in the oocyte are due to increased fatty acid breakdown in the presence of cytochalasin B. Future studies are required to confirm the exclusive role of transzonal projections and their involvement in fatty acid transfer from cumulus cells to the oocyte.

Besides the facilitation of FFA transfer, cumulus cells are able to delay and hence to reduce the transfer of FFA towards the oocyte. Expanded cumulus cells of *in vivo* maturing COCs form a molecular filter that only allows restricted transfer of specific metabolites in the direction of the oocyte (Dunning *et al*., 2012). This proposed filter function of the cumulus layer supports the hypothesis that albumin complexed FFA and HDL -diffusion through the cumulus cell intercellular matrix of intact COCs, is not an important route for oocyte uptake of FFA (for the proposed major route for FFA entry in the oocyte see Fig. 2). The absence of a functional cumulus cell layer appears to simplify the entrance of exogenous fatty acids into the oocyte, as was demonstrated by an increased incorporation of exogenously administered fatty acids during *in vitro* maturation, into TAG in lipid droplets of oocytes that lacked an intact compact cumulus cell layer investment (Lolicato *et al*., 2015). Furthermore, in the presence of saturated stearic acid active removal of cumulus cells, after a first essential oocyte maturation period of 8 h with cumulus cells, resulted in a significant drop in the capacity of maturing oocytes to develop into a blastocyst when compared to intact COCs (Aardema *et al*., 2017). Cumulus cells massively accumulated fatty acids in lipid droplets after being exposed *in vivo* to elevated FFA levels in the follicular fluid of peri-ovulatory follicles, while no effect was measured on the lipid content nor on the developmental competence of the oocyte (Aardema *et al*., 2013). In somatic cell types lipid storage has been proven to be a protection mechanism to avoid cellular stress by fatty acids (Listenberger *et al*., 2003; Coll *et al*., 2008; Henique *et al*., 2010). This indicates that a proper functional expanded cumulus cell layer reduces the transfer of fatty acids and appears to protect the oocyte against abundant exposure to FFA. One may conclude that cumulus cells form an efficient barrier between FFA present in the follicular fluid and the oocyte. Whether or not cumulus cells are able to protect the developing oocyte against elevated levels of FFA during a prolonged period of time is not known and remains an interesting question, especially since metabolic aberrant conditions are most commonly experienced during an extended period of time.

Lipid droplet dynamics in the oocyte

Physiologically, a high amount of lipid droplets is present in porcine, equine and bovine oocytes (Genicot *et al*., 2005; Sturmey *et al*., 2006; Ambruosi *et al*., 2009; Aardema *et al*., 2011). The abundant amount of neutral lipids stored in those lipid droplets seems to serve as a reserve energy deposit for developing oocytes and hence early-stage embryos. This was indicated by induced embryo development *in vitro* when fatty acid breakdown was stimulated and another study that demonstrated development of embryos in the complete absence of nutrients (Kane, 1987; Dunning *et al*., 2010; Sutton-McDowall *et al*., 2012). There appears to be a large variation in the amount of lipid droplets both in oocytes from different mammalian species and even within individual animals (Sturmey *et al*., 2009). A low number of lipid droplets is present in oocytes from mice, and an increasing number is noted for respectively human, sheep, bovine, horse and in porcine oocytes a very high number is present (Genicot *et al*., 2005; Aardema *et al*., 2011; Dunning *et al*., 2014). The differences that exist between mammalian species are thought to correlate with the elaborated duration and thus energy need of the embryo until functional nourishment from the placenta is taken over the energy demands of the implanted embryo (Sturmey *et al*., 2009). This period of time is short in rodents and primates and long in ruminants, equids and pigs; as a consequence, oocytes from the latter mammalian species contain high amounts of stored neutral lipids. An intriguing suggestion, because it suggests a controlled regulation of lipid storage in the oocyte that could be affected by exogenous exposure to certain fatty acids, or by altering lipid metabolism in the oocyte. Lipid droplets are formed at the endoplasmic reticulum and contain a hydrophobic core of neutral lipid, from TAG and cholesterol-esters, surrounded by a monolayer of phospholipids to meet the hydrophobic content with the hydrophilic nature of the ooplasm (Brown, 2001; Robenek *et al*., 2006). Enzymes of the perilipin family are located at the surface of the lipid droplet to control

the storage and release of fatty acids from the lipid droplets (Fig. 3). The surface of lipid droplets in bovine and murine oocytes contains perilipin-2 (formerly named ADRP), a ubiquitously present perilipin; in addition to perilipin-2, perilipin-3 (formerly named TIP47), has also been localized to lipid droplets of porcine oocytes (Aardema *et al*., 2011; Yang *et al*., 2012; Uzbekova *et al*., 2015; Xu *et al*., 2018). Forty-five years ago, lipid droplets were, for the first time, recognized as potential metabolic units, due to their localisation next to mitochondria in the oocyte (Fleming and Saacke, 1972; Kruip *et al*., 1983; Hyttel, *et al*., 1997). The intimate relationship between lipid droplets and mitochondria has been confirmed by fluorescence resonance energy transfer (imaging, which indicates co-localization on a molecular scale of 6-10 nm (Sturmey *et al*., 2006). Fatty acid breakdown, β-oxidation, in mitochondria appears to be of fundamental importance for proper oocyte development (Downs *et al*., 2009; Dunning *et al*., 2010). Inhibition of fatty acid breakdown in the cumulus-oocyte-complex (COC) during IVM, by blocking the entry of fatty acids in mitochondria via the carnitine shuttle with methyl-palmoxirate or etomoxir, results in a sharp decline in the competence of murine, porcine and bovine oocytes to develop into an embryo (Ferguson and Leese 2006; Sturmey *et al*., 2006; Downs *et al*., 2009; Dunning *et al*., 2010;Paczkowski *et al*., 2013). However, studies where fatty acid breakdown in COCs was inhibited, did not discriminate between whether or not the inhibition was specific for cumulus cells and/or for the oocyte. The activity of carnitine-palmitoyl-transferase-I (CPT-I) is rate limiting for the transport of fatty acids into the matrix of the mitochondria and thus also rate limiting for the follow up β-oxidation of the transported fatty acids. The CPT-I activity is significantly higher in cumulus cells when compared to oocytes. Interestingly, when β-oxidation in COCs is stimulated by supplementation of the co-factor L-carnitine an improved developmental competence of the oocyte was observed (Dunning *et al*., 2011, 2014; Somfai *et al*., 2011). The positive association between the level of β-oxidation in COCs and the developmental competence of the oocyte may result from an abundant energy yield in the cell. However, another option for this positive association may be due to the breaking down and hence reduction of potentially toxic fatty acids.

Sensitivity of oocytes for their lipid environment

Obesity in women is an example of a severe metabolic stress condition associated with elevated, potentially toxic, levels of FFA in blood and follicular fluid. Mouse oocytes exposed to lipid rich follicular fluid of obese women during IVM showed an increased amount of intracellular lipid; likewise oocytes retrieved from obese mice demonstrated a higher amount of lipid compared to controls (Wu *et al*., 2010; Yang *et al*., 2012). This suggests increased lipid uptake by oocytes during exposure to elevated levels of FFA. However, in contrast, it was striking that elevated levels of FFA in bovine follicular fluid during a period of negative energy balance did not affect the lipid content of *in vivo* derived oocytes (Aardema *et al*., 2013). While the exposure of *in vitro* maturing bovine COCs to elevated levels of the dominating follicular fluid -FFA resulted in rapid incorporation of fatty acids in lipid droplets in the oocytes (Aardema *et al*., 2015; Lolicato *et al*., 2015). The response of the oocyte to exogenous FFA thus seems to be unpredictable. However, a major metabolic difference exists between the above-described metabolic conditions. During obesity in an energy rich anabolic condition, glucose levels are high in blood and in follicular fluid and likewise during *in vitro* maturation glucose levels in the media are standard high. This situation is in sharp contrast to the catabolic negative energy balance of high yielding dairy cows, when the plasma levels of glucose are low. These distinct glucose concentrations that emerge during a condition with elevated levels of FFA may result in a different response of the oocyte to elevated levels of fatty acids, since the level of glucose coupled to insulin is the main driver for the energy storage in cells. Furthermore, the expression of CD36, the enzyme involved in the extracellular uptake of fatty acids by somatic cells, which is also expressed in both cumulus cells and oocytes, increases in response to elevated glucose and insulin levels (Uzbekova *et al*., 2015; Garbacz *et al*., 2016; Wilson *et al*., 2016; Ly *et al*., 2017). Indeed, it has been suggested that CD36 functions as a protective metabolic sensor in the liver during conditions of lipid overload. Storage of exogenous fatty acids by the oocyte, in the presence of elevated FFA in follicular fluid, thus appears to depend on glucose and consequently on insulin levels. The subsequent impact of elevated levels of FFA on the oocyte may therefore largely depend on the energetic condition of the dam. Furthermore, there appears to be a significant difference in the response of oocytes to distinct classes of FFA: 1) Exposure of bovine COCs to blood and follicular fluid - dominating saturated FFA (palmitic and stearic acid) during *in vitro* maturation resulted in a significant reduction in the number of lipid droplets in the oocyte and had a negative impact on the developmental competence of the oocyte (Aardema *et al*., 2011). 2) In contrast, exposure to the dominating mono-unsaturated oleic acid caused increased numbers of lipid droplets in the oocyte and counteracted the adverse effects of saturated fatty acids by restoring the number of lipid droplets and the developmental competence of the oocyte (Aardema *et al*., 2011). Another study demonstrated that oocytes with a higher intracellular lipid level of oleic acid appear to have increased developmental competence in comparison to oocytes with a higher level of stearic acid (Kim *et al*., 2001). This indicates that apart from an either energy rich or poor metabolic condition, the type and balance of saturated and unsaturated FFA appears to be of fundamental importance to the impact of FFA on oocyte developmental competence.

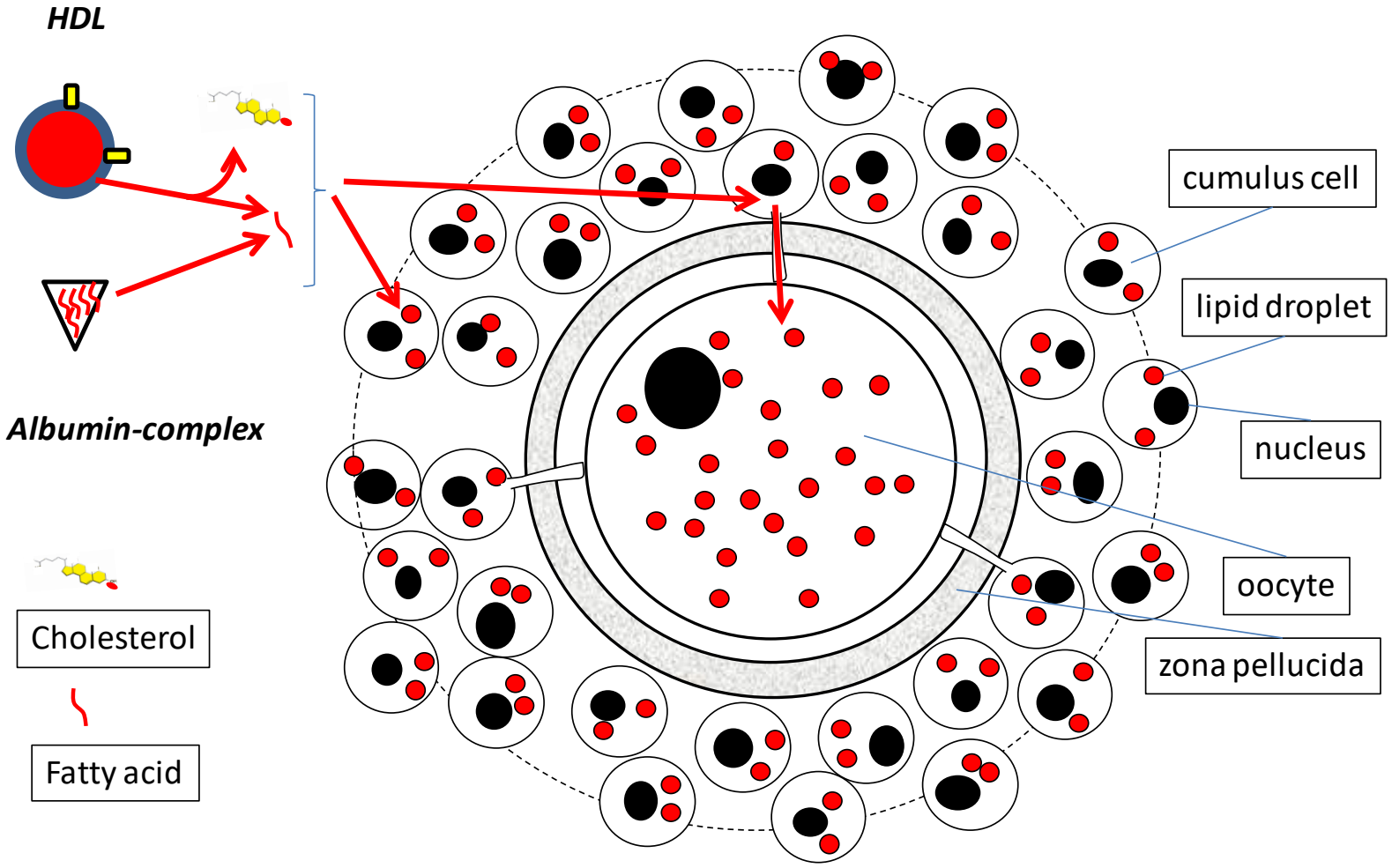

Figure 2. Delivery of fatty acids via HDL and albumin complex at the cumulus cell surface. HDL and Albumin (short hand depiction for details see Fig.1) can deliver their fatty acid via surface exchange to cumulus cells. Exchange of long chain fatty acids is facilitated by the presence of CD36 on the surface of cumulus cells. Cumulus cells can store the fatty acids in lipid droplets from where they can be metabolized for energy production or for building new membranes, or fatty acid may be transported by means of gap-junctional contact via protrusions of cumulus cells through the zona pellucida to the oocyte. The oocyte in turn can use these lipids for storage in lipid droplets or use them for energy by or for building new membranes. Note that besides fatty acids, HDL also exchanges cholesterol, which can be used for steroid production (cholesterol is a precursor). Red circles represent lipid droplets (for more details of them see Fig. 3).

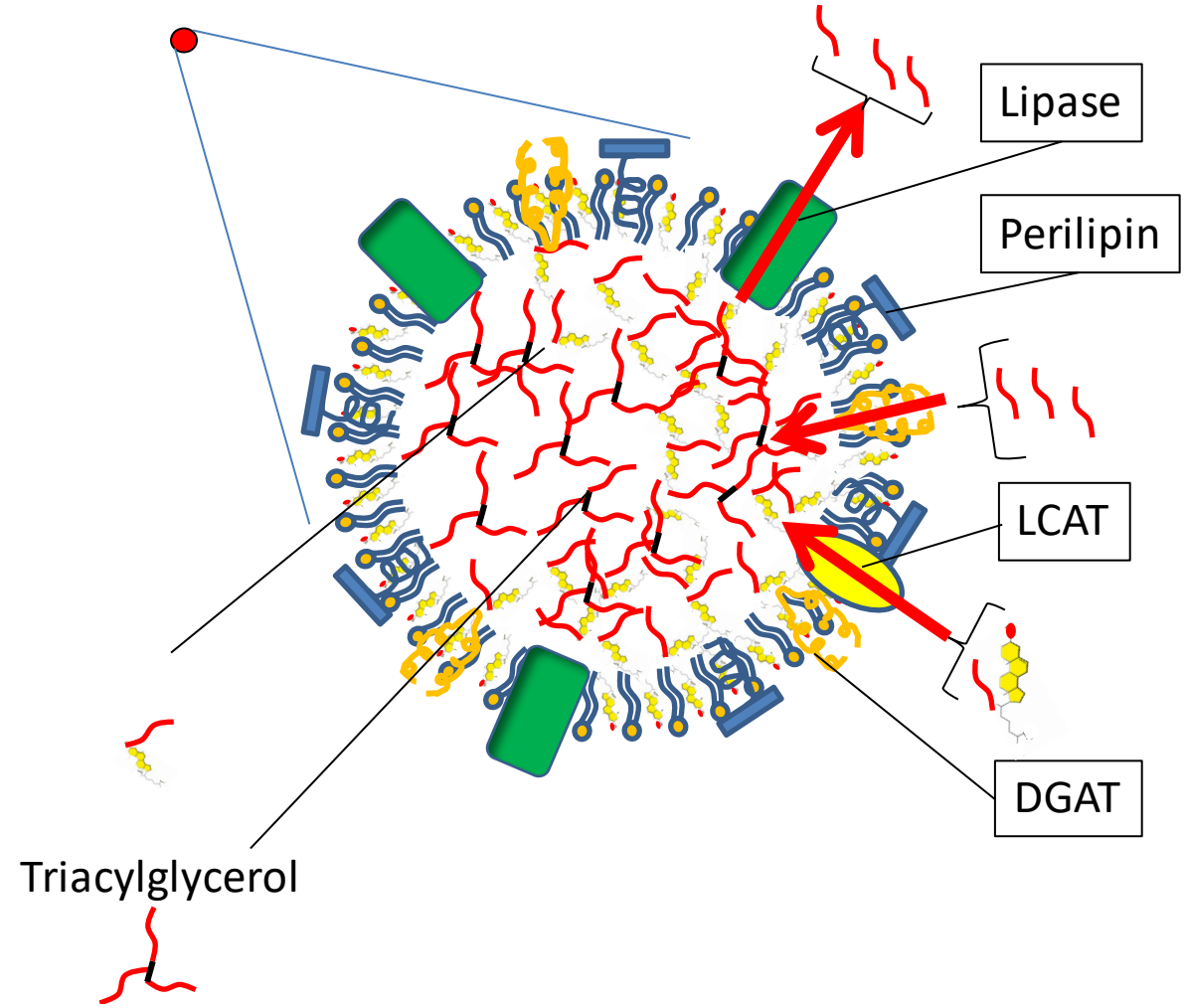

Figure 3. A lipid droplet is a dynamic organelle. Red circles (the lipid droplets from Fig. 2) are in this figure depicted with molecular details. Cholesterol and one fatty acid can be esterified to cholesteryl-ester by lecithin-cholesterol acyltransferase (LCAT) and fatty acids can be esterified to triacylglycerol (last step at the surface of the lipid droplet by the enzyme diacylglycerol acyltransferase (DGAT). Fatty acids from neutral lipids can be hydrolyzed and transported into the cytosol by the hormone sensitive lipase.

Saturated free fatty acids induce lipotoxic stress responses in the COC

Saturated FFA appear to have a detrimental impact on the cumulus cells of the COC resulting in increased levels of apoptosis and hence a reduced developmental competence of the oocyte (Aardema *et al*., 2011, 2017; Leroy *et al*., 2005; Mu *et al*., 2001). Likely, the loss of viable and functional cumulus cells due to apoptosis impairs the 'natural barrier' of cumulus cells, which increases the risk for access of undesired FFA to the oocyte.

Maturing mouse COCs that are exposed to saturated palmitic acid experience a dose-dependent endoplasmic reticulum (ER) stress response, demonstrated by a rise in ER stress markers Atf4, Atf6, Xbp1s and Hspa5 and a reduced mitochondrial membrane potential, indicative for mitochondrial damage (Mu *et al*., 2001). The link between exposure to saturated FFA and lipotoxic events in COCs has also been demonstrated in other somatic cell types (Mu *et al*., 2001; Listenberger *et al*., 2003; Coll *et al*., 2008; Henique *et al*., 2010). The induction of ER stress has been shown to be an important factor in the negative cascade of lipotoxic responses induced by saturated fatty acids. Moreover, ER stress markers are also increased in granulosa cells of obese women and in COCs exposed to a lipid rich follicular fluid of obese women (Wu *et al*., 2010; Yang *et al*., 2012; Sutton-McDowall *et al*., 2016). This observation suggests that saturated FFA induce lipotoxic responses of granulosa cells and the COC during obesity. Apart from inducing ER stress in COCs, saturated fatty acids also increase the level of the apoptosis inducer ceramide and ROS (reactive oxygen species; Lolicato *et al*., 2015). The increased level of ROS is a consequence of electron leakage from the inner mitochondrial membrane during oxidative phosphorylation (Dumesic *et al*., 2015).

Saturated palmitic acid is a potent inducer of an ER stress response and the resulting leakage of ER

calcium stores triggers a cascade of intracellular actions, including unfolding and/or misfolding of proteins in the ER, that results in ROS formation in ER and mitochondria and consequently mitochondrial dysfunction (Pizzo and Pozzan, 2007; Ly *et al*., 2017). Due to mitochondrial damage and the consequently reduced fatty acid breakdown, the toxic impact of fatty acids for the cell is even further increased, which can eventually result in apoptotic cell death after the release of cytochrome-C. On the one hand β-oxidation has been associated with an undesirable increase of ROS production in mitochondria, however, on the other hand a reduction in lipotoxic responses has been observed when β-oxidation was stimulated (Henique *et al*., 2010; Ly *et al*., 2017).

The cascade of actions induced by saturated FFA can be prevented by inhibition at two points (i) inhibition of ER stress by salubrinal in the presence of palmitic acid during IVM results in improved mitochondrial function and cumulus cell morphology and prevents a negative impact on oocyte developmental competence (Wu *et al*., 2012). This indicates that the ER is one of the primary harmed organelles in COCs exposed to palmitic acid, in line with the observed responses towards palmitic acid exposure in other somatic cell types. (ii) Inhibition of ceramide formation by fumonisin-B results in a significant reduction in the ROS formation and a reduction in cumulus cell deterioration and increased cumulus cell expansion, which suggests that the pro-apoptotic ceramide is an important initiator of ROS generation (Lolicato *et al*., 2015). Both ER stress and ceramide formation appear to be key events in the lipotoxic response observed in COCs exposed to palmitic acid (Fig. 4).

The role of CD36 for COCs in relation to their response to palmitic acid may be of interest for future research. Inhibition of CD36 in somatic cell types reduces ROS formation and prevents lipotoxic events during exposure to palmitic acid (Hua *et al*., 2015; Xu *et al*., 2015; Kim *et al*., 2017). Whether or not CD36 is also involved in the cascade of lipotoxic events induced by saturated FFA in COCs needs to be elucidated. Another interesting option to prevent a potential toxic impact by saturated FFA is the safe storage of fatty acids in lipid droplets of the cell, which is stimulated by mono-unsaturated FFA (Listenberger *et al*., 2003; Coll *et al*., 2008; Henique *et al*., 2010).

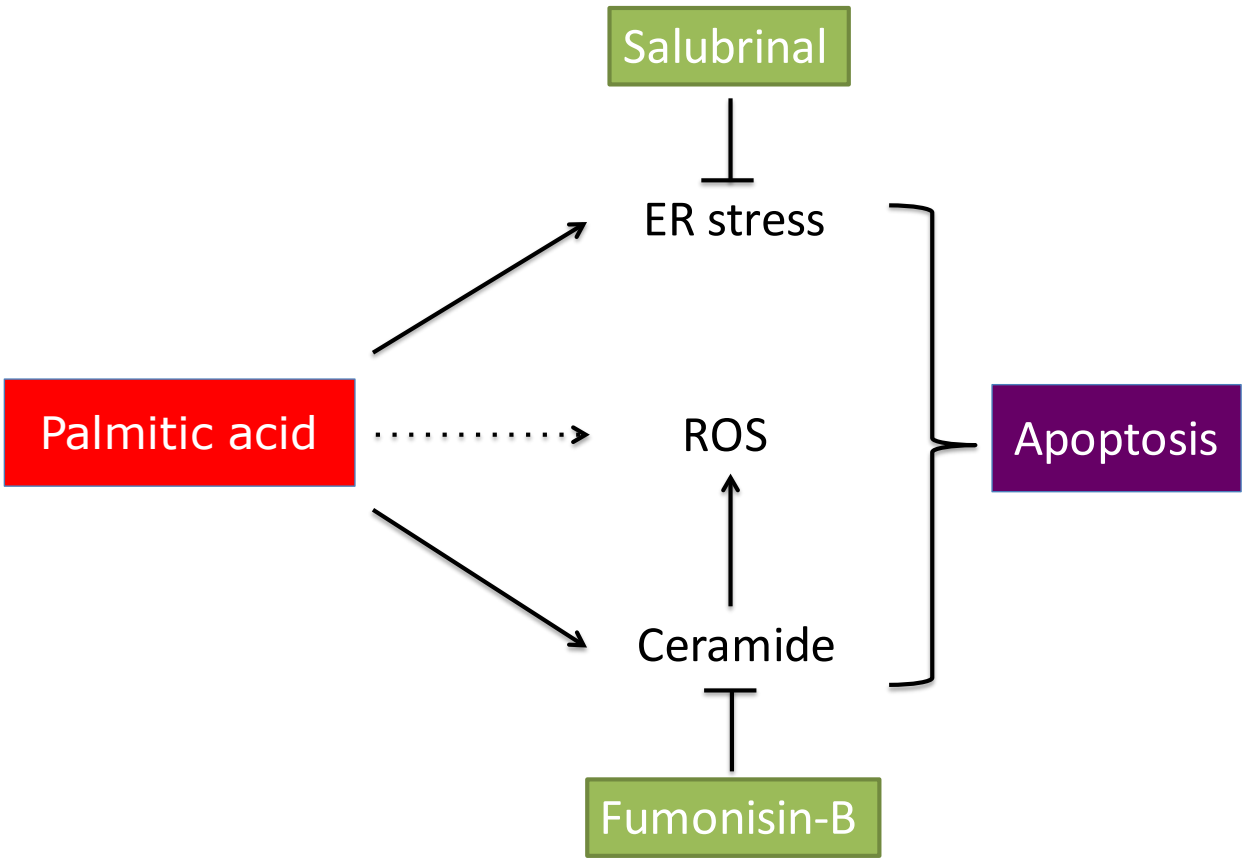

Figure 4. Saturated FFA induce lipotoxic events in COCs and reduce oocyte developmental competence. ER stress and mitochondrial damage are key events in the cascade of actions induced by saturated FFA in COCs. Lipotoxic events can be prevented by inhibition at two points (i) inhibition of ER stress by salubrinal and inhibition of ceramide formation by fumonisin-B.

Mono-unsaturated oleic acid prevents saturated fatty acid stress on oocyte

Mono-unsaturated oleic acid is in contrast to the response of COCs to saturated fatty acids, harmless to COCs and the oocytes. During exposure to high concentrations of oleic acid, oocytes of exposed COCs remain fully competent to develop into an embryo (Leroy *et al*., 2005; Aardema *et al*., 2011; Wu *et al*., 2012). The distinct impact of mono-unsaturated fatty acid versus saturated on cells has been attributed to a different intracellular distribution of the fatty acids. Mono-unsaturated fatty acids like oleic and palmitoleic acid are primarily distributed towards lipid droplets, whereas, saturated fatty acids are directed to apoptotic pathways in somatic cell types (Listenberger *et al*., 2003; Coll *et al*., 2008; Henique *et al*., 2010). Oleic acid does not induce a significant ER stress response in somatic cells, unlike palmitic acid (Ly *et al*., 2017). Interestingly, when oocytes are simultaneously exposed to oleic acid and saturated FFA the negative impact of the saturated fatty acids alone on the oocyte is counteracted and oocyte developmental competence is maintained (Aardema *et al*., 2011). A recent paper demonstrated that alpha-linolenic acid also appears to have the potency to prevent a lipotoxic impact by FFA on the oocyte (Marei *et al*., 2017). However, it is good to note that in contrast to oleic acid, which is not toxic even at high concentrations (500 µm), alpha-linolenic acid has a detrimental impact on oocyte developmental competence above a concentration of 50 µm (Marei *et al*., 2009; Aardema *et al*., 2011). The protective mechanism by which mono-unsaturated FFA reduce the potential stress of saturated fatty acids, is thought to be via redistribution away from apoptotic cascades (ROS and ceramide formation), towards lipid droplets for storage (Listenberger *et al*., 2003; Coll *et al*., 2008; Henique *et al*., 2010). Interestingly, follicular fluid contains a relatively high amount of oleic acid in comparison to the levels in blood (Leroy *et al*., 2005; Aardema *et al*., 2013). Cumulus cells of COCs exposed to elevated levels of FFA during a (mimicked) period of metabolic stress, both *in vivo* and *in vitro*, massively incorporate fatty acids in lipid droplets resulting in a 6-fold increase of TAG storage (composed primarily by esterified oleic acid); moreover, the oocytes of the exposed COCs remain fully competent to develop into an embryo (Aardema *et al*., 2013). Storage of fatty acids in the form of TAG in cumulus cells appears to be an effective route to prevent a toxic impact of FFA in the COC, which is in line with observations in other somatic cell types.

The impact of an elevated concentration of FFA during metabolic stress in cows was investigated *in vitro* by supplementing maturation media with comparable concentrations of the three dominating FFA, saturated palmitic and stearic and mono-unsaturated oleic acid, in two distinct experimental setups. Fatty acids were either solved in ethanol and were stirred (Van Hoeck *et al*., 2011; Sutton-McDowall *et al*., 2016) or were complexed to albumin, which is comparable to the physiological presentation of FFA to the COC (Aardema *et al*., 2013). COCs exposed to a mixture of saturated and mono-unsaturated fatty acids solved in ethanol, demonstrated hampered oocyte developmental competence (Van Hoeck *et al*., 2011; Sutton-McDowall *et al*., 2016), whereas, in the study of Aardema *et al*. no such negative impact of a comparable FFA mixture was observed in line with the observations after *in vivo* exposure of COCs to elevated levels of FFA during metabolic stress (Aardema *et al*., 2013). The expected presentation of fatty acids in micelle formation after ethanol injection or via the physiological presentation in a complex with albumin may account for the distinct outcome between the studies and this certainly needs further investigation. The absence of a negative impact on the oocyte competence after exposure of COCs to a mixture from saturated palmitic and stearic acid and mono-unsaturated oleic acid (Aardema *et al*., 2011; Aardema *et al*., 2013) is in line with observations in other cell types exposed to a combination of saturated and mono-unsaturated FFA (Mu *et al*., 2001; Listenberger *et al*., 2003; Henique *et al*., 2010). The observed distinct impact of saturated and mono-unsaturated FFA on oocyte developmental competence clearly indicates that a balance between the different types of FFA, rather than the total amount of FFA, appears to determine the impact on the COCs.

The follicle creates a protective oleic rich FFA environment for the oocyte

The beneficial environment for the COC thus appears to be follicular fluid rich in mono-unsaturated oleic acid and, therefore, it is intriguing that the follicular fluid contains a relatively high level of mono-unsaturated oleic acid and a relatively low level of stearic acid, compared to blood (Aardema *et al*., 2011, 2013, 2015). Previously, it has been suggested that the distinct FFA composition of follicular fluid originates from selective transfer of FFA over the blood/follicle membrane. There are indeed indications that CD36/FAT (fatty acid translocase), the transmembrane protein that facilitates diffusion of long chain fatty acid, may be selective in the uptake of long chain fatty acids. In this respect, CD36/FAT is expressed in all cell types of the follicle including the oocyte itself (Uzbekova *et al*., 2015). Over expression of chicken CD36/FAT in Chinese hamster ovary cells resulted in an increased uptake of linoleic and arachidonic acid and a reduced uptake of palmitic acid, but overexpression of CD36 had no effect on the uptake of stearic and oleic acid (Guo *et al*., 2013). To this end, selective transfer of fatty acids over the blood-follicle membrane does not seem to explain the relatively high oleic acid levels in the follicular fluid. The distinct FFA composition in follicular fluid could originate from either storage or metabolism by the cells that form the follicle; theca, granulosa and cumulus cells all contain the intracellular machinery for fatty acid uptake and metabolism (Uzbekova *et al*., 2015). Fatty acids that dominate in theca cells, the cells that form the outer layer of the blood-follicle membrane, do reflect the composition of FFA in blood and are dominated by mono-unsaturated

oleic acid (31%) and saturated palmitic (21%) and stearic acid (12%) in sheep (Hughes *et al*., 2011). Granulosa cells, which cells form the inner layer of the blood-follicle membrane, do also reflect dietary supplementation of n-3 or n-6 poly-unsaturated fatty acids (Wonnacott *et al*., 2010). As mentioned before, when COCs are exposed to elevated levels of FFA during IVM cumulus cells respond with intracellular storage of these fatty acids (Wonnacott *et al*., 2010; Aardema *et al*., 2013). This indicates that intracellular storage of fatty acids may affect the composition of FFA in follicular fluid, due to uptake of, formerly free, fatty acids by cells. However, the intracellular lipid that was stored in COCs exposed to elevated levels of FFA was dominated by oleic acid storage (Aardema *et al*., 2013). To this end, this phenomenon cannot explain the relatively high level of oleic acid in follicular fluid, as storage of oleic acid would result in a net reduced level of oleic acid in follicular fluid. Both selective uptake of FFA nor storage and metabolism do seem to provide an explanation for the distinct FFA composition in follicular fluid. There is, however, an interesting third option: the desaturation of saturated fatty acids into mono-unsaturated FFA in follicular fluid. The key enzyme responsible for the conversion of saturated stearic acid into mono-unsaturated oleic acid is SCD, i.e. by the formation of a double carbon bond at the Δ9 position of saturated fatty acid. In the rat ovary SCD type 2 is indeed expressed (SCD type 1-4 are present in murine species) in theca, granulosa and cumulus cells (Moreau *et al*., 2006). Women as well as cows do express SCD types 1 and 5 and both types are also expressed in granulosa and cumulus cells (Feuerstein *et al*., 2007; Aardema *et al*., 2017). Likely, the relatively low stearic and high oleic acid levels in follicular fluid, when compared to blood, may originate from SCD activity in the follicle (Aardema *et al*., 2013, 2015). After entrance of FFA from blood via the blood-follicle barrier, the active conversion of stearic acid into oleic acid in both theca and granulosa cells may explain the relative high level of mono-unsaturated FFA in follicular fluid, at the cost of saturated FFA. To this end, the transfer of FFA from blood to the follicle and the potential desaturation of FFA by follicular cells certainly needs attention in future research, in particular as being a promising route to potentially detoxify the FFA to which the COC is exposed.

Cumulus cells protect the oocyte against potential stress by free fatty acids

Interestingly, cumulus cells appear to be important in regulating the fatty acid transfer towards the oocyte and seem to protect the oocyte against potential stress by free fatty acids. In the absence of cumulus cells, oocytes appear to be extremely prone to elevated levels of FFA (Aardema *et al*., 2013; 2017; Lolicato *et al*., 2015). We investigated whether the enzyme SCD-1, that can detoxify saturated FFA into mono-unsaturated FFA, is expressed in COCs and has a role in protecting the oocyte against FFA. The genes *SCD-1* and *SCD-5* are expressed in human cumulus cells and *SCD* expression was associated with oocyte competence (Feuerstein *et al*., 2007). *SCD-1* mRNA expression in human cumulus cells is increased in the presence of whole and partly delipidified serum, which may indicate that these conditions relate to the competence of the oocyte, as *SCD* expression in cumulus cells has been related with oocyte competence (Mardomi *et al*., 2018). The elevated *SCD* expression in cumulus cells may however also be a direct response to the medium and could reveal a potentially beneficial medium for the development of the oocyte, rather than being a direct marker for oocyte quality. More studies are needed to investigate how *SCD* expression in cumulus cells and oocyte competence are linked. *SCD-1* mRNA expression has also been demonstrated in bovine oocytes (Aardema *et al*., 2018; Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, The Netherlands; unpublished data) and cumulus cells (Aardema *et al*., 2017; Warzych *et al*., 2017a, b). Warzych *et al*., 2017a showed a correlation between FFA composition in follicular fluid and *SCD-1* gene expression in cumulus cells, which may also alter the fatty acids that are transported and metabolized in the oocyte. Bovine cumulus cells abundantly express SCD-1 protein, in contrast to the oocyte with non-detectable levels of SCD-1 protein (Aardema *et al*., 2017). Furthermore, when SCD activity in cumulus cells was inhibited during exposure to a physiological level of stearic acid, the developmental competence of the oocyte was hampered and comparable with the situation where oocytes were exposed to stearic acid in the absence of cumulus cells (Aardema *et al*., 2017). In a recent paper, the negative outcome of SCD inhibition in cumulus cells on oocyte developmental competence was also confirmed in human cumulus cells and also appeared to relate to reduced aromatase gene expression and estradiol production (Fayezi *et al*., 2018). SCD-1 expression has been associated with lipid metabolism in dairy cows and may be linked to reproductive performance (Wathes *et al*., 2012). SCD activity in cumulus cells appears to be of crucial importance to protect the oocyte against fatty acid stress. Furthermore, SCD activity in cumulus cells in the presence of stearic acid resulted in a significant reduction in the rate of apoptosis in the cumulus cells and hence increased lipid droplet storage of fatty acids dominated by oleic acid (Aardema *et al*., 2017). This convincingly demonstrates that the role of SCD activity and the resulting storage of fatty acids in lipid droplets of cumulus cells appears to be an important strategy to protect the oocyte against elevated FFA levels during metabolic stress (Fig. 5).

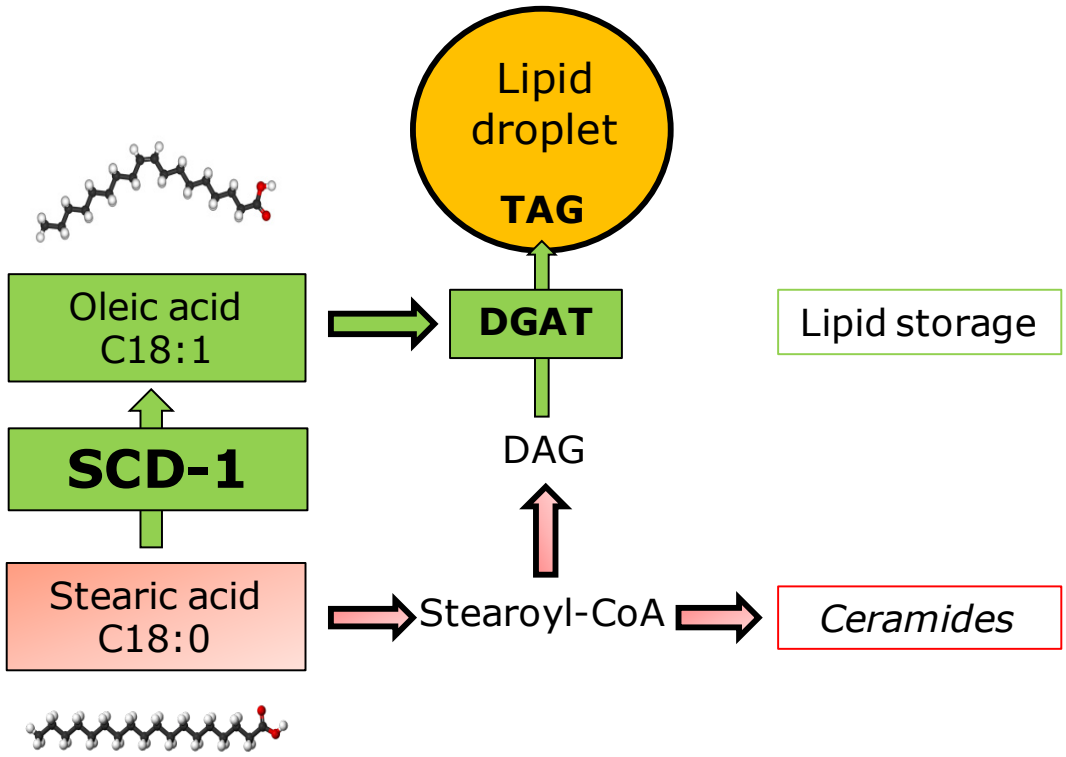

Figure 5. Cumulus cells protect the oocyte by SCD-1 activity and fatty acid storage. Saturated stearic acid induces apoptotic pathways. Conversion of saturated stearic into mono-unsaturated oleic acid by Stearoyl-CoA-Desaturase (SCD-1) and consequent storage of fatty acids in the lipid droplets of cumulus cells protects the oocyte against saturated fatty acids.

Conclusion

The amount and molecular composition of FFA in blood correlate with those in follicular fluid of pre-ovulatory follicles and hence FFA levels increase in both fluids during periods of metabolic stress. The type of FFA to which the maturing COC is exposed largely dictates the impact of elevated FFA levels on the survival of the COC and competence of the oocyte. Mono-unsaturated FFA compensate for a negative impact of saturated FFA on the COC and maintain the developmental competence of the oocyte. Cumulus cells that surround the oocyte regulate fatty acid transfer towards the oocyte, and an intact cumulus cell layer appears to be essential to protect the oocyte effectively against undesired FFA. SCD activity of cumulus cells prevents FFA stress on the oocyte due to conversion of potentially toxic saturated FFA (stearic acid) into harmless mono-unsaturated FFA (oleic acid), which is safely stored in lipid droplets of cumulus cells. At this point it is unknown whether and how presumptive oocytes during stages of early (pre-antral) follicular development, which lack the presence of cumulus cells, are protected during metabolic stress and in the presence of elevated FFA levels. These observations are important directions for future research.

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DOI: 10.21451/1984-3143-AR2018-0036

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Looking at the big picture: understanding how the oviduct's dialogue with gametes and the embryo shapes reproductive success

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Abstract

The oviduct is a tubular organ comprising three distinct anatomical regions (the infundibulum, the ampulla and the isthmus) connecting the ovary and the uterus. Oviductal function is regulated by ovarian hormones, gametes, and embryo-derived factors, for optimally facilitating key reproductive events. A cross-talk is established between the oviduct and the gametes and embryo and this dialogue shapes the microenvironment in which gamete transport, fertilization, and early embryonic development occur. This review aims to address each participant in this conversation in a holistic manner by delineating several advances in the field within the greater context of understanding how oviduct-gamete and oviduct-embryo dialogue shape reproductive success and furthermore how this knowledge can be applied *in vitro*.

Keywords: cattle, embryo, gametes, interaction, oviduct.

Introduction

Successful blastocyst production following *in vitro* fertilization (IVF) and embryo culture (Gordon, 2003), coupled with the fact that pregnancies can be achieved after IVF embryo transfer to non-mated synchronized recipients (Lonergan *et al*., 2016), indicate that oviduct exposure is not essential for early embryo development. Thus, this has led to the view that the oviduct is a passive conduit for gametes and the early embryo(s). However, a significant body of evidence demonstrates that the oviduct is a dynamic organ. The luminal microenvironment is influenced by ovarian hormones, gametes, and embryo-derived factors, for the purpose of *optimally* facilitating key reproductive events – to the extent that a pathophysiological peri-conceptual milieu can result in embryo loss, or even adverse lifelong effects (Fazeli, 2008).

Following ovulation and/or insemination, a cross-talk is established between the oviduct and the gametes and embryo. This dialogue will shape the microenvironment in which gamete transport, fertilization, and, if successful fertilization takes place, early embryonic development occur. Studying each conversation participant in isolation facilitates research; however, to fully understand complex oviduct dynamics, a more holistic view is necessary. This review aims to achieve this by delineating several advances in the field within the greater context of understanding how oviduct-gamete and oviduct-embryo dialogue shape reproductive success.

Oviduct anatomy and physiology: epithelial and fluid cyclic changes

The oviduct is a tubular organ comprising three distinct anatomical regions: 1) the infundibulum, 2) the ampulla, and 3) the isthmus, adjoining the uterus at the utero-tubal junction (UTJ) – all with different, yet equally critical, roles.

The oviduct epithelium comprises secretory (most abundant in the isthmus) and ciliated cells (most abundant in the infundibulum and ampulla; Yániz *et al*., 2000). The cause of the regional variation in cell type distribution is unknown; however, both lineages derive from embryonically-labelled PAX8+ (a secretory cell marker) cells (Ghosh *et al*., 2017). Therefore, secretory cells are the oviduct epithelium progenitors, with the potential to self-renew or differentiate into ciliated cells upon 17β-oestradiol (E2) stimulation (Comer *et al*., 1998).

The ampullar and the infundibular epithelium undergoes cycle-dependent changes – surface fold amplitude, cell populations, gene expression, and overall physiology vary in response to ovarian steroid fluctuations (Yániz *et al*., 2000; Cerny *et al*., 2015). Interestingly, the isthmic epithelium undergoes few changes throughout the cycle (Yániz *et al*., 2000), despite estrogen receptor alpha (ERα) and progesterone receptors A and B (PRA and PRB) being more abundant in the isthmus than the ampulla and infundibulum (Okada *et al.,* 2003). This suggests that the region-specific differences are not only due to differences in epithelial gene expression, but also likely due to the level of exposure to locally secreted factors – specifically from the ovary, ovulated follicle and consequent corpus luteum, via the ovarian artery and the oviductal ostium. This counter-current transfer is thought to underpin oviduct endocrine regulation (Hunter, 2012), and could explain why the ipsilateral oviduct contains higher concentrations of P4 during the luteal phase, relative to its contralateral counterpart (Wijayagunawardane *et al*., 1998; Lamy *et al*., 2016). It is important to note, however, that there is conflicting evidence regarding differences in abundance of other hormones such as E2, between ipsilateral and

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Received: February 22, 2018
Accepted: April 5, 2018

contralateral regions (Wijayagunawardane *et al*., 1998; Lamy *et al*., 2016). This local delivery system may act to coordinate oviductal tissue changes in step with the pre-ovulatory maturation of the oocyte within the Graafian follicle, and the capacitation of sperm. During the follicular phase, infundibular and ampullar folds reach maximum amplitude (greatest surface area to volume ratio) and exhibit numerous ciliated cells in the apical areas (Yániz *et al*., 2000), with secretory cells clustered basally, between folds. At this stage, genes involved in cell cycle, cholesterol biosynthesis, cell division, mitosis, and protein folding – responsible for proliferation and secretory activity – are upregulated (Cerny *et al.*, 2015). High E2, characteristic of the preovulatory phase, is thought to be responsible for proliferative epithelial activity (Steffl *et al*., 2008). Indeed, mitotic activity in the isthmus and ampulla is highest during the follicular phase and around ovulation (Ito *et al*., 2016). In addition, high E2-low P4 treatment induces morphological changes and increased P4 receptor (PR), estrogen receptor 1 (ESR1), oviductal glycoprotein 1 (OVGP1), and heat shock protein ember 90kDa member 1 (HSP90B1) gene expression in porcine oviduct cells (Chen *et al*., 2013). However, *in vitro* studies reported no increase in proliferation after FSH, LH (in baboon and mouse), or E2 treatment (in the baboon, mouse, and pig; King *et al.,* 2011; Chen *et al*., 2013), suggesting that additional factors may participate in epithelial remodelling.

At ovulation, expelled follicular fluid contacts the ipsilateral oviduct (Hansen *et al*., 1991), inducing increased ciliary beat frequency (CBF), therein aiding oocyte transit to the site of fertilization (Lyons *et al*., 2006). Ovulation, furthermore, induces double-strand DNA breaks in the oviduct epithelial cells (OEC), and increases epithelial macrophage infiltration (King *et al*., 2011). Interestingly, these macrophages associate with oviduct epithelia adjacent to the cumulus-oocyte complex (COC), which may be important as they secrete cytokines that could interact with the oocyte and the early embryo (Schäfer-Somi, 2003).

After ovulation, the P4 rise associated with the luteal stage, results in decreased oviduct mucosal fold amplitude, with secretory cells beginning to dominate the luminal landscape (Abe and Oikawa, 1993; Yániz *et al*., 2000). Epithelial exposure to elevated P4 leads to cell atrophy, decreased cell height, secretory granule loss, and cell death (Steffl *et al.*, 2008). The oviduct epithelium during the luteal phase is also characterised by a downregulation of genes involved in cell communication, blood vessel development, innate and humoral immune responses, complement activation, and an upregulation of genes involved in focal adhesion formation, cell growth regulation, and fatty acid metabolism, amongst others (Hess *et al*., 2013). These changes are indicative of an environment required to support semi-allogeneic embryo development.

Oviduct fluid dynamics

Oviduct fluid (OF) formation is a spatio-temporally dynamic process. The spatial secretory profile is influenced by 1) the secretory cell proportion, which increases longitudinally from infundibulum to isthmus (Leese, 1983), and 2) the secretory mucosal surface area, which decreases as the oviduct tapers toward the UTJ. The most pronounced secretory portion of the oviduct is subject to debate. Whilst secretory cells dominate the isthmic luminal landscape (~70%), in contrast to ~50% in the ampulla (Crow *et al*., 1994), primary metabolites have been detected in the ampulla at 1.8 times their isthmic concentration — presumably owing to the relative secretory mucosal surface area of the ampulla being ~1.8 times greater (Leese, 1988). Factoring both surface area and secretory cell population, however, the ampulla has a secretory index of 0.9 (0.5 x 1.8) compared to the isthmic 0.7 (0.7 x 1.0) (adapted from Abe, 1996). This is physiologically counter-intuitive, given that the embryo migrates through the isthmus following fertilisation at the ampullary-isthmic junction.

In addition to spatial variability, OF composition and volume vary temporally as a function of the oestrous cycle, mediated by steroid hormones acting on the oviduct both directly and indirectly (Aguilar and Reyley, 2005). This was functionally demonstrated first by Bishop (1956) who ligated anaesthetised rabbit oviducts at the UTJ and vertically cannulated the ostium, measuring pressure as a function of fluid formed. At oestrous, oviducts produced 0.79 ml over 24 h, whereas ovariectomised subjects secreted 0.14 ml over the same period. Importantly, secretion rates were restored in ovariectomised rabbits following exogenous E2 supplementation, and secretion volume and pressure declined during pregnancy. Hugentobler *et al*. (2008) performed a similar study in heifers by catheterising the exteriorised oviduct during surgery. Whilst secretion rates declined from day 0 (1.9 ± 0.3 µl/min; n = 7 ± SEM) to day 6 (1.2 ± 0.3 µl/min; n = 7 ± SEM), differences were non-significant.

The primary OF formation mechanism is osmotic water transfer secondary to solute transit, the dominant of which is Cl^- (Dickens *et al*., 1993; Leese *et al*., 2001). K^+ flux is also likely important for moving water apically (Dickens and Leese, 1994). The fact that oviduct epithelia exhibit an inherently relatively low transepithelial resistance (Leese and Gray, 1985) is indicative that paracellular fluid transport also contributes to OF formation and composition (Simintiras and Sturmey, 2017). It is also worth noting that, under physiological conditions, OF composition is influenced by peritoneal and follicular fluid entry from the abdominal cavity, and uterine fluid (UF; Leese, 1988.

OF composition

Ions

OF ionic composition is highly conserved across mammals, with K+ consistently elevated relative to plasma levels (Aguilar and Reyley, 2005). In cattle, K+ is highest in OF at oestrus (Olds and VanDemark, 1957), and in mice, more pregnancies were established

by IVF when using a culture medium high in K+ (Quinn *et al*., 1985) – the basis of synthetic oviduct fluid (SOF), now also used for cattle embryo production (Gandhi *et al*., 2000). Ca2+ is also highest in bovine OF around ovulation and is interestingly more abundant in the isthmus than the ampulla (Grippo *et al*., 1992). This longitudinal variation is unlike Mg2+ which does not appear to vary spatially but rather temporally (Grippo *et al.,* 1992). It is also interesting to note that the ionic composition and rate of secretion of bovine OF differs considerably to that of uterine fluid (Hugentobler *et al*., 2007).

Protein

The OF protein source is twofold: 1) basal vasculature 'filtration' and 2) epithelial synthesis and secretion (Aguilar and Reyley 2005). OF protein levels are ~10 - 15% of that of plasma (Leese, 1988), with serum albumin and serum immunoglobulin G comprising approximately 95% of this total (Oliphant *et al*., 1978). Other proteins identified include high-density lipoproteins, secreted during the follicular phase, and presumed to bind sperm membrane cholesterol as part of the capacitation process (Ehrenwald *et al.,* 1990).

Further to spatially-regulated protein secretions, a temporal pattern of protein secretion is evident (Nieder and Macon, 1987; Abe, 1996), as discussed below in the context of the best studied and characterised protein of the oviduct: OVGP1, reported as the major secretory glycoprotein which is synthesized and secreted exclusively by the oviduct (Buhi, 2002). OVGP1 is consistently observed in the ampulla across species and enters the lumen via epithelial secretory granule exocytosis (Avilés *et al*., 2010). OVGP1 has also been identified in *in vitro* derived bovine, porcine, and murine oviduct fluid (Chen *et al*., 2017; Simintiras *et al.*, 2017).

OVGP1 secretion *in vivo* is cycle-dependent and thus correlates with the aforementioned epithelial differentiation states (Verhage *et al*., 1988); however, OVGP1 production and secretion patterns differ between species. In the goat it is expressed in the infundibulum and ampulla during the follicular phase (Abe *et al*., 1995) – *i.e*. around the time of fertilisation but not at the site of fertilisation, whereas in the rat it is secreted predominantly in the isthmus, where the sperm reservoir is located (Abe, 1996). In the bovine, OVGP1 is found in the isthmus and ampulla, the respective sites of sperm capacitation and fertilisation (Lefebvre *et al*., 1997) during the follicular phase. Ovine OVGP1 is exclusively produced by the ampulla (Gandolfi *et al.,* 1991), in greatest amounts at oestrus (DeSouza and Murray, 1995).

Advances in proteomic methods (Simintiras and Forde, 2017) such as mass spectrometry, will undoubtedly lead to a clearer picture of the oviduct proteome, based on empirical data as opposed to gene expression extrapolations. For instance, a recent study by Acuña *et al*. (2017) found almost 5000 genes expressed in the porcine oviduct, of which only 7% corresponded to secretory proteins, and 11% to membrane proteins – *i.e.* products with the potential to directly influence the offspring.

Extracellular vesicles

An additional new area of research lies in luminal extracellular vesicles (EVs). The term EV encompasses different vesicle types, released by somatic cells, that are present in body fluids, and contain bioactive molecules (i.e. mRNAs, small ncRNAs – such as miRNA, proteins, carbohydrates, and lipids; Raposo and Stoorvogel, 2013). EVs are important for intercellular communication, playing a key role in the regulation of physiological and pathological processes (Thery, 2011). EVs can horizontally transfer mRNAs to other cells, which can then be translated into functional proteins at the new location (Hergenreider *et al*., 2012). EVs have been identified *in vivo* in several body fluids including amniotic fluid, urine, and blood (Simpson *et al*., 2008). Until recently, the study of reproductive EVs in mammals was limited to follicular fluid (Silveira *et al*., 2012), uterine fluid (Ng *et al*., 2013; Burns *et al*., 2014), and seminal plasma (SP; Piehl *et al*., 2013). Burns *et al*. (2016) demonstrated that EVs emanate from both the conceptus trophectoderm and uterine epithelia, and are involved in intercellular communication between these tissues during pregnancy establishment in sheep. Recent studies from our group showed that EVs obtained from bovine OECs cultures *in vitro* (Lopera-Vásquez *et al*., 2016) and from bovine OF (Lopera-Vasquez *et al*., 2017) substantially improved *in vitro* produced blastocyst quality, measured in terms of cryotolerance, differentially cell count and mRNA abundance of specific genes. However, it was evidenced that EVs obtained from *in vivo* and *in vitro* bovine OECs differ in their protein content, with some proteins known to be involved in reproductive function differently abundant in EVs from *in vivo* compared to *in vitro* origin (Almiñana *et al*., 2017). Thus, oviductal EVs from different origins may differ in their ability to mediate key processes such as sperm-oocyte binding and fertilization; for greater detail see (Pérez-Cerezales *et al*., 2018).

Oviduct-gamete interactions

The response of the oviduct to sperm or oocytes differs, but both the male and female gamete induce changes in the oviductal proteome (Georgiou *et al*., 2005). Oviduct-gamete communication is an intricate dialogue leading to the fine regulation of sequential processes resulting in successful fertilization. The main oviduct-driven events in gamete physiology are detailed below.

Oocyte transportation to the site of fertilization

At ovulation, the COC is expelled into the peritoneal cavity and guided through the infundibulum into the ampulla of the oviduct. Once contact is established between the COC and the oviduct

epithelium, ciliated cells transport the COC to the ampulla. In addition to the OF current created by ciliary beating, COC adhesion to ciliary cells is essential for gamete transport (Lam *et al*., 2000). Adhesion is mediated by the cumulus cells, as their removal prevents oocyte pick-up, due to the zona pellucida not interacting with the epithelia (Mahi-Brown and Yanagimachi, 1983). The granules and filaments of the cumulus extracellular matrix adhere to the glycocalyx of ciliary crowns at the infundibular ciliary tip (Lam *et al*., 2000). Ciliary beating weakens this adhesive interaction, such that the COC is never completely released, yet rolls into the ampulla. The importance of ciliary cells in this process is highlighted by the fact that women with Kartagener syndrome, a genetic disorder causing defects in global ciliary action, exhibit impaired fertility (Afzelius and Eliasson, 1983; McComb *et al.,* 1986).

Interestingly, mating induces changes in oviduct ER signalling, which is directly involved in oocyte transport acceleration (Orihuela *et al*., 2009). This could be a mechanism for ensuring that the oocyte and sperm meet at an appropriate time, and represents an example of how the sperm, oocyte, and oviduct interact to ensure successful fertilization.

Oviduct sperm reservoir formation

In many mammalian species, sperm bind to the isthmic epithelium to establish a sperm reservoir. Different studies have linked the formation of this storage reservoir to the prevention of polyspermy, or maintenance of sperm motility and fertility until ovulation (Suarez, 2006). Indeed, sperm incubated with OECs are capable of developing hypermotility, and maintain their fertilising capacity for 30 h, in contrast to sperm incubated in isolation (Pollard *et al.*, 1991). In the bovine, sperm-oviduct interactions are mediated by fucose residues present throughout the oviduct during oestrus (Lefebvre *et al*., 1997). Only uncapacitated sperm can bind to the oviduct (Lefebvre and Suarez, 1996). In fact, Ca2+ influx and tyrosine phosphorylation in sperm are reduced or inhibited whilst bound, likely keeping them uncapacitated (Töpfer-Petersen *et al*., 2002). Reservoir release likely occurs via plasma membrane modification, leading to the loss of oviductal binding proteins, and hyperactivation of motility (Suarez, 2006). The signals that maintain sperm quiescence and that activate capacitation remain unknown; however, it is plausible that ovarian cues from the dominant or ovulated follicle stimulate the oviduct epithelium to secrete factors that regulate sperm physiology. This would explain why OF from oestrus cows is more successful in inducing sperm capacitation than fluid collected from other stages of the cycle (Parrish *et al.,* 1989).

Once sperm disengage from the reservoir they still have to make their way to the site of fertilisation. So far, four mechanisms have been proposed to guide sperm to the proximity of the oocyte, all of which are driven by the female environment: peristaltic pumping, thermotaxis, rheotaxis, and chemoattractant gradient (Suarez, 2006). The smooth muscle contractions of the oviduct, especially in the isthmus, not only propel sperm, but also create OF currents (Ishikawa *et al.*, 2016). Bull sperm have been shown to orientate their heads against a current when flow velocity reaches 15 µm/s (Tung *et al*., 2015). While the rate of fluid flow in the bovine oviduct is unknown, in mice it is 18 ± 1.6 µm/s (Miki and Clapham, 2013). In addition to OF flow, thermotaxis has been proposed as a long-range guiding mechanism. In pigs and rabbits, a temperature drop in the isthmus is observed at ovulation (Hunter and Nichol, 1986; Bahat *et al*., 2005). Capacitated sperm seem able to sense temperature differences and orientate their swimming towards warmer temperatures (Bahat *et al*., 2012; Pérez-Cerezales *et al.*, 2015a), leading them to the site of fertilisation. The final guidance system, chemotaxis, is likely limited to short distances, within the order of millimetres (Pérez-Cerezales *et al*., 2015b). Many substances have been proposed as sperm chemoattractants (reviewed by Eisenbach and Giojalas, 2006); however, due to multiple technical difficulties in chemotactic studies, the data are inconclusive.

Sperm capacitation and hyperactivation

The fertilising ability of sperm is suppressed until capacitation, a process comprising physiological changes, which physiologically occurs in the female reproductive tract (Yanagimachi, 1994). These include: flagellar motility hyperactivation, regulation of signal transduction pathways enabling chemoattractant responsiveness and acrosome-oocyte reactivity (Florman and Fisore, 2014).

Capacitation seems to be initiated by cholesterol efflux (Visconti *et al*., 2002). Cholesterol removal requires extracellular bicarbonate and cholesterol acceptors, such as albumin, one of the major OF proteins (Flesch *et al*., 2001). Cholesterol extraction increases membrane fluidity and ion permeability (Flesch and Gadella, 2000; Khorasani *et al*., 2000, and initiates diffusion, and possibly formation, of acrosomal lipid raft-like structures containing ZP-binding molecules (Khalil *et al*., 2006).

In addition to membrane architecture changes, the oviduct can alter sperm motility patterns which can be recapitulated *in vitro*. Hyperactivated motility, seen in most sperm recovered from the ampulla, requires elevated Ca2+ (Colás *et al*., 2010) and enables sperm to penetrate OF, the cumulus intercellular matrix, and the ZP. Plasma membrane Ca2+-ATPase 4a (PMCA4a), the major Ca2+ efflux pump in murine sperm, is present in OF EVs (Al-Dossary *et al*., 2013), and plays an important role in sperm motility, as its absence leads to an inability to hyperactivate (Okunade *et al*., 2004). EV PMCA4a is enzymatically active and can be transferred to sperm, as evidenced by increased activity following EV interaction (Bathala *et al*., 2018). On the other hand, CatSper (cation channel of sperm is the major Ca2+ entry pathway controlling sperm hyperactivation in different mammalian species (Ren *et al*., 2001; Quill *et al*., 2003; Johnson *et al*., 2017). Nanomolar concentrations of P4, diluted ZP preparations, or bovine serum albumin (BSA) can activate CatSper, inducing

increased intracellular Ca2+ (Xia and Ren, 2009a, b; Lishko *et al.,* 2011; Smith *et al*., 2013). Therefore, the emerging theory is that CatSper is essential for sperm hyperactivation and is controlled by oviduct signals, depending on sperm location and phase of the cycle (Kirichok and Lishko, 2011; Johnson *et al*., 2017).

Fertilization

Soon after the oocyte and sperm meet in the ampulla, fertilization occurs – a complex process requiring an intimate association between the gametes, such that the sperm can penetrate the ZP and plasma membrane, and deliver the paternal DNA. Although some of the key players of these interactions remain unknown, several OF-derived factors are thought to be involved. Perhaps one of the most studied is OVGP1. As mentioned above, OVGP1 has been identified in the OF of numerous mammals, and has been shown to bind to the ZP (O'Day-Bowman *et al*., 1996; Coy *et al*., 2008). Interestingly, the role of this protein appears to differ between species. Porcine and bovine oocyte incubation with OF leads to decreased sperm bound to the ZP (Coy *et al*., 2008). Moreover, in the same species, OF-derived OVGP1 and heparin-like glycosaminoglycans seem to increase ZP resistance to enzymatic digestion and sperm penetration, contributing to the control of polyspermy (Coy *et al*., 2008; Algarra *et al*., 2016). OVGP1 can also bind to sperm to mediate changes involved in the process of capacitation and acrosome reaction (Choudhary *et al*., 2017); another example of how the oviduct can synchronise the capacitation status of the sperm to ensure that fertilization occurs under optimal circumstances.

Immune response modulation

The immune system of the reproductive tract is uniquely required to protect the mother against pathogens, whilst allowing symbiosis with allogeneic sperm and the semi-allogeneic embryo and fetus. The mechanisms regulating immunological tolerance towards paternal antigens and the embryo have not been completely elucidated. However, the oviduct epithelium seems to play an important role. Sperm incubation with OEC-conditioned media decreases their phagocytosis by neutrophils *in vitro* (Marey *et al*., 2014). Prostaglandin E2 (PGE2), alpha-1 acid glycoprotein (AGP), (BSA), and the combination of AGP or BSA with other OF components are predicted to regulate this decreased phagocytosis (Kowsar *et al*., 2017). It seems that live sperm are involved in the regulation of this protective response, as sperm binding to OECs induces them to produce PGE2, and the anti-inflammatory cytokines TGFB1 and IL10 (Yousef *et al.,* 2016). In contrast, dead or abnormal sperm fail to induce PGE2 secretion (Kodithuwakku *et al*., 2007).

The role of SP in modulating reproductive immune responses has been gaining interest lately. The absence of SP at insemination in mice leads to decreased embryo development in the oviduct, embryo implantation, and placental development (Bromfield *et al*., 2014). The positive effect of SP is thought to be attributable to its immunoregulatory properties – it induces antigen specific Treg cell expansion, as well as tolerogenic dendritic cell expansion, considered important in immune tolerance to paternal antigens in the embryo (Robertson *et al*., 2009; Guerin *et al*., 2011; Shima *et al*., 2015). In addition, granulocyte-macrophage colony-stimulating factor (CSF2), leukemia inhibitory factor (LIF), interleukin 6 (IL6), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), embryokines important to embryo quality, can be regulated by SP exposure in the oviduct (Bromfield, 2016). Thus, seminal plasma may help shape an optimal environment for the early embryo. However, evidence for a significant role for seminal plasma in pregnancy establishment in cattle is not clear. To date, the only study that has looked at the effect of SP or transforming growth factor beta (TGFβ) (thought to be responsible for the beneficial effects of SP in rodents) in cattle pregnancy outcome, concluded that this factor (but not SP as a whole) had a positive effect only when reproduction was suboptimal (Odhiambo *et al*., 2009).

Oviduct-embryo communication

Following fertilization, the bovine zygote spends ~4 days in the oviduct until migrating to the uterus as a 16-cell stage embryo (Hunter, 2012). During this period, the oviduct provides a nourishing environment conducive to embryo development comprising simple and complex carbohydrates, ions, lipids, phospholipids and proteins (Avilés *et al*., 2010). In addition, the oviduct is also responsible of transporting the embryo to the uterus through muscular and ciliary activity.

Whilst uterine-embryo dialogue has been extensively studied, relatively little is still known about oviduct-embryo communication. Our current understanding is that this phenomenon is a two-way process (Fig. 1), *i.e.* signals can be sent and received from both the oviduct and the embryo; however, these remain largely undefined. Our group has recently described bone morphogenetic proteins (BMPs) as participants in a signalling pathway involved in oviduct-embryo cross-talk *in vitro* (García *et al*., 2017). Embryo-oviduct interaction *in vitro* induces transcriptional changes of BMP signalling components, both through direct and indirect contact (Hamdi *et al*., 2018), indicating that the signal is released in OF. Thus, analysing early embryo-maternal interactions involves studying OF in addition to the embryo, oviduct epithelium, and the direction of the communication.

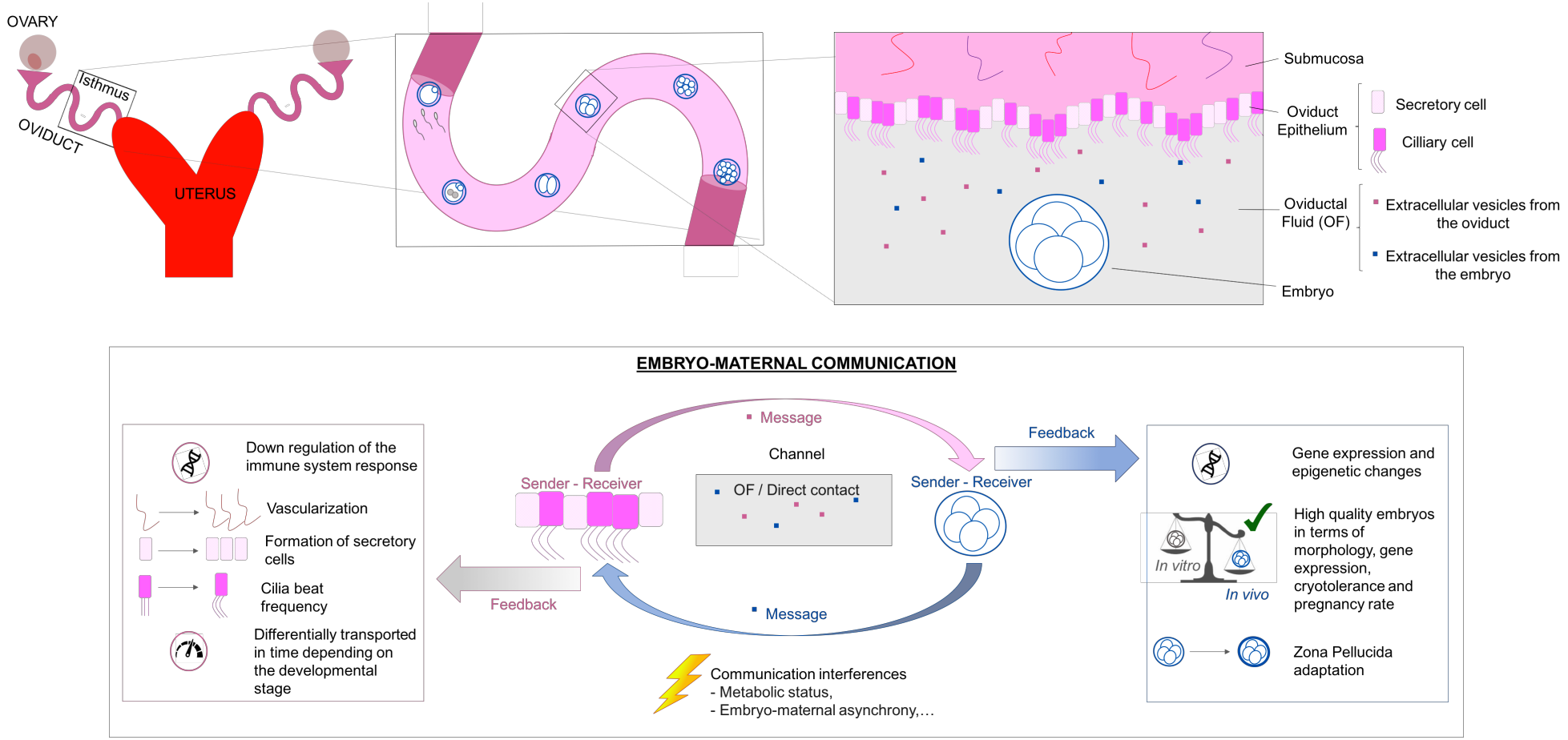

Figure 1. Schematic representation of embryo-maternal communication in the oviduct.

In vivo *modelling*

Although tremendous advances have led to improved *in vitro* models for studying embryo-oviduct interactions, such models remain limited in their ability to fully mimic *in vivo* conditions (Lonergan and Fair, 2008). Therefore, animal models are crucial to holistically understanding the physiology and pathology surrounding early embryo development.

In our laboratory, we have investigated the effect of different embryo culture environments (*in vitro*, *in vivo* in surrogate ovine oviducts, and *ex vivo* in the murine oviduct) on blastocyst development and quality, finding that culture in the oviduct (*in vivo* and *ex vivo*) improves embryo quality in terms of morphology, gene expression, and cryotolerance (Enright *et al*., 2000; Rizos *et al*., 2002; Lonergan *et al*., 2003). Interestingly, heterologous embryo culture can support early embryo development, resulting in the establishment and maintenance of pregnancy, although only the ovine oviduct has been routinely used for bovine embryo culture (Lazzari *et al*., 2010). A new approach for culturing *in vitro* and/or *in vivo* produced embryos in the homologous bovine oviduct *in vivo* by transvaginal endoscopy has been introduced successfully (Besenfelder *et al*., 2010). Using this technique, Wetscher *et al*. (2005) and Havlicek *et al*. (2010), found that short and long-term *in vivo* culture of *in vitro* produced embryos in the homologous bovine oviduct enhanced blastocyst quality, in terms of cryotolerance, relative to blastocysts grown entirely *in vitro*. Moreover, total blastocyst yields were similar to previous data derived using surrogated heterologous oviducts as a temporary incubator (Enright *et al*., 2000; Rizos *et al*., 2002; Lonergan *et al*., 2003; Lazzari *et al*., 2010).

A key milestone in early embryo development is embryonic genome activation (EGA). Using alternative *in vivo* and *in vitro* culture conditions for blastocyst production, Gad *et al*. (2012) demonstrated that *in vitro* conditions at the time of major EGA critically influence the transcriptome of the subsequent blastocysts. Furthermore, the methylation pattern of *in vitro* derived blastocysts differs from *in vivo* counterparts (Gad *et al*., 2012). This was demonstrated in the bovine by Salilew-Wondim *et al*. (2015), who transferred *in vitro* zygotes, 4-cell, and 16-cell embryos into recipient heifer oviducts. Resulting blastocysts were recovered on day 7 and compared with blastocysts produced *in vitro*. The degree of DNA methylation dysregulation in the promoter and/or gene body regions was correlated positively with *in vitro* culture duration.

Due to the early embryo being usually described as somewhat autonomous up to the blastocyst stage (*i.e*. does not need contact with the maternal reproductive tract), one could argue about the influence of maternal-embryonic asynchrony on embryo development. To investigate this further, our group endoscopically transferred day 1 *in vitro* produced bovine zygotes to the oviducts of heifers either synchronous with the embryos (at day 1 post-ovulation), or asynchronous (at day 3 post-ovulation), prior to embryo recovery on day 4 (8- to 16-cell stage), day 7 (morula-blastocyst), and day 15 (elongated conceptus). Interestingly, asynchrony had a negative impact on early embryo survival and development (Rodríguez-Alonso *et al*., 2018a), unlike in the uterus, wherein the transfer of a blastocyst to an advanced uterus results in accelerated embryo development (Randi *et al*., 2016).

Another study from our group assessed the contribution of the oviduct to poor fertility in postpartum dairy cows – a disorder linked with suboptimal follicle development, oocyte quality, sperm transport and fertilization, reproductive tract environment, and/or a combination of these (Lonergan *et al*., 2016) – and found significantly lower blastocyst yields when *in vitro* produced zygotes were transferred to the oviducts of lactating (~ day 60 postpartum *vs*. dry cows (Maillo *et al*., 2012) and heifers (Rizos *et al*., 2010).

Today, most of the studies related to the maternal-embryo interactions in the oviduct reflect the effect on the embryo, meanwhile there are only few reported the converse effect on the oviduct (reviewed by Maillo *et al*., 2016). Even more, most of them have been performed in poly-ovulatory species – *e.g.* murine and porcine – in which the presence of multiple embryos presumably magnifies the signal for altering OEC gene expression (Chang *et al.,* 2000; Lee *et al.*, 2002; Almiñana *et al.,* 2012). In an effort to dissect the directionality of oviduct-embryo dialogue in the mono-ovulatory species, Smits *et al*. (2016) reported a local influence of a single embryo on the transcriptome of the equine oviduct epithelium; while in bovine Maillo *et al*., (2015) was unable to detect differences in the oviduct isthmus transcriptome in the presence of a single embryo. However, when up to 50 embryos were endoscopically transferred into heifer oviducts, with the aim of amplifying embryo-derived signals, OEC transcriptomic differences became apparent, mostly related to the immune system response. Thus, the physiological local embryo-oviduct interaction may be undetectable using current technologies owing to the relatively small and localised response elicited.

To tease this out, we recently isolated ipsilateral oviducts from single-ovulated artificially inseminated heifers post-mortem on day 2.5 post-estrus. These were subsequently sectioned (into 2 cm lengths) and flushed for embryo retrieval (2-cell). The expression of 10 genes previously shown to be differentially expressed between the isthmus of pregnant and cyclic heifers (Maillo *et al*., 2015), was assessed. Differences were found both where the embryo was located and proximally, *i.e.* where the embryo had passed (Rodríguez-Alonso *et al*., 2018b).

In vitro modelling

Owing to technical limitations surrounding OF sampling *in vivo* (see Leese *et al*., 2008) coupled with logistical issues, and the high costs associated with *in vivo* studies, *in vitro* models are pivotal to studying oviduct physiology. *In vitro* modelling furthermore enables investigations of greater environmental

manipulation, (Ulbrich *et al*., 2010). OECs are currently generally cultured *in vitro* as basic monolayers or cell suspensions (Lopera-Vásquez *et al.* 2016), polarized two-dimensional monolayers (Chen *et al*., 2017; Jordaens *et al*., 2017; Simintiras *et al*., 2017), or three-dimensional monolayers (Ferraz *et al*., 2017a, b).

Despite *in vitro* OEC de-differentiation and morphological characteristic loss (Rottmayer, *et al*. 2006), including height reduction, cilia and secretory granule loss, and bulbous protrusions (Thibodeaux, *et al.* 1992, Walter 1995), *in vitro* modelling presents an opportunity to detect essential and functional candidate genes in embryo-maternal dialogue (Schmaltz-Panneau *et al.* 2014) that are difficult to study *in vivo*, and the capacity to investigate OF formation and regulation free from systemic effects (Simintiras *et al*., 2017). The latter, coupled with OEC-conditioned media (Ramos-Ibeas *et al.* 2014), offer scope for improving *in vitro* embryo culture, particularly as co-culture is associated with a lack of reproducibility, biosanitary risk (Guerin *et al.* 1997), do not contain foreign cells, and contain embryotrophic factors (Ramos-Ibeas *et al*., 2014). We recently reported that conditioned media from extended bovine OEC monolayer cultures had a consistently positive effect on blastocyst quality when used during IVC (Lopera-Vásquez *et al.* 2016).

One limitation of *in vitro* work is an inherent behavioural variability between cell populations; however, a promising solution is the use of immortalised cell lines that maintain many primary culture attributes (Ulbrich *et al.* 2010). Another development is the short-term (24 h) epithelial cell suspension culture, in which OECs maintain morphological characteristics as well as gene markers present *in vivo* such as OVGP1, E2 and P4 receptors (Rottmayer, *et al.* 2006). However, suspended cells do not adhere and mitosis does not occur (Walter, 1995).

The OEC polarized system consists of culturing the cells on inserts to allow media access from both basolateral (vasculature mimic) and apical (luminal mimic) sides, therein also maintaining the natural asymmetrical nature of the epithelium. This system preserves detailed morphological features of the porcine oviduct and oviduct-specific markers (Miessen *et al*., 2011). Bovine OECs cultured in this way have been used to model elevated non-esterified fatty acid metabolic stress (Jordaens *et al*., 2015, 2017) in addition to testing the barrier properties of the oviduct epithelium to dietary-derived embryotoxins (Simintiras and Sturmey, 2017). Another category of such polarized culture is the air-liquid interface (ALI) system in which medium is exclusively supplied basolaterally, allowing the formation of oviduct fluid surrogate or *in vitro* derived oviduct fluid (Simintiras *et al*., 2017), in the apical chamber. Epithelia derived from human, porcine, and bovine oviducts maintain polarity and an *in vivo*-like morphology when cultured like this long-term (Chen *et al*., 2013, 2017; Levanon *et al*., 2010).

Moreover, Chen *et al*. (2017) reported that ALI supports development *in vitro* in the OFC, of porcine, murine, and bovine embryos. However, blastocyst rates were inferior to current optimized standard IVP procedures, suggesting a need for further model improvement by simulating physiological hormonal changes, and developing a sequential culture system using oviduct as well as uterine epithelial cells (Chen *et al*., 2017).

Very recently, the use of three-dimensional (3D) printing in combination with microfluidics, has led to the creation of the oviduct-on-a-chip with a U-shaped porous membrane enabling OEC polarization, which can be maintained during long-term culture, therein mimicking tissue and organ-specific micro-architecture (Ferraz *et al*., 2017a, b). It has also been shown that specific tissue morphology and functions are more faithfully mimicked in customized 3D *vs*. 2D systems (Gualtieri *et al*., 2012; Costello *et al*., 2014).

As aforementioned within an *in vivo* context, *in vitro* derived embryos also secrete EVs (Saadeldin *et al*., 2015). These data led us to hypothesize that culture medium supplementation with OEC EVs could initiate a maternal-embryo dialogue beneficial to embryo development. We found that suplementation of *in vitro* embryo culture media with bovine EVs obtained from OECs culture *in vitro* (Lopera-Vásquez *et al*., 2016) and *in vivo* (OF) (Lopera-Vasquez *et al*., 2017) substantially improved *in vitro* produced blastocyst quality.

In addition, the use of OF and UF has been recently used to improve *in vitro* embryo production. One example is a study from our group for which *in vitro* derived embryos were produced and cultured with or without OF and/or UF supplemented media. Low concentrations of OF (days 1 to 4) and UF (days 4 to 8) in serum-free culture indeed supported embryo development and improved embryo quality with OF incorporation resulted in more physiological embryo methylation patterns, whereas UF is thought to have played an antioxidant role (Hamdi *et al.*, 2017).

In conclusion, the oviduct is an important, unique, and interesting secretory organ gaining greater attention owing to increased awareness of embryo-induced changes affecting later stages of development. Answers to fundamental questions foreseeably reside in merging data obtained from advanced complementary *in vivo* and *in vitro* methodologies, all geared at understanding important events of early embryo-maternal communication.

Acknowledgments

The authors' work is supported by the Spanish Ministry of Economy and Competitiveness, AGL2015-70140-R; EU, Horizon 2020 Marie Sklodowska-Curie, REPBIOTECH 675526, Science Foundation Ireland (13/IA/1983), the Irish Research Council (GOIPD/2017/942), and a University College Dublin Career Development Award (54580). Authors are members of the European Cooperation in Science and Technology, Cost Action CA 16119 - *In vitro* 3-D total cell guidance and fitness.

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DOI: 10.21451/1984-3143-AR2018-0028

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Physiological and cellular requirements for successful elongation of the preimplantation conceptus and the implications for fertility in lactating dairy cows

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Abstract

Elongation of the preimplantation conceptus is a prerequisite for maternal recognition of pregnancy and implantation in ruminants. Failures in this phase of development likely contribute for the subfertility of lactating dairy cows. This review will discuss our current understanding of the physiological and cellular requirements for successful elongation of the preimplantation conceptus and their potential deficiency in subfertile lactating dairy cows. Major requirements include the priming of the endometrium by ovarian steroids, reprogramming of trophectoderm cells at the onset of elongation, and intensification of the crosstalk between elongating conceptus and endometrium. Conceptus elongation and survival in dairy cows does not seem to be affected by lactation per se but seem to be altered in subgroups of cows with endocrine, metabolic and nutritional imbalances or deficiencies. These subgroups of cows include those suffering diseases postpartum, anovular cows enrolled in synchronization programs, and cows with low concentration of circulating steroids and IGF1. Success of conceptus elongation starts long before breeding and entails optimization of health and nutrition programs, especially during the transition period, and might be extended to the supplementation of endocrine and nutritional shortages at the time of breeding. Genetic selection will eventually become more important as researchers unravel the molecular control of reproduction and develop new fertility traits focused on pregnancy survival.

Keywords: conceptus elongation, dairy cow, pregnancy loss.

Introduction

Early pregnancy losses are highly prevalent in lactating cows and lessen production efficiency in dairy herds (Ribeiro *et al*., 2012). Approximately half of the zygotes fail to survive the first 4 weeks of development, contributing meaningfully for the low average conception risk of lactating cows (Santos *et al*., 2004; Wiltbank *et al*., 2016; Ribeiro, 2018; Fig. 1). Ultimately, these losses are caused by impaired developmental competence of the zygote and/or inadequate uterine environment, which in turn are influenced by the genetics of the cow and embryo, and by health, nutritional, endocrine, environmental factors affecting ovarian and uterine biology of the cow. Although embryonic losses in the first week are substantial, failures in the peri-implantation stages of conceptus development seem to account for an important portion of pregnancy losses (Fig. 1A). It has been estimated that 39% of day 6 morulas fail to survive by day 28 of development (Ribeiro, 2018). The moderate efficiency of embryo transfer (ET) as breeding strategy for lactating cows further emphasize the significance of the peri-implantation period for pregnancy success (Table 1).

In cattle, the developing morula enters the uterus around day 4 but implantation starts only around day 20. Therefore, formation of the blastocyst, expansion and hatching from the zona pellucida, formation of ovoid conceptus, elongation and initial differentiation of trophectoderm binucleated cells must all be coordinated by uterine histotroph. Over the years, substantial research efforts have been placed to understand the biology of these events and their connection to pregnancy failures in dairy cows. Large emphasis has been given to the elongation phase of conceptus development because of its complexity and necessity for maternal recognition of pregnancy and implantation. This review summarizes our current understanding of the physiological and cellular requirements for successful elongation of the conceptus and discusses the potential contribution of impaired elongation to subfertility of lactating dairy cows.

Physiological and cellular requirements of elongation

Elongation of the preimplantation conceptus entails remarkable expansion of extraembryonic tissues along the uterine lumen in a short window of development (Betteridge, 1980; Wales and Cuneo, 1989). In cattle, elongation starts around day 14 and, within 3 days, the conceptus grows from <5 mm to approximately 250 mm in length and occupies almost the entire extension of the pregnant uterine horn. The exponential increase in tissue mass is explained mainly by rapid proliferation of trophectoderm cells (Wang *et al*., 2009). The augmented rate of proliferation is induced by driver signals and demands substantial supply of nutrients (e.g. lipids, amino acids, sugar, nucleotides) for energy expenditures and synthesis of biomass. The required signals and nutrients are provided by the uterine histotroph, whose composition is modulated by the activity of ovarian steroids and conceptus-derived molecules (Spencer *et al*., 2004). This section reviews scientific data that provide insights on the physiological and cellular events that coordinate conceptus elongation.

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Received: February 14, 2018
Accepted: April 29, 2018

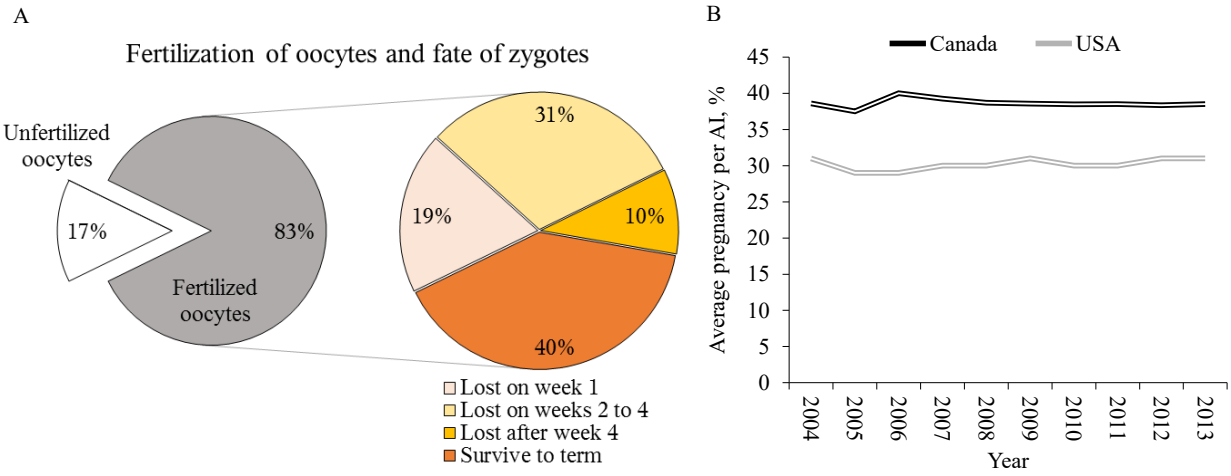

Figure 1. Pregnancy failures and average conception risk in lactating cows in North America. (A) Percentage of fertilize and unfertilized oocytes and fate of zygotes. (B) Average conception risk of DHI herds in Ontario and Western Canada and average conception risk of US Holstein cows in DHI herds. Data from Ribeiro (2018).

Table 1. Pregnancy per embryo transfer in lactating dairy cows according to the type of embryo[1].

Reference	Embryo	Frozen/Fresh	Recipient, n	Pregnant, n	Pregnant, %
Chebel *et al*., 2008	IVP	Fresh	524	269	51.30%
Stewart *et al*., 2011	IVP	Fresh	136	62	45.50%
Mikkola *et al*., 2015	IVP	Fresh	2935	1429	48.70%
Monteiro *et al*., 2015	IVP	Fresh	360	96	26.60%
Ferraz *et al*., 2016	IVP	Fresh	2225	723	32.50%
Pereira *et al*., 2016	IVP	Fresh	2003	837	41.80%
Pereira at al., 2017	IVP	Fresh	323	100	31.00%
Barbosa *et al*., 2018	IVP	Fresh	1097	396	36.10%
Reese *et al*., 2018	IVP	Fresh	240	80	33.30%
Total Fresh IVP			***9843***	***3992***	***40.60%***
Chebel *et al*., 2008	IVP	Frozen	109	48	44.00%
Block *et al*., 2010	IVP	Frozen	142	47	33.10%
Stewart *et al*., 2011	IVP	Frozen	178	56	31.60%
Marinho *et al*., 2015	IVP	Frozen	3392	1241	36.60%
Ferraz et al., 2016	IVP	Frozen	194	56	28.90%
Total Frozen IVP			***4015***	***1449***	***36.10%***
Pereira *et al*., 2013	SOV	Fresh	487	216	44.40%
Ferraz *et al*., 2016	SOV	Fresh	651	303	46.50%
Total Fresh SOV			***1138***	***519***	***45.60%***
Thatcher *et al*., 2001	SOV	Frozen	181	76	42.00%
Kenyon *et al*., 2012	SOV	Frozen	289	84	29.10%
Kenyon *et al*., 2013	SOV	Frozen	135	49	36.20%
Mikkola *et al*., 2015	SOV	Frozen	7762	3283	42.30%
Ferraz *et al*., 2016	SOV	Frozen	1042	356	34.20%
Total Frozen SOV			***9409***	***3849***	***40.90%***
Total			**24405**	**9808**	**40.20%**

[1]IVP = *in vitro*-produced embryo. SOV = *in vivo*-produced embryo.

Priming of the uterus by ovarian steroids

During the course of the estrous cycle, endometrium physiology in ruminants is regulated by changes in concentrations of ovarian steroids in blood circulation and temporal changes in expression of steroid receptors in endometrial cells (Spencer and Bazer, 1995). Activity of steroid hormones in endometrial cells determines the length of the estrous cycle and is essential for pregnancy establishment and maintenance (Thatcher *et al*., 1989, Geisert *et al*., 1992). Classic studies that use replacement of ovarian steroids in ovariectomized ewes were fundamental to demonstrate the importance of these hormones to early conceptus development. Ewes ovariectomized 3.5 days after breeding (Foote *et al*., 1957) or at the time of embryo transfer 4 days after estrus (Moore and Rowson, 1959) were able to support pregnancy to term when exogenous progesterone was administered from the time of the ovariectomy until day 60 of gestation. To be successful, daily doses of at least 10 mg of progesterone were required (Moore and Rowson, 1959; Bindon, 1971). It was observed that exogenous progesterone increased cell height of glandular and luminal epithelial cells of the endometrium (Bindon, 1971) and increased their secretory activity into the uterine lumen (Miller and Moore, 1976; Garrett *et al*., 1988).

Miller and Moore (1976) established a steroid replacement protocol for ovariectomized ewes that would resemble endogenous ovarian secretion during early pregnancy (referred to as maintenance progesterone), around the time of estrus (referred to as estrous estradiol) and during the luteal phase immediately preceding estrus (referred to as priming progesterone). This protocol was successful to support embryo development after ET on day 4. Overall, 72% of the recipient ewes (73 out of 101) had a conceptus on day 25, and 62% of the conceptuses (45 out of 73) were considered morphologically normal. The importance of each component of the established protocol was also investigated (Fig. 2). The complete protocol resulted in 9 pregnant and 8 normal conceptuses out of 13 ewes. As expected, pregnancies were not obtained (0 out of 11) when the maintenance progesterone was omitted. Omission of the estrous estradiol resulted in smaller number of pregnancies (7 out of 11) and increased proportion of abnormal conceptuses (6 out of 7). Similarly, exclusion of the priming progesterone reduced the number of pregnancies (5 out of 11) and increased the proportion of abnormal conceptuses (3 out of 5). Finally, the omission of both priming progesterone and estrous estradiol resulted in no pregnancies (0 out of 11). Investigation of endometrium metabolism revealed that omission of maintenance progesterone reduced synthesis of proteins and RNA to DNA ratio, and omission of estrous estradiol and priming progesterone had additive effects reducing the RNA to DNA ratio. Thus, not only progesterone during the time of conceptus development but also estradiol secretion at time of estrus and the progesterone secreted before estrus have important roles in the establishment of a uterine environment suitable for normal development of embryos. These results and interpretation were reinforced by a second experiment (Miller *et al*., 1977).

The development of high-throughput technologies for transcriptomics has allowed researchers to revisit classic concepts in reproductive biology and expand our understanding of the molecular control of reproduction. For instance, Shimizu *et al*. (2010) used ovariectomized cows to investigate the independent and the interdependent effects of progesterone and estradiol in the endometrium transcriptome. Compared to a control group (no additional treatment), replacement of progesterone alone, estradiol alone, and the combo treatment resulted in 289, 721, and 689 differentially expressed genes (DEG) in the endometrium, respectively. Interestingly, there was little overlap in the endometrial response to progesterone alone compared with the response observed in groups that received estradiol. Contrarily, the response to estradiol alone and the response to progesterone and estradiol combined had 50% overlap. Functional analyses of DEG revealed that the response of the endometrium to combined administration of the two hormones did not simply reflect the net effect of individual treatments, but rather suggested a functional interaction between the two hormones. In general, priming of the endometrium with progesterone caused the amplification of some estrogen-specific responses and created novel transcript responses to the estradiol treatment. Among the progesterone-primed estrogen response were genes involved with cell differentiation, migration and adhesion.

Transcriptome studies of the bovine endometrium were also fundamental to understand the direct regulation of endometrial physiology by progesterone during the time of conceptus development. The magnitude of progesterone effects on endometrium physiology was evidenced by the substantial temporal changes in the transcriptome of endometrial cells at different stages of the estrous cycle (Bauersachs *et al*., 2005; Mitko *et al*., 2008; Forde *et al*., 2009, 2011) and its modulation by exogenous progesterone supplementation (Forde *et al*., 2009, 2011). These studies reported multiple patterns of gene expression throughout the estrous cycle, with both up- and down-regulation of specific transcripts caused by exposure of the endometrium to increasing concentration of circulating progesterone. These changes are likely important for the preparation of an optimal uterine environment for the onset of conceptus elongation, and were frequently associated with transport of molecules, carbohydrate and lipid metabolic processes, extracellular matrix remodeling, growth factors and cytokine signaling, and immune responses. To some extent, these changes observed naturally during the estrous cycle are advanced by the supplementation of exogenous progesterone between days 3 and 8 of the estrous cycle (Forde *et al*., 2009, 2010, 2011; Okumu *et al*., 2010). It is noteworthy that changes in endometrial physiology are generally amplified around day 13 of the estrous cycle with the downregulation of progesterone receptors in epithelial cells, which has important implications for histotroph secretion (Spencer and Bazer, 1995; Okumu *et al*., 2010).

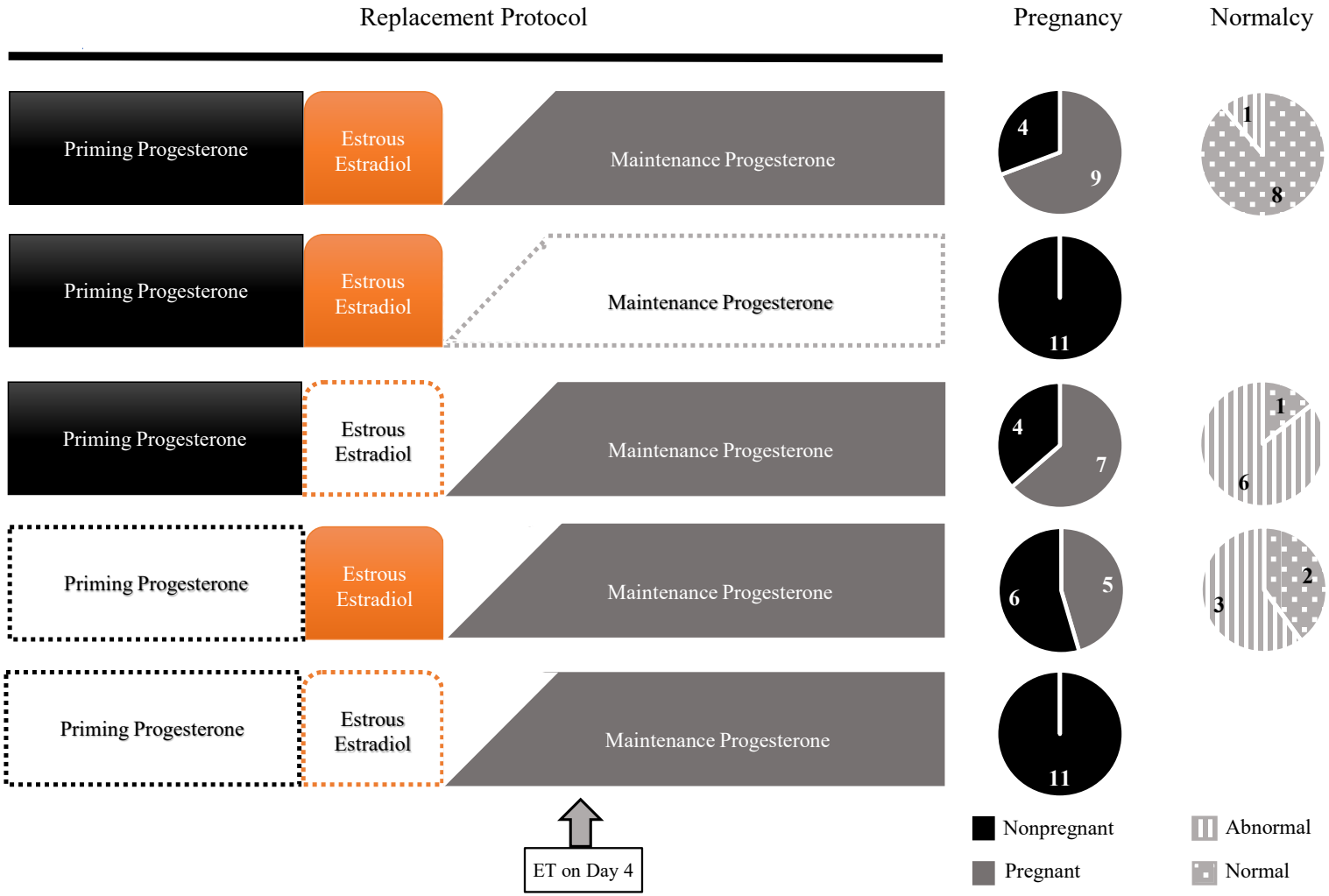

Figure 2. Survival and quality of ovine conceptuses transferred to ovariectomized ewes subject to steroid replacement protocols. Forms without color within the protocol represent the omission the specific treatment from the protocol. Data from Miller and Moore (1976).

The temporal regulation of endometrium physiology affects the composition of the uterine histotroph which is important to meet the evolving cellular requirements of the developing conceptus. Embryo transfer studies have clearly demonstrated the importance of synchrony between donor and recipient for successful establishment of pregnancy (Moore and Shelton, 1964), which is intrinsically associated with the ability of the uterus to provide an adequate uterine environment to a specific stage of development. As the estrous cycle progresses, changes in the uterine environment support the formation of an ovoid conceptus by day 13 and provide signals that augment proliferation of trophectoderm cells and the onset of elongation. For instance, Mullen *et al*. (2012a) compared the proteomics of uterine flushings collected from dairy heifers on days 7 and 13 of the estrous cycle and identified 38 proteins with different abundances. These proteins were associated with tissue remodeling, metabolism, and free radical management, and might be relevant for the onset of elongation.

The composition of histotroph is also affected by temporal changes in the ability of the endometrium to sequestrate and transport metabolites into the uterine lumen. For instance, concentrations of amino acids in the uterine fluid changes from early to mid-diestrus (Hugentobler *et al*., 2007) and are altered by the profile of steroid hormones during the peri-ovulatory period (França *et al*., 2017). Transcript expression of the facilitative glucose *SLC5A1* in endometrial epithelium of heifers was greater on day 13 of the estrous cycle compared with samples collected on days 5 and 7 (Forde *et al*., 2010). This transporter could facilitate the influx of glucose into the uterine lumen according to the demand of the developing conceptus. Similarly, transcript expression in the endometrium and protein concentration in the histotroph of retinol-binding protein 4 (RBP4) were increased on day 13 compared with day 7 of the estrous cycle in heifers (Mullen *et al*., 2012b), which might be important to regulate sequestration of circulating retinol and its availability in the uterine lumen at the onset of elongation. In fact, Costello *et al*. (2010) reported greater concentration of retinol in uterine flushings collected at late-diestrus compared with those collected in metaestrus and early diestrus. Moreover, transcript expression of insulin-like growth factor (IGF) binding protein 1 (*IGFBP1*) in the endometrial epithelium of heifers and cows was undetectable on days 8-10 but highly expressed on days 12-14 of the estrous cycle, which could influence sequestration and bioavailability of IGF1 in the uterine lumen, in addition to its IGF-independent roles in conceptus elongation (Robinson *et al*., 2000; Simmons *et al*., 2009).

Steroid hormones also influence lipid metabolism in endometrial cells. The amount of lipid droplets in the epithelium fluctuates according to the phase of estrous cycle in both, cows (Wordinger *et al*., 1977) and ewes (Brinsfield and Hawk, 1973), being low during metaestrus and increasing during diestrus. Brinsfield and Hawk (1973) administered 25 mg of exogenous progesterone for 5 days in ovariectomized ewes and observed an accumulation of lipids in the endometrium that was comparable to the accumulation observed in a spontaneous estrous cycle, thus concluding that progesterone was the main factor inducing the accumulation of lipids during diestrus. The mechanism by which progesterone influences the formation of lipid droplets is still unknown, but genes reported to be modulated by progesterone in the endometrium of cattle such diacylglycerol O-acyltransferase 2 (*DGAT2*) and lipoprotein lipase (*LPL*) might be important in this process. Transcript expressions of *DGAT2* and *LPL* in epithelial cells are increased and decreased, respectively, during diestrus (Forde *et al*., 2009, 2010, 2011). Their encoded proteins have opposing functions, with the former being involved in synthesis of triglycerides and the later in hydrolysis of triglycerides. Neutral lipids such as triglycerides facilitate the packing of lipids and the formation of lipid droplets (Thiam *et al*., 2013).

Reprogramming of conceptus cells at the onset of elongation

The specific components of the histotroph that drive conceptus elongation are not completely known. The nature of these components could be potentially identified by evaluating the biology of conceptus cells at the onset of elongation and how they sense and respond to the histotroph stimuli. Recently, two independent studies evaluated the transcriptome of bovine conceptuses at early stages of elongation and revealed substantial changes in cell biology with the onset of elongation (Barnwell *et al*., 2016; Ribeiro *et al*., 2016a). Comparison of DEG in the two studies shows ample overlap, which increases the confidence on the methodology used and in the results obtained. Functional analyses of DEG not only revealed important pathways that are modulated during the onset of elongation but also identified potential upstream regulators of the observed changes in the transcriptome, which might represent the uterine signals required for elongation. Changes in the transcriptome of conceptus cells with the magnitude observed at early stages of elongation do not seem to occur at later stages of elongation (Valour *et al*., 2014). Thus, the substantial change in transcriptome at the onset of elongation is suggested here as a reprogramming event of extraembryonic cells required for successful elongation. To some extent, the main findings of these two recent studies are also supported by previous research evaluating the development of bovine conceptuses but using distinct experimental designs and methodology (Hue *et al*., 2007, 2012; Clemente *et al*., 2011; Mamo *et al*., 2011).

A considerable portion of the DEG reported by Barnwell *et al*. (2016) and Ribeiro *et al*. (2016a) referred to cytoskeleton organization and cell adhesion molecules, and reinforced the idea that elongation in ruminants is not the simple proliferation of cells that are molded according to the shape of the uterine lumen but an elaborate event that requires cytoskeleton reorganization and active interaction between cells and the extracellular matrix (Kim *et al*., 2010). Ribeiro *et al*. (2016a) suggested that with the onset of elongation, the

upregulation of multiple transmembrane cell-matrix adhesion proteins increases the capacity of conceptus cells to interact with the extracellular matrix and endometrial epithelial cells, receiving mechanical load and transmitting it to intracellular actin filaments. Enhanced expression of myosins and actin crosslinker proteins would aid actin filaments to generate intracellular mechanical force that could be used for cell motility and force-generated migration. Candidate genes to coordinate these events were identified and include transmembrane 4 L six family member 1 (*TM4SF1*), transgelin (*TAGLN*), and ankyrin repeat domain 1 cardiac muscle (*ANKRD1*).

Ribeiro *et al*. (2016a) also reported IGF1 and protein kinase AKT as upstream regulators of transcriptome changes in conceptus cells during the onset of elongation, both with predicted increased activity. Insulin-like growth factor 1 is a mitogen molecule whose main downstream effects are stimulation of cell proliferation and cell survival. The AKT kinase is not only an essential component in the downstream signaling of the IGF1 receptor (Le Roith, 2001) but is also part of the mammalian target of rapamycin (mTOR) signaling pathway, which has been described to be important for cytoskeletal changes, adhesion and migration of ovine trophectoderm cells (Kim *et al*., 2010). Synthesis and secretion of IGF1 is stimulated by growth hormone (GH) in multiple tissues, although the liver is responsible for production of most of the circulating IGF1 (Le Roith, 2001). The bovine endometrium expresses GH receptor, IGF1, and IGF1 receptor (Robinson *et al*., 2000; Rhoads *et al*., 2008), while the preimplantation bovine conceptus expresses both GH and IGF1 receptors (Yaseen *et al*., 2001; Sawai *et al*., 2007). Therefore, endometrial physiology and the developing conceptus can be affected by systemic GH and IGF1 as well as by the locally produced endometrial IGF1. Of note, the increased expression of *IGFPB7* in conceptus cells during elongation is one of the most consistent outcomes reported in multiple studies (Hue *et al*., 2012; Barnwell *et al*., 2016; Ribeiro *et al*., 2016a). Moreover, the elongating bovine conceptus also seems to express IGFBP1 and IGFBP3 (Sawai *et al*., 2007; Ribeiro *et al*., 2016a).

Lipid metabolism was one of the top molecular and cellular functions associated with the DEG in the two studies described above (Barnwell *et al*., 2016; Ribeiro *et al*., 2016a). In fact, Ribeiro *et al*. (2016a) identified at least 132 genes with known annotation and linkage to this specific function. Among those genes, some were involved with lipid uptake, lipid droplet formation, biogenesis of peroxisomes, activation, oxidation, desaturation and elongation of fatty acids, biosynthesis of phospholipids, mobilization of membrane phospholipids, biosynthesis of prostaglandins, and transport of prostaglandins and other lipids metabolites (Fig. 3). In addition, peroxisome proliferator activated receptor gamma (*PPARG*) not only had transcript expression markedly increased during the onset of elongation but was also listed as an important upstream regulator of the transcriptome changes observed in trophectoderm cells during elongation. In fact, PPARG is a nuclear receptor that functions as ligand-dependent transcription factor and its transcript expression was highly correlated with the expression of other genes known to be involved with lipid metabolism and conceptus development in ruminants. The increase in expression of PPARG in conceptus cells during elongation is likely caused by the accumulation of lipids in the uterine lumen during diestrus (Ribeiro *et al*., 2016d), although a direct effect of progesterone on conceptus cells cannot be discarded (Kim *et al*., 2008; Yang *et al*. 2016).

The PPARG have large binding pockets that interact promiscuously with multiple lipid ligands including unsaturated fatty acids and prostanoid metabolites (Kliewer *et al*., 1997; Nagy *et al*., 1998). Itoh *et al*. (2008) examined crystal structures of PPARG bound to oxidized unsaturated fatty acids and concluded that the large binding pocket of the receptor confers remarkable versatility in ligand binding and could therefore act a cellular sensor of the varying composition of the cellular pool of fatty acid ligands. Binding of fatty acids into the ligand pocket of PPARG causes conformational changes in the receptor that facilitates the formation of heterodimers with retinoid X receptor (RXR) and subsequent binding of the dimer to PPAR response elements (PPRE) in regulatory regions of target genes (Berger and Moller, 2002). Putative PPRE were identified in regulatory regions of several genes transcriptionally regulated during the onset of conceptus elongation, which suggests a direct effect of PPARG on the abundance of the respective transcripts (Ribeiro *et al*., 2016a). Among genes with PPRE in regulatory regions is PPARG, which suggests that activation of PPARG could alter its own expression (Ribeiro *et al*., 2016a, d).

The activity of the nuclear receptor dimer PPARG-RXR as transcription factor depends on the presence and binding of their respective ligands. The increased expression of RBP4 and accumulation of lipid droplets in the bovine endometrium during diestrus likely contribute for this requirement (Wordinger *et al*. 1977; Mullen *et al*., 2012b). In fact, retinol and lipids are constitutive part of the histotroph of cows on day 15 of the estrous cycle (Costello *et al*., 2010; Ribeiro *et al*., 2016a). Moreover, transcript expression of *RBP4* and fatty acids transporters such as *SCL27A6* increases in conceptus cells during elongation, which might facilitate capture of retinol and fatty acids from histotroph (Ribeiro *et al*., 2016a). Research in sheep has shown that the elongating conceptus accumulates lipid droplets within the cytoplasm of trophectoderm cells (Carnegie *et al*., 1985) and causes a reduction in lipid droplet accumulation on endometrial epithelial cells (Boshier *et al*., 1987), suggesting the existence of an active transfer of lipids from the endometrium to the conceptus. Lipids would be used not only for control of gene expression but also for synthesis of biomass, energy, and cell signaling (Ribeiro *et al*., 2016d).

The idea that PPARG is important for elongation of the bovine conceptus is strongly supported by an elegant study performed in sheep. Brooks *et al*.

(2015) performed a loss-of-function study by infusing morpholino antisense oligonucleotides in the uterus of pregnant ewes from day 7 to day 14 after breeding using an osmotic pump affixed surgically in the mesosalpinx. Morpholino antisense oligonucleotides for PPARG resulted in the recovery of growth-retarded conceptuses, while infusion of the designed control or PPAR-delta morpholinos resulted in normally elongated conceptus on day 14. Binding sites of PPARG in ovine conceptus cells were identified and several of them were in close proximity to genes involved in lipid biosynthesis and metabolism also reported to be differently regulated during onset of elongation of bovine conceptus (Ribeiro *et al*., 2016a). Altogether, these studies highlight the importance of lipid metabolism for the onset of conceptus elongation.

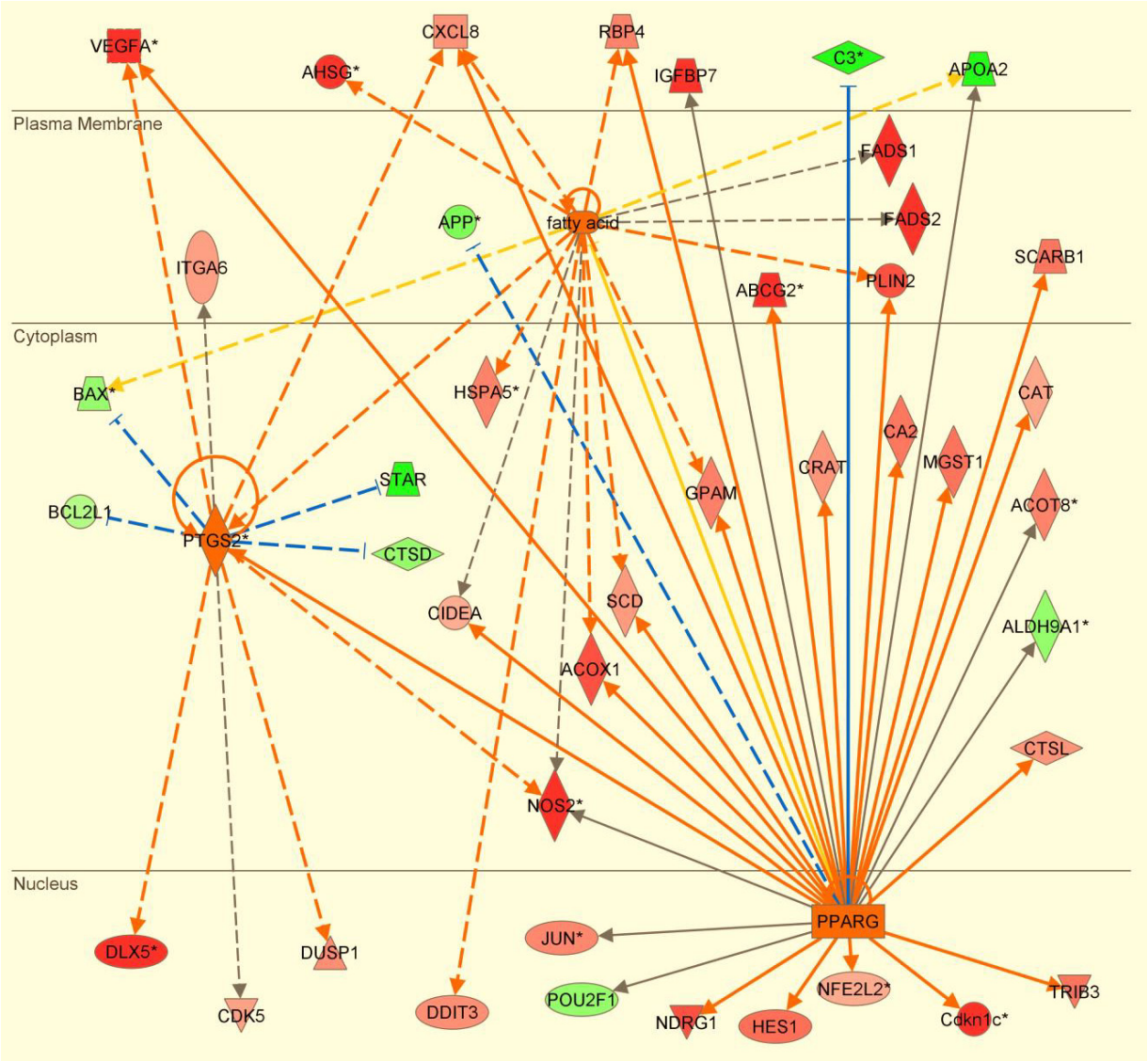

Figure 3. Upstream regulators (orange) and changes in transcriptome (red = upregulated gene; green = downregulated gene) associated with lipid metabolism during the onset of elongation of the bovine conceptus. Data from Ribeiro *et al*. (2016a).

Intensification of the crosstalk between conceptus and endometrium

Physiology of the bovine endometrium is not only influenced by ovarian steroids but also by paracrine effects of conceptus-derived bioactive products. The communication between developing conceptus and endometrium seems to start as early as day 7 of development (Sponchiado *et al*., 2017) and is intensified with the elongation of the conceptus and consequent accumulation of conceptus-derived products in the uterine lumen (Forde *et al*., 2011; Bauersachs and Wolf, 2012). The highly active trophectoderm of the elongating conceptus secrete products such as interferon-τ (IFNT) and prostaglandins that modulate endometrial physiology, establishing a complex crosstalk between the two tissues that coordinate the continuous growth of the conceptus, maternal recognition of pregnancy and maintenance of the corpus luteum, increase blood flow to the pregnant uterus, establishment of uterine receptivity to implantation, and formation of a functional placenta (Spencer *et al*., 2004, 2013; Bauersachs *et al*., 2012; Pinaffi *et al*., 2017). The complete molecular crosstalk between the two tissues is,

however, more complex and not fully understood. Mamo *et al*. (2012) evaluated transcriptome data from bovine conceptuses and endometria and identified a total of 133 conceptus ligands that could interact with corresponding receptors on the endometrium, and 121 endometrium ligands that could interact with corresponding receptors on the conceptus.

The composition of the histotroph during elongation also changes dramatically as a result of conceptus secretions and dynamic responses from the endometrium, and these changes are essential to meet the requirements of the expanding conceptus (Spencer *et al*., 2004). The changes in composition reported in cattle include amino acids (Groebner *et al*., 2011), proteins (Forde *et al*., 2014) and lipids (Ulbrich *et al*., 2009; Ribeiro *et al*., 2016a), and are supported by similar reports in sheep (Gao *et al*., 2009; Romero *et al*., 2017). For instance, Groebner *et al*. (2011) reported a pregnancy-dependent increase in the concentration of all essential amino acids, except for methionine, during the elongation phase. Similar results were observed for the concentrations of prostaglandins (Ulbrich *et al*., 2009). Moreover, Ribeiro *et al*. (2016a) reported reduced amounts of arachidonate, but increased amounts of arachidonate-derivative molecules in uterine flushing of pregnant cows compared with those of nonpregnant cows on day 15. The arachidonate-derivative molecules have been described as ligands of PPARG and may be important for coordination of gene expression in conceptus cells during elongation.

A recent study described a negative association between pregnancy success in dairy cows and size of the uterus (Baez *et al*., 2016). Cows with bigger uterus had reduced P/AI compared with cows with smaller uterus. Although the reasons for such difference in fertility are unknown, the large uterine volume and the spatial regulation of endometrium physiology (Sponchiado *et al*., 2017) might complicate the crosstalk between the developing conceptus and the endometrium and be ineffective to block luteolysis. In addition, two companion studies (Geary *et al*., 2016; Moraes *et al*., 2018) suggested that impaired crosstalk between the elongating conceptus and endometrium contribute to subfertility of beef heifers. In their experimental model, heifers classified as high-fertile and those classified as subfertile presented similar development of ovoid conceptuses by day 14, but remarkable differences in size and transcriptome of conceptuses and in transcriptome of pregnant and nonpregnant endometria on day 17. The reported transcriptome differences in both tissues did not seem to be related exclusively to the observed differences in size of the conceptus but also associated with the capacity of the endometrium to respond to conceptus-derived factors.

Elongation of conceptus in lactating dairy cows

Years of genetic selection for milk production have intensified the homeorhetic control of dairy cow's metabolism to support lactation (Bauman and Currie, 1980). Similar to high-performance athletes, modern high-producing dairy cows have remarkable nutrient demands, with total requirements averaging 4 times the maintenance requirements (Santos *et al*., 2010). Around parturition, however, feed intake is usually depressed and the caloric and nutrient requirements of the cow postpartum are only partially met by feed consumption, causing extensive mobilization of nutrients from body tissues (Drackley, 1999). Adipose tissue is particularly affected by reduced concentrations of glucose and insulin that up-regulate lipolytic signals for hydrolysis of stored triglycerides and increase availability of nonesterified fatty acids (NEFA) to be used as an energy source. The imbalance in energy is extended to nutrients such as amino acids, minerals and vitamins (Goff, 2004; LeBlanc *et al*., 2004). Reduced insulin also impacts the expression of GH receptors in the liver and consequently the circulating concentrations of IGF1 (Butler *et al*., 2003). In addition, the elevated feed intake required for high milk yields result in augmented blood flow to the liver, increased metabolism and reduced concentrations of circulating steroid hormones (Wiltbank *et al*., 2006). All these features have implications for reproductive biology and are often associated with the subfertility presented by lactating dairy cows (Chagas *et al*., 2007). Studies investigating potential risk factors for failures in conceptus development and elongation in lactating dairy cows are discussed below.

Lactation

Multiple studies have investigated the potential impact of lactation on the development of the preimplantation conceptus (Table 2). Their experimental designs included the comparison of conceptus development and endometrial physiology between cows and heifers (Valour *et al*., 2014; Bauersachs *et al*., 2017; Forde *et al*., 2017) and between lactating and non-lactating cows (Cerri *et al*., 2012; Maillo *et al*., 2012; Thompson *et al*., 2012; Bauersachs *et al*., 2017; Forde *et al*., 2017). The latter approach would randomly assign cows to be dried-off right after calving or to continue to be milked until completion of the experiment. The overall hypothesis in these studies was that lactation would cause substantial changes in the metabolism of cows that would alter the uterine environment and impaired conceptus development.

Studies comparing lactating (L) and nonlactating (NL) cows were successful to create differences in metabolism, which included lower concentrations of glucose, insulin, and IGF1, and higher concentrations of NEFA and ketones in serum of L compared with NL during the postpartum period (Maillo *et al*., 2012; Thompson *et al*., 2012). These studies, however, failed to show differences in conceptus development (Table 2). Thompson *et al*. (2012) examined conceptuses on day 17 after timed AI and observed similar length and no differences in gene expression of selected genes. Maillo *et al*. (2012) examined conceptuses on day 14 after transfer of multiple in vitro-produced embryos on day 7 and did not observe differences in conceptus survival or size. Forde *et al*. (2017) investigated the transcriptome of conceptuses and the amino acid composition of uterine fluid in L and NL on day 19 of

pregnancy but no differences were found. Two studies compared the transcriptome of intercaruncular endometrial cells of L and NL cows (Cerri *et al*., 2012; Bauersachs *et al*., 2017). Cerri *et al*. (2012) used endometrium from pregnant and nonpregnant cows on day 17. Using a less stringent cutoff (nominal P value ≤ 0.01), they reported 277 DEG mainly involved with immune responses, cell adhesion and tissue remodeling. Bauersachs *et al*. (2017) compared endometrium of L and NL cows on day 19 of pregnancy after ET on day 7. Using adjusted P value ≤ 0.10 as cutoff, only 28 transcripts were considered differently expressed, and none of them were reported as DEG by Cerri *et al*. (2012). It is important to mention that differences in progesterone concentration in serum between L and NL cows were observed only in the study by Cerri *et al*. (2012) and not in the study by Bauersachs *et al*. (2017), which might also contribute for the slight different outcomes.

Studies comparing transcriptome of conceptus and endometrium between L cows and nulliparous heifers (NH) reported considerable differences (Table 2). The DEG in endometrium reported by Bauersachs *et al*. (2017) were mostly involved with immune responses and cell adhesion and migration. Based on gene expression, Forde *et al*. (2017) predicted that conceptus from NH were more advanced compared with those recovered from L cows. Conversely, Valour *et al*. (2014) found no differences in development based on length of the conceptuses and morphological and molecular staging of the embryonic discs. Nonetheless, 483 transcripts were differently expressed in trophectoderm cells and revealed important differences in lipid and energy metabolism. Although the comparison between L and NH has many confounding factors, the results of these investigations are important to characterize developmental biology in cattle and how it might be affected by distinct uterine environments.

Table 2. Impact of lactation and parity on survival, size, and transcriptome of conceptuses and on transcriptome of endometria in dairy cattle.

Item	Lactating cow	Nonlactating cow	Heifer	Reference
Survival of conceptus on day 14, % (n/n)[1]	39.8 (67/175)	33.3 (65/175)	---	Maillo *et al*., 2012
Length of conceptuses on day 14 , mm	1.6 ± 0.5	1.2 ± 0.5	---	Maillo *et al*., 2012
Pregnant on day 17 after AI, (n/n)	80 (8/10)	50 (6/12)	---	Thompson *et al*., 2012
Length of day 17 conceptuses, mm	251 ± 51	200 ± 72	---	Thompson *et al*., 2012
DEG in day 17 conceptuses [2]	Reference	0	---	Thompson *et al*., 2012
DEG in day 19 conceptuses	Reference	0	269	Forde *et al*., 2017
DEG in day 19 pregnant endometria	Reference	28	238	Bauersachs *et al*., 2017
DEG in day 17 pregnant endometria	Reference	277	---	Cerri *et al*., 2012
DEG in day 18 conceptus	Reference	---	483	Valour *et al*., 2014
Fully elongated conceptus[3], % (n/n)	72.7 (8/11)	---	63.6 (7/11)	Valour *et al*., 2014
Length of day 18 conceptuses (elongated), mm	157 ± 14	---	151 ± 11	Valour *et al*., 2014
Length of day 18 conceptuses (delayed), mm	67 ± 9	---	58 ± 22	Valour *et al*., 2014

[1]Recipients received multiple *in vitro*-derived embryos on day 7. [2]Analyses of candidate genes performed by PCR only. [3]Larger than 150 mm in length.

Diseases postpartum

Clinical diseases caused by microbial infection and tissue injury are prevalent in postpartum dairy cows and have consequences for reproduction (Santos *et al*., 2010; Ribeiro *et al*., 2013, 2016b). Approximately 40% of dairy cows have at least one clinical disease between calving and the first breeding postpartum, and the odds of pregnancy per AI (P/AI) after synchronized ovulation are reduced by 30% in cows affected by clinical diseases postpartum compared with those with no clinical disease (Ribeiro and Carvalho, 2017). Moreover, conception risk is also reduced by clinical diseases in cows receiving a viable embryo on day 7 of the estrous cycle, which indicates that altered uterine environment must account for at least part of the subfertility of cows diagnosed with clinical diseases postpartum (Table 3; Ribeiro *et al*., 2016b).

To evaluate the impact of diseases on conceptus elongation, health information of 148 lactating cows was collected from parturition until first postpartum AI, and uterine flushing for recovery of conceptuses was performed 15 or 16 days after AI. Cows with disease had shorter conceptuses and reduced concentration of IFNT in the uterine flush (Ribeiro *et al*., 2016b). These results were supported by a second experiment that evaluated the transcript expression of IFN stimulated genes (ISGs) in peripheral blood leukocytes (PBL) on day 19 after AI (Ribeiro *et al*., 2016b). As expected, the expression of ISGs was about 2-fold greater in cows later diagnosed pregnant. However, an interaction between pregnancy status and disease category was detected. A significant increase in expression of ISGs by pregnancy was only observed in cows that did not have disease and not in those that had disease before AI. Although expression of ISGs does not depend exclusively of stimulation by IFNT, it has been used by researchers as an indirect method to evaluate conceptus development without the need to terminate pregnancy (Ribeiro *et al*., 2014b, 2016b).

Comparison of the transcriptome of conceptuses recovered from cows having or not non-

uterine diseases before AI revealed small but interesting differences in transcript expression (Ribeiro *et al*., 2016b). Conceptus recovered from cows that had disease before AI presented upregulation of genes associated with inflammatory response, and inflammatory molecules such as lipopolysaccharide, tumor necrosis factor α (TNFα), and IFN-γ were predicted as potential upstream regulators of such differences. In addition, the predicted downstream effects of the transcriptome changes were activation of immune cells and tissue rejection, which in the context of the pregnant uterus could be translated to activation of the maternal immune system in the endometrium and rejection of the allogeneic conceptus.

Altogether, these findings reinforce that disease postpartum is a relevant problem in dairy herds and has a substantial impact on reproduction. It also suggest that elongation of the preimplantation conceptus is impaired in this subgroup of cows and might help explain the difference observed in P/AI and the higher incidence of late embryonic and fetal losses. It is still not clear how diseases occurring early in the postpartum would have a long term impact on uterine environment, but it has been suggested that clinical diseases would lead to exacerbated inflammation and long-lasting effects on energy and lipid metabolism that could influence the composition of the uterine histotroph and consequently conceptus development (Ribeiro, 2018).

Table 3. Reproductive outcomes of first breeding postpartum in dairy cows according to incidence of disease before breeding and breeding technique[1].

Item	Pregnant day 45 (%)	Calving (%)	Pregnancy loses (%)
	-------- Adjusted mean ± SEM[2] --------		
No disease-AI	38.8 ± 1.8	32.9 ± 1.7	12.4 ± 1.5
Disease-AI	31.0 ± 2.1	22.2 ± 1.9	21.3 ± 3.1
No disease-ET	40.7 ± 1.7	35.9 ± 1.7	11.1 ± 1.5
Disease-ET	35.9 ± 2.4	28.2 ± 2.2	22.4 ± 3.4
	---------- P-value ----------		
Disease	<0.01	<0.01	<0.01
Breeding technique	0.12	0.03	0.27
Disease x breeding technique	0.37	0.27	0.59
	Adjusted odds ratio (confidence interval)[3]		
Within AI	0.71 (0.58-0.87)	0.58 (0.46-0.73)	1.92 (1.24-2.98)
Within ET	0.82 (0.65-1.02)	0.70 (0.55-0.90)	2.30 (1.41-3.76)

[1]Data from Ribeiro *et al*. (2016b). [2]Adjusted mean and standard error of the mean for cows that had or not disease before breeding and were bred by artificial insemination (AI) or embryo transfer (ET). [3]Adjusted odds ratio (confidence interval) for the effect of disease within cows bred by AI and within cows bred by ET.

Anovulation at the onset of synchronization programs

Anovulation is a normal and temporary physiological condition of dairy cows during early postpartum. It is characterized by lack of regular estrous cycles and ovulation, although follicle growth is still present (Wiltbank *et al*., 2002). Time for resumption of estrous cyclicity postpartum varies among cows and is directly associated with energy balance in the first weeks of lactation (Butler, 2003). As consequence, 18 to 43% of dairy cows remain anovular at the end of the voluntary waiting period, constituting an important problem in achieving adequate reproductive performance in dairy herds (Rhodes *et al*., 2003; Santos *et al*., 2009). Adoption of timed AI programs maximizes submission to AI and lessens the problem of anovular cows reducing reproductive performance. Nevertheless, P/AI of anovular cows after synchronized estrus or ovulation is reduced compared with that of estrous cyclic herdmates independent of the breed of the cow (Santos *et al*., 2009; Ribeiro *et al*., 2016c).

Submission of anovular cows to synchronization programs creates a novel endocrine scenario where the ovulatory follicle develops under low concentration of progesterone (Bisinotto *et al*., 2010). When ovulation is successfully induced in anovular dairy cows by administration of GnRH, the newly formed corpus luteum (CL) secretes progesterone but it takes approximately 4 days for circulating concentrations to be above 1 mg/ml. As the interval between GnRH and PG shots in most synchronization programs is 7 days, priming progesterone in these cows would act for only 3 days. In the ovariectomized sheep model, priming progesterone replacement need to last at least 6 days in order to be effective (Moore, 1985). Thus, synchronization of anovular cows might represent an alternative model for insufficient priming progesterone with consequences to fertility. In fact, supplementation of progesterone in cows without a CL at the onset of synchronization program improves P/AI (Bisinotto *et al*., 2013). Moreover, priming progesterone is also important for estrogen-induced estrous behavior (Schinckel, 1954; Allrich, 1994) and, interestingly, anovular cows that express estrous behavior on the day of timed AI have P/AI comparable to that observed in estrous cyclic herdmates (Bisinotto *et al*., 2013; Ribeiro *et al*., 2014a). Thus, the requirements for priming progesterone may vary among individuals and estrous behavior might be seen as an indicator of sufficient exposure of the hypothalamus and endometrium to priming progesterone.

Ribeiro *et al*. (2016c) compared conceptus elongation of anovular (HA) and estrous cyclic (HC) Holstein cows subjected to timed AI program. Considering the difference in P/AI between the two groups, it was initially predicted that HA cows would have poorly developed conceptuses. Surprisingly, conceptuses from HA cows were longer and secreted greater amounts of IFNT than conceptuses from HC

cows. The difference in size of the conceptus was likely caused by the distinct progesterone profiles before and after AI. Reduced concentration of progesterone during the synchronization program allowed accelerated development of the dominant follicle, ovulation of a larger follicle and formation a larger CL after AI. The larger CL resulted in greater concentrations of progesterone in the postovulatory period, which is known to advance conceptus elongation (Lonergan and Forde, 2014). Nonetheless, HA cows had reduced concentrations of IGF1 in plasma, and their conceptuses presented remarkable differences in transcriptome. Although the difference in size of the conceptuses influenced the results (Ribeiro *et al*., 2016a), some of the altered transcripts seemed to be unrelated to elongation and suggest that conceptus cells from HA might be under greater cellular stress, which could be associated with greater pregnancy mortality after day 15 of development.

These findings taught important aspects of reproductive biology of anovular cows subjected to synchronization programs but are not conclusive regarding the causes of their subfertility. One could hypothesize that developmental problems in anovular cows occur before day 15 and the conceptuses that survived by day 15 would continue to develop comparably to conceptuses in estrous cyclic cows. In favor of this hypothesis are the phenotype of the conceptus and increased secretion of IFNT, and the potential effects of insufficient priming progesterone on oocyte quality (Santos *et al*., 2016) and early embryo development (Miller *et al*., 1977; Rivera *et al*., 2011). On the contrary, one could suggest that although conceptuses from anovular cows were longer and secreted more IFNT, they might not necessarily be better, and perhaps the difference in transcriptome could indicate imbalances in cell biology that could result in greater incidence of pregnancy loss after day 15. In support of the latter, research has demonstrated increased pregnancy loss in anovular cows (Santos *et al*., 2004) and in those that develop the ovulatory follicle under low concentrations of progesterone (Bisinotto *et al*., 2010). Unfortunately, we are unaware of data of anovular cows subjected to an ET program, which would isolate the potential effects on oocyte quality and early embryo development and inform whether the uterus contributes to the subfertility observed in this subgroup of cows.

Concentrations of circulating ovarian steroids

The reduced concentration of circulating steroids in high-producing dairy cows caused by augmented metabolism and clearance of these hormones (Wiltbank *et al*., 2006) could compromise endometrium physiology and consequently conceptus development. In fact, there are extensive evidences in the literature that progesterone before estrus, estradiol during estrus and progesterone after estrus are associated with fertility in cattle and likely involve effects on endometrial physiology (Wiltbank *et al*., 2014; Madsen *et al*., 2015). For instance, recipient lactating cows that express estrus at the end of synchronization program have increased pregnancy per ET compared with those that did not present estrous behavior (32.7 *vs*. 46.2%; Pereira *et al*., 2016). As discussed above, estrous behavior is a result of adequate exposure of the hypothalamus to priming progesterone during the diestrus preceding estrus and adequate exposure to estrous estradiol. Thus, the reported differences in pregnancy success after ET demonstrate the importance of steroid hormones exposure prior breeding to endometrium physiology in the subsequent estrous cycle and conceptus development in lactating dairy cows. Moreover, faster rise in concentration of progesterone from day 0 to day 7 was also associated positively with pregnancy establishment following ET (Kenyon *et al*., 2013).

If concentration of steroid hormones is a critical factor for endometrial physiology and fertility of dairy cows, then supplementation of these hormones would be a reasonable strategy to overcome this problem. However, fertility responses of lactating cows to steroid supplementation are variable. Bisinotto *et al*. (2015) performed a meta-analysis to evaluate the impact of progesterone supplementation during timed AI programs, and reported an overall 8% increase in risk of pregnancy on day 32 and a tendency to reduce pregnancy losses after day 32. Conversely, supplementation of progesterone during metaestrus and early diestrus, although is the most effective and consistent method to promote conceptus elongation (Lonergan and Forde, 2014), has not proven to be consistent in increasing P/AI or P/ET in lactating dairy cows. In fact, recent large studies show limited success (Monteiro Jr *et al*., 2014) or even negative results (Parr *et al*., 2014; Monteiro Jr *et al*., 2015). In addition, supplementation of estradiol around estrus is generally successful to enhance estrous behavior but not P/AI in dairy cows (Sellars *et al*., 2006; Souza *et al*., 2007; Hillegass *et al*., 2008).

Concentrations of circulating IGF1

Ribeiro *et al*. (2014b) supplemented low doses of GH to lactating dairy cows during pre and peri-implantation periods, between days 0 and 28 relative to AI, with the objectives to increase circulating concentrations of IGF1 and improve conceptus development and survival. Supplementation of low doses of GH during the preimplantation period resulted in increased concentrations of IGF1 in plasma, improved P/AI, and reduced pregnancy losses, resulting in a 28% increase in calving per AI (Fig. 4). Conceptus development was evaluated by expression of ISG in PBL, concentration of pregnancy specific protein B (PSPB) in plasma, and ultrasonographic morphometry of conceptuses. Corroborating with the fertility results, supplementation of GH from days 0 to 28 after AI increased expression of ISGs in PBL, and resulted in earlier rise of PSPB in plasma of pregnant cows and larger conceptuses than untreated controls (Fig. 4). Altogether, these evidences support the role of IGF1 as an important mediator of preimplantation development and the potential use of pharmacological interventions to improve conceptus elongation and survival in lactating dairy cows.

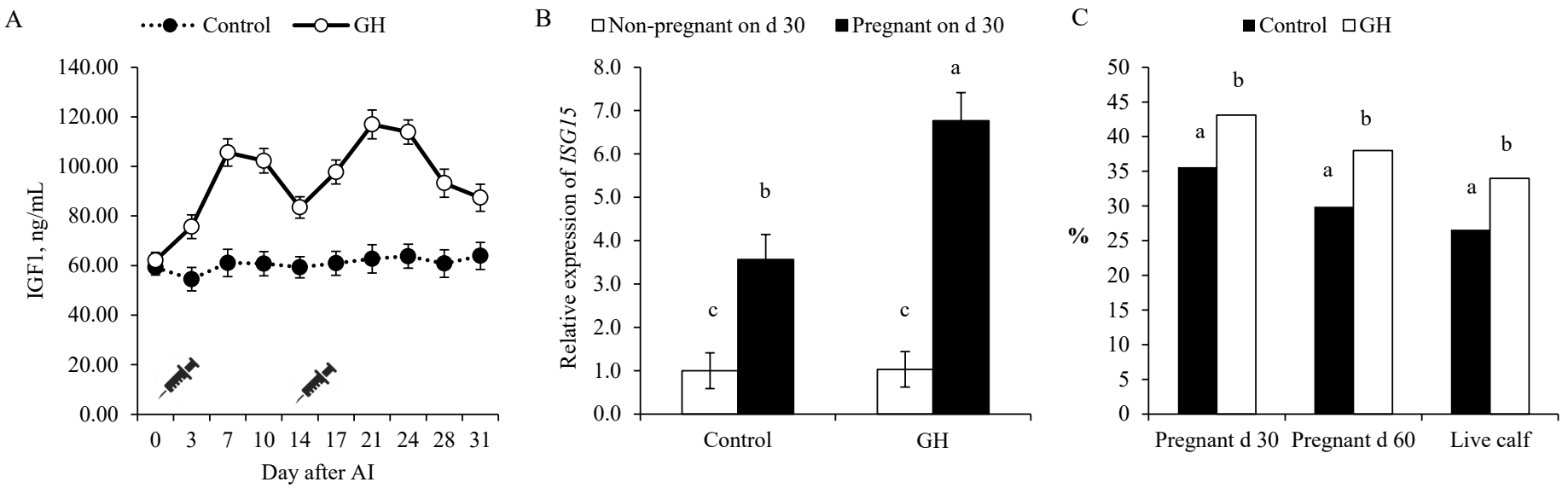

Figure 4. Impact of growth hormone (GH) supplementation on concentration of IGF1 in plasma (A), relative expression of ISG15 in leukocytes (B), and pregnancy and calving per AI (C) in lactating dairy cows. Data from Ribeiro *et al*. (2014b).

Genetics

A conceivably small but important portion of the variation in pregnancy success among dairy cows within and across herds is explained by the genetics of the cow, the genetics of the breeding sire, and the resulting genetics of the embryo (Santos *et al*., 2010; Butler, 2014; Cole *et al*., 2016; Han and Peñagaricano, 2016; Ortega *et al*., 2016). In general, the genetic heritability of reproductive traits is relatively low, generally less than 10%, and suggests that reproductive success is affect mainly by non-genetic factors. Nonetheless, the growing understanding of molecular regulation of reproduction will likely result in the development of new fertility traits and more efficient ways to selected high fertility cows. As depicted in Fig. 1B, conception risk in dairy cows has been low and stagnant for many years and therefore novel traits should target conceptus survival for consistent genetic improvements in conception risk. The contribution of genetics to the success of conceptus elongation, however, is still unknown. Some of the few studies exploring potential effect of genetics on elongation are discuss below.

Meier *et al*. (2009) compared the conceptus development and fatty acid composition of the endometrium in two strains of Holstein-Friesian cows, New Zealand (NZ) and North American (NA), managed in a pasture-based system. In general, NA cows have reduced P/AI compared with NZ cows and conceptus development could contribute for this difference in fertility. Reproductive tissues were obtained on day 17 of the estrous cycle or pregnancy. The average length of the conceptuses was similar between groups, but the variation in size was a 3.6-fold greater in those recovered from NA recipients (NZ: 20.8 ± 2.84 cm; NA: 27.9 ± 10.23 cm). Concentrations of C17:0 and C20:3n-3 were higher in the endometrial tissues from NZ cows, and there was a tendency for higher total concentrations of polyunsaturated fatty acids. Concentrations of C18:1, C20:2, and total monounsaturated fatty acids tended to be less in NZ cows. If the reported differences in lipid composition of the endometrium are reflected in the histotroph, activity of PPARG in conceptus of NZ cows could be enhanced by the greater amount of polyunsaturated fatty acids and reduced amounts of monounsaturated fatty acids as hypothesized by Ribeiro (2018) and potentially contribute for the greater success in pregnancy establishment in NZ cows compared with NA cows in pasture-based systems.

One strategy widely used in commercial herds is crossbreeding of complementary pure breeds not only to obtain a desired phenotype, but also to reduce inbreeding and maximize heterosis. The crossbreeding between Holstein and Jersey cattle, for instance, is common and aims to combine the high milk volume yield of Holsteins with the high solids content in milk of Jerseys. In addition, improvements in reproduction have been reported with this crossbreeding strategy (Ribeiro *et al*., 2011; 2016c). Ribeiro *et al*. (2016c) compared conceptus elongation of Holstein (HC) and Holstein-Jersey crossbred cows (CC) randomly selected in a grazing herd and subjected to the Ovsynch protocol. Compared to HC, CC cows presented advanced conceptus development on day 15 and tended to have greater concentration of IFNT in the uterine flushings. Contrary to HC cows that had some ovoid conceptuses, CC had only elongated conceptuses. Moreover, CC pregnant cows had greater concentration of anandamide in the uterine flush and increased transcript expression of *PPARG* in the conceptus. Crossbred cows also had greater concentrations of circulating ovarian steroids before, around and after AI.

It is interesting to note that both studies investigating conceptus elongation in groups of cows with distinct genetics and fertility reported differences in lipid metabolism of the pregnant uterus, which reinforces the importance of lipid metabolism in conceptus development of dairy cows and supports the overall positive effects of dietary supplementation of fat in reproduction of dairy cows (Santos *et al*., 2008; Rodney *et al*., 2015). In addition, both studies reported differences in concentration of circulating steroids, in which the genetic group of higher fertility (New Zealand Holstein or North American Holstein-Jersey crossbred) had greater concentrations of circulating steroids compared with the genetic group of lower fertility (purebred North American Holsteins). Moreover, a study that compared Holstein cows with high and low estimated breeding values (EBV) for calving interval and similar EBVs for production traits found important differences in concentrations of circulating progesterone and IGF1, estrous behavior, energy metabolism and incidence of uterine diseases postpartum (Butler, 2014), suggesting that genetics could influence multiple physiological and cellular requirements for successful elongation of the preimplantation conceptus in lactating dairy cows.

Conclusions

Successful elongation of the preimplantation conceptus depends on the modulation of endometrium physiology by steroid hormones, which include not only the effects of progesterone after ovulation but also the effects of estradiol during estrus and progesterone during the diestrus preceding estrus. The combined activity of priming hormones eventually leads to an environment that promotes the onset of conceptus elongation. Elongation requires reprogramming of cells in the extraembryonic tissues and involves changes in the cytoskeleton and adhesion molecules, increased in proliferation and migration of cells, and altered metabolism. Histotroph lipids are heavily utilized for synthesis of biomass, energy, production of prostaglandins, cell signaling, and regulation of gene expression though activity of the PPARG-RXR dimer of nuclear receptors. Abundance of PPARG-RXR ligands in the uterine histotroph is likely facilitated by the accumulation of lipid droplets and greater expression of RBP4 in the endometrium during diestrus. The highly active conceptus cells secrete bioactive factors that alter endometrium physiology

and induce further changes in composition of the histotroph that are critical to attend the evolving demands of the expanding conceptus. Impairment of these physiological and cellular requirements could lead to failures in elongation and pregnancy losses (Fig. 5).

In lactating dairy cows, pregnancy losses are substantial and failures in the elongation process likely account for some of these losses. Lactation per se does not seem to impair elongation. However, elongation and survival of conceptuses seem to be affected in subgroups of lactating cows that present endocrine, metabolic and nutritional imbalances or deficiencies. These subgroups of cows include those that had health problems postpartum, anovular cows subjected to synchronization programs without supplementation of progesterone, cows with low concentrations of circulating IGF1, cows with reduced estrous behavior, and cows with reduced concentrations of progesterone after ovulation. In addition, elongation might be affected by genetics of cow and embryo, and by nutritional imbalances of protein, amino acids, minerals, vitamins, and fatty acids. Improving conceptus elongation and survival in lactating cows starts with optimization of health and nutrition programs during the transition period, includes the promotion of cow comfort and intake of complete and balanced diet, and can be extended to supplementation of specific deficiencies or stimulators such as essential fatty acids and somatotropin. Supplementation of steroids are mostly ineffective to promote survival of the conceptus except for progesterone supplementation during synchronization programs. Finally, the growing understanding of molecular regulation of reproduction is expected to result in the development of new fertility traits for genetic selection, which should emphasize pregnancy survival in order to consistently improve conception risk of dairy herds.

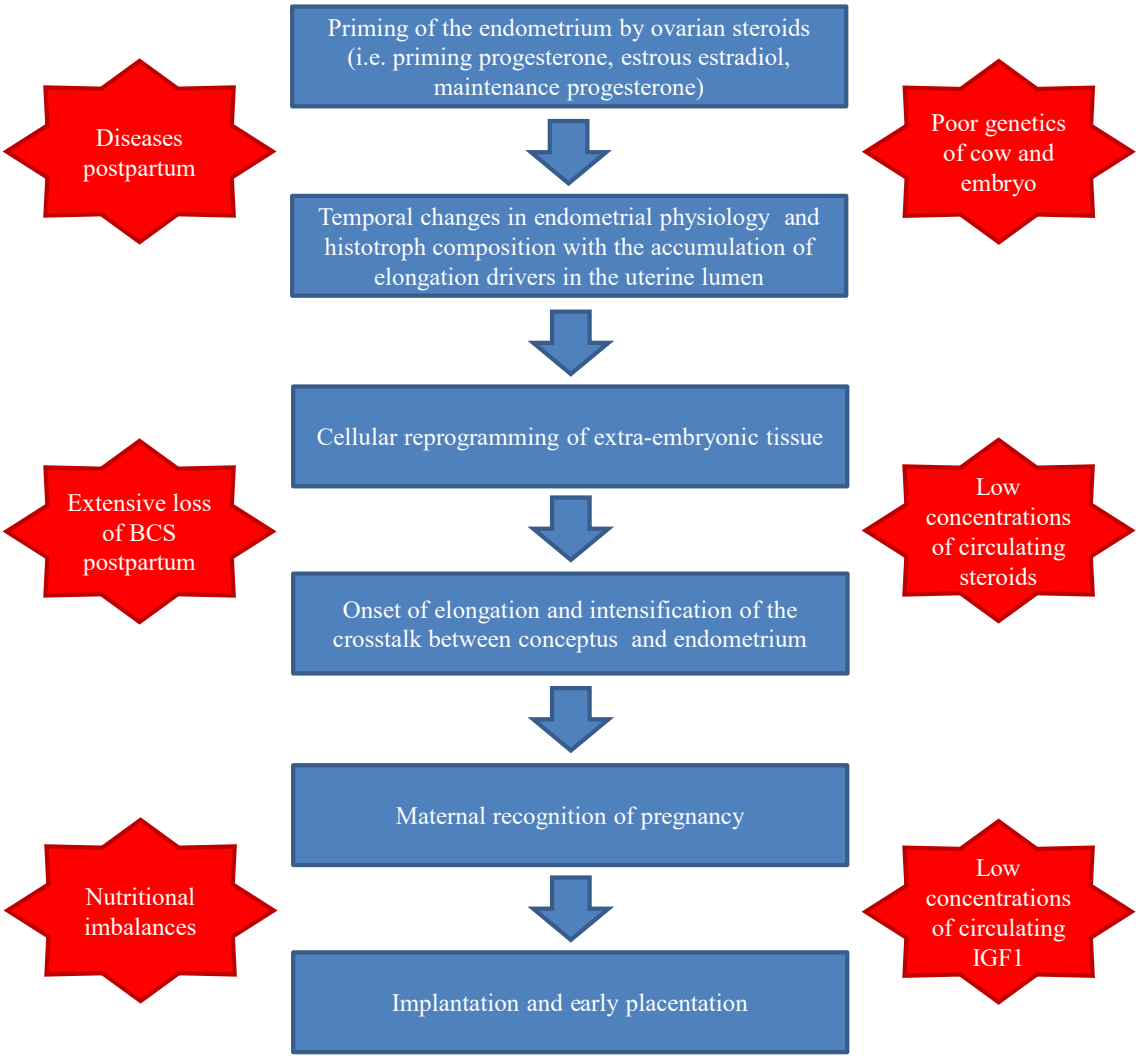

Figure 5. Summary of physiological and cellular requirements for elongation of the preimplantation bovine conceptus (represented in blue) and major factors affecting the success of elongation in lactating dairy cows (represented in red).

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DOI: 10.21451/1984-3143-AR2018-0012

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Modeling early embryo-maternal interactions *in vitro*

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Abstract

Environmental conditions experienced during early embryonic development influence growth, metabolism, and gene expression of the embryo as well as the epigenetic profile of the offspring. The environment of the early embryo consists of the luminal fluid within the oviduct and uterus and the epithelial cells composing this fluid. Whether the embryo is able to shape its own microenvironment by interacting with the epithelial lining of the oviduct/uterus and which factors potentially interfere with or regulate these interactions remains to be elucidated. As early embryonic signals and the respective maternal responses are subtle and local events, it is challenging to study them *in vivo*. Therefore, adequate *in vitro*-models optimally mimicking the contact zone between the maternal reproductive tract and the early embryo are needed to a) elucidate basic mechanisms involved in early embryonic development and b) reduce the number of experimental animals used for such studies. Functional epithelial cells are generally defined by a polarized distribution of organelles and proteins. Proper polarization is tightly connected with physiological cell behavior and *in vivo*-like reactivity of the epithelium. Therefore, this review summarizes current strategies for *in vitro* preservation of epithelial cell polarity. It presents recent advances in 3D culture of female reproductive tract epithelia and embryo-epithelial co-cultures. A special emphasis is set on compartmentalized culture systems, powerful tools for studying early embryo-maternal interactions *in vitro*. In such systems, cultured epithelial cells are manipulable from their basolateral as well as their apical cell pole, allowing concomitant application of embryonic as well as maternal effectors from the appropriate cellular compartment.

Keywords: embryo-maternal interactions, endometrium, oviduct, three dimensional cell culture models.

Introduction

Early embryos of eutherian mammals reside within the oviduct (species-specific up to the 4-cell, 8-cell, morula or blastocyst stage) before they transition into the uterus. It is now well established that the environmental conditions experienced during early embryonic development (zygote to blastocyst stage) influence growth, metabolism, and gene expression of the embryo as well as the epigenetic profile of the offspring (recently reviewed by Fazeli and Holt, 2016; Rizos *et al*., 2017).

So far, clear evidence is given for a mutual, reciprocal interaction between the female reproductive tract (FRT) and the developing conceptus during maternal recognition of pregnancy and implantation. However, the biological relevance of earlier interactions of the embryo with the upper FRT is still a matter of debate.

Studies in litter bearing species like pigs and mice show that the oviduct responds to preimplantation embryos, long before the embryonic signal for maternal recognition of pregnancy (Lee *et al*., 2002; Alminana *et al*., 2012; Li *et al*., 2015). In the monovulatory cow, where only one single embryo resides in the oviduct, this could not be confirmed *in vivo* at the transcriptomic level (Maillo *et al*., 2015), but first hints exist for an early embryonic influence on the oviduct fluid proteome in this species (Maillo *et al*., 2016). There is clear *in vivo* evidence for early embryo-maternal communication in another monovulatory species, the horse, where embryonic prostaglandin E2 causes relaxation of the oviductal isthmus and allows selective transport of the embryo to the uterus (Weber *et al*., 1991; Freeman *et al*., 1992). Differential transcriptomic profiles between pregnant and non-pregnant oviducts also suggest an impact of one single early embryo on the innate immune response in the equine FRT (Smits *et al*., 2016).

However, whether the embryo is actually able to shape its own microenvironment by interacting with the epithelial lining of the oviduct/uterus and which factors potentially interfere with or regulate this fine-tuned interactome (Fazeli, 2011) remains to be elucidated.

As early embryo-maternal interactions are presumably subtle and local events, they are challenging to study *in vivo* (not only, but especially in monovulatory species), both from a technical and an ethical point of view. Biological variation, very limited numbers of possible replicates and the unfavorable signal-to-noise ratio might hamper the success of such *in vivo* studies.

Therefore, adequate *in vitro*-models optimally mimicking the contact zone between the maternal reproductive tract and the early embryo could help to elucidate basic mechanisms involved in early embryonic development and programming. At the same time such models reduce the number of experimental animals needed for basic research and might proof useful also for reproductive toxicity testing (Simintiras and Sturmey, 2017).

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Received: January 30, 2018
Accepted: March 16, 2018

The environment of the early embryo

The environment of the early embryo consists of the luminal fluid within the FRT and the epithelial cells composing this fluid.

It is suggested that the oviduct and uterine luminal fluids represent the optimal milieu for the early embryo. Its basic composition has been analyzed with respect to protein composition, ion content and energy substrates using *in situ* and *ex vivo* techniques (reviewed in Leese *et al*., 2008). Differences in the fluid composition have been documented regarding the estrous cycle stage and sampling region. However, the mechanisms of oviductal and uterine fluid formation and its regulation are far from being understood.

The epithelial cells assembling the luminal surface of the oviduct and uterus compose this fluid in accordance to the developmental needs of the embryo (Absalon-Medina *et al*., 2014) and directly get in contact with the early embryo.

This contact zone is a simple epithelium which not only nourishes the early embryo but also provides protection from or clearance of unfavorable molecules and (in case of the oviduct) transport of the embryo towards the uterus.

Epithelia of the FRT: developmental origin and structural hallmarks

Oviduct and uterus both derive from a pair of Mullerian ducts (MDs), which consists of three elements: an inner epithelium layer, surrounding mesenchyme, and the external Mullerian coelomic epithelium. During MD development, the opening cranial end forms into the oviduct, and the caudal ends of left and right MD fuse and give rise to the uterus. Epithelia in the oviduct and uterus both develop from the MD epithelium, while the stromal compartment of uterus evolves from the MD-surrounding mesenchyme (Kurita, 2011).

Epithelia are generally defined by a polarized distribution of organelles and proteins within each cell. The paracellular space between adjacent epithelial cells is sealed by cell-cell junctions at the apical part of the lateral plasma membrane. Especially tight junctions are essential for epithelial polarity and functionality as they form both a paracellular barrier (regulating selective paracellular permeability) as well as a barrier within the membrane which restricts the exchange of membrane components between apical and basolateral cell surface domains. In recent years it became evident that beside their role as simple diffusion barriers, tight junctions are cellular signaling platforms which are regulated by diverse physiological and pathological stimuli (reviewed in (Zihni *et al*., 2016). Loss of cellular polarity, in turn, is a pathological condition frequently seen in cancer development, which alters specific cell functions and responsiveness to external signaling events (Ellenbroek *et al*., 2012).

The basal part of the cell membrane of an epithelial cell is attached to the basement membrane, which separates the epithelium from the underlying connective tissue. With this basolateral cell pole, it takes in systemic effector molecules and nutrients as well as molecules secreted by the connective tissue. In contrast, the contact to gametes or embryos takes place on the apical surface of the cell, which is morphologically and functionally different from its basolateral counterpart in terms of membrane properties and abundance of receptor molecules.

Therefore, proper polarization of the epithelial cells in the FRT is tightly connected with their physiological behavior and *in vivo*-like reactivity towards systemic maternal as well as embryonic stimuli.

Modelling the contact zone: oviductal and uterine epithelial cells *in vitro*

Maintenance of epithelial polarity during culture is an important prerequisite for *in vitro* investigations concerning the fine-tuned interactions possibly taking place between the early embryo and the maternal organism.

Under standard culture conditions (2D, adherent on cell culture plastic ware), which are most frequently used to explore embryo-maternal interactions *in vitro*, epithelial cells (primary or cell lines) from the FRT attach to the plastic surface and are submerged in medium. Even if cells build cell-cell contacts and a certain level of cellular polarization under such conditions, they receive nutrition from the apical pole. This leads to rapid adaptation processes within the cells and to marked changes in their morphological and functional integrity (Fig. 1A). Polarization, expression of marker genes and ciliation get lost (Danesh *et al*., 2016). A well-known example for the loss of marker gene expression during *in vitro* culture is the oviductal glycoprotein 1 (OVGP1), one of the most abundant glycoproteins in the oviduct of most mammals (Coy *et al*., 2008). Under 2D culture conditions, OVGP1 is promptly down-regulated and cannot be triggered by ovarian steroids anymore (Briton-Jones *et al*., 2002, 2004; Schoen *et al*., 2008; Danesh *et al*., 2016).

Cell culture conditions preserving epithelial cell polarity in vitro

Suspension culture

Suspension culture (Fig. 1B) is frequently used for primary oviduct epithelial cells (OEC), especially for analysis of sperm binding in mammalian species (De Pauw *et al*., 2002; Waberski *et al*., 2005; Henry *et al*., 2015). Cells are obtained by squeezing out or scraping off the oviduct epithelium, and later maintained in suspension culture dishes as cell clusters (also termed explants) with their cilia directed outwards. Suspension culture is a particularly useful tool for short-term experiments as it preserves oviduct specific characteristics for approximately 12 h before first signs of de-differentiation are observed (Rottmayer *et al*., 2006).

3D Organoids

Epithelial cells cultured in extracellular matrix (ECM) substitutes can form three dimensional structures with a lumen (Fig. 1C). An organoid culture system from clonal cells was established from the human oviduct, which showed fully *in vivo*-like epithelial differentiation including OVGP1 expression (Kessler *et al*., 2015). Likewise, three dimensional human glandular endometrial cultures were recently developed, leading to highly differentiated, hormone-responsive organoids, which secreted uterine marker proteins like glycodelin and osteopontin (Turco *et al*., 2017). In these models stemness was maintained within the cell population and therefore allowed long-term culture. Differentiation is induced via exogenous factors. Gland-like endometrial spheroids have also been used to study human endometrium-trophoblast interaction and trophoblast invasion (Buck *et al*., 2015). However, manipulation of the organoid lumen, e.g. applying an embryo or embryonic effector molecules on the apical cell pole, is not possible. Yet such organoid cultures enable propagation of differentiated FRT epithelia *in vitro* (e.g. from one individual). Organoid cultures represent self-organizing 3D systems which are genetically stable and contain progenitor and/or stem cells as well as differentiated cells. Therefore, they can be used as a substitute for freshly isolated primary cells in other culture systems.

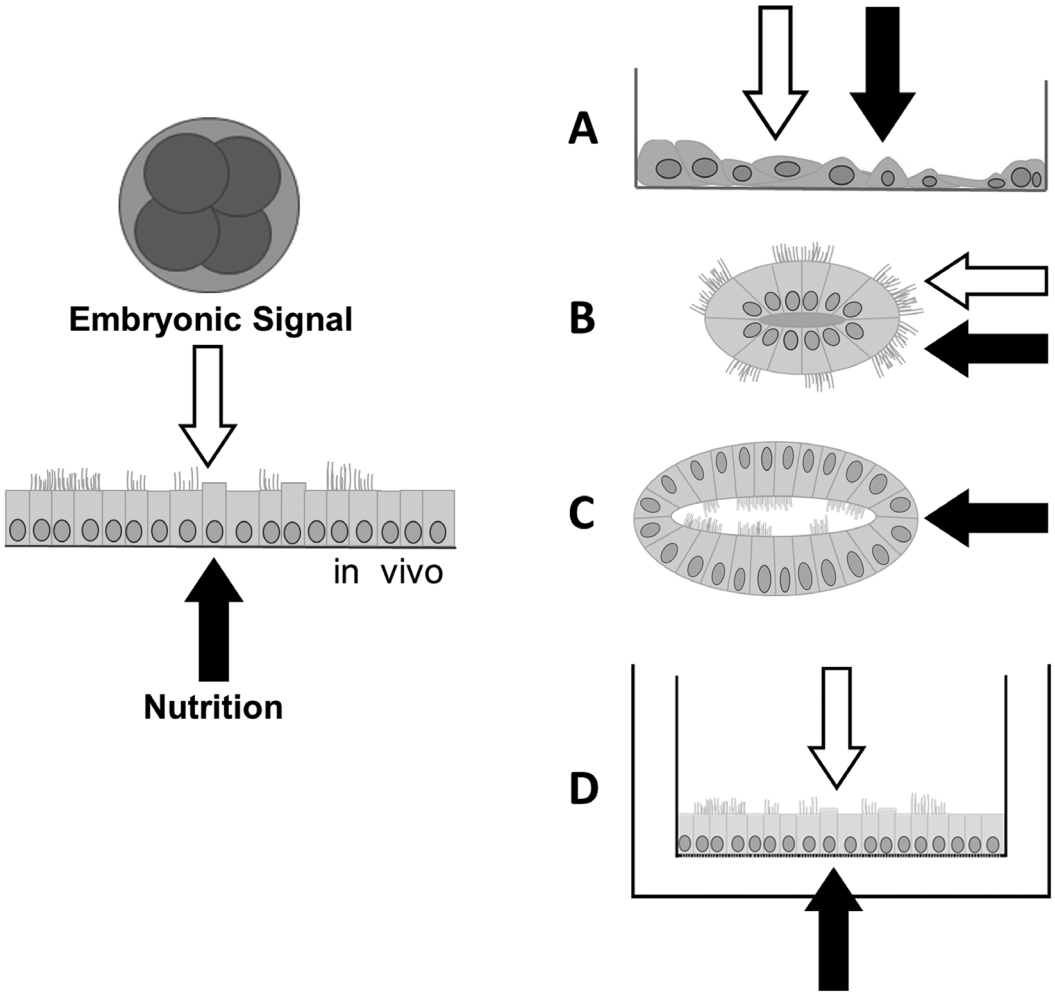

Figure 1. Schematic illustration of possible reception routes for embryonic signals and nutrition in different culture systems of epithelial cells. (A) Standard 2D adherent submerged culture; embryonic signals and nutrition from apical side. (B) Epithelial sphere in suspension culture; signals and nutrition from outside of the sphere (apical epithelial cell pole). (C) Epithelial 3D organoid; nutrition is provided from outside the organoid; embryonic signal is not applicable. (D) Compartmentalized culture system; nutrition from the basolateral side, embryonic signal could be given from the apical side.

Compartmentalized culture systems

In compartmentalized culture systems, cells are grown in inserts on either porous membranes or scaffolds (Fig. 1D). Placing the insert into the cell culture medium mimics the *in vivo* nutrient supply from the basolateral side of the cell, and thereby supports epithelial differentiation and polarization. The compartmentalized system can be used to grow cells submerged (liquid-liquid interface) with either the same or two different media in the basolateral and apical compartment (e.g. creating a serum gradient over the

membrane). Porous filter supports can also be used to grow FRT epithelia at the air-liquid interface (ALI), i.e. with no medium in the apical compartment. The ALI method is primarily known from differentiated long-term cultures of skin and airway epithelia. When applied to FRT epithelia, ALI culture systems engendered excellent long-term differentiation of oviduct (Levanon *et al*., 2010; Miessen *et al*., 2011; Gualtieri *et al*., 2012) and uterine epithelial cells (Munson *et al*., 1990; Classen-Linke *et al*., 1997). In general, as known for airway epithelia, ALI culture seems to better support differentiation of FRT epithelia than submerged conditions. Furthermore, ALI models allow acquisition and analysis of the luminal fluid created by the epithelia. Initial analysis of oviductal fluid surrogates obtained from ALI cultures showed apparent similarities to oviductal fluid *in vivo* (Simintiras *et al*., 2016; Chen *et al*., 2017). This makes the ALI approach a promising tool to investigate effectors regulating or modifying the environment of the early embryo.

In compartmentalized culture systems, cell polarization can be further enhanced by coating the membrane or scaffold with ECM components like collagens (Munson *et al*., 1990; Chen *et al*., 2017). Especially collagen IV, a structural protein present in the apical *lamina densa* of the basement membrane, could facilitate the initial attachment rate of isolated cells during seeding and therefore prevent dedifferentiation due to excessive proliferation (Aumailley and Timpl, 1986). Also conditioning the basolateral medium with homo- or heterologous fibroblasts enhances the structural differentiation of the cultured epithelial cells (Munson *et al*., 1990; Ostrowski and Nettesheim, 1995; Miessen *et al*., 2011).

3D models combining epithelial and stromal cells

Stromal cells regulate cell fate, morphology, and function of epithelia through epithelial-mesenchymal interactions (Cunha *et al*., 1985; Kurita *et al*., 2001). Interactions between epithelial and stromal cells are evident in the oviduct (Umezu and Tomooka, 2004) as well as in the uterus, where they have been extensively studied in human models for decidualization and embryo invasion (reviewed in Weimar *et al*., 2013). Stromal cells seem to translate systemic signals within the maternal organism (e.g. from steroid hormones) and to modulate their effect on the epithelial lining (Pierro *et al*., 2001; Qi *et al*., 2012).

Stromal cells can be co-cultured with epithelial cells in compartmentalized culture systems with or without ECM resembling scaffolds (e.g. hydrogel, agarose or matrigel; Fig. 2). While 3D models of the oviductal or luminal endometrial epithelium with the underlying stroma can relatively easily be constructed (Arnold *et al*., 2001; Simintiras *et al*., 2016; Fig. 2A, B), modeling the endometrium with both its glandular and luminal epithelium in 3D is a more complex endeavor. In contrast to the luminal epithelium, the glandular endometrial epithelium finds its niche within the interstitial tissue (Fig. 2C). To our knowledge, there is only one report documenting the capability of uterine epithelial cells to form both the endometrial luminal epithelium as well as glands *in vitro*. Primary epithelial endometrium cells were co-cultured with stromal cells seeded in fibrin-agarose and gland formation occurred spontaneously (Wang *et al*., 2012).

More recent epithelial-stromal co-culture models are based on *ex vivo* ECM scaffolds as the de-cellularized endometrium (Olalekan *et al*., 2017) or novel artificial scaffold structures (MacKintosh *et al*., 2015) which provide a more *in vivo*-like 3D environment for the stromal cells.

Perfused culture systems and microfluidic devices

The FRT epithelium of most mammalian species undergoes dramatic morphological and functional changes throughout the estrous cycle. In the luteal phase (progesterone dominance), the epithelium exhibits a regressed status; conversely, cells re-enter proliferative status in the follicular phase (estradiol dominance), including a rise in epithelium height and increased secretory activity.

However, the exact and time resolved impact of hormones (as well as many other dynamic systemic maternal cues) on luminal fluid formation and epithelium responsiveness to embryonic signals are not elucidated yet. Even if different stages of the estrus cycle can be mimicked in compartmentalized models of the FRT epithelium (Chen *et al*., 2013), these systems are still static. Devices which allow constant perfusion of the cell culture vessel therefore provide much better options for modeling the dynamic changes induced by maternal cues and to elucidate their effects on the embryonic environment and the embryo itself. Perfusion approaches were already used to model FRT epithelia, and were proven to enhance structural differentiation (Reischl *et al*., 1999). Lately, new dynamic culture systems, which enable long-term maintenance of differentiated and hormone responsive epithelia, have been established. An organ-on-a-chip model of the bovine oviduct (suitable for live cell imaging) recapitulates the oviduct epithelium over extended culture periods (up to six weeks). In co-culture experiments, epithelial interactions with sperm and oocytes as well as fertilization events were observed (Ferraz *et al*., 2017). Beyond that, organ modules of the murine ovary, fallopian tube, uterus, cervix and liver, with a sustained circulating flow between all tissues, were recently coupled in a multiple unit microfluidic platform. This system simulated not only the female reproductive tract, but also the endocrine loops between different organs (Xiao *et al*., 2017). In the era of 3D (bio-)printing, these approaches surely represent the next generation of *in vitro* models for studying early embryo-maternal interactions.

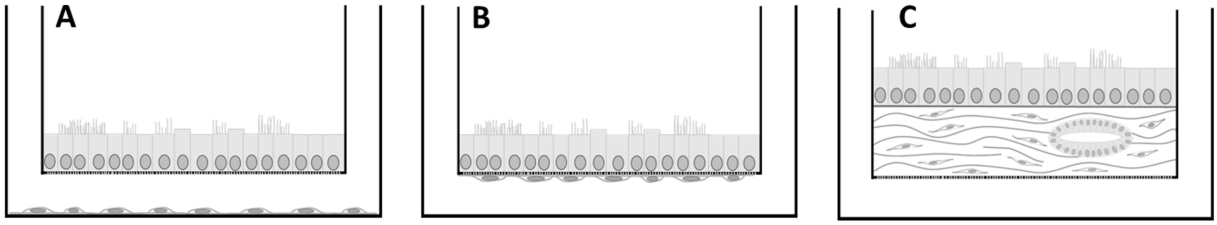

Figure 2. Schematic illustration of strategies for co-culturing stromal cells with FRT epithelial cells in compartmentalized culture systems. (A, B) Stromal and epithelial cells grown in separate compartments. (A) Stromal cells seeded in the basal culture dish. No direct epithelial-stromal cell contact; (B) Stromal cells seeded on the basal side of the insert directly beneath epithelial cells; (C) Stromal and epithelial cells within the same compartment. Stromal cells embedded in ECM support, which may permit the formation of glandular structures.

Co-culture of embryos with epithelia of the FRT

FRT epithelial cells cultured under standard 2D submerged conditions were widely used as feeder layers to improve IVP outcomes and showed a positive effect on *in vitro* embryo quality (Locatelli *et al*., 2005; Cordova *et al*., 2012, 2014; Schmaltz-Panneau *et al*., 2015). In terms of their embryo supporting capacity, however, no difference was detected between oviduct and uterine epithelial cells and oviduct stromal cells (Goff and Smith, 1998). Feeder origin also had no influence on bovine embryonic development and transcriptome when bovine oviduct epithelial cells were compared with a primate kidney cell line as feeder layer (Carvalho *et al*., 2017). In recent years, co-cultures have also been applied to examine early embryo-maternal dialogue. In several studies bovine embryos were co-cultured with OEC to mimic the maternal environment *in vitro.* These experiments demonstrated bi-directional responses at the transcriptional level from both the maternal and embryo side (Schmaltz-Panneau *et al*., 2014; Garcia *et al*., 2017). However, OEC were subjected to multiple embryos and/or prolonged co-incubation, which does not ideally imitate the *in vivo* situation in the monovulatory cow.

Taking advantage of the ALI approach (see paragraph compartmentalized culture systems), co-culture experiments for the first time demonstrated embryo development on OEC up to the blastocyst stage without supplementation of any embryo culture medium (Chen *et al*., 2017). However, more *in vivo*-like mRNA expression of bovine embryos could not be proven as a result of co-culture (van der Weijden *et al*., 2017). We deduce that to enhance embryo quality in co-culture including a dynamic hormonal stimulation procedure might be necessary to better mimic the *in vivo* oviductal environment.

Conclusion

Depending on the researcher's specific scientific question, different culture models are available to reconstruct the upper FRT *in vitro*, either for the short or long term. In compartmentalized culture systems, epithelia are manipulable from their basolateral as well as apical surface, allowing co-culture of embryos/zygotes on the apical and concomitant application of maternal effectors to the basolateral compartment. This makes them powerful tools for studying early embryo-maternal interactions. Stromal cells and ECM components can be incorporated, which is of special interest for modeling the endometrium *in vitro*. Considering that the oviduct and uterus are highly dynamic, hormone responsive organs, perfused culture systems or microfluidic devices allow a more *in vivo*-like recapitulation of the early embryonic environment. The recent advances achieved in these model systems provide the basis for deciphering the possibly fine-tuned interactions between the single early embryo and the maternal organism as well as their effects on offspring development and health.

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DOI: 10.21451/1984-3143-AR2018-0038

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Applications of large-scale molecular profiling techniques to the study of the corpus luteum

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Abstract

The corpus luteum (CL) is vital for the establishment and maintenance of pregnancy. Throughout the history of luteal biology, cutting-edge technologies have been used to develop a thorough understanding of the functions of specific luteal cell types, the signaling pathways that result in luteal cell stimulation or demise, and the molecules that regulate specific functions of luteal cells. The advent of large-scale profiling technologies such as transcriptomics, proteomics, and metabolomics, has brought with it an interest in discovering novel regulatory molecules that may provide targets for manipulation of luteal function or lifespan. Although the work to date is limited, transcriptomics have been effectively used to provide a global picture of changes in mRNA that relate to luteal development, steroidogenesis, luteolysis or luteal rescue. Some studies have been reported that profile microRNA (miRNA) and proteins, and although not yet published, metabolomics analyses of the CL have been undertaken. Thus far, these profiling studies seem to largely confirm earlier findings using targeted approaches, although previously unstudied molecules have also come to light as important luteal regulators. These molecules can then be studied using traditional mechanistic techniques. Use of profiling technologies has presented physiologists with unique challenges associated with analyses of big data sets. An appropriate technique for balancing the risks associated with type I (false discoveries) and type II (overlooking a real change) statistical error has not yet been developed and many big data studies may have potentially important differences that are overlooked. Also, it is imperative that attempts be made to integrate information from the various -omics studies before drawing conclusions based on expression of only one class of molecule, to better reflect the interdependency of molecular networks in cells. Currently, few analysis programs exist for such integrations. Despite challenges associated with these techniques, they have already provided new information about the biology of the CL, notably allowing identification of a key regulator of acquisition of luteolytic capacity and providing a big-picture view of the subtle changes that occur in the CL during early pregnancy. As these technologies become more accurate and less expensive, and as analysis becomes more user-friendly, their use will become much more widespread and many new discoveries will be made. This review will focus only on relevant studies in which these technologies were used to study the CL of ruminants.

Keywords: bovine, corpus luteum, molecular profiling.

Introduction

In the 1600's, Regnier de Graaf described his observation of transient yellow globules that form from emptied ovarian follicles after coitus, noting that the number of globules was the same as the number of fetuses (Jocelyn and Setchell, 1972), and Marcello Malpighi first called this structure a corpus luteum, latin for yellow body. The function of the corpus luteum (CL) remained a mystery for 300 years, when experimental evidence was obtained that the CL was necessary for the maintenance of pregnancy (Simmer 1971; Frobenius 1999). This was followed by the discovery of the primary secretory product of the CL, progesterone, in the 1930's. Despite a slow beginning to the understanding of the function of the CL, once it was identified that this small structure was absolutely essential for the establishment and maintenance of pregnancy in all mammals, it captured the attention of reproductive biologists, and none more so than those interested in reproduction of domestic ruminants. Thus, in the last 50 years, great advances have been made in understanding luteal function, much of which came from studies in cows and sheep. While the goal of this research was to enhance fertility of these species, the knowledge of the basic biology of the CL could be generally applied to nonruminants, and because of its ephemeral nature, the CL has served as a model for many aspects of cellular biology, including angiogenesis, tumor development, steroidogenesis, roles of tissue-resident immune cells, and pathways of cellular death.

During the mid- to late 20th century, the hormonal regulators, second messenger molecules and biochemical reactions in steroidogenesis were elucidated. Sources of cholesterol as substrate for progesterone synthesis and intracellular signaling pathways were defined. Refined procedures to separate cells based on size led to a race to determine the origins and distinct functions of the small and large steroidogenic cells, and the discovery that oxytocin is produced in the CL prompted a flurry of research to determine if luteal oxytocin is necessary for uterine prostaglandin (PG)F2A release during luteolysis. The cellular heterogeneity that characterizes the CL also intrigued researchers, whose work revealed the contributions of endothelial cells, fibroblasts, pericytes and immune cells to development, function, and regression of the CL. The advent of technologies for identifying and quantifying steady state concentrations of mRNA in cells and tissue, including northern blotting, PCR, and qPCR, brought about a revolution in targeted-approach experimentation to elucidate how

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Received: February 26, 2018
Accepted: May 15, 2018

changes in luteal functions are driven by changes in gene expression. For more information about these discoveries and the general biology of the CL, the reader is referred to a number of reviews on ruminant luteal function (Niswender *et al*., 2000; Pate *et al*., 2012; Wiltbank *et al*., 2012; Miyamoto *et al*., 2013; Smith and Meidan, 2014).

Transcriptomic profiling in the corpus luteum

Using the technologies mentioned above, studies of mRNA concentrations in the CL have been hypothesis-driven, searching for the key changes in mRNA relative to receptor activation, signal transduction, steroidogenesis, cytokine production, and cell death pathways. Much has been learned about which pathways and genes were regulated during development and regression of the CL using this type of approach. However, more recently, researchers have used high throughput technologies to profile many (microarray) or all (sequencing) of the transcripts present in the CL from selected times or physiological states. This approach was at first criticized as being a fishing expedition, but identification of potentially important molecules that led to new hypotheses about luteal regulation has enhanced acceptance of these powerful approaches. Transcriptomic analyses have largely confirmed our understanding of luteal functions as determined by more targeted approaches, lending further support to previously drawn conclusions. Perhaps more importantly, they have also shed light on unexplored or potentially new cellular pathways and functions.

Development of the ruminant CL involves differentiation of follicular steroidogenic cells and it was suggested from cell-labeling studies that the small luteal steroidogenic cells (SLC) are derived from the thecal cells of the follicle, whereas the large cells (LLC) originally differentiate from granulosal cells (Alila and Hansel, 1984). Romereim *et al*. (2017) used microarrays to profile the transcriptomes of isolated granulosal, thecal, and separated luteal cells. This approach supported the existing model of differences between SLC and LLC, including identification of the LHCGR in greater abundance on SLC and the PTGFR in greater abundance on LLC. Additionally, it allowed for identification of six novel cell lineage markers each for the thecal cell-SLC lineage and the granulosal cell-LLC lineage. These lineage markers include molecules involved in ion and molecular transport and lysosomal function in LLC and are primarily molecules involved in signaling in SLC. Further, the transcriptome of the large steroidogenic cells indicated that these cells likely function in recruitment of immune and endothelial cells, activities that had not previously been ascribed to a particular luteal steroidogenic cell type. Baddela *et al*. (2018) reported 1276 differentially abundant mRNA in small and large luteal cells. The small luteal cells were enriched in mRNA responsible for immune cell recruitment, whereas the profile of large luteal cell mRNA suggested functions in regulating folliculogenesis, luteolysis, and small molecule metabolism. The reported purity of the separated cell populations was similar in these two studies, so the clear discrepancy between them may be due to the cell-type comparisons made. Baddela *et al*. used days 11-12 CL (n = 4) from timed estrous cycles. The stage of the cycle from which CL (n = 3) were collected in the study of Romereim *et al*. was not described. Because differentiation of the small and large cells is a somewhat continuous process, it is possible that functions associated with small and large cells are stage-dependent. Although it remains to be determined which steroidogenic cell type is responsible for recruiting immune cells, other differentially abundant mRNA and predicted functions of small and large cells were fairly consistent between the two studies.

Differentiation and maximal steroidogenic capacity of the ruminant CL is dependent on luteinizing hormone. As might be expected, gonadotropic stimulation of the CL resulted in upregulation of genes related to lipid metabolism, cholesterol metabolism and progesterone production (Fatima *et al*., 2012). The most upregulated mRNA was fatty acid binding protein 5 (FABP5), which can transport lipids within cells to lipid droplets and mitochondria. To our knowledge, this potentially important regulator of steroidogenesis has not been studied in the CL. Transcriptomic analysis of day 4 and day 11 bovine CL also indicated that steroidogenic and cholesterol biosynthetic genes are upregulated in the midcycle CL, along with genes involved in immune response, whereas the day 4 CL is characterized by genes related to cell cycle, DNA replication and metabolic processes (Kfir *et al*., 2018). This analysis also revealed that the developing CL expresses angiogenesis-promoting genes, whereas the mature CL expressed genes related to cessation of blood vessel sprouting.

As the CL develops, it must gain the capacity to regress in response to PGF2A (Tsai and Wiltbank, 1998). The inability of the developing CL to regress, despite clear responses to PGF2A, has intrigued ovarian biologists for decades, leading to comparison of the transcriptomes of early (day 4) and midcycle (dday 10) CL in response to exogenous PGF2A. Using microarrays, Goravanahally *et al*. (2009) found 167 differentially expressed genes, most of which were upregulated in the day 10 CL, likely reflecting differentiation and development of pathways for maximal steroidogenesis. This group then focused on the 20 genes that were associated with cell signaling pathways, as these genes could be regulators of luteal responsiveness to PGF2A. Collection of CL 24 h after a luteolytic injection of PGF2A showed upregulation of CAMKK2 in day 9, but not day 4, CL. Although the CL in this study were collected 24 h after the PGF2A injection, when luteolysis is advanced, this research group later showed that CAMKK2 is indeed a component of the PGF2A signaling pathway in day 10, but not day 4, CL (Bowdridge *et al*., 2015), providing a good example of how a profiling experiment led to the discovery of a molecule that could potentially be targeted to regulate luteal function. Differences in PGF2A regulation of gene transcription were further

delineated by Mondal *et al* (2011) who also used microarrays to determine differentially abundant genes in early (day 4) and midcycle (day 11) CL collected following administration of PGF2A. Prostaglandin-regulated genes were detected in both types of CL, but the response was much more robust in the midcycle CL. Genes that were upregulated by PGF2A in day 11, but not day 4, CL indicated activation of biological processes involved in receptor activity, cellular death, and immune cell-related genes, and many were genes that are under the control of ETS family transcription factors. This is a large family of transcription factors that is associated with regulation of many cellular functions, including apoptosis. Of note, almost all of the listed biological processes upregulated by PGF2A within 4 h in the midcycle, but not day 4 CL, include genes normally associated with an immune response, such as cytokines, chemokines and adhesion molecules. A macrophage marker, CD14, was upregulated by PGF2A on day 11, but not day 4, suggesting differential recruitment of immune cells into the mature CL, after induction of luteal regression. The profiling study of Mondal *et al*. (2011) also led to further studies that defined the response of angiogenic factors to PGF2A in day 4 and day 11 CL and their functional roles on luteal endothelial cells (Zalman *et al*., 2012). Overall, these studies support the earlier work from Wiltbank's group (Tsai and Wiltbank, 1998) showing that the day 4 CL is not unresponsive to PGF2A, rather the responses of the day 4 CL differ from those of an older CL that has acquired luteolytic capacity.

Shah *et al*. (2014) and Talbott *et al*. (2017) used microarrays to investigate temporal changes in gene expression during luteal regression, using water buffalo and cows, respectively. As in previous studies, changes in transcripts related to steroidogenesis, LH receptor signaling, and apoptosis were observed. Talbott *et al*. (2017) also confirmed earlier studies in which progesterone declined prior to any decrease in transcripts related to steroidogenesis, and in which luteolysis was characterized by changes in transcripts related to cholesterol availability. However, most of these transcripts also changed coincident to, not before, the decrease in progesterone. Transcription factor mRNA and transcripts indicating activation of cytokine signaling were altered prior to the decrease in progesterone, further supporting the growing evidence that inflammatory-like events are key mediators of PGF2A-induced luteolysis (Mondal *et al*., 2011; Atli *et al*., 2012; Shah *et al*., 2014; Talbott *et al*., 2017). Shah *et al*. also reported downregulation of CYP19A1 and differential abundance of estrogen-responsive genes, a novel finding. Although estrogen synthesis by the bovine CL is low, a role of intraluteal estrogen and estrogen receptor signaling in PGF2A-induced luteolysis was proposed. This would extend previous findings that estrogen can induce premature luteolysis (Wiltbank *et al*., 1961) and that follicular estradiol may be necessary for timing normal luteolysis (Villa-Godoy *et al*., 1985).

Atli *et al*. (2012) developed a model of repeated intrauterine infusions of physiological concentrations of PGF2A, coupled with luteal biopsies, to evaluate temporal changes in the CL during luteal regression. This study, which used qPCR to profile transcripts, indicated that activation of genes related to immune response and prostaglandin metabolism were necessary to ensure the progression of luteolysis. This model was further used to determine if PGE2, which is thought to be involved in luteal rescue during early pregnancy, could suppress PGF2A-induced gene expression. The magnitude of effect of PGF2A pulses on gene expression in the CL, as assessed using RNAseq, was quite large (Ochoa *et al*., 2018). Compared to saline infused controls, 572 mRNA were altered by PGF2A, with an additional 373 mRNA that differed from PGE2 and PGF2A + PGE2 infusions. Transcripts most significantly regulated by PGF2A included those associated with steroidogenesis, apoptosis, and signal transduction, as expected. These data also indicated that ceramide signaling may be associated with luteolysis. Remarkably, compared to saline controls, there were no differentially abundant mRNA in the CL following intrauterine infusions of PGE2 or PGE2 + PGF2A, and these CL did not regress, demonstrating that PGE2 can completely prevent PGF2A-induced changes in mRNA that would ensure luteolysis. A summary of the pathways associated with the stages of luteal development, maintenance and regression, as determined by RNA profiling of luteal tissue, is depicted in Fig. 1.

Little is known about changes that occur within the CL to facilitate its rescue during early pregnancy. When the CL of pregnancy was compared to midcycle (days 10-12) CL, differentially abundant mRNA gradually increased throughout pregnancy (Sakumoto *et al*., 2015), indicating that, once rescued, the CL is not static, but is actively regulated by either intrinsic or extrinsic factors that alter mRNA abundance to facilitate luteal survival and progesterone production. In this study, large changes in chemokine mRNA in the CL of pregnancy were noted, particularly a more-than 10-fold decrease in lymphotactin, a chemokine that recruits T cells, and a more-than 100-fold increase in eotaxin, a chemokine that recruits eosinophils. This study also noted a more modest increase in growth factor-related mRNA during early pregnancy (Sakumoto *et al*., 2015). Similarly, a microarray study performed by Romero *et al*. (2013) demonstrated that in the ovine CL of early pregnancy, there is stabilization or upregulation of pathways related to interferon and cytokine signaling, cell-cell adhesion, and cytoskeleton, as compared to the late and regressing CL. Pentraxin-3, which is produced by several immune cell types, was stabilized in early pregnancy, but reduced during luteal regression. The authors suggest that this molecule may increase cellular resistance to stress (Romero *et al*., 2013). Overall, these and other studies demonstrate that chemokines and cytokines appear to be key regulators of both luteal regression and luteal survival during pregnancy.

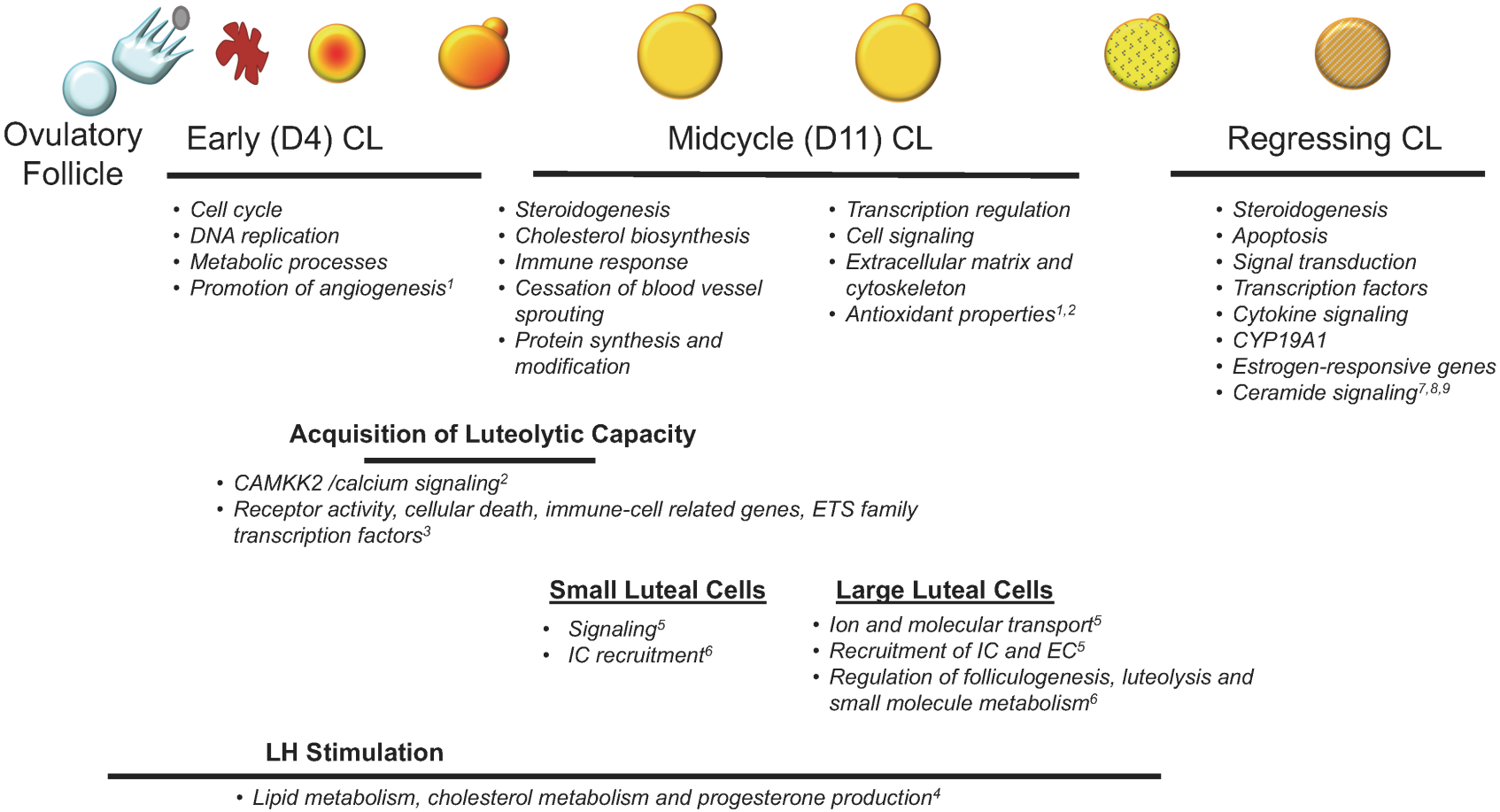

Figure 1. Pathways associated with stages of luteal development, maintenance and regression as revealed by transcriptomic profiling of ruminant CL. Superscripts refer to references as follows: [1]Kfir *et al*., 2018; [2]Goravanahally *et al*., 2009; [3]Mondal *et al*., 2011; [4]Fatima *et al*., 2012; [5]Romereim *et al*., 2017; [6]Baddela *et al*., 2018; [7]Talbott *et al*., 2017; [8]Shah *et al*., 2014; [9]Ochoa *et al*., 2018.

The advent of RNAseq provided more sensitive and more accurate detection of mRNA, allowing for more comprehensive studies of the luteal transcriptome, and providing the opportunity to reveal potentially important transcripts that were previously unrecognized. In a recent RNAseq study comparing transcript abundance in bovine CL of day 17 of the estrous cycle and day 17 of pregnancy, 144 differentially abundant mRNA were reported and immune signaling pathways were among those predicted to be modulated in early pregnancy, as well as novel potential regulators of luteal rescue, including PPAR signaling and PDGF signaling (Hughes *et al*., 2018; Penn State University, Center for Reproductive Biology and Health, University Park, PA USA; unpublished data) Moore *et al*. (2016) used RNAseq to determine if CL and endometria of cattle of low or high genetic merit for fertility contained differentially abundant mRNA. Only 9 mRNA were different in the endometrium, whereas 560 mRNA were different in the CL, suggesting an important relationship between luteal function and fertility. Of the 560 DE mRNA, 85% were lesser in abundance in the CL from low fertility cows, indicating a general reduction in luteal activity. These included genes related to steriodogenesis, extracellular matrix and RNA replication, indicating compromised luteal development and steroidogenic capacity. Conversely, mRNA related to PGF2A response were greater in CL from low fertility cows.

Although a primary focus in statistical analysis of transcriptomic datasets has been on the reduction of type I error, due to the large number of statistical tests performed, this problem has been largely corrected by the Benjamini Hochberg false discovery rate correction, which allows a researcher to choose a threshold (typically between 5 and 15%) of false discoveries that they are willing to tolerate (Benjamini and Hochberg, 1995). However, while this method reduces type I error, it increases type II error and, in some studies, may cause researchers to overlook many genes, proteins, or metabolites that change in a biologically relevant way (Mudge *et al*., 2017). Recent experiments have demonstrated that in an RNAseq study of the CL of the estrous cycle and pregnancy, mRNA that were well below the false discovery rate cutoff were still differentially expressed ($P < 0.05$) by two-fold or more when measured by qPCR (Hughes *et al*., 2018; Penn State University, Center for Reproductive Biology and Health, University Park, PA USA; unpublished data; Fig. 2). Given the cost and time associated with gene expression profiling experiments, the amount of information lost due to this increased type II error is concerning. One proposed solution to this problem is an optimized P-value cutoff, based on power and relative cost of type I and type II error, for each big data study (Mudge *et al*., 2017).

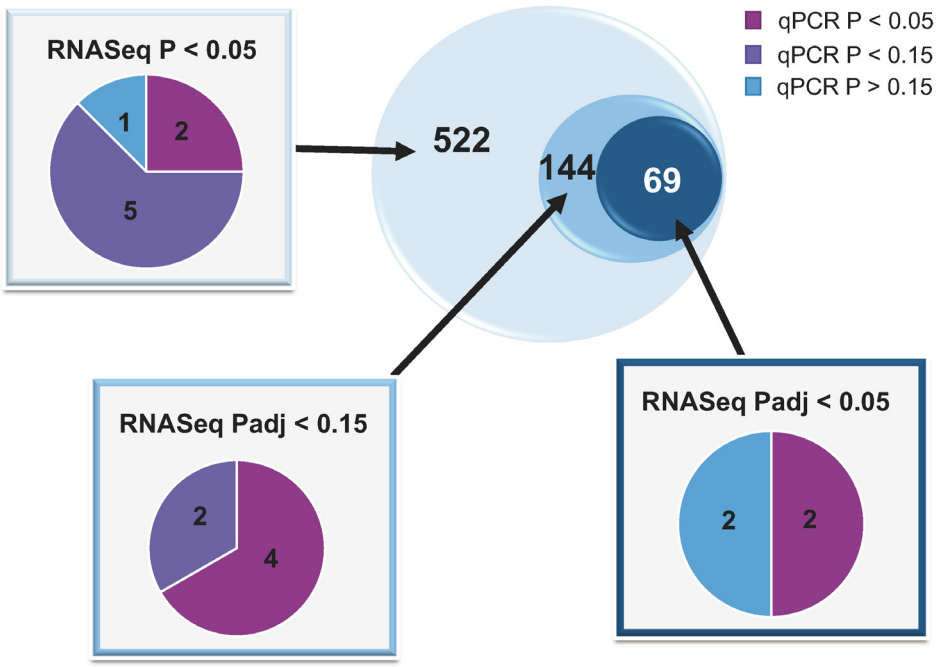

Figure 2. mRNA that were differentially abundant in a transcriptomics study (Hughes *et al*., 2018; Penn State University, Center for Reproductive Biology and Health, University Park, PA USA; unpublished data). Three P-value cutoffs were used ($P < 0.05$, padj < 0.15, padj < 0.05), with 522, 144, and 69 mRNA in each group. Padj-values are P-values that have been adjusted for false discovery rate of 5% false discoveries. These three groups are represented by the three concentric circles. A subset of mRNA from each group was analyzed using qPCR ($n = 6$); total number of mRNA analyzed by qPCR in each subset is represented within each pie chart as significantly ($P < 0.05$), or with a tendency to be ($P < 0.15$), differentially expressed, or not DE ($P > 0.15$).

MicroRNA profiling and regulation of the corpus luteum

MicroRNA are single-stranded noncoding RNA, approximately 22 nt in length, that serve as posttranscriptional regulators of gene expression. Following transcription, the precursor forms of miRNA undergo nuclear and cytoplasmic cleavage to form the mature miRNA. The mature miRNA binds to Argonaute 2 (AGO2), is then incorporated into the RNA-Induced silencing Complex (RISC), and the complex is targeted to the 3'UTR of mRNA with sufficient complementarity to the miRNA. This results in loss of mRNA translation to protein, either via translational repression or degradation of the mRNA. Individual miRNA can have numerous mRNA targets, and individual mRNA can be targeted by many miRNA, which makes elucidation of miRNA-regulated signaling pathways quite complex. MicroRNA expression and activity can be both tissue- and stage-specific, and in some cases can even enhance, rather than suppress, synthesis of specific proteins. The complexity of miRNA biology necessitates confirmation of a proposed regulatory role in a particular tissue at a specific developmental or functional stage. For information on microRNA biology, the reader is referred to reviews by He and Hannon (2004), Treiber *et al*. (2012), Catalanotto *et al*. (2016), Maalouf *et al*. (2016a), and Tesfaye *et al*. (2018).

MicroRNA have captured the attention of biologists in many disciplines, but in particular, seem to have improved the understanding of the dynamic nature of reproductive tissues. Most studies of ovarian miRNA have focused on follicles and oocytes of nonruminant species (reviews: Christenson, 2010; Hossain *et al*., 2012; Li *et al*., 2015; McGinnis *et al*., 2015; Maalouf *et al*., 2016a). Hossain *et al*. (2009) cloned and sequenced a small RNA library derived from bovine ovary and found that some miRNA were dissimilar in abundance among cortex, follicles and CL. Using a screened target set of 115 genes likely to be regulated by abundant ovarian miRNA, pathway analysis indicated that the selected miRNA and their predicted targets were indeed involved in functions indicative of the dynamic nature of ovarian components, such as growth factor signaling, cellular growth and development, and cellular death.

Expression of miRNA in the CL may be developmentally regulated, because greater abundance of miRNA in the mature than in the developing CL has been reported (Maalouf *et al*., 2016b; Baddela *et al*., 2017), and the functions regulated by luteal miRNA shift from cellular metabolism and growth in the day 4 CL to cell cycle, cell death, and gene expression in the midcycle CL (Maalouf *et al*, 2016b). One of the upregulated miRNA, miR34a, targeted NOTCH1 and YY1 and promoted luteal cell progesterone production while suppressing proliferation of luteal cells (Maalouf *et al*., 2016b), consistent with a role in inhibition of growth while enhancing differentiated function. McBride *et al*. (2012) reported that 9 miRNA decreased and 8 increased during the follicular-luteal transition in sheep, and their predicted targets are involved in cellular development, differentiation, proliferation and survival. The difference in number and direction of DE miRNA between these two studies is likely due to method of detection (microarray *vs*. Sanger sequencing) and comparison of mature CL to follicular (McBride *et al*., 2012) *vs*. immature luteal (Maalouf *et al*., 2016b) cells. When water buffalo CL of 3 estrous cycle stages were compared to granulosal cells using miRNAseq, more miRNA were CL-specific than granulosal cell-specific and 39 of 43 differentially abundant miRNA were greater in abundance in CL. Interestingly, 93% of the luteal-unique miRNA mapped to a 0.7 Mb region of buffalo chromosome 20 (chromosome 21 of cows) and it was proposed that this miRNA cluster suppresses 20-alpha hydroxysteroid dehydrogenase, and thus progesterone metabolism, during luteinization (Baddela *et al*., 2017). In one study, a greater abundance of miRNA was found in follicular cells compared to luteal cells (Mohammed *et al*., 2017). Perhaps there is a robust expression of miRNA in developing and preovulatory follicles, that is then generally downregulated around the time of ovulation and early luteinization. Subsequent upregulation of miRNA during latter developmental stages of the CL would negatively regulate growth and support maximal steroidogenesis. It should be noted that in one recent study, there was no change in the number of miRNA in the midcycle compared to the early CL (Gecaj *et al*., 2017). It is unclear why this study differs from the previous ones.

Ma *et al*. (2011) compared nonregressed to regressed CL and reported 13 DE miRNA, 7 being less abundant and 6 being more abundant in regressed CL. The most downregulated miRNA in regressed CL was miR378, and its expression appeared to be inversely correlated to its predicted target, IFNGR1 protein, suggesting that it may serve to repress IFNG-mediated cell death in the nonregressed CL. Using next generation sequencing, Maalouf *et al*. (2014) identified 544 known and 46 novel miRNA in the bovine CL. To determine if miRNA may be involved in luteal rescue during maternal recognition of pregnancy, CL collected on day 17 of the estrous cycle were compared to CL collected on the same day of pregnancy. Fifteen miRNA were found to be differentially abundant. The predicted targets of these 15 miRNA represent genes involved in immune-related events and apoptosis, reminiscent of pathways predicted to regulate luteal survival in the transcriptomic studies mentioned above. One of the miRNA targets associated with the top pathways in this study was CAMKK1, which, along with the mediator of acquisition of luteolytic capacity discussed previously, CAMKK2, plays a role in the calcium/calmodulin-dependent (CaM) kinase cascade. This study indicated that miRNA are also likely to play in role in luteal rescue (Maalouf *et al*., 2014).

A comprehensive study of miRNA expression using miRNAseq has shown that some miRNA are highly abundant throughout luteal lifespan, whereas others are found in abundance only at specific stages (Gecaj *et al*., 2017). The dynamic and transitory nature of the CL makes it an exemplary tissue for demonstration of stage specificity of miRNA expression. The top 20 most abundant miRNA (based on mean reads from all stages of the cycle studied in each study) were identified for

four miRNA profiling studies (Maalouf et al., 2014, 2016b, Baddela et al., 2017, Gecaj et al., 2017). MiRNA found in only one study, or common to two, three, or all four studies are listed in Table 1. The five miRNA common to at least three of these studies were analyzed in mirPath version 3 (Vlachos et al., 2015) and the top 10 gene ontology (GO) terms associated (P < 10^-325) with their predicted targets, using the Tarbase database, are listed in Table 2. The top 10 GO terms indicate that the most abundant miRNA in the CL are likely to be involved in regulation of the cell cycle, protein synthesis, and immune function. Notably, all five miRNA have predicted targets associated with each of the top 10 GO terms, demonstrating possible redundancy in miRNA functions.

Finding significant differential expression of miRNA using profiling techniques may be affected by sample size, statistical analyses, isomiR distribution, relative abundance, and degree of variation among biological replicates. Thus, it is not surprising when some discrepancies in lists of DE miRNA in different studies occur. Researchers must use caution when drawing definitive biological conclusions based on somewhat arbitrary cutoffs for significance and variation in how data are handled.

Although profiling technologies have significantly enhanced understanding of miRNA in the corpus luteum, the targeted approaches that have sprung from these miRNA profiling studies have yielded important functional information about specific miRNA in the CL. Dai *et al*. (2014) reported an increase in the abundance of miR126 during luteal development, during which time its expression was inversely correlated to Talin2, suggesting that miR126 may regulate cellular interactions with extracellular matrix during final maturation of the CL. Maalouf *et al*. (2016b) also observed greater abundance of miR126 in midcycle compared to developing CL. miR96 is upregulated in the early CL compared to the follicle and supports survival of luteal cells by directly targeting FOXO1 (Mohammed *et al*., 2017). Angiogenesis in the developing CL is at least partially regulated by miR221 targeting thrombospondin 1 in luteal endothelial cells (Farberov and Meidan, 2017).

Table 1. miRNA identified among the top 20 most abundant miRNA in at least one of four miRNA profiling studies.

Number of studies	miRNA
Four	let-7a-5p
Three	mir-21-5p, let-7f, mir-26a, let-7b
Two	let-7c, let-7d, let-7e, let-7g, let-7i, mir-100, mir-103, mir-10b, mir-125b, mir-143, mir-148a, mir-202, mir-30d, mir-320a, mir-3600
One	let-7j, mir-107, mir-126-3p, mir-126-5p, mir-127, mir-140, mir-145. mir-148b, mir-151-3p, mir-154c, mir-1839, mir-186, mir-199a-3p, mir-214, mir-2284x, mir-23b, mir-24a, mir-26c, mir-27b, mir-29a, mir-30a-5p, mir-30e-5p, mir-320b, mir-320c, mir-342, mir-378, mir-423-5p, mir-450a, mir-486, mir-503-5p, mir-99a-5p, mir-99b

Table 2. Top 10 gene ontology (GO) terms associated with predicted targets of the 5 miRNA common to at least 3 of the studies.

GO Category	Number of predicted target genes associated with GO category
G1/S transition of mitotic cell cycle	69
G2/M transition of mitotic cell cycle	64
Mitotic cell cycle	188
Protein binding transcription factor activity	199
Nucleic acid binding transcription factor activity	292
Toll-like receptor signaling pathway	51
Immune system process	430
MyD88-independent toll-like receptor signaling pathway	42
Molecular function	4339
RNA binding	702

Proteomic profiling in the corpus luteum

Inverse correlation of miRNA and mRNA abundance is often used as evidence that a particular mRNA is a target of a miRNA of interest. However, it is not always the case that a miRNA-mRNA interaction results in degradation of the target mRNA. If instead, the interaction simply causes translational repression of the mRNA, the steady state concentration of the mRNA may remain unchanged, or may increase if expression of the gene continues. Therefore, confirmation of a miRNA target requires determination of a change in protein concentration when the miRNA concentration is altered. An example of this is NOTCH1 regulation by miR34a in the CL (Fig. 3). Both miR34a and *NOTCH1* mRNA are greater in the midcycle than the developing CL, but the increase in *NOTCH1* is not reflected by an increase in NOTCH1 protein, indicating either translational repression or enhanced protein degradation. In cultured luteal cells, a miR34a mimic clearly decreased NOTCH1, confirming translational repression by this miRNA. Therefore, global investigation of miRNA targets will be more reliable using proteomic, rather than transcriptomic, analyses.

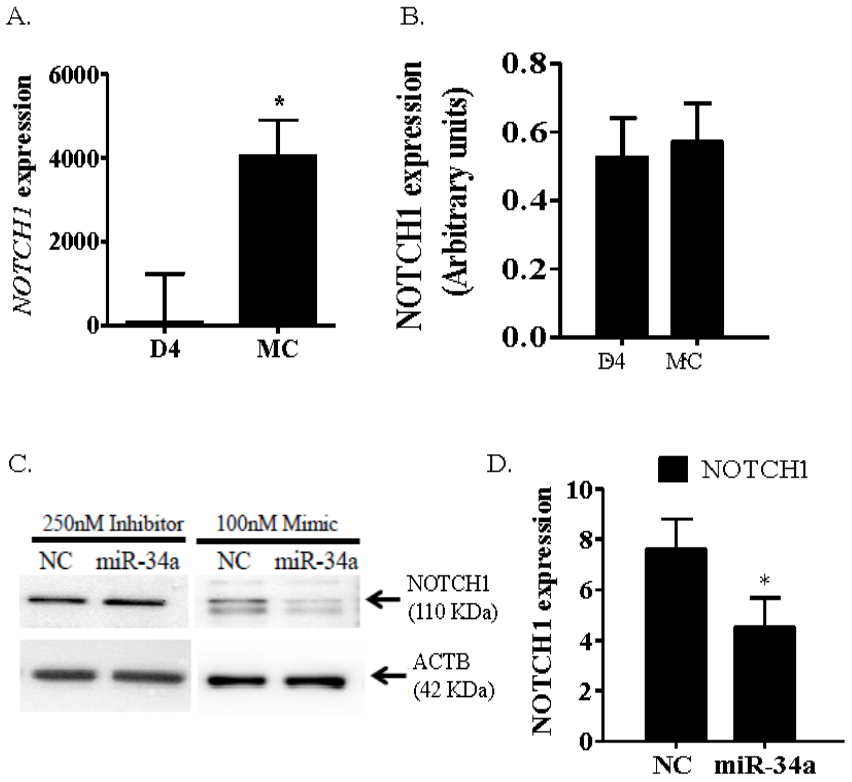

Figure 3. Relative expression of *NOTCH1* (mRNA, A) and NOTCH1 (protein, B) in developing (day 4) and fully functional (MC=midcycle, days 10-12) CL. C) Representative western blot depicting downregulation of NOTCH1 in response to a miR-34a mimic compared to a negative control (NC) scrambled sequence RNA, and D) Mean (n = 3) NOTCH1 protein abundance in response to miR-34a mimic. Adapted with permission from Maalouf *et al.* (2016b).

Few proteomics studies of the CL have been conducted and proteomic studies present challenges in terms of sensitivity. Often, identified proteins represent only the most highly abundant fraction of total proteins in a tissue. The two published studies of proteomics in the ruminant CL used two-dimensional polyacrylamide gel electrophoresis and MALDI-TOF-MS to identify proteins that changed during the estrous cycle or pregnancy. Arianmanesh *et al*. (2011) found that 139 proteins were upregulated and 69 were downregulated in the ovine CL as it progressed from day 12 to day 16 of the cycle. On day 16, plasma progesterone was low, indicating that the CL was regressed, but upregulated proteins included those involved in signal transduction, oxidative stress and structural integrity, indicating that the events of luteolysis are coordinated to induce cell death without a massive inflammatory response, rather than simply being a cessation of all cellular functions. In the progression from day 12 to day 16 of pregnancy, 52 proteins were upregulated and 14 were downregulated, suggesting that the presence of an embryo suppressed the changes in protein abundance that were apparent when the CL regressed. Upregulated proteins during pregnancy are involved in signal transduction, protein synthesis, electron transfer, steroidogenesis, and cytokine signaling (Arianmanesh *et al*., 2011).

Chung *et al*. (2012) compared the CL of day 90 of pregnancy to midcycle (days 6-13) CL from nonbred cattle. Analysis of the 2D gels revealed differences in protein abundance represented by 32 spots, and from these, 23 proteins were identified, of which 6 were more abundant and 17 were less abundant in CL of pregnancy. Differences in proportions of up- and down-regulated proteins in these two studies are likely due to the different stages from which the CL of pregnancy were collected.

We have also used this procedure to compare bovine CL collected on day 18 of the cycle or pregnancy. The number of differentially expressed proteins was undetermined, but 18 spots that were clearly different in CL from cyclic or pregnant cattle (Fig. 4) were sequenced. Identified differentially abundant proteins included vimentin, adrenodoxin, 3-hydroxymethylglutaryl-CoA synthase, apolipoprotein A1, annexin and glutathione S-transferase. There was considerable similarity in identified proteins between this study and the previous two. Six proteins were differentially abundant in at least two of these three proteomics studies (Arianmanesh *et al*., 2011; Chung *et al*., 2012; Pate *et al*., 2018; Penn State University, Center for Reproductive Biology and Health, University Park, PA USA; unpublished data). Commonly identified proteins among at least two of these three studies are

listed in Table 3. Gene family was assigned by Ingenuity Pathway Analysis (IPA; Qiagen). The Diseases and Functions feature of IPA was used to identify functions associated with the commonly differentially abundant proteins. All significant (P< 0.005) functions involving all six differentially abundant proteins and relevant functions involving four or five differentially abundant proteins are shown in Table 4. This analysis demonstrates that proteins that are modulated in the CL during early pregnancy are likely involved in regulating apoptosis and cell survival and maintaining steroidogenesis. Interestingly, all six common proteins were predicted to be involved in synthesis of lipid, which can be addressed in metabolomic studies.

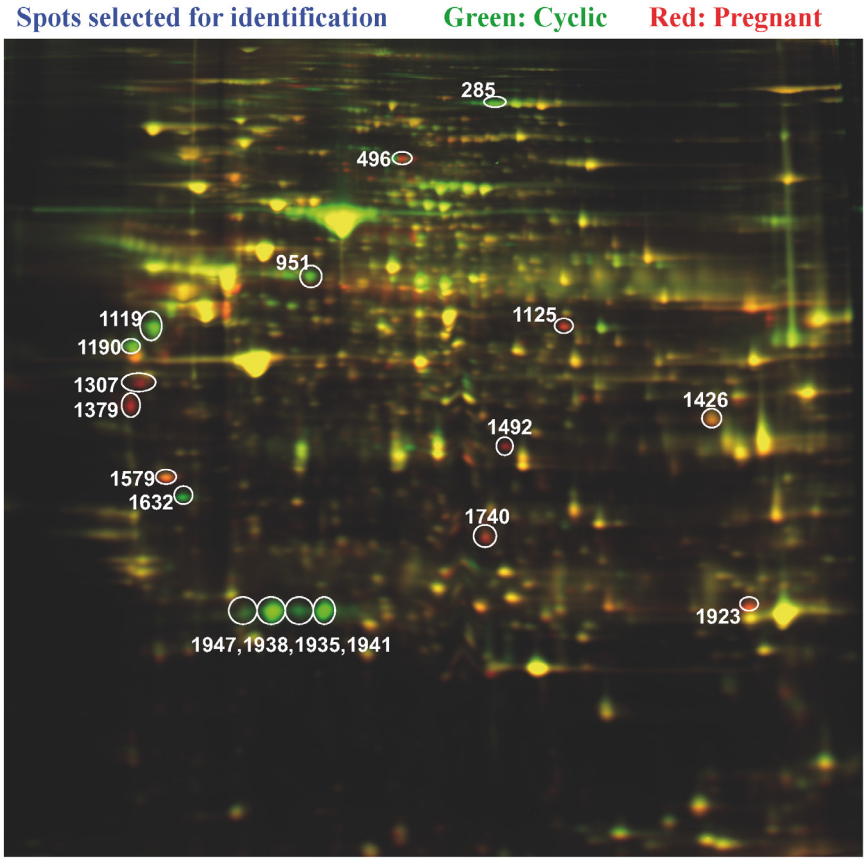

Figure 4. Representative 2D gel of proteins from CL collected on day 18 of the estrous cycle (green dye) and day 18 of pregnancy (red dye). Yellow indicates proteins that were of similar abundance in both treatment groups.

Table 3. Proteins identified as differentially abundant during the estrous cycle and pregnancy in at least two proteomics studies.

Protein	Gene symbol	Gene family
Vimentin	VIM	other
Apolipoprotein A1	APOA1	transporter
Annexin (5 or A1)	ANXA1, ANXA5	enzyme
Adrenodoxin reductase	FDXR	enzyme
Glutathione S-transferase	GSTA1	enzyme
Superoxide dismutase	SOD1	enzyme

Table 4: Functional analysis of proteins differentially abundant in the estrous cycle and pregnancy in at least two proteomics studies

Functions involving all 6 common proteins	Apoptosis, necrosis, synthesis of lipid
Relevant functions involving 4 or 5 common proteins	Fatty acid metabolism, migration of cells, synthesis of reactive oxygen species, synthesis of steroid hormone, vasculogenesis

Metabolomic studies of the corpus luteum

Metabolomics is a broad term that can refer to measurement of metabolites of any biochemical processes, including amino acids, sugars, and lipids. Changes in specific lipid metabolites, such as prostaglandins, phospholipids, and steroidal molecules, as the CL progresses from development through luteolysis have been achieved using various targeted approaches, such as chromatography and radioimmunoassay. However, no comprehensive metabolomic study of the CL has been reported. We have recently completed a comprehensive analysis of lipid metabolite concentrations in the CL during the estrous cycle, luteolysis and maternal recognition of pregnancy (Hughes *et al*., 2018; Penn State University, Center for Reproductive Biology and Health, University Park, PA USA; unpublished data). Among 79 lipids measured, there were 24 lipids that differed in abundance during the estrous cycle, all being less abundant on day 4 than on day 11, with nine remaining high on day 18 of the cycle. During a 24-h time-course of luteolysis, 35 lipids changed, and as might be expected, represented arachidonic acid metabolism and prostaglandin signaling. In the early period of maternal recognition of pregnancy, only subtle changes in mRNA, miRNA and proteins are detectable in the CL, and this was reflected in the metabolic profile of the bovine CL on day 18.

While there are many programs available for functional and pathway analysis of transcriptomics and proteomics data, fewer such programs are available for analysis of metabolomic data and integration of metabolomic and transcriptomic data. Using the data integration feature in the program MetaboAnalyst (Xia and Wishart, 2016), pathways including sphingolipid (ceramide) metabolism, propanoate metabolism, and pyruvate metabolism were indicated as differentially regulated in the CL of pregnancy. However, close examination of these results revealed that these pathways were modulated by either genes or lipids, but not both, indicating that this program resulted in a listing of potential metabolic pathway modulation, without lipid-metabolite integration. In IPA, the Network and Diseases and Functions features were much more useful in demonstrating functions that may be regulated by combinations of differentially abundant genes and lipids. The top networks containing both differentially abundant genes and lipids were Lipid metabolism, molecular transport, and small molecule biochemistry and DNA replication, recombination and repair, cell death and survival, cellular function and maintenance. Further, differentially abundant mRNA and lipids were expected to be involved in cell movement and migration; among significant diseases or functions annotations, these pathways included the greatest total number of molecules, including both mRNA and lipids (Fig. 5). Other pathways were related to cell interaction and to immune cell differentiation.

Conclusion

Although large-scale molecular profiling studies have largely supported existing hypotheses about luteal function, they have also allowed identification of novel signaling pathways that could be targeted to support luteal function. In particular, these studies have contributed to our understanding of the CL of pregnancy, which, because of the subtlety of the changes that occur in these CL, have been difficult to study using targeted approaches in the past. Figure 6 shows a summary of what large-scale molecular profiling and pathway analysis has revealed about functions that are modulated in the CL during early pregnancy. Notably, each of these technologies, except proteomics, have indicated that luteal immune cell function may be modulated during early pregnancy (with proteomics suggesting cell migration, which is likely migration of immune cells), a compelling finding in light of the growing body of evidence that immune cells are intricately involved in regulation of luteal functions.

Application of large-scale molecular profiling to study the corpus luteum is still in a nascent stage, and laboratory experiments to confirm functions suggested by these kinds of studies are necessary to elucidate specific regulation of luteal function. However, improvements in statistical analysis techniques, as well as advances in the profiling technologies themselves, to improve accuracy and precision with which miRNA, mRNA, proteins, and metabolites can be measured, will continue to drive these kinds of studies forward and allow generation of high-quality, high-resolution data, to more completely understand luteal function.

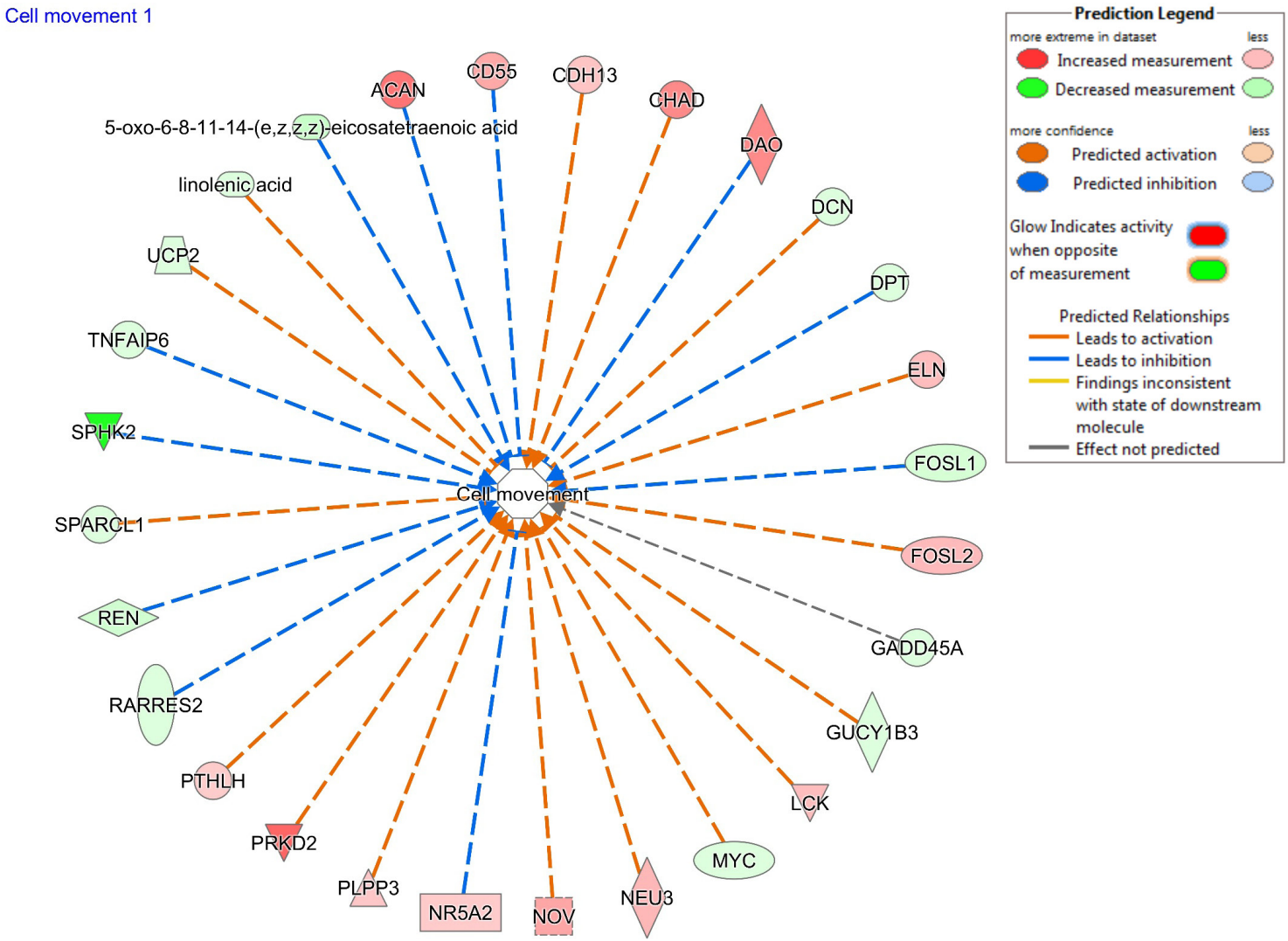

Figure 5. The network from the Diseases and Functions feature of Ingenuity Pathway Analysis (Qiagen) with the greatest total number of molecules, including both differentially abundant genes and lipids from early pregnancy. Red indicates a molecule greater in pregnancy, while green indicates lesser in pregnancy. An orange line indicates activation of cell movement by a molecule, while a blue line indicates inhibition of cell movement.

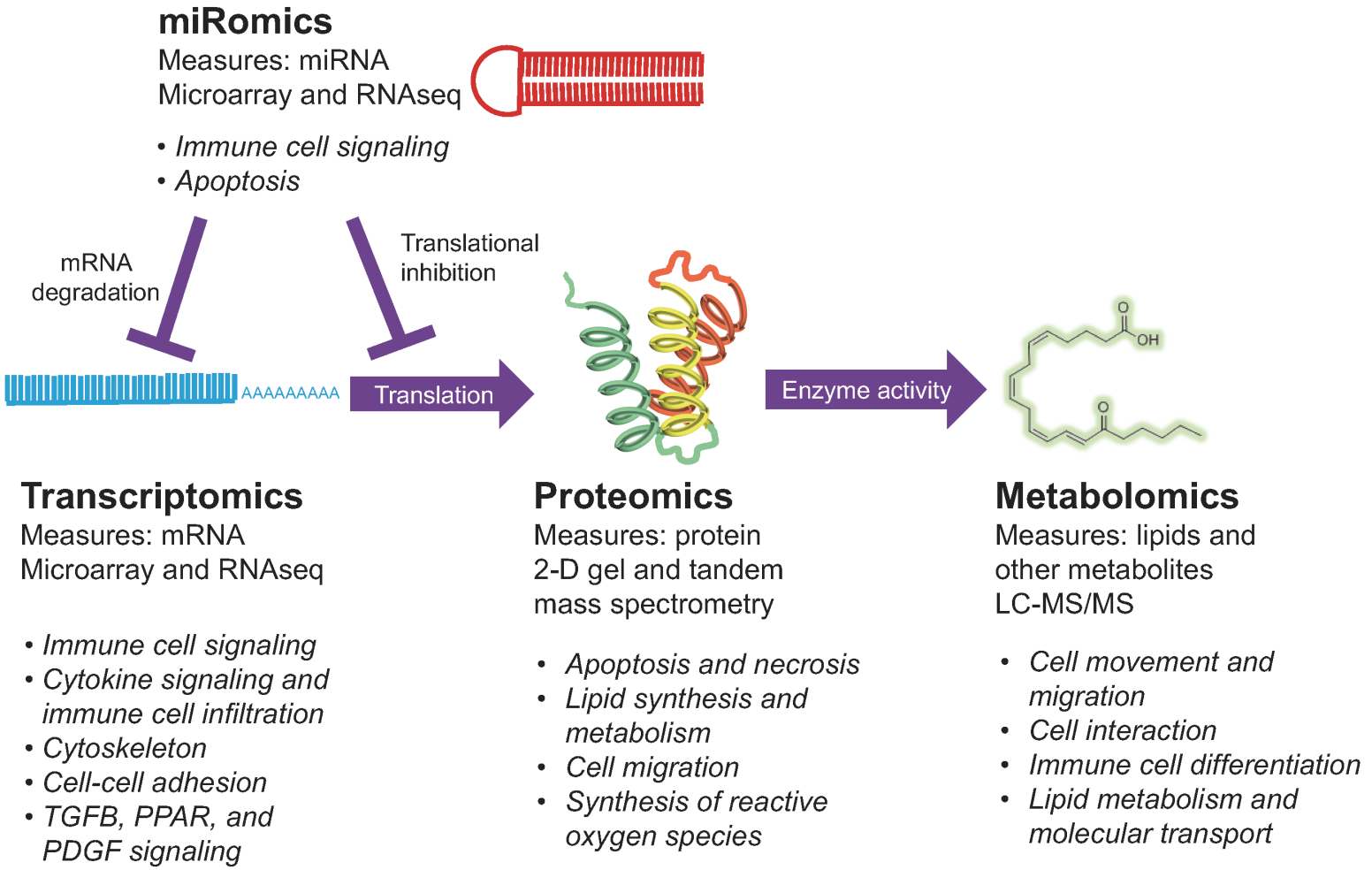

Figure 6. miRomics, transcriptomics, proteomics, and metabolomics have all been used to study the CL of pregnancy. Each technology is shown, with functions modulated in early pregnancy that have been revealed by each technology in italics. miRNA may lead to mRNA degradation or to translational inhibition. mRNA are translated into proteins and proteins mediate the production of lipids and other metabolites that may have key signaling functions.

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DOI: 10.21451/1984-3143-AR2018-0045

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Physiological mechanisms involved in maintaining the corpus luteum during the first two months of pregnancy

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Abstract

Maintenance of the corpus luteum (CL) during pregnancy is essential for continuing the elevated circulating progesterone (P4) that is required to maintain pregnancy. The mechanisms that protect the CL during early pregnancy when the non-pregnant animal would typically undergo CL regression have been extensively investigated. It is clear uterine prostaglandin F2α (PGF) causes regression of the CL in non-pregnant ruminants and that maintenance of the CL during early pregnancy is dependent upon secretion of interferon-tau (IFNT) from the elongating embryo. A number of specific mechanisms appear to be activated by IFNT. Most studies indicate that there is an inhibition of oxytocin-induced secretion of uterine PGF. There is also evidence for increased resistance to PGF action, perhaps due to secretion of PGE2 and PGE1 or direct endocrine actions of circulating IFNT. These mechanisms occur concurrently and each may help to maintain the CL during the first month of pregnancy. However, during the second month of pregnancy, IFNT is no longer secreted by the embryo. Attachment of the embryo to the uterus and subsequent placentome development have been linked to silencing of expression from the IFNT gene. In addition, there is some evidence that oxytocin responsiveness of the uterus returns during the second month of pregnancy leading to substantial basal secretion of PGF and perhaps PGF pulses. There is also no evidence that the CL during the second month of pregnancy is resistant to the actions of PGF as observed during the first month. Thus, this manuscript attempts to compare the mechanisms that maintain the CL during the first and second months of pregnancy in ruminants and provides a new, speculative, physiological model for maintenance of the CL during month two of pregnancy that is distinct from the previously-described mechanisms that maintain the CL during the first month of pregnancy.

Keywords: corpus luteum, interferon-tau, pregnancy.

Introduction

In ruminants, progesterone (P4) is produced by the corpus luteum (CL) and is essential for the original establishment and subsequent maintenance of pregnancy throughout gestation (Wiltbank *et al.,* 2014). Likewise, the embryo/placentomes is essential for maintaining the CL after the first two weeks of pregnancy (Bazer *et al.,* 1997; Spencer *et al.*, 2007; Giordano *et al.*, 2012). Thus, the CL and the pregnancy have a co-dependent relationship that involves both long distance (systemic) and adjacent (local pathways) interactions (Fig. 1). It is well-established that production of IFNT by the elongating embryo maintains the CL during the classical maternal recognition of pregnancy period in the first month of pregnancy (Bazer *et al.,* 1997; Spencer *et al.*, 2007; Wiltbank *et al.*, 2016a). During the second month of pregnancy and beyond, the CL is maintained by mechanisms that remain to be elucidated (Wiltbank *et al.,* 2016b).

Maintenance of pregnancy or, conversely, pregnancy loss can be viewed from two general perspectives (Giordano *et al.*, 2012). First, the embryo/pregnancy may be defective and therefore it is lost, which may be positive for reproductive efficiency because, it is unlikely that a viable offspring would be produced from that pregnancy. The sooner the pregnancy can be recognized as non-viable and discarded, the sooner a new, potentially viable, pregnancy can be initiated. Alternatively, pregnancy loss could occur because the CL inappropriately regresses, resulting in loss of a viable pregnancy, with a subsequent delay in the establishment of a new viable pregnancy, and therefore a reduction in reproductive efficiency. Previous studies have quantified the amount and timing of pregnancy loss in lactating dairy cows, beef cattle, and recipients of *in vitro*-produced (IVP) or *in vivo*-derived (IVD) embryos (Santos *et al.*, 2004; Diskin *et al.*, 2016; Wiltbank *et al.*, 2016a). However, no studies have clearly differentiated if a defective embryo or inappropriate CL regression underlies pregnancy loss in month one or two of pregnancy.

Pregnancy loss during the second month of gestation is substantial (Diskin and Morris, 2008; Santos *et al.*, 2009; Diskin *et al.*, 2016; Wiltbank *et al.*, 2016a). To determine the current magnitude of the problem, we analyzed recent data or recently published studies that evaluated pregnancy loss in lactating dairy cattle (Table 1) or in embryo transfer recipients (Table 2).

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Received: March 4, 2018
Accepted: April 16, 2018

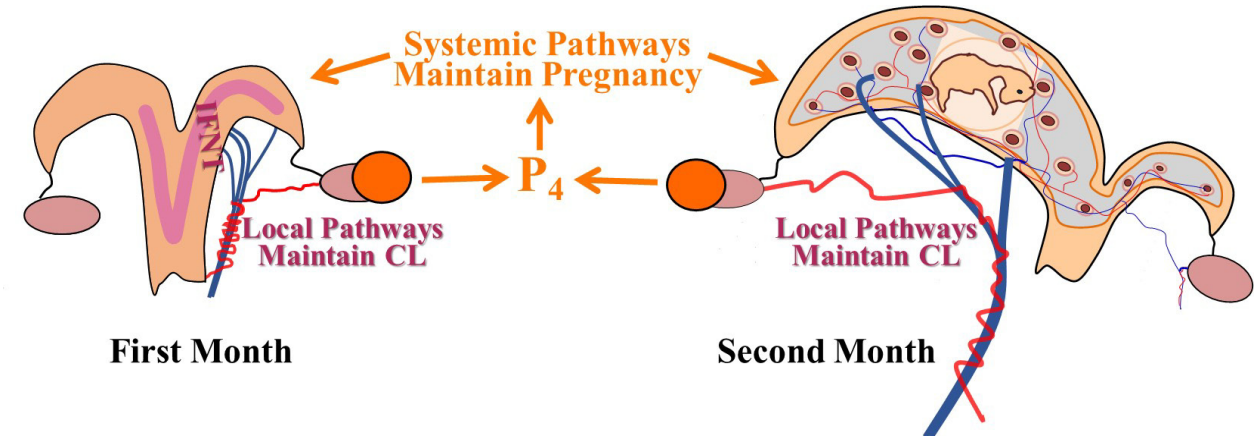

Figure 1. Simplified diagram illustrating that progesterone (P4) maintains the pregnancy through systemic pathways while the pregnancy, either during the first month or second month of pregnancy maintains the CL through local pathways within the utero-ovarian system.

Table 1. Studies that evaluated pregnancy/AI (P/AI) and pregnancy loss between the first (27-34 days after AI) and second pregnancy diagnosis (53-74 days after AI) in primiparous and multiparous cows or overall pregnancy loss in dairy cows from 2005 to 2017.

		Pregnancy diagnosis, day		P/AI at 1st diagnosis, % (n breedings)		Pregnancy loss % (n pregnancies)		Overall pregnancy loss % (n/n)
Reference[1]	Year	1st	2nd	Primiparous	Multiparous	Primiparous	Multiparous	
Baez	2016	32	67	52.2 (289)	42.8 (327)	4.6 (153)	8.6 (140)	6.5 (19/293)
Bartolome	2005	30	55	-	-	-	-	8.5 (10/118)
Bartolome	2009	30	55	29.1 (302)	39.1 (416)	11.4 (88)	10.4 (163)	10.8 (27/251)
Bartolome	2005	27	55	-	-	-	-	18.7 (67/358)
Bilby	2013	32	60	-	-	-	-	10.5 (60/569)
Bisinotto	2015	32	60	-	-	-	-	9.7 (68/699)
Bisinotto	2015	32	60	-	-	-	-	8.0 (56/704)
Bruno	2009	31	66		35.4 (717)		20.1 (254)	20.1 (51/254)
Carvalho	2014	32	70	51.0 (224)	46.0 (377)	5.2 (115)	7.0 (172)	6.3 (18/287)
Carvalho	2015	32	67	57.5 (240)	53.3 (553)	9.4 (138)	9.7 (289)	9.6 (41/427)
Dirandeh	2015	32	60	-	41.0 (900)	-	15.4 (369)	15.4 (57/369)
Dirandeh	2014	32	60	-	28.1 (459)	-	6.2 (129)	6.2 (8/129)
Dirandeh	2015	32	60	-	17.7 (1374)	-	7.0 (243)	7.0 (17/243)
Giordano	2013	29	74	53.8 (519)	40.7 (565)	-	-	15.5 (79/509)
Giordano	2012	29	74	-	-	-	-	15.4 (101/654)
Giordano	2012	29-32	53	-	-	-	-	7.8 (58/737)
Giordano	2015	31	67	-	-	-	-	11.3 (37/326)
Hernandez	2012	28-32	56-60	-	-	12.7 (173)	19.5 (339)	17.0 (88/512)
Karakaya	2014	31	62	47.6 (126)	28.4 (176)	13.3 (60)	8.0 (50)	10.9 (12/110)
Lima	2009	28-32	56	-	-	-	-	12.7 (108/849)
Lima	2012	32	60	-	-	-	-	16.8 (68/405)
Lopes Jr	2013	32	64	-	-	-	-	5.1 (20/394)
Martinez	2016	32	60	45.3 (168)	43.1 (274)	8.3 (76)	11.6 (118)	10.3 (20/194)
Melo	2016	32	60	-	-	-	-	14.3 (52/363)
Monteiro	2015	32	60	35.9 (298)	28.6 (370)	18.7 (107)	17.9 (206)	18.3 (39/213)
Monteiro	2014	34	62	-	-	-	-	8.0 (39/487)
Pereira	2014	32	60	-	-	-	-	11.1 (37/333)
Pereira	2013	32	60	-	-	-	-	15.7 (40/254)
Pereira	2013	30	71	-	-	-	-	11.8 (40/338)
Pereira				-	-	-	-	10.7 (104/968)
Pontes	2015	31	62	-	-	-	-	16.3 (46/283)
Ribeiro	2012	30	65	-	-	-	-	9.5 (96/1016)
Santos	2017	33	63	54.6 (196)	38.4 (318)	6.5 (107)	9.0 (122)	8.3 (19/229)
Toledo	2017	28	61	65.0 (126)	66.9 (157)	13.8 (80)	13.0 (100)	13.3 (24/180)
Vieira-Neto	2017	32	60	40.3 (3242)	36.8 (4853)	9.1 (1306)	14.4 (1784)	12.2 (376/3090)
Overall[2]				43.0[x] (2509/5830)	36.1[y] (4280/11836)	9.5[w] (229/2403)	13.4[z] (586/4378)	11.7 (2002/17145)

[1]Only first author listed. [2]Overall P/AI and pregnancy loss of primiparous vs multiparous cows differ ($P < 0.01$).

Table 2. Recent studies (since 2009) that evaluated pregnancy/embryo transfer (P/ET) and pregnancy loss between the first (28-38 days of pregnancy) and second pregnancy diagnosis (50-60 days of pregnancy) in various breeds and categories of cattle.

		Pregnancy diagnosis, day					
Reference[1]	Year	1st	2nd	Breed	Category	P/ET at 1st diagnosis % (n/n)	Pregnancy loss % (n/n)
In vitro produced (IVP)							
Breukelman	2012	36	50	Holstein	Dry cows	52.4 (76/145)	14.5 (11/76)
Garcia-Guerra	2017	32	60	Holstein	Heifers	43.4 (6539/15052)	19.8 (1293/6539)
Gatea	2018	30	60	Girolando	Lactating cows	NR[2]	16 (21/131)
Munhoz	2014	30	60	Gyr	Heifers/Lactating cows	52.6 (950/1807)	17.9 (170/950)
Pereira	2017	32	60	Holstein/Girolando	Lactating cows	31.0 (100/323)	19.0 (19/100)
Pereira	2016	32	60	NR	Lactating cows	41.8 (838/2003)	19.5 (163/838)
Pohler	2016	31	59	Girolando	Lactating cows	NR	16.5 (47/285)
Pontes	2009	30	60	Nelore	Heifers	37.4 (341/910)	10.5 (36/341)
Pontes	2011	30	60	Nelore x Simmental	Heifers	36.6 (1974/5398)	9.4 (186/1974)
Randi	2015	30	60	NR	NR	43.5 (2065/4749)	6.1 (127/2065)
Rasmussen	2013	32-35	60	Holstein	Lactating cows	25.9 (57/220)	26.8 (15/56)
Total						43.5 (10966/25209)	15.6 (2088/13355)
In vivo derived (IVD)							
Baruselli	2011	30	60	Holstein	Lactating cows	39.3 (2109/5364)	20.5 (432/2109)
Breukelman	2012	36	50	Holstein	Dry cows	53.9 (62/115)	4.8 (3/62)
Pereira	2013	28	60	Holstein	Lactating cows	44.4 (216/487)	17.6 (38/216)
Pontes	2009	30	60	Nelore	Heifers	45.6 (132/289)	9.0 (12/132)
Rodrigues	2010	30	60	Holstein	Lactating cows*	43.4 (159/366)	7.5 (12/159)
Vasconcelos	2011	28	60	Holstein	Lactating cows	45.4 (298/657)	16.4 (49/298)
Wallace	2011	31-33	55/59	Beef mixed	Mature cows	55.6 (198/356)	3.5 (7/198)
Total IVD						41.6 (3174/7634)	17.0 (541/3174)

*Repeat breeders. [1]Only first author listed. [2]NR = not reported.

In lactating dairy cows, analysis of studies from 2005 to 2017 demonstrated an average pregnancy loss of 11.7% (2,002 losses of 17,145 confirmed pregnancies). In 10 of these studies, there was a direct comparison of pregnancy per AI (P/AI) and pregnancy loss in primiparous and multiparous cows. Primiparous cows had ~20% greater P/AI than multiparous at first pregnancy diagnosis (43.0 *vs*. 36.1 = 6.9% absolute difference; 6.9/36.1 = 19.1% relative difference). However, the difference was even greater at second pregnancy diagnosis (primiparous = 38.9% *vs*. multiparous 31.3%) due to ~40% greater pregnancy loss in multiparous than primiparous cows (3.9/9.5 = 41.1%).

Pregnancy loss is also a substantial problem in embryo transfer recipients (Table 2). Based on data from over 25,000 embryo transfers, researchers that transferred IVP embryos, had over 40% P/ET at the first pregnancy diagnosis with subsequent loss of 15.6% of confirmed pregnancies during the second month. Published studies with IVD embryos also had over 40% P/ET at first pregnancy diagnosis and 17% pregnancy loss during second month of pregnancy. These studies were not direct comparisons of IVP *vs*. IVD embryos but are shown to illustrate that pregnancy loss is a substantial problem in either IVP or IVD embryo transfer. Clones have even more problems in this period, with ~50% of confirmed pregnancies lost during the second month (Sala *et al.*, 2018; University of Wisconsin-Madison and ST Technology; unpublished; 51.9%; 82/158). Thus, the second month of pregnancy is a pivotal period for pregnancy loss and could be a substantial area of opportunity, particularly if the problem is due to inappropriate regression of the CL during this period.

In cattle, if pregnancy does not occur, regression of the CL is initiated between day 16 and 25 after ovulation due to secretion of pulses of PGF from the uterus in response to circulating oxytocin pulses (Ginther *et al.*, 2012; Spencer and Hansen, 2015; Wiltbank *et al.*, 2016b). In pregnant cattle, during this same time period (i.e. during the first month of pregnancy) the CL does not undergo regression due to the actions of IFNT which is secreted by the elongating embryo near the time when luteolysis would be expected to occur (Thatcher *et al.*, 1989; Plante *et al.,* 1991; Meyer *et al.*, 1995; Spencer and Bazer, 1996).

However, the mechanisms that maintain the CL during the second month of pregnancy are incompletely defined. This is despite the potential practical value of this research since it could lead to a rational method for reducing the substantial and economically-costly effects of pregnancy loss during this pivotal period. Unraveling these mechanisms could also provide intriguing fundamental biological information that could be of value in other species, including humans. This review will explore four key principles related to maintenance of CL during the first month of pregnancy including: 1) involvement of local pathways, 2) role of IFNT, 3) patterns of uterine PGF secretion, and 4) resistance to PGF action. We will then review these same potential mechanisms in maintaining CL during month two of pregnancy and perhaps later pregnancy. Our purpose in writing this review is to stimulate research on maintenance of the CL after the original maternal recognition of pregnancy period.

Local utero-ovarian pathways in protection of the CL during pregnancy

First month of pregnancy

The local pathways for maintenance of the CL during pregnancy are interconnected with the local pathways that produce regression of the CL during the normal estrous cycle. Early studies demonstrated that the non-pregnant uterus was the initiator of the luteolytic process in the guinea pig (Loeb, 1927). Studies in ruminants also found that removal of the uterus greatly prolonged, perhaps indefinitely, the lifespan of the CL, demonstrating the pivotal role of the uterus in luteolysis (Wiltbank and Casida, 1956). In addition, ipsilateral (uterine horn on same side as CL) hysterectomy invariably prolonged the lifespan of the CL, while contralateral (opposite side from CL) hysterectomy consistently failed to affect CL lifespan (Inskeep and Butcher, 1966). This clearly demonstrated that local pathways between uterus and ovary were involved in initiating CL regression. The ovarian artery in ruminants is extremely convoluted and in close apposition to the uterine vein, thus allowing transfer of the uterine luteolysin, PGF, to the ovarian artery (Ginther and Delcampo, 1974; Mapletoft *et al.*, 1976a). Elegant vascular anastomoses studies were done by exchanging the uterine veins after unilateral hysterectomy. These studies demonstrated that the intact uterine horn secreted a luteolysin into the uterine vein that subsequently diffused, through a local pathway, to the ovarian artery and caused luteolysis (Mapletoft *et al.*, 1976a). Nevertheless, well-designed physiological studies done in sheep during the late luteal phase and following infusion of a low dose of oxytocin indicate that there are multiple pathways by which PGF secreted from the uterus can reach the CL (Bonnin *et al.*, 1999). Determinations of PGF flow rates from the uterus, into the lungs and subsequently arriving at the ovary were done by catheterizing the uterine vein, pulmonary artery, femoral artery, and ovarian artery near the ovarian hilus (distal ovarian artery). Treatment with oxytocin increased PGF concentrations in uterine vein (3,811 pg/ml), pulmonary artery (before lungs; 224 pg/ml), femoral artery (after lungs; 18 pg/ml), and distal ovarian artery (42 pg/ml). Only 0.05% of uterine-secreted PGF reached the ovary (1/2000 of PGF released) with one-third of the PGF arriving rapidly by a systemic route (PGF not metabolized in lungs) and two-thirds arriving by slower routes involving local diffusion (Bonnin *et al.*, 1999). Thus, the majority of PGF involved in regressing the CL arrives through local mechanisms, although some PGF may arrive from systemic circulation during a PGF pulse.

There are now multiple types of evidence that uterine-derived PGF is the definitive initiator of CL regression in ruminants and that PGF crosses from uterine vein to ovarian artery through local pathways that initiate the luteolytic process (Knickerbocker *et al.*,

1988; Bonnin *et al.,* 1999; Wiltbank *et al.*, 2016b). Studies using [^{3}H]-PGF demonstrated that during PGF pulse peak, sufficient PGF is transported from uterine vein to ovarian artery to initiate the luteolytic process (Lamond *et al.*, 1973; McCracken *et al.*, 1981). Indeed, intrauterine treatment with pulses of PGF, that mimic the natural PGF pulses, can induce CL regression that resembles natural luteolysis (Schramm *et al.*, 1983; Ginther *et al.*, 2009; Atli *et al.*, 2012; Ochoa *et al.*, 2018). Transport of PGF between the utero-ovarian vein and the ovarian artery involves a specific PG transporter, termed SLCO2A1 or OATP2A1 (Kanai *et al.,* 1995; Schuster, 1998, 2002) that has 12-transmembrane domains and provides efficient and specific transport of PGF and PGE between these vessels (Lee *et al.*, 2010, 2013; McCracken *et al.*, 2011). The remainder of PGF continues to be transported by the uterine vein into the systemic circulation, the heart, with eventual passage through the lungs in which ~88% of PGF will be metabolized (Bonnin *et al.*, 1999), primarily to the inactive PGF metabolite (PGFM) by the enzyme prostaglandin dehydrogenase (PGDH). Thus, luteolysis is initiated at days 17-20 of the normal bovine estrous cycle due to uterine secretion of PGF pulses. Some PGF arrives at the ovary, primarily through local pathways, and activates important molecular and cellular pathways that ultimately lead to CL regression (Davis and Rueda, 2002; Atli *et al.*, 2012; Maalouf *et al.*, 2014; Ochoa *et al.,* 2018; Pate and Hughes, 2018).

Similar vascular anastomoses experiments demonstrated that pregnancy also maintains the CL through local pathways involving transport from uterine vein to ovarian artery. For example, transfer of embryos into a surgically-isolated uterine horn resulted in CL regression if the embryo was transferred contralateral to CL, but CL was maintained if embryo was transferred ipsilateral to the pregnancy in cows (Del Campo *et al.*, 1977) or ewes (Moor, 1968). In surgically-isolated horns, anastomoses of uterine veins from gravid to non-gravid side resulted in maintenance of CL on non-gravid side in both ewes (Mapletoft *et al.*, 1975) and cows (Del Campo *et al.*, 1980). This demonstrated that the pregnancy signal was local and not systemic and was carried in the local uterine vein. Other experiments were done with anastomoses of the ovarian artery from gravid to non-gravid side resulting in CL maintenance on non-gravid side (Mapletoft and Ginther, 1975; Mapletoft *et al.*, 1976b) demonstrating that the pregnancy signal passed from uterine vein to ovarian artery, only on the same side as the pregnancy (Fig. 1).

Local pathways for CL maintenance during second month of pregnancy

Previous researchers stated (Bridges *et al.,* 2000), "a local relationship between the ovary bearing the CL and the embryo/fetoplacental unit still exists during the 2nd month of pregnancy" and we support this idea. The primary evidence that there is a local relationship comes from studies that have induced accessory CL on the contralateral or ipsilateral ovary during pregnancy. In one study (Lulai *et al.*, 1994), ten pregnant beef heifers were treated on days 30-35 of pregnancy with progestin implants, to maintain pregnancy, the original CL was regressed using cloprostenol, and heifers were given two treatments with LH to induce accessory CL. All heifers ovulated and had an accessory CL; however, half of these heifers regressed this CL by 15-17 days after LH treatment (day 45-50 of pregnancy). Of particular interest, the accessory CL was contralateral to the pregnancy in all five of the heifers that regressed, whereas, four of five of the heifers that maintained their CL had an accessory CL that was ipsilateral to the pregnancy (Lulai *et al.*, 1994). Similarly, induction of an accessory CL on days 29 to 59 of pregnancy was sufficient to maintain the pregnancy in 20 of 27 cows that had accessory CL that were ipsilateral to the pregnancy but maintained pregnancy in 0 of 5 cows that had accessory CL contralateral to the pregnancy (Bridges *et al.*, 2000). Thus, an induced CL contralateral to the pregnancy is not maintained and does not maintain the pregnancy during the second month.

Our research group induced accessory CL in lactating cows by treatment with GnRH on day 5 after AI (Baez *et al.,* 2017). In this model, 65.4% of cows (234/358) ovulated to the GnRH and formed an accessory CL. In pregnant cows, the accessory CL ipsilateral to the pregnancy rarely regressed (8/67), however when the accessory CL was contralateral to the pregnancy, most CL regressed (66.2%; 86/130) even though the pregnancy and original ipsilateral CL were maintained. Timing of contralateral CL regression is particularly relevant with only 25.6% (22/86) of contralateral CL regressions occurring during the first month and 74.4% (64/86) happening during the second month of pregnancy ($P < 0.0001$; Baez *et al.*, 2017). One interesting question is why some accessory CL regression happened during the normal time of maternal recognition of pregnancy (i.e. first month of pregnancy) while most happened during the second month. By day 18-20 of pregnancy, the whole gravid horn is filled by the elongating embryo and it extends into the contralateral horn during the next few days of pregnancy (Chang, 1952). It seems likely that the elongating embryo and IFNT will readily migrate into the contralateral horn in cows with a normal size uterus, such as heifers and primiparous cows. Interestingly, cows that had accessory CL regression during the first month of pregnancy were primarily multiparous cows. Multiparous cows have a much larger uterus than primiparous cows and fertility decreases as uterine size increases (Baez *et al.*, 2016; Young *et al.,* 2017). The coverage of the contralateral horn by embryonic membranes might be affected by uterine size and this may determine if early contralateral CL regression will occur. Nevertheless, most cows had contralateral, accessory CL regression during the second month of pregnancy (Baez *et al.*, 2017). We also induced accessory CL in heifers and, although no ipsilateral accessory CL regressed, almost all contralateral accessory CL regressed and this occurred primarily during the second month of pregnancy (Baez, Garcia-

Guerra, Wiltbank, 2018, University of Wisconsin-Madison : unpublished). Thus, there is a second distinct period of either luteolysis or CL protection happening between days 30-60 of pregnancy, regulated by local, but likely distinct, pathways from those that have been described during the first month of pregnancy.

Role of embryonic production of IFNT in maintenance of CL

In cyclic ewes, intrauterine infusion of homogenates or secreted proteins from day 14-15 embryos extended CL lifespan, while, homogenates of day 21-25 embryos did not alter CL lifespan (Rowson and Moor, 1967; Godkin *et al.*, 1984b), demonstrating the limited interval during pregnancy when the CL-maintaining signal is secreted by the conceptus. The active principal in the homogenates was heat and protease-labile, and had properties consistent with a low molecular weight protein (Rowson and Moor, 1967; Martal *et al.*, 1979; Godkin *et al.*, 1982). Later studies showed that a single protein, initially called ovine or bovine trophoblast protein-1 but later IFNT, was solely responsible for maintenance of the CL during pregnancy in ruminants (Godkin *et al.*, 1984a, 1997; Thatcher *et al.*, 1984).

Thus, during the critical period, days 17 to 25 in cattle or day 13-21 in sheep, the embryo is dramatically elongating and secreting IFNT, the definitive signal for CL maintenance during early pregnancy (Roberts, 1996; Bazer *et al*., 1997). In the uterus, INFT acts in a paracrine manner to prevent expression of estrogen receptor alpha and oxytocin receptor in luminal epithelial cells of the endometrium and superficial glandular epithelium, thereby altering response to oxytocin and release of luteolytic pulses of PGF (Spencer *et al.*, 2007). Interferon-tau also stimulates expression of specific genes, termed interferon-stimulated genes (ISG) in the uterus (Johnson *et al.*, 1999; Hansen *et al.*, 2013) although their role in maintenance of the CL has not been clearly defined.

In sheep, IFNT is not secreted by morula stage embryo but expression is detected in day 8 blastocysts with dramatic increases as the embryo begins to expand and elongate (Farin *et al.*, 1990; Ealy *et al.*, 2001). Substantial IFNT protein secretion is observed on day 12-13 of pregnancy, with 27-fold increases between days 13-17, with a subsequent decrease of 50% by day 21 (Hansen *et al.*, 1985). The mRNA for IFNT is maximal on day 14 of ovine pregnancy and is related to expression of two genes, IFNT1 and IFNTc1 (Kim *et al.*, 2018). The transcription regulatory pathways for induction of IFNT in the elongating embryo are well-described with ETS2 as the master regulator, combined with activation of a nearby AP-1 site and a DLX3 binding site (Ezashi and Imakawa, 2017). Maximal IFNT protein secretion occurs 2-3 days later with subsequent decreases in both IFNT mRNA and protein expression (Nojima *et al.*, 2004). There is disappearance of IFNT mRNA and protein by days 20-23 of ovine pregnancy (Godkin *et al.*, 1982; Guillomot *et al.,* 1990).

One of the more interesting recent findings is the clear demonstrations that IFNT escapes the uterus and induces ISG expression in peripheral tissues such as the CL (Oliveira *et al.*, 2008; Bott *et al.*, 2010) and peripheral blood cells (Gifford *et al.*, 2007; Shirasuna *et al.*, 2012). Elegant experiments were done in which IFNT was delivered into the uterine vein (20 µg/day) or jugular vein (200 µg/day). These treatments increased expression of a number of genes in the CL including ISGs and cell survival genes (BCL2L1, Bcl-xL, AKT, and XIAP) and decreased PGF receptor expression (Antoniazzi *et al.*, 2013). In addition, ewes were challenged with a single injection of PGF (4 mg/58 kg body weight, i.m.). Ewes receiving infusions of BSA, as a control, had a decrease in circulating P4 to about 30% of control ewes that were not treated with PGF; however, IFNT-treated ewes that were also treated with PGF had only a small decrease in circulating P4 and a return to control values by 48 h after PGF treatment (Antoniazzi *et al.*, 2013). Thus, IFNT has clear actions within the uterus to decrease uterine PGF secretion. Embryonic IFNT also escapes the uterine lumen, acting directly on the CL, potentially to directly suppress the luteolytic actions of PGF on the CL. The actions of IFNT directly on the CL could be independent or synergistic with the actions of IFNT in the uterus.

Studies indicate decreased IFNT production after 3 weeks of pregnancy in sheep and an absence of IFNT in the second month of pregnancy in both cattle and sheep (Ealy and Yang, 2009; Ezashi and Imakawa, 2017). This sudden drop in IFNT mRNA occurs concurrently with attachment of the trophectoderm to the uterus (Guillomot *et al.*, 1990). The mechanisms are temporally linked to placental development and uterine attachment and these processes may mediate the reduction in IFNT expression. It is known there is increased DNA methylation in the 5' flanking region of the IFNT gene in CpG islands that are proximal to the IFNT-010 gene (Nojima *et al.*, 2004). Culture of ovine embryos with a DNA methylation inhibitor results in no change in IFNT secretion in day 14 embryos but increased IFNT mRNA in day 17 embryos (Nojima *et al.*, 2004). Conversely, acetylation of histone H3 was observed in the IFNT gene near the CDX2-binding site on days 14-16 when IFNT expression was elevated but acetylation dramatically declined as the embryo attached to the uterine wall and IFNT declined (Sakurai *et al.*, 2010). Finally, the transcription factor EOMES has also been implicated in IFNT gene suppression since it increases near time of IFNT gene silencing and binds the coactivator CREBBP, thereby disrupting activation of the AP1 binding site in the 5' flanking region of IFNT genes (Sakurai *et al.*, 2013). Thus, increased EOMES protein combined with declining histone acetylation and increased DNA methylation of the upstream region of the IFNT gene are linked to silencing of IFNT gene transcription as the embryo develops beyond day 17 of ovine pregnancy (Ezashi and Imakawa, 2017).

In cattle, there are some results that corroborate the timing of IFNT expression and subsequent silencing during early pregnancy. Expression of ISGs has been used as an indirect pregnancy marker at 16 to 22 days

after AI (Romero *et al.,* 2015; Wijma *et al.*, 2016; Wiltbank *et al.*, 2016a) with a subsequent decrease near the expected time of conceptus attachment to the uterine lining. Kizaki *et al*. (2013) collected bovine blood samples from days 0 to 28 after AI and evaluated expression of ISGs using qPCR and microarray of mRNA from peripheral blood leukocytes. As pregnancy progressed (between days 21-28), peripheral blood leukocytes had a decrease in expression of ISGs, such as ISG15, MX1, MX2, and OAS1. Moreover, in another study (Pugliesi *et al.*, 2014), the abundance of ISG mRNA in maternal peripheral blood mononuclear cells (PBMC) peaked on day 20 and then had a sharp decrease, especially after day 30 post-AI in pregnant cows. Thus, in both cattle and sheep, most of the evidence indicates that IFNT is high during the maternal recognition of pregnancy period but then decreases to minimal secretion prior to the second month of pregnancy.

Alterations in oxytocin-induced PGF pulses during pregnancy

Patterns of PGFM during first month of pregnancy

The PGF patterns in ruminants are the result of communication between multiple organs using multiple hormones. The uterus is the central player and the source of PGF secretion. However, the posterior pituitary that releases pulses of oxytocin throughout the day is the source of the pulsatile pattern. Levels of oxytocin receptor in the uterus vary throughout the cycle, decreasing mid cycle, and increasing at day 17 through 21 in non-pregnant cows, with temporal association to uterine PGF secretion (Fuchs *et al.*, 1990; Mann and Lamming, 2006). The cascade of events that ultimately leads to expression of uterine oxytocin receptors involves an initial down-regulation of endometrial P4 receptors after continuous exposure to P4 during the luteal phase (Wathes *et al.*, 1996; Spencer *et al.*, 2004). This produces an up-regulation of uterine estrogen responsiveness, which, after activation of the estrogen receptor (ESR1) by circulating estradiol (E2) from a developing follicle, generates increased expression of oxytocin receptors (Ivell *et al.*, 2000; Mann *et al.*, 2001; Kieborz-Loos *et al.*, 2003; Fleming *et al.*, 2006). The key role of follicular E2 is highlighted by the delay in luteolysis when follicular E2 is reduced by either follicular aspiration (Araujo *et al.*, 2009) or inhibition of follicle growth with steroid-stripped follicular fluid (Salfen *et al.*, 1996). The essential role of oxytocin is evidenced by inhibition of PGF secretion and delay in luteolysis by treatment with an oxytocin receptor antagonist (Mann *et al.*, 2003). Binding of oxytocin to its receptor in the uterus stimulates PGF production via stimulation of both Phospholipase A2 and C (Burns *et al.*, 1997a), releasing arachidonic acid that is subsequently converted to PGH2 by prostaglandin H synthase-2, which is also induced by oxytocin (Burns *et al.,* 1997b).

Pulsatile secretion patterns of PGF have generally been evaluated by serial evaluations of the PGF metabolite (PGFM) throughout the process of CL regression (Wolfenson *et al.*, 1985; Silvia and Raw, 1993; Shirasuna *et al.*, 2004). While the pulsatile nature of these patterns is a consistent finding in all of these studies, the number of pulses and day when the pulses begin varies between animals. While CL regression can be triggered with a single large dose of exogenous PGF, the pulsatile nature of PGF secretion in the physiologic state has been shown to be required for regression when using smaller doses of PGF which more closely reflect the physiologic amounts present during regression (Ginther *et al*., 2009).

The PGFM concentrations have also been evaluated in early pregnant ruminants, near the time when luteolysis would normally occur in non-pregnant animals but fewer studies and inconsistent results have been reported. In one study (Lewis *et al.*, 1977), there were no detectable differences in mean concentrations of PGFM in blood from the jugular vein in pregnant vs non-pregnant ewes from day 11-16. However, in another study (Zarco *et al.*, 1988) mean concentrations of PGFM in pregnant ewes were greater than non-pregnant ewes on days 13-17. This study also provided some evidence for PGFM pulses in pregnant ewes, though they were of lower amplitude and less frequent than pulses documented in non-pregnant ewes (Zarco *et al.*, 1988). In the cow, one study (Kindahl *et al.*, 1976), failed to observe increases in PGFM concentrations or pulses of PGFM in pregnant animals during the time when luteolysis would be occurring in non-pregnant animals. However a recent study (Pinaffi *et al.*, 2018), described statistically-detectable PGFM pulses in pregnant heifers during this same time period, although the amplitude of PGFM pulses was much lower in pregnant than non-pregnant cows. There have been fewer studies done in goats, however, no pulses of PGFM were detected in pregnant does (Fredriksson *et al.*, 1984).

While not directly measuring PGFM production, there is documentation that from days 17-21 of bovine pregnancy there are decreased endometrial oxytocin receptors (Fuchs *et al.*, 1990). In ruminants, IFNT during the first month of pregnancy decreases expression of oxytocin receptors in endometrium and this mechanism appears to underlie the decrease in uterine oxytocin receptors during early pregnancy and inhibition of oxytocin-induced PGF secretion from the uterus (Jenner *et al.*, 1991; Asselin *et al.*, 1997; Dorniak *et al.*, 2011; Antoniazzi *et al.*, 2013; Romero *et al.*, 2015). It appears that IFNT does not prevent oxytocin receptor gene transcription directly, however, IFNT inhibits ESR1 transcription *in vitro*, preventing estrogen from being able to upregulate oxytocin receptor, and thereby blocking luteolytic amounts of PGF from being synthesized in pregnant animals (Spencer and Bazer, 1996; Spencer *et al.*, 1996; Fleming *et al.*, 2006). Consequently, there is decreased PGF in the uterine vein and ovarian artery and decreased pulsatile secretion of PGF by the uterus during early pregnancy (Arosh *et al.*, 2004; Dorniak *et al.*, 2011). Thus, in Fig. 2 is shown the postulated difference in PGFM secretion patterns during the period encompassing the normal time of luteolysis in non-pregnant cows or a similar time of early pregnancy (Fig. 2).

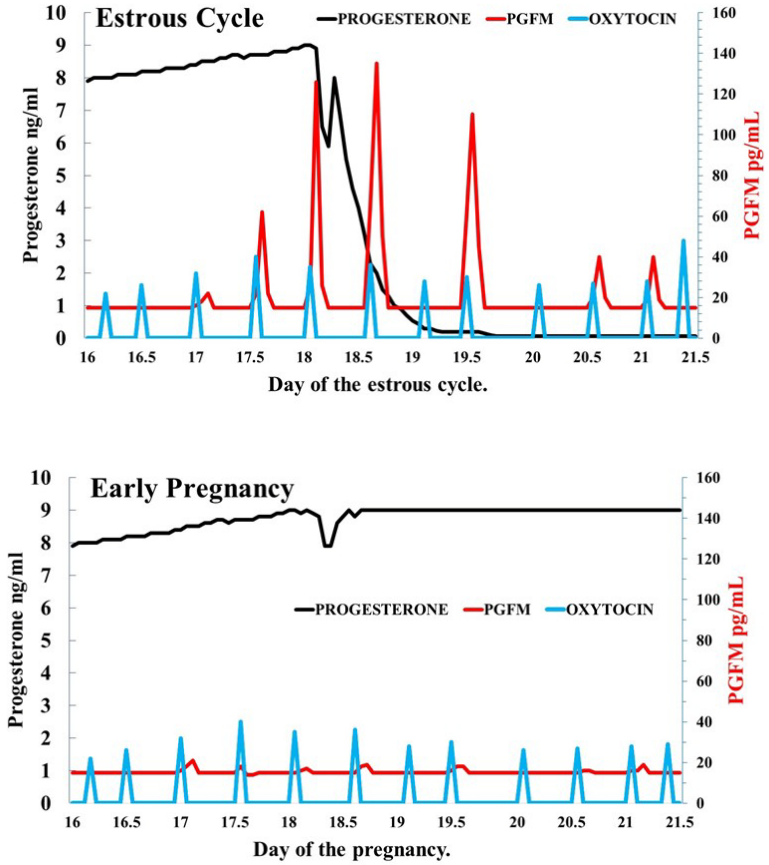

Figure 2. Simplified model for the patterns of PGFM, progesterone, and oxytocin in cattle during the late estrous cycle when luteolysis is occurring or during the same time in early pregnancy.

Patterns of PGFM during second month of pregnancy

Very little characterization of PGF patterns has occurred in ruminants during the second month of pregnancy. There is evidence that signaling pathways involved in PGF production become reestablished in the uterus and are present during month two of pregnancy. This idea is primarily supported by studies that used oxytocin challenges. During early pregnancy, oxytocin challenge on day 18 increased PGFM at 60 min in non-pregnant but not in pregnant heifers (Parkinson *et al.*, 1990; Robinson *et al.*, 1999). One study evaluated if this oxytocin response returns after the first month of pregnancy. Fuchs *et al.* challenged cows at known stages of gestation (50, 150, 250, or 280 days) with 10 or 100 IU oxytocin, and measured circulating oxytocin and PGFM during the next 3 h (Fuchs *et al.*, 1996). Circulating oxytocin concentrations in plasma were not affected by gestation length, however PGFM increased after oxytocin challenge as dose and time of pregnancy increased. For example, at day 280 there was 7-fold greater increase in circulating PGFM than on day 50, although on day 50 there was a clear increase in PGFM after 100 IU of oxytocin. In addition to the oxytocin challenge, presence of intercaruncular endometrial oxytocin receptors were found at day 50 of pregnancy, suggesting the uterus is capable of responding to oxytocin stimulation with PGF production during the second month of pregnancy (Fuchs *et al.*, 1996).

Another study evaluated this topic by maintaining pregnancies for the first month of pregnancy using exogenous progestins and by inducing an accessory CL during the second month of pregnancy. Cows on days 31-35 of pregnancy had a tendency to maintain the pregnancy at a greater rate if they produced more PGFM, as measured in blood from the posterior vena cava, and these cows also had greater circulating P4 (Bridges *et al.*, 2000). This study also reported constant secretion of PGF, rather than secretory episodes in samples taken hourly during a 10 h period (Bridges *et al.*, 2000). However, it is possible that the multiple treatments done in this study, such as regression of the original CL and formation of an accessory CL, may have had some residual effects on the PGF secretion patterns.

Recent data from our group (Drum, Wiltbank, Sartori, 2018; University of Wisconsin-Madison; unpublished) showed that the upregulation of oxytocin receptors occurs even earlier during pregnancy. By day 25 after AI in pregnant lactating dairy cows, there was 3-fold increase in circulating PGFM concentrations 30 min after treatment with 50 IU oxytocin i.m. but no detectable PGFM increase at day 18 in pregnant cows. The response to oxytocin challenge was increased as pregnancy progressed until day 53-60. Moreover, no cow aborted after oxytocin treatment, despite substantial increases in circulating PGFM. These intriguing data argue that in the absence of IFNT after day 18 to 21 of pregnancy, endometrial oxytocin receptors increase, allowing for PGF release in response to oxytocin.

Thus, there are two distinct models for

circulating PGFM during second month of pregnancy: 1) PGFM pulses are present during the second month of pregnancy (Fig. 3A), consistent with the presence of uterine oxytocin receptors and oxytocin-induced PGFM secretion in pregnant cows after day 25 (Fuchs *et al.*, 1996) or 2) no pulses of PGF during the second month of pregnancy, consistent with (Bridges *et al.*, 2000), in spite of pulsatile oxytocin secretion (Fig. 3B). Further research will be necessary to determine which model is correct for month two of pregnancy.

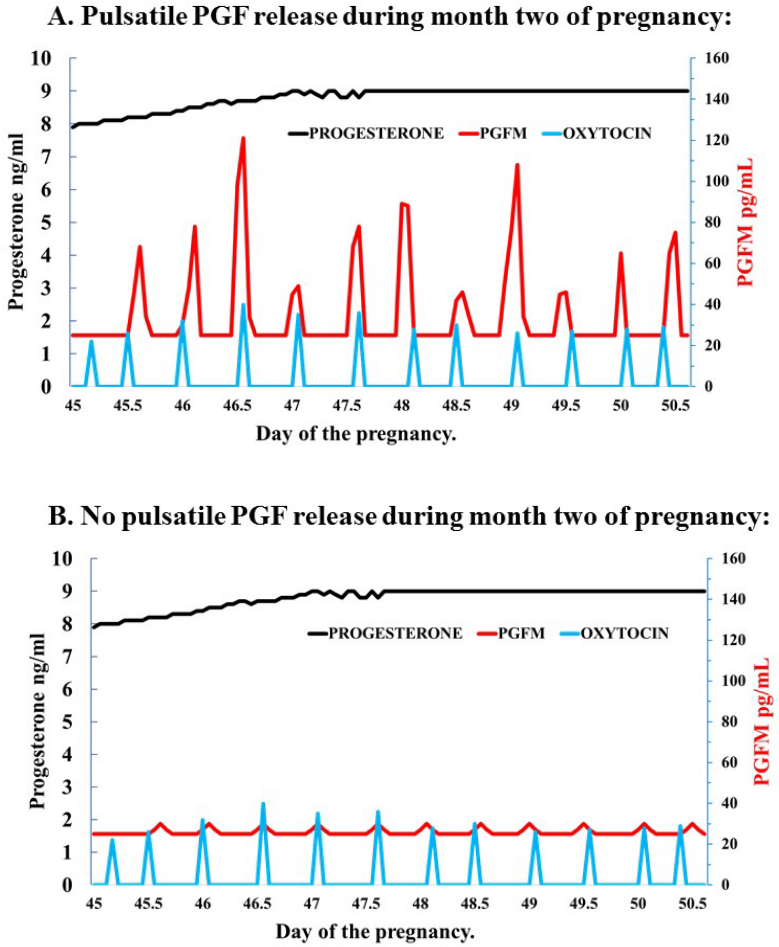

Figure 3. Speculation on two different models for the patterns of PGFM, progesterone, and oxytocin in cattle during the second month of pregnancy.

Protection from PGF action during first and second month of pregnancy

A third mechanism that may protect the CL of pregnancy is protection from luteolytic effects of the uterine luteolysin, PGF. This idea was based on the results of experiments primarily done with pregnant and non-pregnant ewes starting in the mid-1970s by three research groups. At University of Wisconsin, the research group of OJ Ginther showed that a pregnancy substance was present in either the utero-ovarian vein (UOV) or ovarian artery (OA) on the same side as the early pregnancy (before day 20) but not in the contralateral vessels and this substance had a luteotropic effect or, more accurately in our opinion, an antiluteolytic effect (Mapletoft *et al.*, 1975,1976b). In these intricate experiments it was clearly shown that the pregnant uterus produces a substance that is transported in the UOV and subsequently in the OA and that this substance protects the CL from the luteolytic actions of endogenous or exogenous PGF.

At University of West Virginia two studies were done using a model in which a low dose of PGF was injected into the largest follicle near the CL in pregnant or non-pregnant ewes (Inskeep *et al.*, 1975; Pratt *et al.*, 1977a). A dose of 200 µg of PGF, given on day 12 of estrous cycle or pregnancy, induced luteolysis in 11/14 non-bred ewes and in 8/9 mated ewes that did not have an embryo, however, it induced luteolysis in only 3/8 mated ewes with an embryo (2 ewes with CL regression had a very small embryo; Inskeep *et al.*, 1975). In a second experiment (Pratt *et al.*, 1977a), ewes on day 13 of the estrous cycle or pregnancy received 270 µg of PGF-Tham salt or saline in the ipsilateral dominant follicle and luteolysis was induced in 8/8 non-bred ewes and in 5/5 mated ewes without an embryo but in none of the 6 mated ewes that had an embryo.

Finally, a series of experiments were done at Colorado State University, by Bill Silvia and Gordon Niswender to evaluate whether the CL of early

pregnancy was resistant to the effects of exogenous PGF. First, various doses of PGF were tested and they found that a dose of 4 mg PGF/58 kg of body weight i.m., given on day 13, caused CL regression in 5/8 non-pregnant ewes but in 0/9 pregnant ewes (Silvia and Niswender, 1984). In a subsequent experiment, they used this same PGF dose in pregnant and non-pregnant ewes on day 10 or 13 (Silvia and Niswender, 1986) and found that PGF decreased P4 in both pregnant and non-pregnant on day 10 but only regressed the CL in non-pregnant ewes on day 13. In the final experiment, ewes were treated on various days of pregnancy with the same PGF dose (Silvia and Niswender, 1986). They found no effect of PGF at 36 h after treatment on P4 in ewes on days 13 or 16 of pregnancy but major decreases in P4 on days 10, 26, and 30 of pregnancy. Day 19 and 22 of pregnancy had an intermediate response to this low dose of PGF. Thus, resistance to PGF is only present during a limited period of pregnancy, specifically when IFNT is being secreted by the embryo, and prior to that time or after that period there is no detectable resistance to PGF action caused by pregnancy. In this regard, a similar model was utilized to evaluate PGF resistance during endocrine delivery of IFNT in non-pregnant ewes on day 10 of the estrous cycle (Antoniazzi *et al.*, 2013). Similar to early pregnancy, endocrine delivery of IFNT inhibited the action of PGF on circulating P4, suggesting that actions of IFNT cause the PGF resistance of early pregnancy.

There is also evidence from field studies that there is PGF resistance during early pregnancy in ewes. In one study, with 270 presumably pregnant ewes, 90 ewes were treated with PGF (125 μg cloprostenol) at 22-23 days post-service. The remaining 180 bred ewes were used as lambing controls (Reid and Crothers, 1980). Almost all controls (98.9%) subsequently lambed, whereas, only 36.7% of the PGF-treated ewes lambed. Thus, PGF was effective in aborting most pregnant ewes (63.3%) but about one-third of pregnant ewes displayed a resistance to PGF treatment at this stage of pregnancy. Another research group (Nancarrow *et al.*, 1982), treated ewes on day 21 of pregnancy with 100 μg of cloprostenol and found that 15/23 of these ewes maintained their pregnancy. Interestingly, the ewes that had PGF resistance (i.e. maintained their pregnancy) had significantly more CL (3.2) than ewes that had CL regression (1.8) suggesting that a greater mass of embryonic tissue provides greater PGF protection. The number of CL, alone, did not provide protection from PGF since 93.5% (43/46) of non-pregnant ewes had CL regression to this dose of PGF, and all non-pregnant ewes with more than one CL regressed their CL. Thus, the CL of early pregnancy is somewhat resistant to PGF action due to the action of a substance coming from the embryo.

A number of reports indicate that the locally-active, luteoprotective factor is likely to be PGE2 or PGE1 (PGE) secreted from the endometrium in response to pregnancy or IFNT (Huie *et al.,* 1981; Arosh *et al.*, 2004,2016; Weems *et al.*, 2011). One type of evidence is that during pregnancy, the bovine or ovine uterus produces much greater amounts of PGE than during a similar time period in non-pregnant animals (Danet-Desnoyers *et al.*, 1995; Arnold *et al.*, 2000; Lee *et al.*, 2012). In addition, PGE is also produced by ovine (Hyland *et al.,* 1982; Charpigny *et al.,* 1997) and bovine (Saint-Dizier *et al.,* 2011) embryos during early pregnancy. It has also been found that PGEs (Huie *et al.,* 1981; Lee *et al.,* 2012) can diffuse through the utero-ovarian plexus and provide local protective effects on the CL during establishment of pregnancy in sheep. Of particular importance, many studies have reported that treatment with PGE can inhibit the luteolytic actions of PGF in ovine and bovine CL (Henderson *et al.*, 1977; Pratt *et al.*, 1977b; Huie *et al.*, 1981; Reynolds *et al.*, 1981). Recently we have found that pulses of extremely low doses of PGF, delivered into the uterus, can cause complete CL regression and expression of an intriguing cascade of gene expression that is likely to be critical for luteolysis (Atli *et al.*, 2012; Ochoa *et al.*, 2018). Of special importance, simultaneous intrauterine infusion of PGE with PGF completely blocked the actions of PGF on CL regression and PGF-induced luteal gene expression. These results indicate that either PGE blocks transport of PGF to the CL, the model that we favor, or PGE completely blocks PGF action at the CL level. Thus, the temporal secretion pattern of PGE during early pregnancy, the chemical nature of PGE that is consistent with local delivery of the antiluteolytic substance, and the biological actions of PGE to block PGF action are consistent with PGE being responsible for the observed resistance to PGF action during early pregnancy.

In contrast to early pregnancy, the CL during the second month of pregnancy does not seem to be resistant to PGF action. This is evidenced by the finding that the CL is resistant to PGF action during day 13-16 of pregnancy but loses this resistance by day 26 of pregnancy in sheep (Silvia and Niswender, 1986). Further, treatment with PGF (500 μg cloprostenol) during the second month of pregnancy (day 45-60) caused abortion in 97.9% of pregnant cows (47/48); although this same dose of PGF has not been evaluated in cows during early pregnancy (Thain, 1977).

Thus, there is substantial evidence that PGF resistance occurs during early pregnancy (IFNT period) but little evidence for PGF resistance during the second month of pregnancy (See representation in Fig. 4: CL sensitivity to PGF). Most of this evidence is from studies using pregnant ewes with a paucity of research evaluating whether PGF resistance occurs in cattle during early pregnancy. The mechanisms that underlie this PGF resistance has been investigated with no evidence for changes in PGF receptors (Wiepz *et al.*, 1992) but substantial evidence that there are decreases in PGF synthesis and increased PGF degradation (increased PGDH enzyme) in early pregnant ewes compared to ewes at similar stages of the estrous cycle (Silva *et al.*, 2000; Costine *et al.*, 2007; Lee *et al.*, 2012).

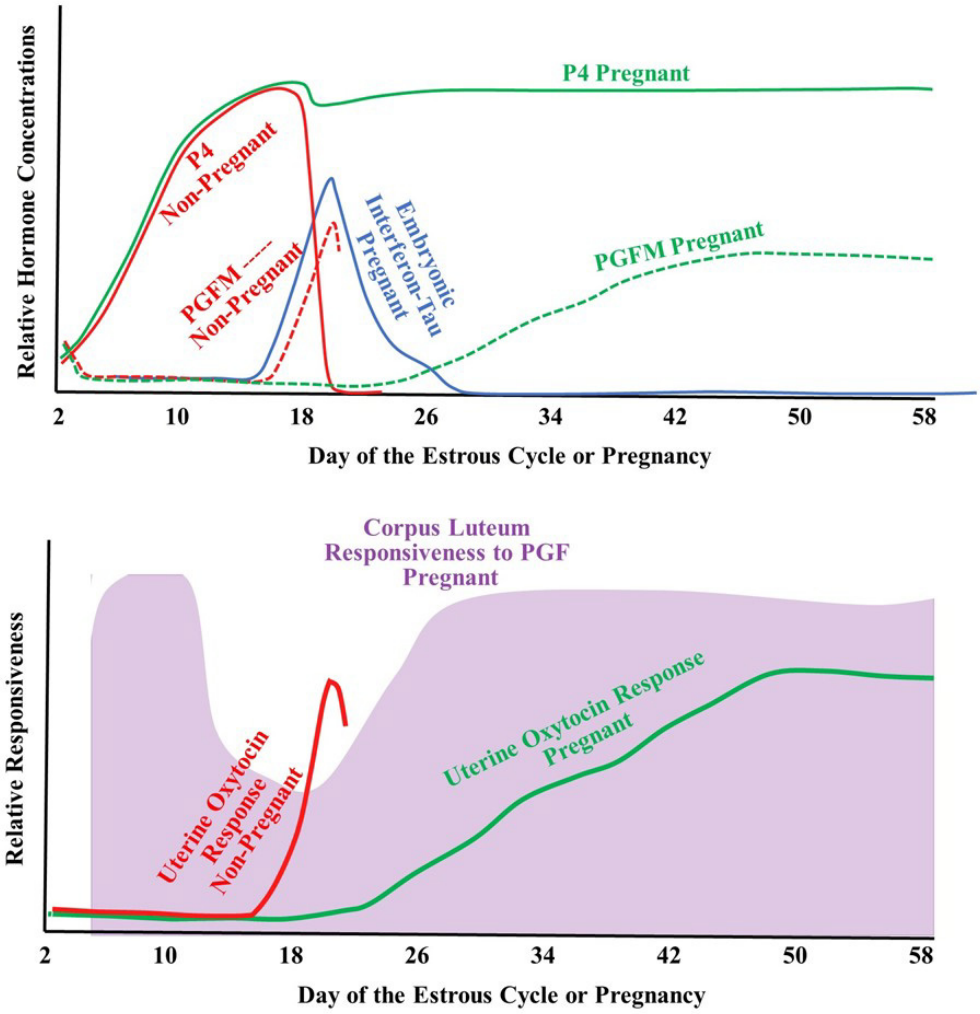

Figure 4. Model of relative changes in hormonal concentrations (upper graph) and relative changes in responsiveness (lower graph) of the CL to PGF (based on extrapolated data from sheep in Silvia and Niswender (1986) or the uterus to oxytocin in pregnant and non-pregnant cows.

Conclusions on maintenance of the CL during first or second month of pregnancy

In Fig. 4 is shown a summary of results discussed in the previous sections and normalized to the events that occur during bovine pregnancy. During early pregnancy, circulating P4 is increasing in both pregnant and non-pregnant cows. Near day 18 of the estrous cycle, there is an increase in uterine responsiveness to oxytocin and a dramatic increase in PGFM pulses in non-pregnant cows that leads to rapid CL regression and initiation of a new estrous cycle. In pregnant cows during this same time period there is an increase in IFNT secretion by the early embryo which inhibits the induction of oxytocin receptor expression in the pregnant uterus and therefore uterine responsiveness to oxytocin is inhibited and oxytocin-induced PGFM pulses are inhibited. In addition, there is some evidence from early pregnant sheep that the CL becomes relatively unresponsive to PGF, perhaps due to PGE secretion from the pregnant uterus. Thus, the CL of early pregnancy is maintained by multiple mechanisms that are initiated by IFNT, seem to be primarily mediated by local pathways, and seem to involve decreased PGF secretion, perhaps decreased PGF transport, and clearly less PGF action at the CL. Figure 5 illustrates a physiological model for the three main mechanisms that are maintaining the CL during the classical maternal recognition of pregnancy that occurs in the first month of pregnancy.

However, the mechanisms that are protecting the CL of early pregnancy do not appear to persist into the second month of pregnancy. It appears that IFNT is no longer secreted by the embryo after the apposition and attachment process are initiated. Therefore, it appears that uterine responsiveness to oxytocin returns during the second month of pregnancy, basal PGF secretion increases and, perhaps, oxytocin-induced PGF pulses are reinitiated during the second month of pregnancy. In addition, CL responsiveness to PGF appears to return during the second month of pregnancy after the loss of IFNT. Thus, current evidence is consistent with the idea that none of the mechanisms that protect the CL during the first month of pregnancy are protecting the CL during the second month of pregnancy.

In our opinion, there are three logical explanations that could explain the maintenance of the CL during the second month of pregnancy. First, there

is a luteoprotective substance that is secreted by the pregnancy during month two and it protects the CL either directly or by inhibiting arrival of active PGF at the CL. Since the evidence from contralateral CL regression indicates that luteal protection is locally mediated (ipsilateral CL protected, contralateral CL generally regresses), a compound like PGE would be logical. A second possibility is that PGF is secreted from the non-gravid horn in response to oxytocin but there is no PGF secretion from the gravid horn during the second month of pregnancy. This idea has not been tested and therefore remains a possibility.

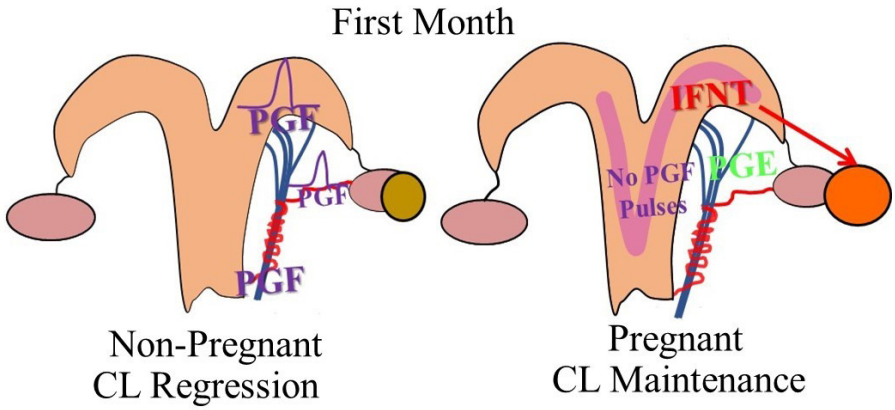

Figure 5. Physiological model depicting the three potential mechanism protecting the CL during the first month of pregnancy in ruminants. In non-pregnant ruminants, the uterus secretes prostaglandin F2α (PGF) which is secreted in discrete pulses and arrives at the corpus luteum (CL) via transport from the utero-ovarian vein to the ovarian artery. In the pregnant ruminant, the elongating embryo secretes interferon-tau (IFNT) which acts to maintain the CL by: 1) suppressing PGF pulses, 2) increasing prostaglandin E (PGE) production, 3) IFNT can escape from the uterus and act directly on the CL.

The physiological model that we currently favor for protection of the CL during the second month of pregnancy is shown in Fig. 6. This model postulates that the increase in uterine blood flow that accompanies development of the placentomes also inhibits the local transport of PGF into the ovarian artery. This could be due to changes in the PGF transporter or may be related to decreased diffusion times or increased diffusion distances for PGF from the UOV to the OA. In the non-gravid horn, a reduced uterine blood flow, allows local transport of PGF which is increasing during the second month of pregnancy. This leads to regression of the CL when PGF pulses that reach the ovary are of sufficient magnitude and frequency. Obviously, future research is needed to definitively evaluate the many aspects of this intriguing but highly speculative physiological model.

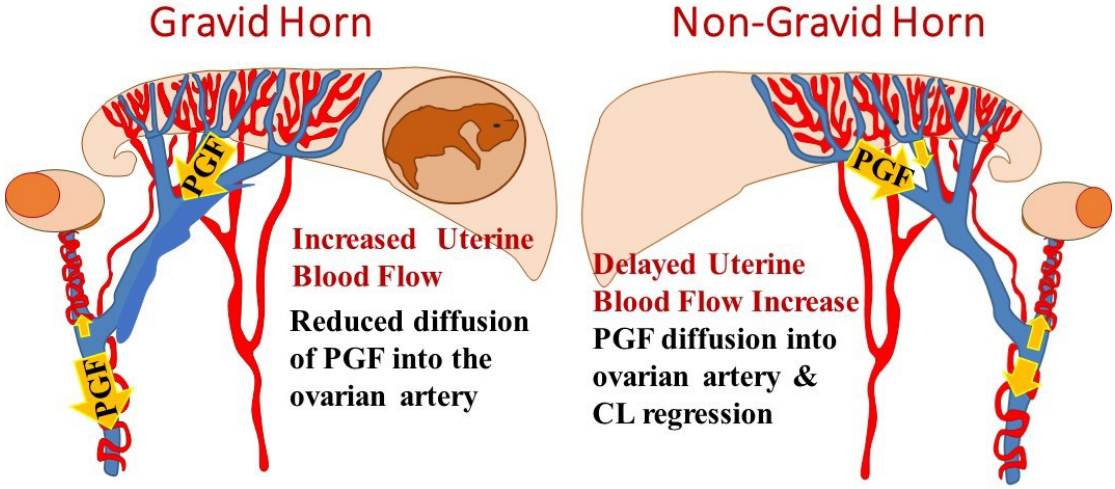

Figure 6. Current speculative physiological model depicting a mechanism that could be protecting the CL during the second month of pregnancy and possibly later pregnancy in ruminants. The gravid horn has an increased uterine blood flow during pregnancy and this high blood flow may inhibit transfer of uterine-secreted PGF to the ovarian artery. Therefore the CL would not regress because it would not be exposed to sufficient PGF, even during pulses of PGF, due to the reduction or loss of the local PGF transport system. In the contralateral horn, there is a delay in development of placentomes and the pregnancy-induced increase in uterine blood flow. Thus, contralateral CL because the local transport system for PGF will still be functional, allowing contralateral CL regression during the second month of pregnancy, as observed in previous studies.

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DOI: 10.21451/1984-3143-AR2018-0015

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Placental contribution to the endocrinology of gestation and parturition

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Abstract

In addition to many other functions, the placenta is a source of a vast number of autocrine, paracrine and endocrine factors. However, the spectrum of placental regulatory factors, their concentrations, gestational profiles and roles may differ considerably even between phylogenetically closely related species. Depending on the species, placental regulatory factors of a broad range of molecule classes have been found including (glyco-)proteins, peptides, steroids and prostaglandins. Local placental regulatory factors are especially important for the dialogue between the fetal and the maternal compartment immediately at the feto-maternal borderline and for the control of growth, differentiation and functions of the placenta itself. Moreover, placental hormones in a proper sense may also have effects in more remote targets within the maternal compartment, serving functions such as pregnancy-specific adaptations of maternal circulation, provision of hemotrophe to the fetus or the development and function of the mammary gland. Functions of placental hormones in the fetus proper are less clear but may be especially important before the establishment of a functional fetal endocrine system and near term within the highly species-specific networks of signals preparing and initiating parturition. This review takes a comparative view on the situation in different domestic animals focusing on ruminants and on placental hormones occurring at significant concentrations in the maternal circulation.

Keywords: gonadotrophins, placenta, placental lactogen, relaxin, steroids.

Introduction

There is probably no other organ which shows such a structural diversity comparable to that of the placenta (Leiser and Kaufmann, 1994; Wildman *et al*., 2006). However, its functions are in general widely similar between species. The placenta anchors the fetus in the maternal uterus, induces the local immunotolerance preventing rejection of the fetal allograft, provides oxygen and nutrients originating from the maternal compartment and disposes fetal waste products. Moreover, the placenta is a rich source of signal molecules which may have important effects in the maternal or fetal compartment including the placenta itself. Although the overall functions of placental signaling molecules are in general widely conserved between species, the occurrence of individual placental messenger substances and their specific roles may differ significantly between species. The production of numerous signaling molecules in placental tissues is commonly summarized as the placental endocrine function. Endocrine effects in a strict sense are exerted by molecules which are produced by specialized glands and released into the systemic circulation to reach their mostly remote target cells, where they activate specific receptors. However, it is very obvious that the concept of a classical endocrine factor holds true only for a minority of placental signaling molecules. The majority act as local regulators of growth, differentiation and functions via para-, auto-, juxta- or intracrine mechanisms in the placenta itself or in the adjacent endometrium. Moreover, a certain signaling molecule may exert its effects in different types of target cells by more than only one of the above-mentioned mechanisms (e.g. sex steroids or members of the prolactin/growth hormone family). Typically, in postnatal life the release of hormones is regulated by feed-back mechanisms. However, in many cases it is unclear or unknown whether the expression of a certain placental signaling molecule is regulated or just follows a genetically determined program. Depending on the species, profiles of several placental hormones continuously increase in the maternal circulation starting at a specific stage of gestation (e.g. progesterone in sheep, estrogens in domestic ruminants, relaxin in cat, dog and horse, placental lactogen in sheep and goat) and thus obviously reflect to a considerable extent the gain in placental mass. However, important factors which influence placental endocrine function are stress such as nutrient restriction or hypoxia mediated by an increased exposure of the placenta to glucocorticoids (Fowden and Forhead, 2009), and the signals targeting the placenta during the initiation of parturition (see section: The initiation of parturition: the placenta as a target and source of endocrine signals). Under pathological conditions, placental hormone production may be altered in cases of impaired pregnancy including fetal abnormalities, intoxications or placentitis (Hoffmann *et al*., 1996; Ryan *et al*., 2001, 2009).

Due to the large variety of placental signaling molecules and considerable species specific peculiarities, a detailed review on placental endocrine function in a larger number of species would clearly go beyond the scope of this article. Thus, here we focus

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Received: January 31, 2018
Accepted: May 18, 2018

predominantly on domestic ruminants (cow, sheep, goat) and those regulatory factors which have been measured at significant concentrations in fetal or maternal blood. According to the specific expertise of the authors special emphasis is placed on placental steroidogenesis.

The placenta as an endocrine organ during pregnancy

Basic features of the ruminant placenta

In the ruminant placenta the intimate feto-maternal contact is restricted to multiple discrete structures named placentomes. These are formed during placentation by interactions of the chorion with preformed placentation sites of the endometrium, whose number and placement varies considerably between ruminant species (Hradecky *et al*., 1988). Placentomes are composed of fetal chorionic villi (cotyledon) and the maternal caruncle. In the placentomes fetal villi arising from the chorionic plate of the cotyledon interdigitate with a corresponding system of widely ramified caruncular crypts. Based on initial histomorphological studies the bovine trophoblast was described to be composed of uninucleated cells (UTCs) and larger binucleated cells (BNCs). Later it was realized that in the bovine trophoblast throughout gestation terminally differentiated trophoblast giant cells (TGCs) differentiate continuously from UTCs. The TGCs, which generally possess two octaploid nuclei, become binucleate and subsequently polyploid by a series of acytokinetic mitoses and several stages of TGC development can be observed, which differ in size, location within the trophoblast epithelium and presence of cytoplasmic granules (Klisch *et al*., 1999). As during bovine TGC differentiation binucleated intermediates with lower ploidy levels occur, in this article the term TGC refers to mature trophoblast giant cells. TGCs may fuse with cells of the maternal caruncular epithelium. This fusion leads to short-living trinucleate feto-maternal hybrid cells in cattle and buffaloes (Cavalho *et al*., 2006) and to larger syncytial plaques in sheep and goat (Wooding, 1992). The function of this fusion is thought to be the exocytotic release of fetal derived mediator substances into the maternal compartment (Fig. 1). Due to this fusion the ruminant placental barrier is now classified as synepitheliochorial (Wooding and Wathes, 1980; Wooding, 1992; Klisch *et al*., 1999; Carvalho *et al*., 2006).

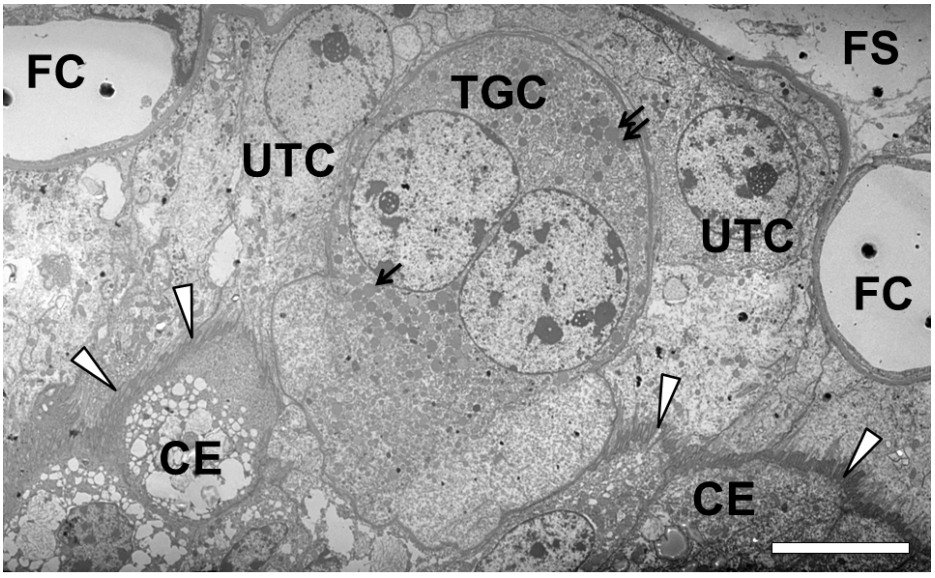

Figure 1. Mature bovine binucleate trophoblast giant cell (TGC) invading the caruncular epithelium (CE; lower part of the micrograph). The TGC is surrounded by several uninucleate trophoblast cells (UTCs). In the TGC cytoplasm numerous secretory granules (small arrows) are visible which are released into the maternal compartment after fusion of a TGC with an individual caruncular epithelial cell. In the secretory granules different signaling molecules have been detected, e.g. placental lactogen and prolactin-related protein-1. The feto-maternal borderline is labeled with arrowheads. FS: fetal stroma; FC: fetal capillaries. Gestation day 150; Bar = 10 μm.

Signals from the periimplantation trophoblast

Already prior to implantation and placentation in many species the trophoblast is a source of signals essential for the establishment and progression of pregnancy with partially considerable differences between individual species, especially with regard to the maternal recognition of pregnancy. In polyoestric spontaneously ovulating species generally a specific signal is necessary to prolong luteal function, which may be luteotrophic (e.g. humans) or antiluteolytic (e.g. domestic ruminants, pig, horse; Spencer *et al*., 2004a; Bazer, 2015). In many species the identity of this/these signal/s or their way of action are still largely unclear or completely unknown. In domestic ruminants trophoblast-derived interferon Tau (IFT) has been identified as the crucial factor for the maintenance of luteal function during early pregnancy. By suppressing

endometrial expression of oxytocin receptors, IFT interrupts a positive feedback loop between corpus luteum and endometrium, which would otherwise lead to the exposition of the corpus luteum to luteolytic prostaglandin F2α of endometrial origin (Spencer and Hansen, 2015; Hansen *et al*., 2017). Different from polyoestric species in the monoestric dog a specific luteotrophic or antiluteolytic signal during early gestation is obviously unnecessary as the luteal phase in cyclic bitches lasts longer than gestational length (Kowalewski *et al*., 2015). A different situation is also present in species with induced ovulation (e.g. rabbit, cat, ferret, camelids). During early pregnancy, in addition to ensuring progesterone supply, numerous other essential processes must be initiated and maintained. For example, nutrient supply by the endometrium, endometrial receptivity for implantation, endometrial differentiation, onset of placentation and local immunotolerance, are induced and controlled by a complex network of signals involving the fetal and maternal compartment (embryo-maternal dialogue). Here again, on the fetal side the trophoblast must be considered as the predominant source of signals. Concerning the embryo-maternal dialogue during early pregnancy the reader is referred to excellent and comprehensive reviews (e.g. Bazer *et al*., 2010; Mathew *et al*., 2016).

The placental endocrine function

Placental steroidogenesis

With respect to the regulation of reproductive functions, sex steroids (progestogens, androgens, estrogens) are considered as a prominent class of hormones. However, they are also involved in the regulation of numerous processes outside the reproductive system (Camacho-Arroyo and Montor, 2012), which could also be important in the mother or the growing fetus during pregnancy. Steroidogenic activity has been found in the placenta of many but not all mammalian species, with the dog being an example for a species without any detectable placental steroidogenesis (Hoffmann *et al*., 1994; Nishiyama *et al*., 1999). Research in placental steroidogenesis has mainly focused on the production of progesterone and estrogens and, to a lesser extent, of androgens. However, it is rather likely that bioactive steroids other than classical estrogens, androgens and progestogens may be produced in steroidogenic placentae and may exert important functions during pregnancy.

Progesterone and other bioactive progestogens

Progesterone is commonly considered indispensable for mammalian pregnancy, as it is involved in the control of numerous essential pregnancy-related functions including endometrial differentiation, myometrial quiescence, closure of the cervix and local immunotolerance in the pregnant uterus (Chwalisz and Garfield, 1997; Spencer *et al*., 2004b; Arck *et al*., 2007). Initially, the corpus luteum is the source of progesterone in all mammals. In some species the corpus luteum remains the only relevant source of progesterone throughout gestation with minimal (e.g. goat, pig) or no (dog) placental contribution, whereas in other species the placenta adopts this role after a species-specific time of gestation (luteo-placental shift; e.g. in sheep, horse, man; Meyer, 1994; Mitchell and Taggart, 2009). With regard to the relevance of luteal vs. placental progesterone the cow has an intermediate position. Throughout gestation the bovine placenta contributes – if at all - only to a minor extent to the maternal progesterone levels which are predominantly of luteal origin. However, placental progesterone is generally able to maintain pregnancy when luteal progesterone supply is eliminated between day 180 until day 240 by application of luteolytic prostaglandins or surgical removal of the ovaries. After this period, treated cows may immediately abort, exhibit a shortened gestational length or may continue pregnancy until normal term (Estergreen *et al*., 1967; Day, 1977; Johnson *et al*., 1981). Because of these observations bovine placental progesterone synthesis was considered to be a transient phenomenon. However, observations of undiminished placental progesterone tissue concentrations and 3β-hydroxysteroid dehydrogenase activities until term (Tsumagari *et al*., 1994) point to an increased demand of progesterone during late gestation rather than a decrease in placental progesterone synthesis. The role of placental progesterone in species with predominantly luteal progesterone synthesis throughout gestation remains unclear but it may be important for the generation of high local concentration at the feto-maternal interface, which could be necessary for some concentration-dependent progesterone effects (Hansen, 1998). Otherwise, placental progesterone could merely be an intermediate or side product arising from the synthesis of other steroids, especially estrogens. From an evolutionary point of view it is tempting to speculate whether there was an evolutionary pressure for placental progesterone to allow for longer gestation, or whether placental steroidogenesis initially served other functions and subsequently enabled the reduction of luteal lifespan. Commonly, progesterone is considered as the universal master hormone of pregnancy. However, early observations of unusually low or undetectable progesterone levels in individual species (horse, zebra, rock hyrax, elephant) challenged the concept of progesterone as the sole physiological progestogen (Conley and Reynolds, 2014). For the horse it was shown that 5α-dihydroprogesterone circulating at high concentration after the massive onset of placental steroidogenesis is a potent progestogen with a bioactivity comparable to progesterone. It is likely that a systematic investigation into the occurrence of structurally related steroids during pregnancy will result in the discovery of other bioactive progestogens (Scholz *et al*., 2014). The above-mentioned essential functions imply that during pregnancy the maternal compartment is the predominant target of progesterone. Depending on the species, progesterone could also serve as a local regulatory factor in the placenta itself as suggested by the detection of progesterone receptors in the human

(Oh *et al*., 2005) or equine chorion (Abd-Elnaeim *et al*., 2009). However, no classical nuclear progesterone receptors were detectable in the fetal part of bovine placentomes (Schuler *et al*., 1999) or in the canine placenta (Vermeirsch *et al*., 2000). Similar to estrogens, on a molecular level the view on the spectrum of possible progesterone effects has become very complex due to the existence of more than one classical nuclear receptor isoform, the existence of membrane-bound receptors and other nonclassical modes of signalling (Garg *et al*., 2017).

Placental estrogens

In many mammalian species, especially in primates and ungulates, the placenta produces significant amounts of estrogens. However, observations made so far with respect to the type of estrogens formed, concentrations, gestational profiles in maternal blood and synthetic pathways point to significant species differences. Different from follicular steroidogenesis with estradiol-17β generally being the sole relevant estrogen identified so far, during pregnancy frequently other estrogens are quantitatively dominating in maternal blood. In many ungulate species estrone is the major estrogen and sulfonated forms frequently exceed by far the concentrations of their free counterparts in maternal blood (e.g. sheep: Nathanielsz *et al*., 1982; horse: Hoffmann *et al*., 1996; cattle: Hoffmann *et al*., 1997; llama, alpaca: Aba *et al*., 1998). Moreover, during human (estriol) and equine pregnancy (equilin, equilenin), the formation of species specific placental estrogens is observed (Raeside, 2017). In humans (Loriaux *et al*., 1972; De Hertogh *et al*., 1975), domestic ruminants (Nathanielsz *et al*., 1982; Hoffmann *et al*., 1997) and camelids (Aba *et al*., 1998) maternal estrogen concentrations increase steadily during gestation, whereas horse and donkey mares exhibit a pronounced peak around midgestation (Hoffmann *et al*., 1996; 2014; Crisci *et al*., 2014). The bovine placenta is capable of producing estrogen autonomously from cholesterol (Schuler *et al*., 2008). In contrast, the human and equine placentae depend on the provision of C19 precursors due to a lack of significant CYP17A1 expression. In these species the essential precursors for placental estrogen production are provided by the maternal and fetal adrenal (humans) or fetal gonads (horse), respectively (Raeside, 2017). This interdependence of fetus and placenta for pregnancy associated steroidogenesis led to the term feto-placental unit (Diczfalusy, 1964).

Despite many observations on the biological roles of placental estrogens, definite information is still sparse and their functions may differ considerably between species. It has been suggested that during primate pregnancy placental estrogens have numerous functions such as trophoblast differentiation, autoregulation of placental steroidogenesis, regulation of the maternal cardiovascular system, utero-placental blood flow, placental neovascularization and mammary gland development (Pepe and Albrecht, 1995). However, the dramatic decrease of maternal estrogen levels in cases of placental steroid sulfatase deficiency generally resulted in an only mild impairment of fetal maturity and process of labor, whereas fetal development and placental progesterone production seemed normal. Nevertheless, maternal estrogen levels still remained clearly above basal level and may have been considerably higher locally in placental tissue. Thus, the absence of severe clinical symptoms does not necessarily mean that placental estrogens are unimportant for the above-mentioned functions (Lykkesfelt *et al*., 1984; Pepe and Albrecht 1995). To elucidate the role of placental estrogens in the mare, Pashen and Allen (1979) gonadectomized four equine fetuses between days 197 and 253 of pregnancy, which induced an immediate fall in maternal estrogen levels. The foals were born lighter and their musculature was less developed compared to sham-gonadectomized controls. In a different experimental approach Esteller-Vico *et al*. (2017) applied the aromatase (CYP19A1) inhibitor letrozole to pregnant mares starting on day 240 until parturition. This treatment suppressed maternal estrogen levels by approximately 90% compared to untreated controls but had no effect on uterine artery hemodynamics, normal placental development, maintenance of pregnancy, or neonatal viability. However, neonates from letrozole-treated mares had significantly lower birth weights than controls, pointing to a role of placental estrogens in fetal growth which is not mediated through regulation of uterine blood flow. As discussed above for humans, other roles of placental estrogens may have remained undetected as suppression of placental estrogen production was incomplete. Janowski *et al*. (1996) applied the estrogen receptor antagonist tamoxifen to pregnant cows starting on day 240 until parturition to block the effects of placental estrogens. Animals of the treatment group had significantly lower progesterone concentrations between days -9 to -2 before parturition, but no effects were observed on gestational length, calving, neonatal viability, incidence of placental retention and placental histomorphology. Again, also in this study effects of placental estrogens may have been missed as it remained unclear whether the blockage of estrogen receptors was complete. On a molecular level the effects of placental estrogens are difficult to estimate. Different estrogens may differ substantially in their binding affinities to classical nuclear estrogen receptors (ESR) or may differentially bind to the two ESR paralogues, ESR1 and -2. Weak estrogens act as agonists in the absence of potent ESR ligands, whereas they may have antagonistic effects in the presence of potent ESR ligands. Moreover, the effects of estrogens may be differentially modulated by the specific cellular context, especially by the presence of ESR coactivators or –repressors. Eventually functions mediated by membrane bound receptors and other non-classical signaling pathways also must be taken into account (Zhu *et al*., 2006; Cheskis *et al*., 2007). Another factor contributing to the complexity of estrogenic effects during pregnancy is metabolism, especially the local fine tuning of estrogenic activity by sulfonation of bioactive estrogens and the hydrolysis of estrogen sulfates (Mueller *et al*.,

2015; see also section: Placental estrogens - observations from the cow). Commonly, the maternal compartment (uterus, birth canal, mammary gland) is considered as the predominant target of placental estrogens. However, the localization of estrogen receptors in the chorion of various species (e.g. human: Bukovsky *et al*., 2003a, b; cow: Schuler *et al*., 2005; horse: Abd-Elnaeim *et al*., 2009) clearly points to an involvement of placental estrogen in the control of placental differentiation and functions (see also section: Placental estrogens - observations from the cow).

Placental estrogens – observations from the cow

In cows the role of placental estrogens has been predominantly seen in the preparation of the birth canal for parturition, in the myometrial excitability at term and in mammary gland development during late gestation. However, maternal estrogen levels start to increase significantly between days 120-150 (Hoffmann *et al*., 1997), and in fetal fluids significant estrogen concentrations have been measured as early as day 30 (Eley *et al*., 1979). Moreover, CYP19A1 transcripts were detected in bovine blastocysts as early as day 7 after insemination, which were exclusively produced from the placenta-specific promoter P1.1 (Fürbass, 2018; Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany; unpublished data). These observations indicate that during bovine gestation estrogens are produced in the conceptus/placenta starting practically from the very beginning of gestation until term. Apart from late gestation in cows the roles of placental estrogens are still unclear and no definite information is available on their effects on a molecular level. In an attempt to identify possible local target cells of placental estrogens, the expression of ESRs was characterized in bovine placentomes by immunohistochemistry between day 150 and term. ESR1 expression was exclusively detected in the maternal part of the placentomes (Schuler *et al*., 2002), where it was especially prominent in caruncular epithelial cells (CECs). As indicated by the proliferation marker Ki67, CECs exhibit a continuously high proliferation, even during late gestation when placentomal growth is minimal. This suggests that proliferation in CECs clearly exceeds the demand resulting from placentomal growth and remodeling (Schuler *et al*., 2000). Light microscopic observations indicate that numerous cells exhibiting features of apoptosis exfoliate from the caruncular epithelium and are phagocytosed in the trophoblast. Thus, in addition to molecules brought by the maternal blood and crossing the placental barrier by diffusion or specific transport mechanisms (hemotrophe), in bovine placentomes degenerating CECs may be an additional important source of nutrients. According to this concept bovine caruncles can be regarded as modified holocrine glands which are colonized by resorbing chorionic villi. Possibly, placental estrogens are an important stimulator of the high proliferation observed in CECs (Schuler *et al*., 2000). Different from ESR1, ESR2 was expressed in various cell types both in the fetal and maternal part of placentomes (Schuler *et al*., 2005). Interestingly ESR2 was significantly up-regulated during the process of TGC differentiation. Evidence for a possible role of placental estrogens in TGC differentiation also comes from the localization of steroidogenic enzymes. The production of estrogens from cholesterol requires the activities of CYP11A1, CYP17A1, HSD3B1 and CYP19A1. Whereas in the trophoblast expression of CYP11A1 and CYP17A1 have been exclusively observed in UTCs, CYP19A1 expression was undetectable in UTCs but was found to increase steadily after the entry of UTCs into TGC differentiation reaching maximal levels in mature and invasive TGCs. The expression pattern of HSD3B1 in bovine trophoblast cells is less clear. However, there is evidence from *in situ* hybridization that its expression is up-regulated during TGC differentiation (Schuler *et al*., 2008). These observations suggest that in bovine trophoblast cells the expression of ESR2 and steroidogenic enzymes is coupled to the process of TGC differentiation and the expression of steroidogenic enzymes is up-regulated in differentiating TGCs concomitant with the availability of their specific substrates. However, the concept of placental steroids as intracrine regulators of TGC differentiation is challenged by the observation that between D180 and late gestation the expression of CYP17A1 is largely restricted to the trophoblast of chorionic stem villi (Schuler *et al*., 2006b), whereas TGC differentiation obviously occurs at any localization within the chorionic villous tree. A role of estrogens in the control of trophoblast cell differentiation has also been suggested in the human placenta (Bukovsky *et al*., 2003a, b).

In domestic ruminants including the cow sulfonated estrogens clearly dominate over free forms throughout gestation except for the immediate pre- and intrapartal period (Hoffmann *et al*., 1997). In bovine gestation, expression patterns of CYP19A1 and of the estrogen specific sulfotransferase SULT1E1 in placentomal tissue provide clear evidence that placental estrogens are to a significant extent sulfonated in the trophoblast immediately after their production in TGC. Intriguingly, in the bovine trophoblast SULT1E1-mRNA was predominantly detected in TGCs, whereas the corresponding protein was virtually exclusively found in UTCs (Khatri *et al*., 2013; Polei *et al*., 2014). Sulfonation of steroids abolishes their interaction with nuclear receptors. Moreover, as sulfonation of steroids markedly increases their polarity, different from lipophilic free steroids they are practically incapable of crossing biological membranes by passive diffusion, which considerably reduces their distribution volume. Thus, traditionally sulfonation of steroids has been primarily regarded as a mechanism leading to their inactivation and facilitating their elimination. On the other hand, sulfonated steroids commonly circulate at significantly higher concentrations compared to their free counterparts, and may (re-)enter the pool of free steroids by the activity of steroid sulfatase (STS). Thus, sulfonated steroids are now increasingly considered as substrates for the local production of bioactive steroids in specific target tissue (sulfatase pathway of

steroidogenesis), with their cellular uptake by steroid sulfate transporters and hydrolysis being potential additional levels for the local control of activity (Mueller *et al* 2015). In bovine placentomes a high expression of STS was found in the caruncular epithelium. Moreover, the expression of significant levels of mRNA encoding the steroid sulfate transporter SLC10A6 were also detected in caruncles (Greven *et al*., 2007; Greven, 2008). In conclusion, the expression of CYP19A1, ESRs, SULT1E1, STS and SLC10A6 in close proximity to each other points to a role of placental estrogens as local regulators in bovine placentomes and indicates that their effects are tightly controlled by local mechanisms. However, definite information on their roles and mode of action are still missing.

Androgens

Androgens in a strict sense are defined as steroids promoting male secondary sex characteristics. However, the term androgen is commonly also used for their metabolites or C19 steroids such as androstenedione, dehydroepiandrosterone (DHEA) or sulfonated DHEA (DHEAS) which are devoid of any noteworthy activity at the androgen receptor but may serve as precursors for the synthesis of bioactive androgens. It is clear that in species exhibiting placental estrogen production aromatizable C19-steroids must be available as precursors. Due to a lack of considerable CYP17A1 expression, the human and equine placentae depend on the provision of C19-steroids from extraplacental sources (Raeside *et al*., 2017), whereas placentae in other species such as the cow (Schuler *et al*., 1994, 2006a) or the sheep (Mason *et al*., 1989; Gyomorey *et al*., 2000) are capable of efficiently converting C21-steroids into C19-precursors for the production of estrogens. However, as bioactive androgens could disturb sex differentiation in female fetuses or cause virilization in mothers, their unrestricted transfer in relevant amounts into the fetal or maternal circulation must be prevented. In humans one mechanism for androgen inactivation is obviously aromatization (Bulun, 2014). However, the detection of androgen receptors in the steroidogenic placentae of different species, such as humans (Uzelac *et al*., 2010), cattle (Khatri *et al*., 2013), horse (Davis *et al*., 2017) or pig (Wieciech *et al*., 2013) indicates that placental androgens may be more than just precursors for estrogen synthesis. However, the mere presence of androgen receptors in placental tissue does not necessarily imply a function of androgens, as sex steroid receptors may also exert steroid-independent activities due to constitutionally active transcription activation functions of their N-terminal domain or cross-talk with other signaling pathways (Davey and Grossmann, 2016). A completely different situation in comparison to primates or ungulates is present in mouse and rat pregnancy, where placental C19-steroids serve as precursors for the production of estrogens in the ovary, which play an important role in the maintenance of luteal function (Jackson and Albrecht, 1985).

Chorionic gonadotrophins

The pituitary gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) play an outstanding role in the hormonal regulation of gonadal function. Together with thyroid stimulating hormone (TSH), they belong to the family of glycoprotein hormones and are composed of a common α-subunit and a hormone-specific β-subunit. Depending on the species, LH-related glycoproteins are also expressed in the trophoblast. For a long time, chorionic gonadotrophin (CG) expression has only been reported in equids and primates (Cahoreau *et al*., 2015). However, more recently the expression of LH-like substances has been described in a bottlenose dolphin placenta (Watanabe *et al*., 2007). Human CG is composed of the common α-subunit of the glycoprotein hormone family and a specific β_{CG}-subunit, which is highly similar to the human β_{LH}-subunit but is generated from one of several separate β_{CG}-genes. As mentioned above, during early pregnancy hCG from the blastocyst is an essential signal for the maternal recognition of pregnancy as it stimulates the maintenance of luteal function via the binding to luteal LH-receptors until the placenta takes over the role as the relevant source of progesterone. Moreover, the involvement of hCG in other functions during human pregnancy has been identified such as endometrial angiogenesis, quiescence of the myometrium, maternal intrauterine immunotolerance and control of syncytiotrophoblast formation (Perrier d'Hauterive *et al*., 2007; Fournier *et al*., 2015). The CG biology in horses is clearly different from CG biology in humans. The β-subunit of equine CG (eCG) is a product of the β_{LH}-gene. However, the eCG molecule differs significantly from equine LH in that it is intensely and differentially glycosylated. In contrast to hCG, expression of eCG starts clearly after the maternal recognition of pregnancy (day 35-36). In the otherwise epitheliochorial equine placenta, eCG expression is restricted to a specialized trophoblast subpopulation which invades the maternal endometrium to form discrete structures named endometrial cups. By its strong and long-lasting LH activity in horses, eCG induces the transformation of large follicles, which develop during early pregnancy under the influence of pituitary FSH, into accessory corpora lutea (Murphy and Martinuk, 1991; Allen, 2001; Antczak *et al*., 2013).

Pregnancy-specific members of the prolactin and growth hormone family

Growth hormone (GH, somatotropin) and prolactin (PRL) are structurally-related signaling molecules which have evolved from a common precursor. Their receptors (GHR, PRLR) are also structurally related. Whereas in most mammals GH and PRL are evolutionarily conserved, in anthropoid primates, ruminants and muroid rodents either GH, PRL or both have undergone considerable evolutionary change and GH- (primates) or PRL- (rodents, ruminants) gene derivatives are expressed in the trophoblast (Gootwine, 2004; Soares, 2004; Haig, 2008). Moreover, in the

elephant placenta the production of an immunoreactive PRL has been described in the trophoblast (Yamamoto *et al*., 2012), whose characterization on a molecular level is still pending. In primates, ruminants and rodents depending on the individual species lineage and gene, the placental GH- or PRL paralogues may exert their effects via the GHR, PRLR, GHR/PRLR dimers or may have non-classical actions (Gootwine, 2004; Soares, 2004; Haig, 2008). The nomenclature of GH/PRL family members is partly confusing. Depending on the individual molecules, the PRL family members in rodents and ruminants have been given a variety of names such as placental lactogen (PL), PRL-like proteins (PLPs), PRL-related proteins (PRPs) and proliferin (PLF). However, the PRL-derived genes encoding ruminant and rodent PLs and PRPs are not orthologous. GH-family members occurring in primates are referred to as PL, GH-variant (GH-V) and chorionic somatomammotropins (CS; Soares *et al*., 2007).

In ruminants both GH- and PRL-encoding genes have undergone considerable evolution. Duplication of the PRL gene has been shown in all well-studied ruminants, leading to the formation of a cluster consisting of PRL, PL and PRP genes. Most ruminant species including cattle possess a single GH-like gene, which despite the absence of gene duplication has also undergone accelerated evolution. However, domestic sheep and goat have been found to be polymorphic for a GH-duplication (GH1/GH2-N and GH2-Z). Whereas in the bovine placenta there is no evidence for GH expression, in the ovine placenta GH was found to be expressed between days 35-75. In ovine haplotypes with two GH genes, one gene was expressed in the pituitary (GH2-N) and the other one in the placenta (GH2-Z). In addition to a role in the control of pregnancy-specific uterine gland differentiation and functions, GH transiently expressed in the ovine placenta has also been suggested to have effects in the fetal compartment before the onset of GH expression in the fetal pituitary (Gootwine, 2004; Reicher *et al*. 2008).

Ruminant PRL and GH signal through their corresponding receptor, respectively. Ruminant PL (syn.: chorionic somatomammotropin hormone, CSH) acts as agonist at PRLR-homodimers and at PRLR-/GHR-heterodimers, whereas it has antagonistic effects at GHR-homodimers (Gootwine 2004). Expression of bovine PL commences in the trophoblast cells around implantation. After the onset of placentation ruminant PL and PRP-expression is virtually confined to TGCs, where it is strongly up-regulated during TGC differentiation from UTCs and is maintained beyond the migration of TGCs into the caruncular epithelium or fusion of TGCs with CECs (TGC-endometrial heterokaryons). Generally, ruminant PLs have been considered as regulatory factors predominantly involved in the control of uterine and mammary gland development and nutrient delivery from the maternal to the fetal compartment (Gootwine, 2004; Soares, 2004; Haig, 2008). PL-deficient ovine pregnancies generated by the application of the lentiviral-mediated short hairpin RNA technique targeting CSH-mRNA to blastocysts followed by their transfer to recipients resulted in phenotypes consistent with that of intrauterine growth restriction, probably by impairment of placental development beginning early in gestation. Moreover, the results suggested that PL-deficiency impacted fetal liver development and function (Baker *et al*. 2016; Jeckel *et al*. 2018). Consistent with a role in endometrial gland differentiation and function, PRLR expression was found in glandular epithelial cells of the ruminant endometrium. Ovine PL has been shown to bind to endometrial PRLR and stimulate the secretion of uterine milk into the uterus lumen (uterolactation). The stimulatory effect of ruminant PL on uterine histiotrophe delivery is obviously mediated by a paracrine mechanism not requiring PL to enter the maternal systemic circulation (Gootwine, 2004; Soares, 2004; Haig, 2008). However, in pregnant sheep (Handwerger *et al*., 1977; Chan *et al*., 1978) and goats (Currie *et al*., 1990) PL concentrations start to increase substantially during the second trimester, reaching peak levels around 1-2 µg/ml during late gestation suggesting endocrine PL effects in the maternal compartment also beyond uterine tissues. Different from rodents in sheep luteotropic and/or luteoprotective actions of PL could not be demonstrated (Al-Gubory *et al*., 2006).

In contrast to sheep and goat, maternal PL concentrations in cattle were only in the lower ng/ml range throughout gestation (Wallace, 1993). In steroid-primed dairy heifers a mammogenic effect of PL was demonstrated (Byatt *et al*., 1997). As no considerable effect of a PRL inhibitor or a PRL antiserum on bovine luteal function could be demonstrated (Hoffmann *et al*., 1974), like in sheep also in cattle a luteotropic action of PL is unlikely. Concentrations in the bovine fetal circulation were somewhat higher than in the maternal with mean values decreasing from about 25-30 ng/ml in early gestation to 10-20 ng/ml prior to parturition (Holland *et al*., 1997; Alvarez-Oxiley *et al*., 2007), suggesting that bovine PL may have effects in the fetus proper.

Ruminant PLs have been postulated as factors involved in the normal physiological adjustments occurring during pregnancy in concert with other regulators. Observations especially from rodents suggest that activities of PRL paralogues may not be particularly in demand in unimpaired pregnancy but may become important during adaptations to stress (Gootwine, 2004; Soares, 2004; Soares *et al*., 2007; Haig, 2008). Concerning the specific biology of GH or PRL paralogues in anthropoid primates and muroid rodents the reader is referred to the excellent reviews cited here and the references included herein.

In addition to PL, in ruminants another distinct subfamily of PRL-related placental transcripts has been identified predicting glycoproteins structurally related to PL and PRL, named Prolactin-Related Proteins (PRPs). However, these non-classical members of the PRL/GH family are quite different in amino acid sequence from PL. In cattle on the protein level the expression of only PRP-1 has been confirmed, while mRNAs from more than ten different PRP genes are transcribed. The function of PRP-1 produced in TGCs (Fig. 2) throughout gestation is unclear, as it does not bind to PRL or GH receptors. Results from *in vitro* studies suggest that it may stimulate placentomal angiogenesis (Patel *et al*. 2004; Ushizawa *et al*. 2010).

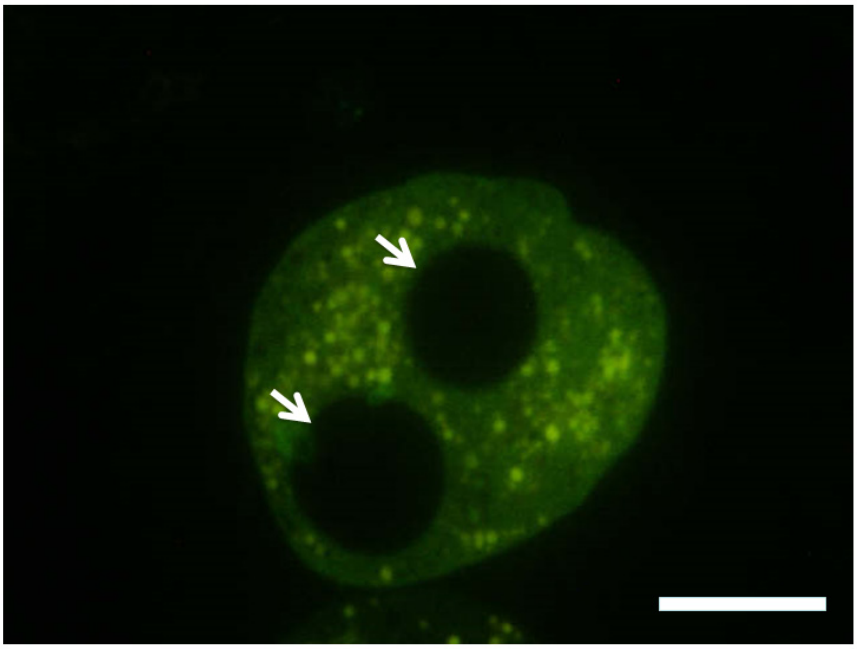

Figure 2. Immunolabelling (green) of prolactin-related protein-I (PRP-I) in secretory granula of a bovine TGC. The two TGC nuclei (arrows) are devoid of immunostaining. Gestation day 110; Bar = 10 µm.

Relaxin/insulin-like family peptides

After the discovery of relaxin by its softening effect on the pubic ligament in virgin guinea pigs (Hisaw, 1926), subsequent research revealed a complex hormone system. In placental mammals it consists of four receptors (RXFP1-4), which are members of the rhodopsin G protein-coupled receptors, and multiple ligands of the relaxin/insulin-like (RLN/INSL) family. Together with the family of the insulin-like growth factors (IGFs) the RLN/INSL family forms the insulin-relaxin superfamily. Evolutionary research provided evidence that the RLN/INSL family traces back to a single progenitor gene in the common ancestor of vertebrates. The diversification of RLN/INSL genes found in contemporary vertebrates resulted from two whole genome duplications in early vertebrate evolution followed by differential additional gain of genes by small scale duplications and gene losses in the individual linages of species (Hoffmann and Opazo, 2011; Yegorov *et al*., 2014). In mammalian species the RLN/INSL gene family generally consists of relaxin 1 (RLN1), relaxin 3 (RLN3) and genes encoding the insulin-like peptides (INSL) 3-6. In an ancestor of humans and great apes a duplication of RLN1 yielded RLNH1 and RLNH2. The latter gene functionally corresponds to RLN1 in other mammals, whereas the function of human RLNH1 is at present widely unclear. RLN/INSL family members are pleiotropic polypeptide hormones which are involved in the regulation of a broad variety of physiological processes. However, RLN1 (human RLNH2), INSL3, INSL4 and INSL6 are clearly associated with reproductive functions in males and females, whereas RLN3 and INSL5 are predominantly associated with the neuroendocrine system and the gut, respectively (Bathgate *et al*., 2013; Anand-Ivell and Ivell, 2014; Arroyo *et al*., 2014). When the term relaxin (RLN) is used in this article the reproductive relaxin (RLN1; human RLNH2) is meant.

Depending on the context RLN may act as a systemic hormone or as a local regulatory factor. In many mammalian species a pregnancy-specific increase of maternal RLN concentrations is observed. Generally, regarding quantities the corpus luteum and/or the placenta are the most important sources. However, their relative contributions to maternal blood levels may differ significantly between species. In a considerable number of species including rabbit, dog, cat, camelids and horse the placenta is the major or sole source of RLN at advanced stages of gestation. Different from the aforementioned species, in humans, rodents and pigs circulating RLN is of luteal origin throughout gestation. However, local effects of placental RLN may also occur in the absence of substantial concentrations in the maternal circulation. A multitude of RLN effects have been described during pregnancy such as decidualization, immunomodulation, quiescence of the myometrium, maternal circulatory adaptations of pregnancy, stimulation of angiogenesis, uterine and vaginal growth, development of the mammary gland and/or nipples. In the prepartal period, RLN has been related to the relaxation of the pubic ligament and the preparation of the maternal birth canal for parturition. However, observations concerning pregnancy-specific roles of RLN point to considerable differences between species (Parry and Vodstrcil, 2007; Bathgate *et al*., 2013; Klein, 2016). The fact that expression of the cognate RLN receptor RXFP1 has been detected in in human (Yamasato *et al*., 2017) and canine trophoblast cells (Nowak *et al*., 2017) indicates that at least in these species RLN may have local effects in the placenta itself. Moreover, the detection of RXFP1 expression in human fetal placental vascular endothelial cells suggests that RLN may have a role in the control of placental perfusion and thus could affect feto-placental growth (Yamasato *et al*., 2017). In addition to direct effects,

RLN may also have indirect effects e.g. by the induction of other mediators such as VEGF or nitric oxide (Palejwala *et al*., 2002; Leo *et al*., 2017). In addition to RLN1, also INSL3 was found to serve important reproductive functions in males and females. However, no evidence was found in the literature for a considerable placental expression of INSL3.

Concerning the physiology of the RLN/INSL family domestic ruminants hold a special position among mammals as they do not express RLN1. In cattle the RLN1 gene has obviously been lost during evolution and in goat, sheep and other ruminants only a pseudogene was identified. However, the bovine expresses fully functional receptors for RLN1 and INSL3, RXFP1 and RXFP2, respectively. Thus, it was suggested that in ruminants other members of the RLN/INSL family or non-relaxin ligands could compensate for the missing RLN1 (Dai *et al*., 2017; Malone *et al*., 2017). However, studies to corroborate the concept of a relaxin physiology in ruminants applying porcine relaxin to late pregnant heifers yielded inconsistent results (Musah *et al*., 1986; Bagna *et al*., 1991; Smith *et al*., 1996, 1997).

The initiation of parturition: the placenta as a target and source of endocrine signals

Initiation of parturition in domestic animals

Mammalian parturition is controlled by a complex network of signals involving the fetus, the mother and the placenta. Important processes related to parturition are the withdrawal of progesterone (or progesterone effects), softening of the birth canal, opening of the cervix, increase in the excitability of the myometrium, release of uterotonic substances, rupture of the fetal membranes, expulsion of the fetus and finally the timely release of the placenta. Another process immediately linked to the prepartal endocrine changes is the onset of lactation. However, initiation and control of parturition has been studied in detail only in a limited number of species with the exact sequence or network of events being still widely unknown in most of them. Although some common motifs have been encountered in several species investigated, observations available so far indicate that even between closely related species significant differences may exist (Jenkin and Young, 2004; Mitchell and Taggart, 2009). As the placenta is an important player in the network of signals controlling initiation and the process of parturition, the diversity of its endocrine function significantly contributes to the considerable differences in these signal cascades between species.

According to the current general concept of initiation of parturition in domestic animals, after gradual maturation the fetus reaches a state of readiness for parturition. At this point a rapid mechanism of progesterone withdrawal is activated (Mitchell and Taggart, 2009). The effects induced by the return of progesterone to basal level allow for the final expeditious processes resulting in the onset of myometrial activity and the stretchability of the birth canal eventually accomplishing the expulsion of the fetus(es). One reason for the significant variability between species concerning the underlying chain of events is the difference in progestogen supply during late gestation, thus requiring different mechanisms of prepartal withdrawal. In species with the corpus luteum being the sole or major source of progesterone at the end of gestation (e.g. dog, cat, pig, goat, cattle, mouse, rat and rabbit) luteolysis is considered as a prerequisite for the onset of physiological parturition. In species with only or predominantly placental synthesis of progestogens (sheep, horse), their withdrawal at parturition may come about by the channeling of C21-precursors of progestogen synthesis into a different pathway (Whittle *et al*., 2001; Fowden *et al*., 2008; Mitchell and Taggart, 2009). However, in humans parturition occurs when maternal progesterone levels of placental origin are maximal (Smith *et al*., 2009) and a significant drop of maternal progesterone concentrations is only observed with the release of the placenta. Thus, in humans the concept of a functional progesterone withdrawal was put forward, possibly based on the local withdrawal of progesterone in relevant target tissues by metabolism or changes on the progesterone receptor level or post-receptor signaling mechanisms. However, during late human pregnancy the role of progesterone and the definite mechanisms leading to the cessation of progesterone effects at parturition are still unclear (Zakar and Hertelendy, 2007; Mitchell and Taggart, 2009). Moreover, different from the situation in species with a prepartal progesterone withdrawal initiating rather single-stranded chains of events, in humans it has been suggested that the onset of birth results from a protracted parallel destabilization of pregnancy in several compartments of the fetal-placental-maternal unit synergizing in the transformation of the uterus from a quiescent to a contractile phenotype (modular accumulation of physiological systems; Mitchell and Taggart, 2009). Similar to the situation in humans, also in guinea pigs parturition occurs in the presence of high progesterone levels of placental origin (Illingworth *et al*., 1971). It is evident that a broad variety of placental signaling molecules participates in the feto-maternal dialogue during the period between the initiation of parturition and the timely release of the placenta, including numerous locally acting factors (Streyl *et al*., 2012). For the sake of manageable size also this part of the article is widely limited to placental hormones detectable in significant amounts in the fetal and/or maternal circulation.

Initiation of parturition with in species with a prepartal collapse of placental progestogen production (sheep, horse)

For predominantly historical reasons (Liggins, 1968, 2000) to date the sheep is practically the only species in which a detailed and largely experimentally confirmed concept for the initiation of parturition has been established (Fig. 3), as this species has served for many years as a model to study the physiology of parturition in humans (Whittle *et al*., 2001). As the

decisive process triggering physiological initiation of parturition, a progressive maturation and activation of the fetal hypothalamus-pituitary-adrenal (HPA) axis during late gestation has been identified, resulting in a considerably increased cortisol release from the fetal adrenals. The increased fetal cortisol levels stimulate a substantial up-regulation of cyclooxygenase 2 (PTGS2) expression in UTCs, causing an enhanced synthesis of PGE_2. Besides promotion of the maturation of the fetal HPA axis by a positive feedback loop, placental PGE2 stimulates a pronounced up-regulation of the steroidogenic key enzyme CYP17A1 co-localized with PTGS2 in UTC. The increase in CYP17A1 expression enhances the synthesis of estrogens at the expense of progesterone production (Whittle *et al*., 2000, 2001). However, due to the inefficiency of ruminant CYP17A1 to exert the lyase reaction after 17α-hydroxylation of progesterone, the collapse of placental progesterone synthesis and the concomitant increase of estrogen production does not result from a direct conversion of progesterone to estrogens but rather from an increased channeling of the common precursor pregnenolone into the Δ5-pathway of steroidogenesis (Fig. 4; Mason *et al*., 1989). The prepartal increase in placental estrogen levels is considered as an important signal to the maternal compartment stimulating the up-regulation of contraction-associated proteins (CAPs; e.g. oxytocin receptors, prostaglandin receptors, gap junctions) in the myometrium and the production of uterotonic PGF2α in the endometrium. In concert with effects induced by the concomitant progesterone withdrawal, the prepartal increase in placental estrogens brings about myometrial excitability and stimulation of myometrial activity, finally resulting in the expulsion of the fetus (Whittle *et al*., 2000, 2001). A prepartal decline of placental progestogen levels is also observed in the mare. However, the chain of events leading to parturition in the horse is much less clear. Similar to the sheep, in the equine fetus adrenocorticotropic hormone (ACTH) and cortisol increase significantly during late gestation after maturation of the fetal hypothalamus-pituitary system. However, clearly different from the sheep, the equine placenta lacks a considerable CYP17A1 expression, and placental estrogen synthesis, which depends on C19-precursors provided from the fetal gonads, substantially decreases between midgestation and parturition. Moreover, equine placental progestogen synthesis depends on C21-precursors provided by the fetal compartment with the fetal adrenals generally considered as the relevant source. Thus, for the prepartal collapse of equine placental progestogen synthesis a concept has been put forward according to which the rise of fetal ACTH concentrations during the final phase of gestation stimulates a steep increase of cortisol synthesis in the fetal adrenal at the expense of C21-precursors for placental progestogen synthesis (Thorburn, 1993; Fowden *et al*., 2008). However, the concept of the adrenals as the sole relevant source of fetal C21-precursors for equine placental progestogen synthesis has recently been challenged (Conley, 2016; Legacki *et al*., 2016, 2017).

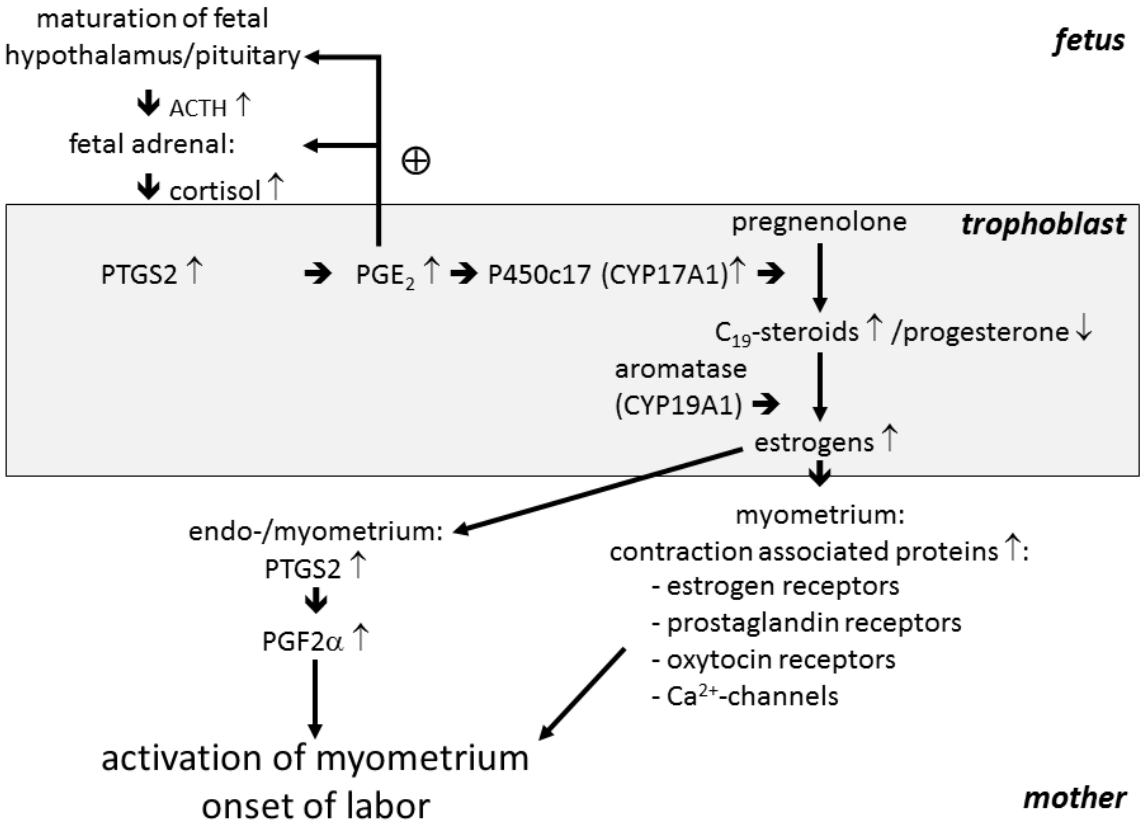

Figure 3. Concept of the endocrine events initiating parturition in the sheep (according to Whittle *et al*., 2001). For description see section: Initiation of parturition with in species with a prepartal collapse of placental progestogen production (sheep, horse) in the text.

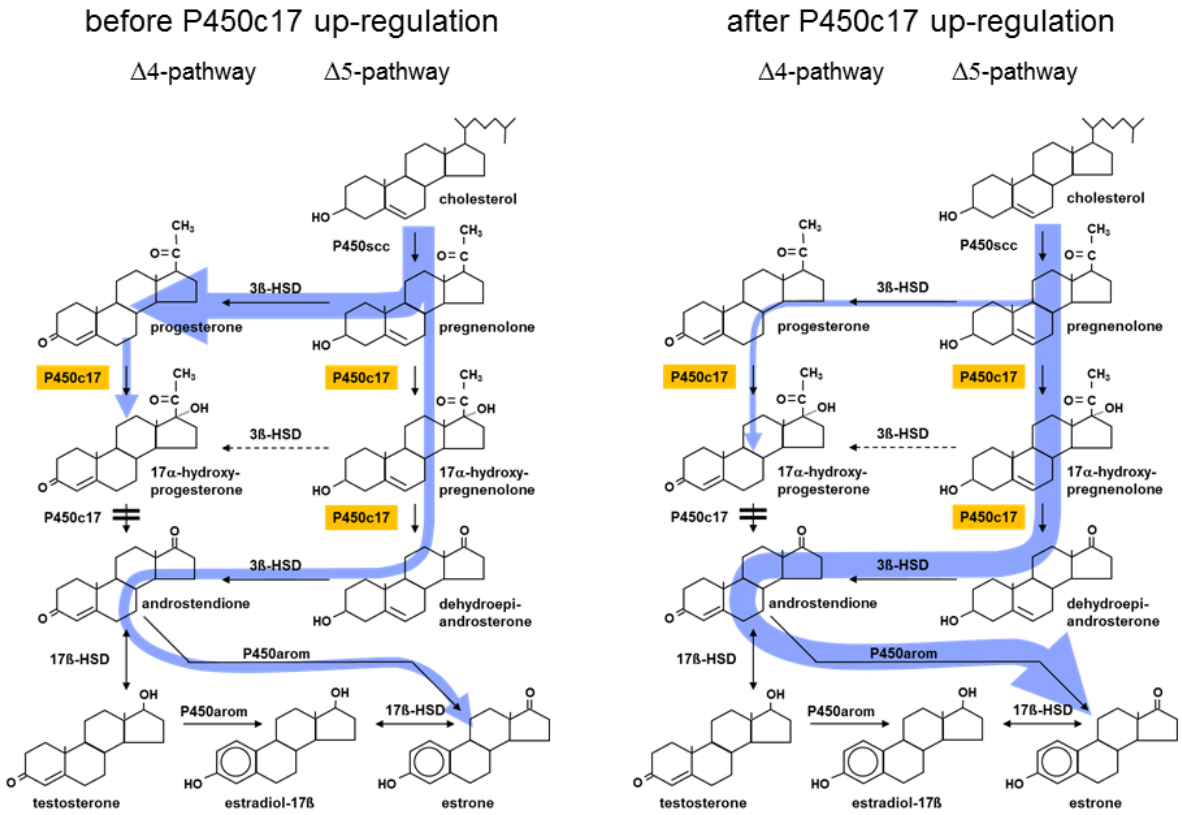

Figure 4. Concept of the prepartal switch in ovine placental steroidogenesis resulting from the up-repulation of CYP17A1 expression in the trophoblast induced by the increase of fetal cortisol levels (see Fig. 3). Due to the minimal lyase activity of ruminant CYP17A1 on the Δ4-pathway, the collapse of placental progesterone synthesis does not result to a noteworthy extent from a direct conversion of progesterone into estrogens but rather from the channeling of the common precursor pregnenolone into the synthesis of estrogens via the Δ5-pathway of steroidogenesis. A considerable up-regulation of placental CYP17A1 expression and activity has also been demonstrated in prepartal cows (Schuler *et al*., 1994; 2006b; Shenavai *et al*., 2012). However, different from the substantial pre- and intrapartal increase of placental estrogens in sheep in cattle a considerable increase in maternal concentrations of placental estrogens does not occur near term. P450scc: cytochrome P450 side-chain-cleavage enzyme (CYP11A1); P450c17: 17α-hydroxylase-C17,20-lyase (CYP17A1); 3ß-HSD: 3ß-hydroxysteroid dehydrogenase-Δ5/4-isomerase (HSD3B1); 17ß-HSD: 17ß-hydroxysteroid dehydrogenase (HSD17B); P450arom: aromatase (CYP19A1).

Placenta and prepartal luteolysis

In species with the ovary as the sole or predominant source of progesterone during late gestation, prepartal luteolysis is commonly regarded as the decisive step for the initiation of parturition. Due to the fact that in many polyoestric spontaneously ovulating species PGF2α of endometrial origin has been identified as the luteolytic signal during the ovarian cycle and the observation that in species with luteal progesterone during late gestation parturition may be readily induced with PGF2α or analogues (Kindahl *et al*., 1984; Weems *et al*., 2006), prostaglandins are commonly considered as the luteolytic agent also at term. However, although seemingly obvious, a definite confirmation is still pending and information on the source of the prepartal luteolytic signal is still sparse. Moreover, the regulatory mechanisms for the generation of the luteolytic signal are still widely unclear or unknown.

Despite the fundamental difference concerning progesterone supply during late gestation in sheep and cattle, important steps of the signal cascade initiating parturition in the sheep has also been confirmed for the cow (see Fig. 3-5). Observations from pathological prolongation of gestation point to the importance of HPA axis maturation also with respect to termination of bovine pregnancy (Kennedy *et al*., 1957; Buczinski *et al*., 2007; Cornillie *et al*., 2007). Also in the bovine fetus a significant prepartal increase in cortisol concentrations has been demonstrated (Comline *et al*., 1974; Hunter *et al*., 1977), and also in the bovine placenta cortisol stimulates a considerable upregulation of CYP17A1 in UTCs, resulting in the collapse of placental progesterone production (Schuler *et al*., 1994; Shenavai *et al*., 2012). However, as in the late pregnant cow progesterone is mainly of luteal origin, the prepartal decline of progesterone levels observed in maternal circulation closely reflects luteolysis (Hoffmann *et al*., 1979). Thus, the question arises

concerning a link between the prepartal changes in bovine placental endocrine function induced by the increase in fetal cortisol and luteolysis. In the ovine pregnant uterus, two different ways have been identified for the production of prostaglandins at the onset of parturition, a cortisol-dependent/estrogen-independent mechanism within the trophoblast leading to the rise in fetal plasma PGE2, and a mechanism stimulated by placental estrogens within the maternal endometrium bringing about the massive release of prostaglandins considered relevant for myometrial activity (Whittle *et al*., 2000). However, during late gestation and at parturition, in the bovine caruncles PTGS2 was undetectable by immunohistochemistry (Schuler *et al*., 2006a), and expression in the intercaruncular endometrium and in the myometrium was low and did not significantly change during the period in question. A strong up-regulation of PTGS2 in the bovine endometrium was detected only on the day after parturition (Fuchs *et al*., 1999; Arosh *et al*., 2004; Schuler *et al*., 2006a; Wehbrink *et al*., 2008). These observations suggest that UTCs, in which PTGS2 is dramatically up-regulated by fetal cortisol around the time of luteolysis, are the major source of luteolytic prostaglandins in the prepartal cow (Fig. 5). This concept is further corroborated by the detection of a significant expression of AKR1B5 in UTCs, an enzyme considered as the relevant prostaglandin F synthase in cyclic cows (Madore *et al*., 2003; Schuler *et al*., 2006a). However, any weak PTGS2 expression in the uterus may not be neglected due to the size of the organ in late pregnant animals. A problem commonly arising in studies concerning the endocrine changes leading to prepartal luteolysis in cows and other species is the exact definition of the time point when luteolysis is initiated, which is commonly based on the time when the decline of maternal progesterone levels becomes obvious. In many cases a precise determination is dubious due to the substantial variability of prepartal progesterone profiles between individual animals and considerable diurnal fluctuations. However, an exact determination of the initiation of luteolysis is crucial for the assessment whether or not an increase in placental or uterine prostaglandin production may be considered as the prepartal luteolytic signal. In prepartal cows, concentrations of PGF2α or of its major metabolite15-keto-13,14-dihydro PGF2α (PGFM) increase substantially concomitant with the onset of labor (Fairclough *et al*., 1975; Edqvist *et al*., 1978; Bosu *et al*., 1984, Meyer *et al*., 1989; Shenavai *et al*., 2012). However, the massive prepartal increase of PGF2α/PGFM levels in the maternal circulation is obviously primarily related to the stimulation of myometrial activity. They may certainly contribute to the final stages of luteolysis, whereas during initial stages, i.e. 36-48 h prior to birth, their rise over basal level is, if at all, only minimal. Moreover the PGFM profile in maternal peripheral blood is distinctly different from luteolysis in cyclic cows (Königsson *et al*., 2001), where prostaglandin spikes of endometrial origin are observed before and during luteolysis (Peterson *et al*., 1975; Kindahl *et al*., 1976; Vighio and Liptrap, 1986). However, marginal PGF2α/PGFM levels during the onset of prepartal luteolysis do not necessarily exclude the role of PGF2α produced in the uterus or placenta as the prepartal luteolytic agent. Similar to a proposed scenario in cyclic cows, PGF2α may reach the ovary predominantly by a local supply system possibly predominantly based on lymphatic vessels. Thus, the local availability of luteolytic prostaglandins at the ovaries may be barely reflected by the profiles showing up in the maternal systemic circulation (Hein *et al*., 1988; Krzymowski and Stefańczyk-Krzymowska, 2008).

The question of the nature and the origin of the prepartal luteolytic signal was also extensively studied in the goat, which exhibits distinct similarities to cattle with respect to estrogen profiles during pregnancy (Sawada *et al*., 1995; Engeland *et al*., 1999; Probo *et al*., 2011), the relative role of luteal vs. placental progesterone (Currie and Thorburn, 1977b; Sheldrick *et al*., 1980) and the prepartal alterations of placental steroidogenesis in response to a prepartal increase of fetal cortisol (Currie and Thorburn, 1977a, b; Flint *et al*., 1978). Results from the studies concerning prepartal luteolysis in goats or their interpretation were partially conflicting. Ford *et al*. (1998, 1999) measured PGF2α or PGFM in late pregnancy in systemic maternal plasma and utero-ovarian venous plasma and did not detect relevant changes in prostaglandin concentrations around the expected time of luteolysis. Therefor they concluded that the results did not support the concept of PGF2α being the principal luteolysin in the pregnant doe at term. However, as in the late pregnant cow (Hein *et al*., 1988), the transport of luteolytic prostaglandins to the ovary by the lymphatic system could not be excluded. Generally, in prepartal goats a gradual decrease of maternal progesterone concentrations starts 3-4 days before parturition. Probo *et al*. (2011) observed a slight increase of maternal PGFM levels starting four days before parturition concomitant with the decline of progesterone. Although the increase of maternal PGFM levels became statistically significant only on the day before parturition, taking into account other observations from the literature (Currie *et al*., 1988) they suggested that luteolysis in prepartal goats is initiated by increasing levels of luteolytic prostaglandins, whereas the onset of their massive production is only possible after the decline of progesterone levels. However, in the literature no information was found which allows identifying a certain cell type in the placenta or uterus as a relevant source of luteolytic prostaglandins in prepartal goats.

A similar situation as described for the cow and the goat is also present in other species which exhibit exclusively or predominantly luteal progesterone supply during late gestation. In these species prepartal luteolysis is considered to be associated with a slight increase of prostaglandins in the maternal blood which precedes their massive rise concomitant with the onset of myometrial activity. Like in the late pregnant cow, a considerable up-regulation of PTGS2 has been found during late gestation in the trophoblast of other species exhibiting prepartal luteolysis such as the dog or cat

(Kowalewski *et al*., 2010; Simieniuch *et al*., 2014). However, a definite role of the trophoblast in the generation of the luteolytic signal and possible contributions from other sources in the late pregnant uterus remain to be confirmed. For the prepartal withdrawal of progesterone in the sheep, goat and cow a direct connection with the late gestational rise in fetal cortisol was demonstrated. A rise in fetal cortisol during the prepartal period, which is essential for final maturing processes in several organs, is obviously highly conserved between mammals. However, a direct linkage to the mechanisms initiating parturition is probably unique to ruminant species among Eutherian mammals (Jenkin and Young, 2004).

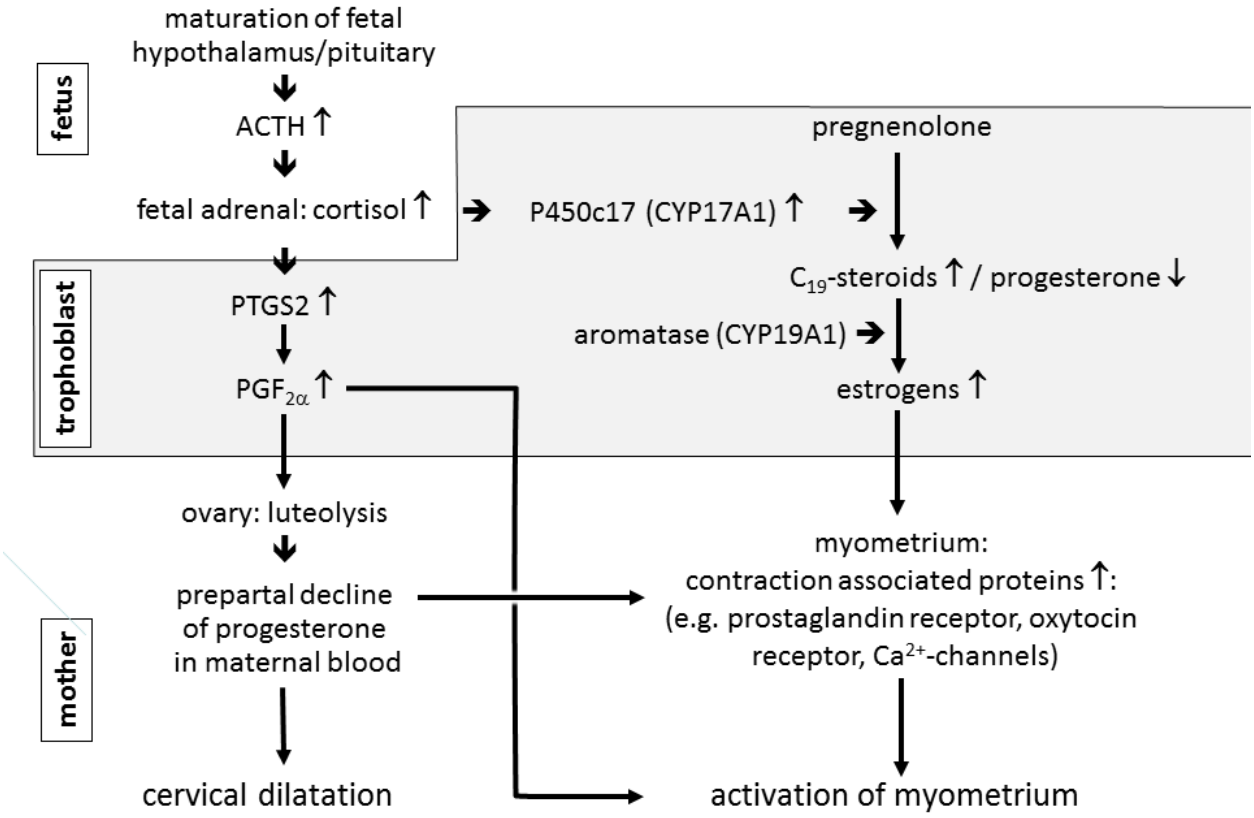

Figure 5. Concept of the initiation of parturition in cattle (according to Shenavai *et al*., 2012). Prepartal endocrine changes similar to the sheep have been confirmed in the prepartal cow (see Fig. 4 and 5). However, different from the late pregnant sheep with the placenta as the only relevant source of progesterone, in cattle the corpus luteum is the predominant source of progesterone throughout gestation and the prepartal decline in maternal progesterone level is clearly associated with luteolysis.

Prostaglandins

Around parturition, across mammalian species prostaglandins are considered as important factors involved in the stimulation of cervical relaxation and myometrial activity, and a massive increase of maternal prostaglandin levels concomitant with the onset of labor is obviously a common trait in mammals (Jenkin and Young, 2004). The key enzyme of inducible prostaglandin synthesis, PTGS2, has been shown to be expressed in the trophoblast of several mammalian species, where it is substantially up-regulated prior to parturition (e.g. dog: Kowalewski *et al*., 2010; cat: Simieniuch *et al*., 2014; cow: Schuler *et al*., 2006b; sheep: McLaren *et al*., 2000). However, the definite contribution of placental prostaglandins vs. prostaglandins from other sources such as the maternal endometrium and myometrium to the massive pre- and intrapartal increase of maternal prostaglandin levels is currently unclear and may differ between species. In the ovine fetus during late gestation PGE2 produced in the trophoblast has been suggested to accelerate maturation of the hypothalamus-pituitary system in response to the rising cortisol levels as a positive feedback mechanism (Whittle *et al*., 2001).

Estrogens

Another group of hormones produced in significant amounts in the placentae of many species and considered to be involved in the control of parturition are estrogens. However, similar to pregnancy (see sections: Placental estrogens and Placental estrogens – observations from the cow), the situation of placental estrogens around parturition is very complex, which obscures the understanding of their definite effects. Moreover, information so far available demonstrates significant differences between species.

As mentioned above in the dog, no placental estrogen production is detectable at all. No other estrogen than free estradiol-17β has been described during canine gestation. However, maternal concentrations of estradiol-17β increase only slightly with gestational age but on the average hardly exceed basal levels. As they tend to decline during the last two weeks of gestation with a final drop concomitant with prepartal luteolysis, they are obviously of luteal origin (Hoffmann *et al*., 1994). Thus, in the bitch a significant role of estrogens for parturition related processes is rather unlikely, and the question arises which other factors in the dog serve the roles exerted by placental estrogens in ungulates or primates. In domestic animals placental estrogens are commonly considered as important factors which, prior to parturition, stimulate the softening of the birth canal and induce myometrial excitability and the release of uterotonic prostaglandins. However, a substantial increase in bioactive free estrogens in maternal blood during the immediate prepartal period has only been found in a rather limited number of species such as the sheep (Challis, 1971; Tsang, 1974) or goat (Sawada *et al*., 1995; Probo *et al*., 2011). In other species such as cattle (Hoffmann *et al*., 1997; Shenavai *et al*., 2010, 2012) or camelids (Leon *et al*., 1990; Riveros *et al*., 2009) free estrogens increase, if at all, only moderately throughout late gestation or even decrease markedly prior to parturition as observed in horses and donkeys (Hoffmann *et al*., 1996, 2014). However, as demonstrated in cows (Greven *et al*., 2007; Khatri *et al*., 2011; Polei *et al*., 2014) placental estrogens may be subject to significant metabolism in the pregnant uterus and as the bioactivity of estrogens could be controlled locally in potential target cells (Mueller *et al*., 2015), profiles in maternal blood may not necessarily reflect their local activities. In those ungulates which have been investigated so far during the prepartal period, placental estrogens occur in the maternal circulation predominantly as sulfonated forms and as free estrone, whereas estradiol-17β, the most potent endogenous estrogen, circulates only at much lower concentrations. Moreover, the accuracy of data on estradiol-17ß in pregnant ungulates remains generally unclear, as measurements were mostly performed using immunological methods in the presence of a large excess of other structurally closely related steroids. Thus, even in cases of a weak cross-reactivity of the applied antiserum with other estrogens, published concentrations of estradiol-17β may be considerably overestimated. Nevertheless, observations on estradiol-17β concentrations in maternal blood indicate that they generally exceed the levels of females at estrus and thus must be considered as biologically relevant. Observations in cattle, sheep and goats provide evidence that in these species the udder contributes significantly to estradiol-17ß levels circulating in the maternal compartment during late gestation and parturition, and CYP19A1 activity was demonstrated in mammary gland tissue *in vitro* (Maule Walker *et al*., 1983; Peaker and Taylor, 1990; Janowski *et al*., 2002). The fact that estradiol-17β concentrations decline rapidly after parturition suggests that the mammary gland could utilize precursors provided by the placenta.

Observations after the experimental elimination of placental estrogen synthesis or blocking of estrogen effects around parturition are rare and partly controversial. Pashen and Allen (1979) gonadectomized equine fetuses between days 197 and 251, which led to an immediate drop of maternal free and conjugated estrogens to basal levels, followed by low estrogen levels throughout the remaining time of pregnancy. In the treated mares parturition started spontaneously. However, uterine contractions were described as weak and inefficient. Correspondingly, the explosive increase of maternal PGFM levels normally occurring in mares during labor was virtually absent. In a different experimental approach Esteller-Vico *et al*. (2017) applied the CYP19A1 inhibitor letrozole to block placental estrogen synthesis in mares throughout the last trimester. However, no cases of dystocia were reported in treated mares, possibly due to the fact that the treatment significantly reduced maternal estrogen levels but blockage of estrogen production was still incomplete. This observation suggests that placental estrogens may have permissive roles, rather than being a regulatory factor and symptoms of deficit may only occur in cases of a virtually complete withdrawal. To elucidate the role of placental estrogens during the initiation of parturition in goats, Currie *et al*. (1976) applied estradiol-17β to late pregnant does. The treatment induced a release of prostaglandin F, regression of corpora lutea, lactogenesis and premature parturition. However, it remains unclear whether the estradiol-17β concentrations in treated animals were in a physiological range and whether the effects induced by the treatment followed the sequence of signals initiating spontaneous parturition.

Relaxin

As discussed earlier (see section: Relaxin/insulin-like family peptides), in many mammalian species pregnancy-associated increases of maternal RLN concentrations have been measured with the ovary and/or the placenta being the relevant source(s) depending on the individual species and stage of gestation. On the one hand, together with progesterone, generally RLN is considered as a pregnancy-retaining factor. On the other hand, towards the end of gestation, RLN is considered important for the preparation of the pelvic symphysis and the birth canal for parturition. The softening effect on connective tissue is obviously resulting from remodeling of the extracellular matrix brought about by the increased activity of collagenases (Bathgate *et al*., 2013; Klein, 2016).

Conclusions

Despite many studies in the field our knowledge on the endocrinology of pregnancy and parturition including the “placental endocrine function” is still very fragmentary or virtually lacking in most species, with the exception of the sheep. Due to

significant species specific particularities, concepts put forward based on observations in a certain species may be largely inappropriate in others. The complex interdependencies in the endocrinology of pregnancy and parturition frequently involving different compartments and intricate hormone systems, which are themselves composed of several ligands and receptor types, are difficult to elucidate. Moreover, hormone measurements in the systemic fetal or maternal circulation may not provide the appropriate information about local roles of hormones, which may depend on local mechanisms of transport, activation of inactive precursors or inactivation. Although some aspects may be successfully studied in the refined *in vitro* models nowadays available, for a significant progress in the field animal experiments performed in the respective target species are still considered indispensable. However, experiments in livestock animals are very expensive due to the high costs for the necessary facilities, purchase and keeping of experimental animals or large amounts of compounds applied, e.g. enzyme inhibitors or receptor blockers. Significant progress may be expected from the availability of new or refined analytical methods, e.g. mass spectrometry based methods for the specific simultaneous determination of multiple analytes in blood and tissues, or new efficient technologies to specifically suppress the expression of target genes in the placenta.

Acknowledgments

Recent reviews have been preferentially cited to avoid an exhaustive list of primary research reports. We apologize to scientists who have significantly contributed to the research in the fields addressed and whose work has not been cited. Nevertheless, their contributions to our current knowledge are highly appreciated.

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DOI: 10.21451/1984-3143-AR2018-0014

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Cellular events during ovine implantation and impact for gestation

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Abstract

The establishment of pregnancy in sheep includes elongation of the blastocyst into a filamentous conceptus, pregnancy recognition, production of histotroph, attachment of the conceptus to the endometrium for implantation, and development of synepitheliochorial placentation. These processes are complex, and this review describes some of the molecular events that underlie and support successful pregnancy. The free-floating sheep blastocyst elongates into a filamentous conceptus and metabolizes, or is responsive to, molecules supplied by the endometrium as histotroph. Amongst these molecules are SPP1, glucose and fructose, and arginine that stimulate the MTOR nutrient sensing system. The placental trophectoderm of elongating conceptuses initiate pregnancy recognition and implantation. The mononucleate cells of the trophectoderm secrete IFNT, which acts on the endometrial LE to block increases in estrogen receptor α to preclude oxytocin receptor expression, thereby preventing oxytocin from inducing luteolytic pulses of PGF2α. In addition, IFNT increases expression of IFN stimulated genes in the endometrial stroma, including ISG15, a functional ubiquitin homologue. Implantation is the initial step in placentation, and includes sequential pre-contact, apposition, and adhesion phases. Implantation in sheep includes downregulation of Muc1 and interaction of GLYCAM1, galectin 15 (LGALS15) and SPP1 with lectins and integrins (αvβ3). Sheep have synepitheliochorial placentation in which mononucleate trophectoderm cells fuse to form binucleate cells (BNCs). BNCs migrate and fuse with endometrial LE cells to form trinucleate syncytial cells, and these syncytia enlarge through continued BNC fusion to form syncytial plaques that form the interface between endometrial and placental tissues within the placentome. The placentae of sheep organize into placentomal and interplacentomal regions. In placentomes there is extensive interdigitation of endometrial and placental tissues to provide hemotrophic nutrition to the fetus. In interplacentomal regions there is epitheliochorial attachment of endometrial LE to trophectoderm, mediated through focal adhesion assembly, and areolae that take up histotroph secreted by endometrial GE.

Keywords: conceptus, endometrium, placentation, pregnancy, sheep.

Introduction

Domestic animal models for research are generally underappreciated (Roberts *et al*., 2009); however, sheep offer unique characteristics of pregnancy, as compared to rodent or primate models, and studies of sheep have provided significant insights into the physiology of implantation including: 1) elongation of the blastocyst into a filamentous conceptus; 2) the protracted peri-implantation period of pregnancy when the conceptus is free within the uterine lumen requiring extensive paracrine signaling between conceptus and endometrium, as well as nutritional support provided by uterine secretions; 3) a protracted and incremental attachment cascade of trophectoderm to endometrial epithelium during implantation; and 4) development of a synepitheliochorial placenta that utilizes extensive endometrial and placental vasculatures for hemotrophic nutrition, and placental areolae for histotrophic support of the developing fetuses. Our understanding of the complex molecular events that underlie successful pregnancy recognition in ruminants, the attachment phase of implantation that occurs across all species, and placentation in livestock species have been, and will likely continue to be, advanced by studies of sheep as agricultural and biomedical research models.

Elongation of the blastocyst into a filamentous conceptus

The early stages of embryonic development in sheep proceed in a manner similar to other mammalian species (Fig. 1). After fertilization within the oviduct, the zygote undergoes the first cleavage division to form the 2-cell embryo, and cleavage divisions continue through the 8-16 cell stage, when transcriptome activation occurs. These divisions culminate in formation of the solid mass of cells, called the morula (16-32 cells), that remains encased in the zona pellucida of the original oocyte. The morula remains in the oviduct before entering the uterus on day 3 or 4 in sheep. Embryos of most species fail to develop beyond the early blastocyst stage if confined to the oviduct, and it is speculated that is due to the absence of critical factors required for embryonic development that are supplied by the uterus. The developing embryo next forms the blastocyst by day 6. At this point the pluripotent blastomeres begin to differentiate into the

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Received: January 31, 2018
Accepted: April 17, 2018

inner cell mass (ICM) and trophectoderm to begin the cell lineages that will eventually become the embryo/fetus proper (primitive ectoderm, mesoderm and endoderm), and the placental trophectoderm/chorion, respectively, and are collectively termed the conceptus (Bazer *et al*., 2005). The blastocyst hatches out of the zona pellucida between days 8 and 9 (200 µm in diameter and containing about 300 cells), and increases in size (400-900 µm in diameter and containing about 400-900 cells) before undergoing a rapid morphological transition called elongation (Fig. 1). The small spherical conceptus grows into a tubular form by day 11, followed by a phase of rapid growth and elongation between days 12 and 16 to form the mature filamentous conceptus (10-22 mm on day 12, 10 cm on day 14, and 25 cm on day 17). During the early elongation period, the conceptus remains unattached to the uterine endometrium and dependent on nutrients in the uterine lumen. Conceptus elongation substantially increases the surface area of placental trophectoderm that will subsequently directly attach to, and interact in a close paracrine manner with the uterus. This provides increased surface area for nutrient exchange between the conceptus and endometrium, and maximize the paracrine effects of the conceptus to prevent luteolysis for pregnancy recognition (Spencer *et al*., 2004a).

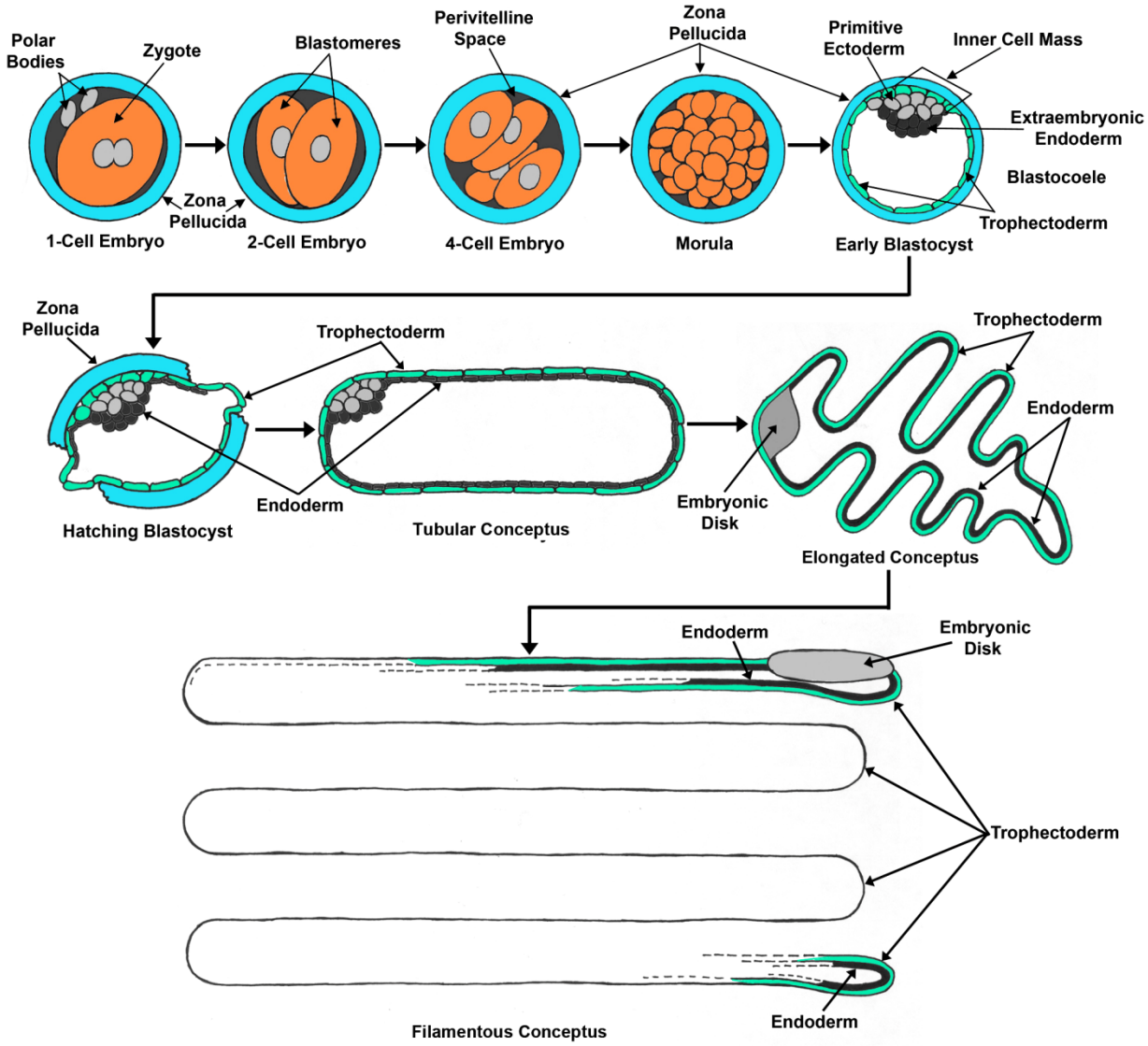

Figure 1. Elongation of the blastocyst into a filamentous conceptus. Sheep embryos enter the uterus at about day 3 or 4, develop into spherical blastocysts and then, after hatching from the zona pellucida, transform from spherical to tubular and filamentous conceptuses (embryos and associated placental membranes) between days 12 and 15 of pregnancy, with placental membranes extending into the contralateral uterine horn between days 16 and 20 (Bazer *et al*., 2005). Conceptus elongation ensures maximum area of surface contact between the conceptus trophectoderm and endometrial luminal epithelium (LE).

As sheep conceptuses elongate they metabolize, or are responsive to, significant concentrations of molecules supplied in the form of histotroph, a complex mixture of hormones, enzymes, growth factors, cytokines, transport proteins, adhesion factors, nutrients and other substances that plays roles in

conceptus nourishment, implantation and placentation. Mammalian cell growth in general, and particularly in cells of the conceptus, is regulated by growth factors and the availability of nutrients. The mechanistic mammalian target of rapamycin (MTOR) cell signaling pathway plays an important role in regulation of cell growth and metabolism in response to growth factors and nutritional status to affect biological and physiological responses of cells and organs. The MTOR pathway is a nutrient sensing system stimulated by molecules that include Secreted Phosphoprotein 1 (SPP1), also called osteopontin (OPN), the hexose sugars glucose and fructose, and select amino acids, including arginine (Nielsen *et al*., 1995; Martin and Sutherland, 2001; Kim *et al*., 2010), to support blastocyst/conceptus development. The MTOR cell signaling pathway is a prominent component of the peri-implantation intra-uterine environment in sheep (summarized in Bazer *et al*., 2012b). FK506-binding protein 12-rapamycin complex-associated protein 1 (FRAP1), G protein β subunit-like (LST8), mitogen-activated protein kinase-associated protein 1 (MAPKAP1), regulatory-associated protein of mechanistic mammalian target of rapamycin (RAPTOR), rapamycin-insensitive companion of mechanistic mammalian target of rapamycin (RICTOR), tuberous sclerosis 1 (TSC1), tuberous sclerosis 2 (TSC2), ras homolog enriched in brain (RHEB) and eukaryotic translation initiation factor 4E-binding protein 1 (EIF4EBP1) are expressed by the endometrium and trophectoderm of sheep conceptuses between days 13 and 18 of pregnancy, and increases in abundance of RICTOR, RHEB, EIF4EBP1 and RHEB are coordinate with rapid growth and development of ovine conceptuses during the peri-implantation period (Gao *et al*., 2009a). Further, progesterone (the hormone of pregnancy) and interferon tau (IFNT; the pregnancy recognition signal in sheep) stimulate expression of RHEB and EIF4EBP1 in the endometrium of sheep (Gao *et al*., 2009a), and MTORC1 is abundant in the cytoplasm, and phosphorylated MTOR is abundant in the nuclei of sheep trophectoderm cells (oTr; Kim *et al*., 2011b).

SPP1 is a prominent component of the uterine environment during pregnancy in sheep (Johnson *et al*., 2014). SPP1 induces motility in human trophectoderm cells through MTOR signaling (Al-Shami *et al*., 2005) and rapamycin inhibits F-actin reorganization and phosphorylation of focal adhesion proteins stimulated by IGF1 (insulin-like growth factor 1) such as focal adhesion kinase (FAK; Liu *et al*., 2008). Those results suggested a role for SPP1-induced MTOR complex signaling during conceptus elongation in sheep. Therefore, we identified relationships and crosstalk between multiple membrane and intracellular cell signaling cascades activated by SPP1, including MTOR and integrin binding to ovine trophectoderm cells. These interaction potentially participate in controlling proliferation, migration and attachment of oTr cells of the conceptus to endometrial luminal epithelium (LE) during the peri-implantation period of pregnancy (Kim *et al*., 2010). Results of these studies demonstrated that SPP1 binds αvβ3 and possibly α5β1 integrin heterodimers to induce focal adhesion assembly, a prerequisite for adhesion and migration of oTr cells, through activation of: 1) P70S6K via crosstalk between FRAP1/MTOR and MAPK pathways; 2) MTOR, phosphoinositide 3 kinase (PI3K), MAPK3/MAPK1 (ERK1/2) and MAPK14 (P38) signaling to stimulate oTr cell migration; and 3) focal adhesion assembly and myosin II motor activity to induce migration of oTr cells (Kim *et al*., 2010).

Arginine is a prominent component of the uterine environment during pregnancy in sheep, and is highly stimulatory to proliferation, migration and protein synthesis in an established oTr ovine cell line (Kim *et al*., 2011a, b); therefore, pathways whereby arginine mediates it effects in oTr cells were studied. The major findings were that arginine: 1) increases phosphorylation of ribosomal protein S6 kinase (RPS6K) in a dose dependent manner; 2) increases phosphorylated forms of RAC-alpha serine/threonine-protein kinase (AKT1), RPS6K and RPS6 over basal levels 3) increases nuclear phosphorylated RPS6K and cytoplasmic phosphorylated RPS6; and 4) stimulates proliferation and migration of oTr cells (Kim *et al*., 2011a). Further, phosphorylation of RPS6K and RPS6 is blocked by inhibitors of both PI3K and MTOR cell signaling, and L-arginine, but not D-arginine, activates MTOR cell signaling via phosphorylation of RPS6K and RPS6 (Kim *et al*., 2011b). Experiments were also conducted to determine whether effects of arginine on cell proliferation are due to its metabolism to nitric oxide (NO) via NO synthase 1/2 (NOS1/NOS2) or due to its metabolism by arginase to ornithine which is converted by ornithine decarboxylase 1 (ODC1) to the polyamines putrescine, spermidine and spermine (Kim *et al*., 2011c). Two NO donors, S-nitroso-N-acetyl-DL-penicillamine (SNAP) and diethylenetriamine NONOate (DETA), increased proliferation of oTr cells as did putrescine. Both N-nitro-L-arginine methyl ester hydrochloride (L-NAME: a NOS inhibitor to reduce NO synthesis) and N-hydroxy-nor-L-arginine (nor-NOHA; an arginase inhibitor to block synthesis of putrescine) decreased oTr cell proliferation. Therefore, both NO and polyamines can stimulate proliferation and migration of trophectoderm cells essential to conceptus elongation in sheep. In practice, exogenous administration of arginine enhances embryonic survival in sheep, as in other mammals such as pigs, mice and rats (Wu *et al*., 2013; Wu *et al*., 2017).

In sheep uteri, total recoverable glucose increases 12-fold between days 10 and 15 of pregnancy, glucose transporter 1 (GLUT1) increases in the endometrium of ovariectomized sheep in response to progesterone and an additional 2.1-fold in response to IFNT, and GLUT3 is expressed by conceptus trophectoderm (Gao *et al*., 2009b). In addition, fructose is detected in uterine flushings of pregnant gilts as early as day 12 of pregnancy and maximum concentrations of fructose rise to between 11.1 and 33 mM (Bazer *et al*., 2011; Kim *et al*., 2012). Further, the glucose that is not metabolized via metabolic pathways for production of ATP (glycolysis) or pentose phosphate pathway products in sheep is converted to fructose by the

trophectoderm. Using our oTr cells we found: 1) that fructose and glucose are equivalent in stimulating cell proliferation via the MTOR pathway; 2) that phosphorylation of RPS6K and EIF4EBP1 in response to fructose requires both PI3K and MTOR, and glutamine-fructose-6-phosphate transaminase 1 (GFPT1); and 3) that inhibition of the hexosamine biosynthesis pathway by azaserine blocks MTOR-RPS6K and MTOR-EIF4EBP1 signaling and the ability of fructose to stimulate proliferation of oTr cells (Wang *et al*., 2016a). We now propose that fructose and glucose support rapid growth and development of the sheep placenta through a process whereby trophectoderm cells enter into the serinogenesis pathway for one-carbon metabolism for synthesis of: 1) purines, required for the synthesis of nucleotides and nucleic acids; 2) thymidine, required for the synthesis of DNA; and 3) S-adenosylmethionine (SAM), the principal biological methylating agent for epigenetic modifications. Due to the possibility that oTr cells in culture may have an altered phenotype, effects of glucose were evaluated using day 16 sheep conceptus explant cultures. Glucose stimulated increased abundance of total and phosphorylated forms of the MTOR cell signaling pathway proteins, as well as ODC1, NOS2 and GTP cyclohydrolase 1 (GCH1) proteins (Kim *et al*., 2011c).

Pregnancy recognition, production of histotroph, and induction of classical IFN stimulated genes (ISGs) in the endometrium

The peri-implantation period of mammals is complex, involving the overlapping events of pregnancy recognition and remodeling for implantation/placentation necessary for embryonic survival during early pregnancy. During early pregnancy in sheep, the mononuclear cells of the placental trophectoderm synthesize and secrete IFNT, the signal for maternal recognition of pregnancy (Spencer *et al*., 2004a). IFNT acts on the endometrial LE and superficial glandular epithelium (sGE) to block increases in transcription of estrogen receptor α to preclude estrogen receptor α interactions with Sp1 and/or AP-1 that otherwise stimulate oxytocin receptor expression, thereby preventing oxytocin from inducing release of luteolytic pulses of prostaglandin $F_2\alpha$ (Fig. 2A and 2B; Fleming *et al*., 2005). This results in maintenance of the corpus luteum, the source of progesterone required for successful pregnancy (Spencer *et al*., 2004a). During the period of pregnancy recognition, progesterone down-regulates expression of progesterone receptors in the endometrial LE and glandular epithelium (GE). The loss of progesterone receptor expression by these epithelia appears to be prerequisite for progesterone to stimulate production and secretion of histotroph, a mixture of hormones, growth factors, nutrients, and other substances required for growth and development of the conceptus and implantation (Fig. 2A and 2C; Bazer *et al*., 2012a). The consensus is that the role of progesterone in producing histotroph is mediated via progesterone receptor (PGR); however, PGR are not expressed in uterine epithelia that secrete histotroph during the peri-implantation period (Spencer *et al*., 2007). It is clear that regulation of gene expression in the endometrium by progesterone during the peri-implantation period is complex. Induction of genes in uterine epithelia may require that progesterone down-regulate PGR, thereby eliminating PGR-dependent inhibition of expression of progesterone-regulated genes. However, another explanation is that progesterone induction of expression of genes in uterine epithelia is mediated by a paracrine-acting factor(s) (progestamedin) produced by the PGR-positive stromal cells (Spencer *et al*., 2007). The endometrial GE have primary responsibility for the production of histotroph in sheep. Indeed, uterine gland knockout UGKO) ewes lack endometrial GE and exhibit a peri-implantation defect and loss of pregnancy that is associated with the absence of the synthesis and secretion of key components of histotroph (Gray *et al*., 2001, 2002).

In addition to its antiluteolytic effects, IFNT also increases expression of several ISGs in the stroma and GE of the sheep endometrium (Fig. 2A and 2D). The list of ISGs known to be upregulated in the endometrium of sheep has grown from one (ISG15; Johnson *et al*., 1999c), to 15 that have actually been localized to the endometrial stroma of sheep (reviewed in Spencer *et al*., 2007; genes listed in Johnson *et al*., 2008; Hansen *et al*., 2017). Although the temporal and spatial expression within the endometrial stroma of pregnant sheep varies slightly among genes, they for the most part follow the expression pattern first described for ISG15 (Johnson *et al*., 1999c). ISG15 is first detectable in the endometria LE and stratum compactum stroma on day 13 of pregnancy (immediately prior to implantation); then expression extends to the stratum spongiosum stroma by day 15 (time of implantation). Expression is maintained throughout the stroma through day 25, then declines by day 30 of pregnancy, with expression limited to patches of the stratum compactum stroma along the maternal-conceptus interface where it remains throughout pregnancy (Johnson *et al*., 1999c; Joyce *et al*., 2005). Interestingly, most classical ISGs, including ISG15, are not induced or upregulated by IFNT in the endometrial LE of the sheep endometrium during early pregnancy, most likely due to the expression of interferon regulatory factor 2 (IRF2) in the endometrial LE (Choi *et al*., 2001). IRF2, a potent transcriptional repressor of ISGs (Taniguchi *et al*., 2001), is expressed specifically in the endometrial LE and represses activity of IFN-stimulated response element (ISRE)-containing promoters. It is hypothesized that the lack of ISG induction and silencing of ISGs, such as major histocompatability complex class 1 (MHC1) and beta 2 microglobulin (B2M), may be involved in the prevention of immune rejection of the semi-allogeneic conceptus (Choi *et al.* 2003; Joyce *et al*., 2008). MHC1 molecules are polymorphic cell surface glycoproteins expressed on most somatic cells that present peptide antigens derived from self proteins or from proteins of intracellular pathogens to cytotoxic T lymphocytes; therefore, they are involved in immune recognition of foreign pathogens and transplanted

allogeneic tissues. The laws of transplantation biology dictate rejection of the conceptus as a semiallogeneic tissue with paternal as well as maternal histocompatibility antigens. It is reasonable to project that downregulation of these molecules in the endometrium benefits pregnancy by blinding cytotoxic T lymphocytes to the presence of the foreign alloantigens within the conceptus trophectoderm to prevent immune rejection (Joyce *et al*., 2008).

At present, we can only speculate on the roles of classical ISGs within the pregnant endometrium. The best characterized of these genes, ISG15 is a functional ubiquitin homologue that has the C-terminus Leu-Arg-Gly-Gly amino acid sequence common to ubiquitin, allowing conjugation to intracellular proteins (Haas *et al*., 1987). Conjugation of proteins either targets them for rapid degradation in the proteasome, or stabilizes the proteins for long-term modification (Wilkinson, 2000). ISG15 does indeed form stable conjugates with endometrial proteins of sheep and cows, indicating a biologically active molecule that is responsive to the IFNT signal from the trophectoderm that can temporally target proteins for pregnancy-associated regulation and/or modification (Johnson *et al*., 1998; Joyce *et al*., 2005). ISG15 is expressed in the decidua of mice (Austin *et al*., 2004; Bebington *et al*., 1999a), and women (Bebington *et al*., 1999b), and in the endometrial stroma of pigs (Johnson *et al*., 2009). The decidua has been hypothesized to play roles in hormone secretion, conceptus nutrition, fetal allograft protection, uterine remodeling, limiting of conceptus trophectoderm invasion, and as a defender against infectious inflammatory insults through generation of a local cytokine environment. Therefore it is possible that ISG15 conjugation/ISGylation in decidua is involved in one or more of these processes (Hansen and Pru, 2014).

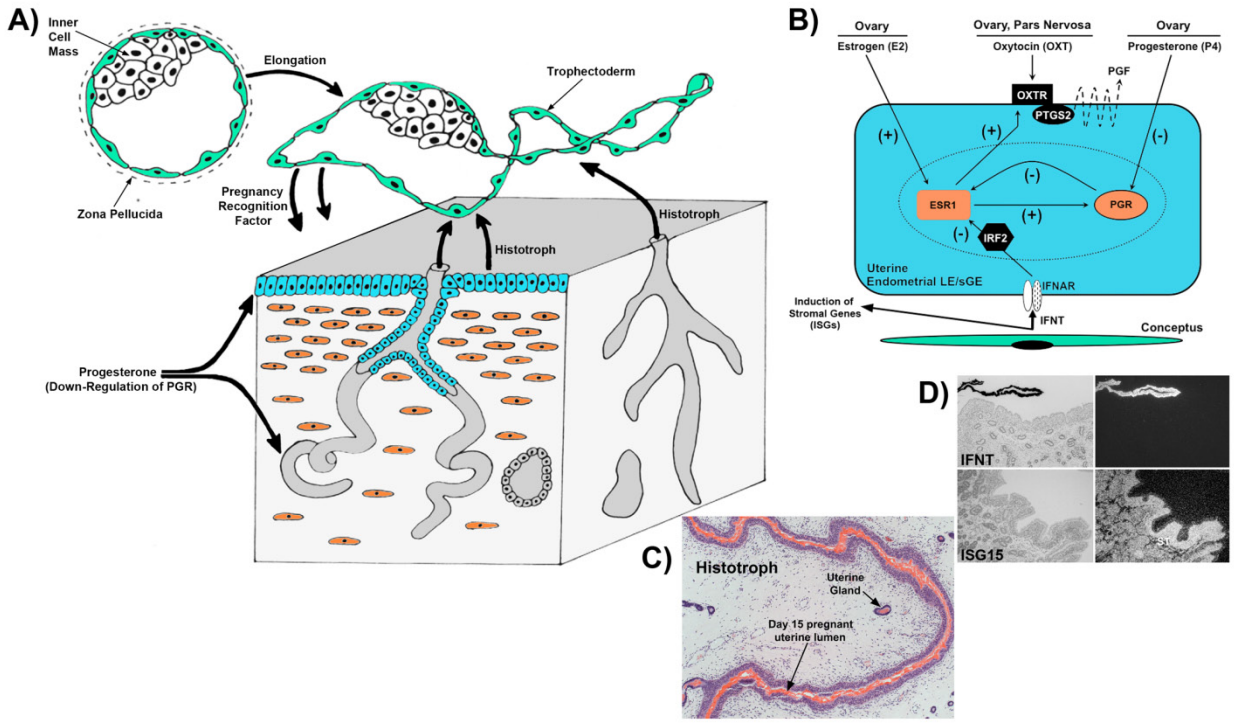

Figure 2. Pregnancy recognition, production of histotroph, and induction of classical IFN-stimulated genes. A) Generalized diagram of paracrine effects between the conceptus (primarily the pregnancy recognition signal IFNT) and the endometrium. B) Model for pregnancy recognition in the sheep in which IFNT silences expression of estrogen receptor α and this prevents expression of estrogen dependent expression of oxytocin receptor. C) Hematoxylin and Eosin (H&E) staining illustrating protein (histotroph) in the endometrial lumen of a pregnant sheep. D) *In situ* hybridization illustrating the expression of IFNT by conceptus trophectoderm and induction of ISG15 in the endometrium.

Attachment cascade of the conceptus to the endometrium for implantation

Implantation is the initial step in placentation, and proper attachment of the conceptus to the endometrium is critical to successful pregnancy (Denker, 1993; Burghardt *et al*., 2002). The process of implantation is highly synchronized, requiring reciprocal secretory and physical interactions between a developmentally competent conceptus and the endometrium during a restricted period of the uterine cycle termed the window of receptivity. These initial interactions between the apical surfaces of endometrial LE and conceptus trophectoderm cells follow an attachment cascade. This attachment cascade is similar to that observed during the extravasation of white blood cells from the vasculature through the endothelium and into connective tissues, and includes sequential non-adhesive or pre-contact, apposition, and adhesion phases, resulting in formation of a placenta that supports fetal-placental development throughout pregnancy (Fig. 3; Carson *et al*., 2000). Conceptus attachment first

requires the removal of large mucins from the glycocalyx of the endometrial LE that would otherwise sterically inhibit adhesion (Aplin *et al*., 2001). The removal of these mucins allows for direct physical interactions between carbohydrates and lectins at the apical surfaces of the opposing endometrial LE and conceptus trophectoderm cells (Kimber *et al*., 1995). These low affinity contacts are then replaced by firm adhesion between integrins and extracellular matrix (ECM) proteins (Burghardt *et al*., 2002; Lessey, 2002).

The term implantation is somewhat a misnomer for the sheep, but nevertheless, it is used to describe the initial stages of placentation in the livestock species. In sheep, the filamentous conceptus is closely associated with the endometrial LE and appears to be immobilized within the uterine lumen by day 14, although the conceptus can still be recovered intact from the uterus by lavage with only superficial damage. Apposition begins near the inner cell mass, and spreads towards the ends of the elongated conceptus, and by day 16 the conceptus trophectoderm is firmly attached to the endometrial LE with significant interdigitation between the microvilli on endometrial LE and conceptus trophectoderm cells, as well as between placental papillae that extend down into the lumen of the ducts of endometrial glands. The conceptus attaches to both the caruncular and intercaruncular regions of the endometrium, and attachment is complete by day 22 (Guillomot *et al*., 1981; Spencer *et al*., 2004b). The current consensus for the attachment cascade in sheep includes downregulation of mucin 1 (MUC1) across the entire endometrial surface, which unmasks glycosylation dependent cell adhesion molecule 1 (GlyCAM-1), LGALS15 and SPP1 for interaction with lectins and integrins. Initial attachment is likely mediated by GLYCAM1 and galectin-15, and firm attachment is likely mediated by SPP1 (Spencer *et al*., 1999, 2004b; Johnson *et al*., 1999a, 2001, 2003, 2014; Gray *et al*., 2004; Muñiz *et al*., 2006). Although progesterone downregulates Muc1 in pigs, progesterone does not appear to decrease Muc1 expression on the apical surface of the endometrial LE of sheep (Bowen *et al*., 1996; Johnson *et al*., 2001). Integrins are constitutively present on endometrial LE and conceptus trophectoderm during the peri-implantation period (Johnson *et al*., 2001). LGALS15 expression is induced by progesterone and LGALS15 expression is further increased by IFNT (Gray *et al*., 2004). SPP1 expression is induced by progesterone (Johnson *et al*., 2000).

Interestingly, SPP1 is not directly synthesized by the endometrial LE of sheep, but is a component of histotroph secreted from the endometrial GE into the uterine lumen of pregnant ewes as early as day13. It is not secreted by the endometrial GE of cyclic ewes (Johnson *et al*., 1999b, 1999a). SPP1 mRNA is expressed by some endometrial GE as early as day 13, and is present in the majority of the endometrial GE by day 19 of gestation (Johnson *et al*., 1999b). Progesterone induces expression of SPP1 in the endometrial GE, and induction is associated with a loss of progesterone receptor in the endometrial GE (Johnson *et al*., 2000). Analysis of uterine flushings from pregnant sheep identified a 45 kDa fragment of SPP1 with greater binding affinity for αvβ3 integrin receptor than native 70 kDa (Johnson *et al*., 2000; Senger and Perruzzi, 1996). This 45-kDa SPP1 cleavage fragment is exclusively, continuously, and abundantly present along the apical surface of the endometiral LE, on the apical surface to the trophectoderm/chorion, and along the entire uterine-placental interface through day 120 of pregnancy (Johnson *et al*., 2003). Comparison of the spatial distribution of SPP1 mRNA and protein by *in situ* hybridization and immunofluorescence analyses of cyclic and pregnant sheep uterine sections has provided significant insight into the physiology of endometrial SPP1 during pregnancy. SPP1 mRNA increases in the endometrial GE during the peri-implantation period; however, it is not present in endometrial LE or conceptus trophectoderm (Johnson *et al*., 1999b). In contrast, immunoreactive SPP1 protein is present at the apical surfaces of endometrial LE and GE, and on conceptus trophectoderm where the integrin subunits αv, α4, α5, β1, β3, and β5 could contribute to the assembly of several SPP1 receptors including αvβ3, αvβ1, αvβ5, α4β1, and α5β1 heterodimers which are expressed constitutively on the apical surfaces of conceptus trophectoderm and endometrial LE (Johnson *et al*., 1999a, 2001). Affinity chromatography and immunoprecipitation experiments have determined whether αv, α4, α5, β1, β3, β5, and β6 integrins expressed by oTr cells directly bind SPP1. Successful immunoprecipitation of labeled oTr integrins occurred with antibodies to αv and β3 integrin subunits, as well as an antibody to the integrin αvβ3 heterodimer. Antibody to the αv integrin subunit also precipitated a β chain, presumed to be the β3 integrin subunit, as an antibody to the β3 integrin subunit precipitated an α chain at the same relative size as the bands precipitated by an antibody to the αvβ3 heterodimer. Thus, the αvβ3 integrin on oTr cells binds SPP1 (Kim *et al*., 2010). SPP1 binding to the αvβ3 integrin receptor induced *in vitro* focal adhesion assembly, a prerequisite for adhesion and migration of trophectoderm, through activation of: 1) P70S6K via crosstalk between FRAP1/MTOR and MAPK pathways; 2) MTOR, PI3K, MAPK3/MAPK1 (Erk1/2) and MAPK14 (p38) signaling to stimulate trophectoderm cell migration; and 3) focal adhesion assembly and myosin II motor activity to induce migration of conceptus trophectoderm cells (Kim *et al*., 2010). Recently we reported that SPP1 binds integrins to form focal adhesions that activate the MTORC2 pathway for cytoskeletal reorganization in both adhered and migrating oTr cells, and that SPP1 cooperates with arginine to increase oTr cell adhesion and migration (Wang *et al*., 2016b). Collectively, results indicate that SPP1 binds αvβ3 integrin receptor to activate cell signaling pathways that act in concert to mediate adhesion, migration and cytoskeletal remodeling of conceptus trophectoderm cells essential for expansion and elongation of conceptuses and their attachment to endometrial LE for implantation (Johnson *et al*., 2014).

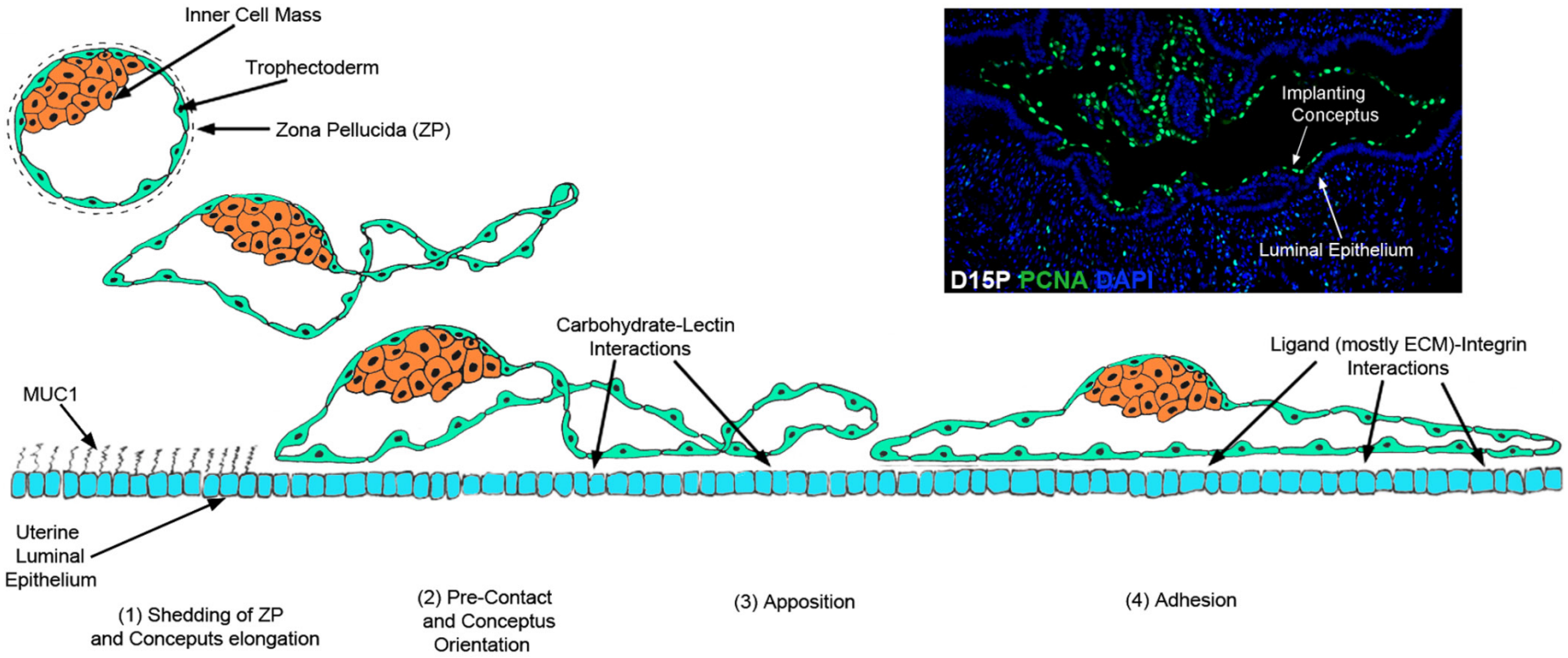

Figure 3. Attachment cascade of the conceptus to the endometrium for implantation. A generalized diagram of implantation in epitheliochorial and synepitheliochorial species. Implantation in sheep extends from days 11-16 and includes four phases that overlap and involve increasingly complex interactions between conceptus trophectoderm and endometrial luminal epithelium (LE). The inserted picture depicts immunostaining of proliferating trophectoderm cells (green color) at an implantation site. The current consensus for the attachment cascade in sheep includes downregulation of mucin 1 (MUC1) across the entire endometrial surface, which unmasks glycosylation dependent cell adhesion molecule 1 (GLYCAM1), galectin-15 and SPP1 for interaction with lectins and integrins. Initial attachment is likely mediated by GLYCAM1 and LGALS15, and firm attachment is likely mediated by SPP1 and the αvβ3 integrin receptor (Spencer *et al*., 2004).

Development of synepitheliochorial placentation

As ruminants, sheep demonstrate synepitheliochorial placentation in which fusion of conceptus trophectoderm with endometrial LE occurs. Two morphologically and functionally distinct cell types, mononucleate trophectoderm cells and binucleate trophoblast giant cells (BNCs), are present in the trophectoderm of ruminant placentae (Fig. 4). The mononucleate cells constitute the majority of the trophectoderm cells and BNCs begin to differentiate from the mononucleate trophectoderm cells in concert with trophectoderm outgrowth during conceptus elongation. BNCs first appear between days 14 and 16 of gestation in sheep conceptuses, and comprise 15-20% of the trophectoderm during the apposition and attachment phases of implantation. BNCs migrate and fuse with individual endometrial LE cells to form trinucleate syncytial cells, beginning on about day 16 of pregnancy in sheep, thereby assimilating the endometrial LE. The syncytia of sheep subsequently enlarge through continued BNC migration and fusion to form syncytial plaques (Fig. 4). The syncytial plaques are conceptus-maternal hybrid cells that are composed of endometrial LE cells and conceptus BNCs, and they eventually form the epithelial interface between endometrial and placental tissues within the placentome. In sheep, the syncytial plaques are a consistent feature in the placentomes throughout pregnancy (Wooding, 1984; Wooding and Burton, 2008).

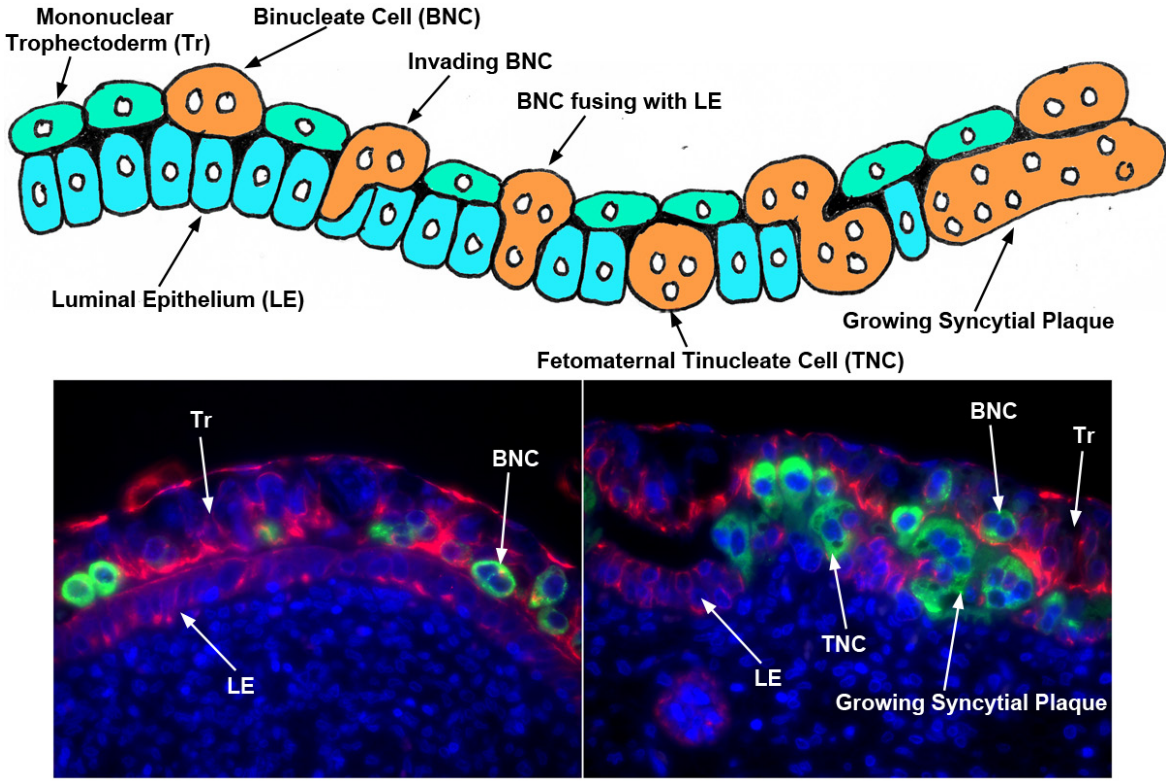

Figure 4. Syncytialization at the uterine-placental interface of sheep (Wooding *et al*., 1984; Wooding and Burton, 2008). Illustrated are a cartoon of the current consensus for syncytia formation in the sheep and immunofluorescence staining for pregnancy associated glycoprotein (PAG; Szafranska *et al*., 1995); stains BNCs, TNCs and syncytial plaques green) and CDH1 (epithelial cadherin (E-Cad); stains mononuclear Tr and endometrial LE cells red).

Following successful elongation of the conceptus, trophectoderm outgrowth, and implantation, the placentae of sheep organize into placentomal and interplacentomal regions (Fig. 5). During placentome development, highly branched villous placental folds, termed cotyledons, initially form by day 30 of gestation in sheep. Cotyledonary chorioallantoic villi lined by syncytial plaques then begin to protrude into crypts in the maternal endometrial caruncular tissue (aglandular areas of endometrium consisting of stroma covered by a single layer of epithelium), resulting in extensive interdigitation of endometrial and placental tissues by day 40. Placentomes provide a conduit for hemotrophic nutrition to the fetus where maternal and placental blood vessels are in close proximity for exchanging oxygen and micronutrients, and there is a close correlation between the placentomal mass and the birthweight of the fetus. In contrast, interplacentomal areas exhibit epitheliochorial attachment of endometrial LE to conceptus trophectoderm, and contain areolae that take up histotroph secreted by the endometrial GE for transport into placental vasculature that rings each of the areolae (Fig. 5; Wooding and Burton, 2008).

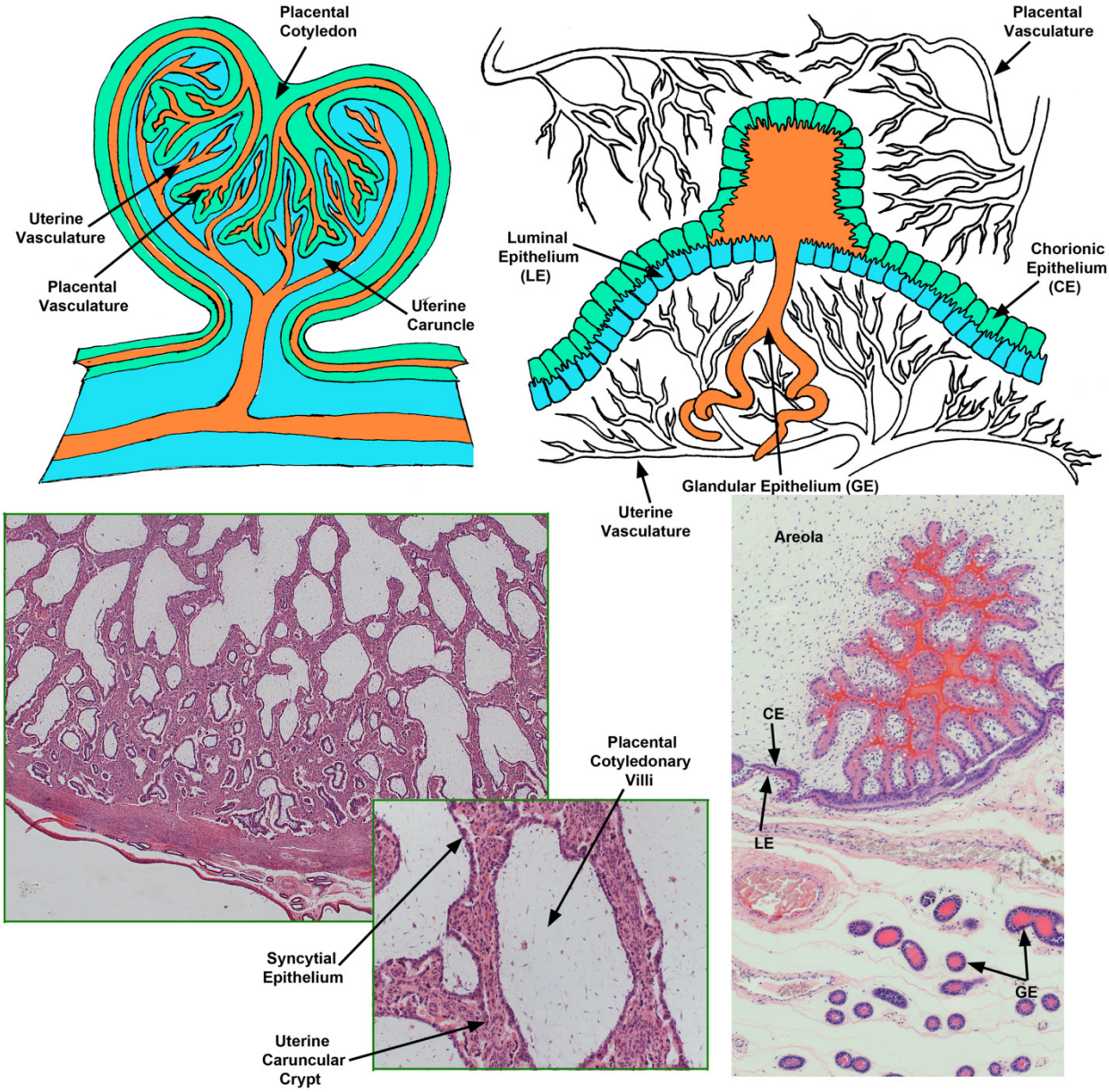

Figure 5. The endometrial-placental interface of mature placentation in the sheep illustrating the placentome and the areola. Illustrated are cartoons depicting the structure of the sheep placentome (Top Left) and areola (Top Right), and H&E staining of a paraffin embedded thin section of a placentome (Bottom Left) and an areola (Bottom Right).

Focal adhesions (FAs), the hallmark of activated integrins, are prominent structures of cells grown in culture; however, they are rarely observed *in vivo*. It is noteworthy that large aggregations of FA-associated proteins, that have been interpreted to be *in vivo* FAs, are present at the endometrial-placental interface of sheep (Johnson *et al*., 2003; Burghardt *et al*., 2009; Fig. 6). By day 40 of pregnancy in sheep, the punctate apical surface staining of integrin receptor subunits identified in peri-implantation endometrial LE and conceptus trophectoderm is replaced by scattered large aggregates of αv, α4, β1, and β5 subunits in interplacentomal endometrial LE and conceptus trophectoderm/chorion cells (Johnson *et al*., 2001; Burghardt *et al*., 2009). Integrin aggregates are observed only in the gravid uterine horns of unilaterally pregnant sheep, demonstrating a requirement for conceptus trophectoderm/chorion attachment to endometrial LE, and aggregates increase in number and size through day 120 of pregnancy. In some regions of the interplacentomal interface, greater subunit aggregation occurs on the endometrial side, in other regions it is predominant on the placental side; whereas in some other regions, both endometrial and placental epithelia exhibit prominent FAs. However, by day 120 of pregnancy, extensive FAs are present along most of the endometiral-placental interface (Burghardt *et al*., 2009). The placentomes, which provide hemotrophic support to the fetus and placenta, exhibited diffuse immunoreactivity for these integrins compared with interplacentomal regions possibly due to extensive folding at this interplacentomal interface and the 3D nature of the ECM within placentomes. It is noteworthy that interplacentomal endometrial stroma, only within the gravid horn of

unilaterally pregnant sheep, also exhibits robust but punctate immunostaining for αv and β3 integrins and ECM proteins including the native 70 kDa (rather than the 45 kDa fragment of SPP1), fibronectin, vitronectin and several other members of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family beginning around day 40 of pregnancy and increasing through day 120 (Burghardt *et al*., 2009). Stromal cells in this same tissue compartment of the gravid horn also exhibited upregulation of smooth muscle actin, desmin and vimentin indicative of myofibroblast differentiation. These stromal/myofibroblasts are surrounded by a connective tissue matrix that is more strain shielded due to crosslinking of ECM in three dimensions (3D) compared to the complex forces focused at the maternal conceptus interface (Burghardt *et al*., 2009). These results suggest that FA assembly at the endometial-placental interface and within placentomes and stromal compartments reflects dynamic adaptation to increasing forces caused by the growing conceptus. Cooperative binding of multiple integrins to SPP1 deposited at the endometrial-placental interface form a strong adhesive mosaic to maintain a tight connection and increased tensile strength and signaling activity between endometrial and placental surfaces along regions of epitheliochorial placentation in sheep.

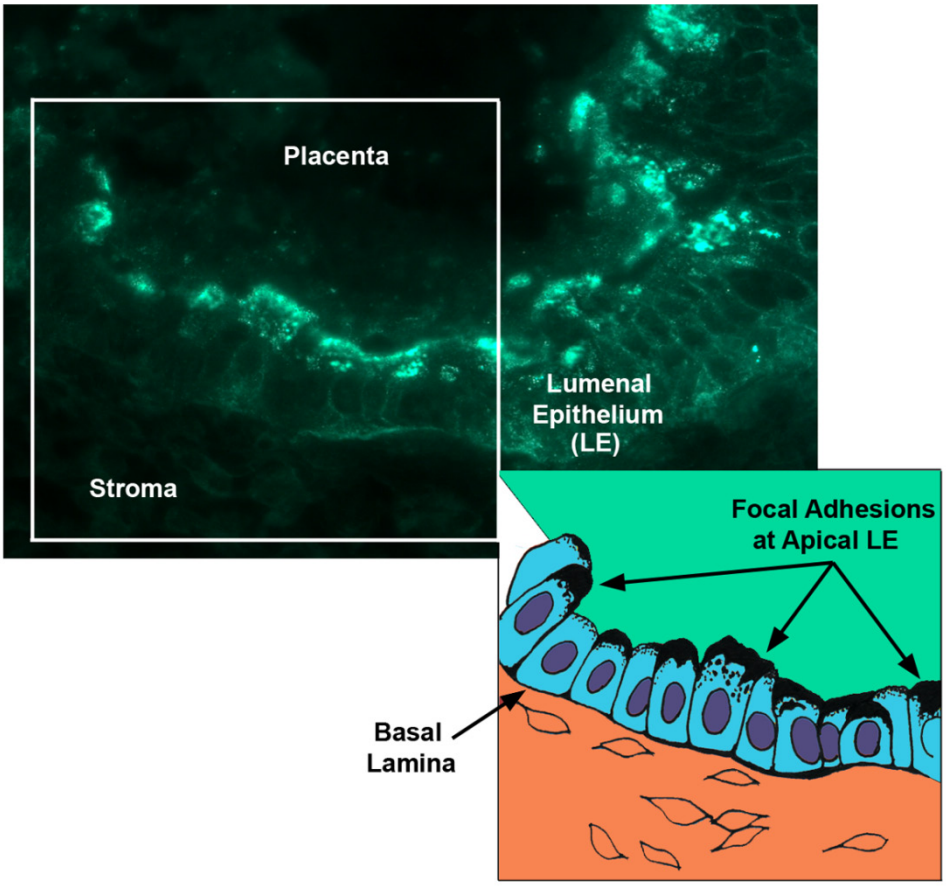

Figure 6. Focal Adhesions at the endometrial-placental interface of interplacentomal regions of placentation in the sheep (Burghardt *et al*., 2009). Illustrated is immunofluorescence staining (Top Panel) for αv integrin and a cartoon (Bottom Panel) depicting the localization of the focal adhesions that form at the interface between endometrial LE cells and the chorion.

Conclusions

Elongation and implantation in sheep are complex events that require significant energy, the substrates for which are primarily supplied as histotroph from the uterus. Embryonic mortality during this complex, energy consumptive, peri-implantation period of pregnancy remains a major constraint to improving reproductive efficiency and profitability in livestock enterprises. Sheep are a strong, if niche, livestock industry within the U.S., and consumer demand for lamb meat and wool products is strong. However, the U.S. sheep industry supplies less than half of this demand because reproductive inefficiency in ewes hampers the ability of the U.S. sheep industry to generate quality meat and wool at a viable profit margin (Shiflett *et al*., 2007). Further, sheep are a compelling animal model for the study of cow placental biology. Sheep are less expensive and more easily manipulated experimentally than cattle, while the formation of syncytia in ovine placentomes is thought to be very similar to the initial formation of the syncytial trophoblasts of cow placentae. In cattle, the fertilization rate is 90%, yet the calving rate to a single fertilization is only 55%. This constraint on cattle production is a considerable burden to the U.S. cattle industry. Our

understanding of the complex molecular events that underlie successful implantation and placentation across species has been and will likely continue to be advanced by studies of sheep as biomedical research models and to increase reproductive success in animal agriculture enterprises providing high quality protein for humans.

Acknowledgments

Research supported by USDA-NRICGP 98-35203-6337 to F.W.B. and R.C.B., NRSA-DHHS/NIH 1-F32-HDO 8501 O1A1 to G.A.J., USDA-NRI 2006-35203-17199 to G.A.J., USDA-NRI 2011-67015-20028 to F.W.B., G.A.J. and G.W., USDA-NRI 2015-67015-24958 to F.W.B., G.A.J. and G.W., and USDA-NRI 2016-67015-24955 to G.A.J. and F.W.B.

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DOI: 10.21451/1984-3143-AR2018-0047

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

New insights on the neuroendocrine control of puberty and seasonal breeding in female sheep

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Abstract

Timing of puberty has a great influence on animal productivity. For example, reproduction in sheep can be affected by seasonality, leading to fluctuations in availability of animal products. Therefore, optimization of birth dates would improve reproductive success in sheep. Since the discovery of the major role of kisspeptin and Kiss1R, its cognate receptor, in reproductive function, there are new opportunities for interventions. Repeated or continuous administration of native kisspeptin are able to hasten puberty and induce ovulation during breeding and non-breeding seasons of sheep. However, due to the short half-life of kisspeptin, protocols involving native kisspeptin are usually proof of concept, but not practical under field conditions. Consequently, there are efforts to develop kisspeptin analogues capable of replicating effects of repeated/continuous administration of native kisspeptin. In this review, we intended to provide a comprehensive summary of the neuroendocrine requirements for puberty onset and ovulation in adult ewes, focusing on kisspeptin, its physiological effects and responses to its analogues on reproductive function in ewes.

Keywords: kisspeptin, ovulation, puberty onset, sheep reproduction.

Introduction

In sheep, the onset of puberty occurs when there are metabolic cues that sufficient growth has occurred and when photoperiod becomes permissive. During this period, the hypothalamus become less sensitive to the negative feedback of estradiol (E2), stimulating increased pulse frequency for both gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH). This increase in GnRH/LH pulse frequency increases E2 production by growing ovarian follicles, inducing an LH surge and ovulation. The timing of puberty onset has a great influence on animal productivity. Hence, a detailed understanding of mechanisms underlying initiation of puberty represents an important target for animal reproduction management, with implications for treating disorders in humans linked to anticipated or delayed puberty.

There is a clear need to improve reproductive success in livestock to provide enough products (i.e. milk and meat) to sustain a world population expected to reach 10 billion people by 2050. As livestock reproduction can be affected by seasonality, leading to fluctuations in availability of animal products, induction of ovulation during the non-breeding season is of great importance, as well as ability to control ovulation during the breeding season.

Efforts to achieve this goal have resulted in the use of molecules that activates the hypothalamo-pituitary-gonadal axis such as synthetic GnRH agonists, extracts of the reproductive hormones from human or equine origin (e.g. human chorionic gonadotropin [hCG], human menopausal gonadotropin [hMG], and equine chorionic gonadotropin [eCG]) or synthetic steroid hormones. Specific methods applying these treatments have been developed for managing livestock reproduction. However, these treatments are not entirely satisfactory. Concerning small ruminants, GnRH agonists are used rarely or not at all. Although hCG and eCG are frequently used in reproductive management, they can induce antibodies which reduce their effectiveness. In addition, production of eCG, obtained from pregnant mares, is highly questioned by animal welfare organizations and by the European Union. Therefore, efficacious, animal welfare-friendly and cost-effective novel treatments are clearly needed to improve control of livestock reproduction.

New players in reproductive function

Among signals converging on GnRH neurons, and therefore involved in reproductive function, kisspeptin (Kp) is a recent and exciting discovery. In 2003, Kp was identified as a potent secretagogue of GnRH, based on mutation of its cognate receptor *Kiss1R* also named GPR54 (de Roux *et al*., 2003; Funes *et al*., 2003; Seminara *et al*., 2003), or of Kp gene itself (*Kiss1;* d'Anglemont de Tassigny *et al*., 2007; Dungan *et al*., 2007), resulting in hypogonadic hypogonadism and infertility. Conversely, gain-of-function mutations of *Kiss1R* cause precocious puberty (Teles *et al*., 2008).

Kisspeptins (Kps) are a group of peptides with varying numbers of amino acids (longest forms: Kp54 in human, Kp53 in sheep, or Kp52 in rodents, and smaller forms: Kp16, Kp14, Kp13 and Kp10), all derived from a common, 145 amino acid precursor. All Kps share the identical C-terminal 10 amino acids within each species, and representing the minimum endogenous sequence that activates the Kiss1R. The Kp10 sequence is relatively similar among species even if some variations can occur, suggesting a conserved physiological function (Oakley *et al*., 2009).

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Received: March 8, 2018
Accepted: June 25, 2018

The gonadotropin releasing action of Kp may be due to a direct stimulatory action upon GnRH neurons at the level of hypothalamus. In sheep, this hypothesis is supported by dramatic increases in GnRH concentrations in the cerebrospinal fluid, with a parallel rise in serum LH, after intracerebroventricular (icv) administration of Kp10 (Messager *et al*., 2005). In addition, peripheral Kp10 administration can stimulate GnRH secretion (Caraty *et al*., 2013). GnRH neurons extend complex, highly branched dendritic trees beyond the blood brain barrier (BBB) into the organum vasculosum of the lamina terminalis (OVLT; Herde *et al*., 2011). This suggest a possible additional site of action of Kp other than GnRH cell bodies, via terminals of GnRH neurons in the median eminence (ME) or OVLT.

An additional site of action at the level of pituitary has also been suggested (Richard *et al*., 2009; Gahete *et al*., 2016 for revue). In sheep, Kiss1R is present in pituitary, and LH secretion increased after addition of Kp10 to pituitary cell cultures. However, Kp10 failed to induce LH release in ewes with hypothalamo-pituirary disconnection, whereas GnRH induced a significant LH release (Smith *et al*., 2008b), questioning involvement of those receptors in LH secretion. In contrast, recent data, mostly from rodents, suggest a putative role of Kp at the level of ovary, controlling follicular development, oocyte maturation, steroidogenesis and ovulation (Hu *et al*., 2017 for revue). Similarly, in a recent study, there was enhanced *in-vitro* maturation of ovine oocytes when Kp10 was added to media supplemented with follicle-stimulating hormone (FSH), LH, and E2 (Byri *et al*., 2017).

In the hypothalamus, two distinct populations of neurons expressed Kps, the anteroventral periventricular or preoptic area (AVPV or POA) according to species, and the arcuate nucleus (ARC). These two populations are in close contact with GnRH cells (Kinoshita *et al*., 2005; Clarkson and Herbison, 2006) or their dendrons (Herde *et al*., 2011). A subpopulation of Kp neurons in the ARC have been described as co-expressing neurokinin B (NKB) and dynorphin (Dyn; Goodman *et al*., 2007) and were named KNDy neurons (Fig. 1).

NKB is also implicated in onset of puberty because mutation of *NKB* or its receptor (*NK3R*) blocked pubertal development in human (Topaloglu *et al*., 2009). In sheep, an agonist of NKBR, senktide, stimulated LH release (Nestor *et al*., 2012) whereas an antagonist of NKBR supressed GnRH/LH pulses (Clarke *et al*., 2018). In the presence of an NKBR antagonist, continuous Kp10 infusion restored GnRH/LH pulses, suggesting that Kp action is downstream of NKB signalling (Clarke *et al*., 2018). In addition, GnRH neurons do not express NK3R (Amstalden *et al*., 2010). Conversely, KNDy neurons express NK3R (Billings *et al*., 2010). These data supported the assertion that NKB acts in an autocrine/paracrine manner, indirectly influencing GnRH secretion.

Dyn, another co-expressed neuropeptide in the arcuate KNDy neurons, is an endogenous opioid peptide that selectively binds the k-opioid receptor (KOR). KOR is expressed in GnRH and KNDy neurons in ewes (Weems *et al*., 2016). There is strong evidence that Dyn tone terminates each GnRH pulse and limits amount of GnRH released during the secretory phase of the pulse (Goodman *et al*., 1995). Dyn has been implicated as a potential mediator of progesterone negative feedback effect on pulsatile GnRH secretion in ewes (Foradori *et al*., 2005) and prepubertal lambs (Lopez *et al*., 2016). However, whether this effect was due to Dyn secreted by KNDy neurons itself or by other populations, remains to be determined.

Corroborating the hypothesis of opposing effects of Dyn *vs*. Kp/NKB, Dyn expression is higher during the early follicular phase, whereas Kp/ NKB expression peak during the surge (Fergani *et al*., 2017). Based on these data, it has been suggested that KNDy neurons of the ARC nucleus could be the GnRH pulse generator.

Another recently discovered neuropeptide, GnIH (Gonadotropin-inhibitory hormone), may have a role in physiological control of reproduction, due to its inhibitory effect on GnRH release in quails (Tsutsui *et al*., 2000). However, effects of its mammalian ortholog, RFamide-related-peptide (RFRP), on GnRH/ gonadotropin secretion, is less evident. The *Rfrp* gene encodes RFRP-1, -2, and -3 peptides, but only RFRP-1 and RFRP-3 are functional peptides, with RFRP-1 stimulating prolactin secretion, and RFRP-3 modulating gonadotropin secretion. Its receptor, GPR147, was expressed in 15-33% of murine GnRH neurons (Rizwan *et al*., 2012) and in a subpopulation of Kp neurons in AVPV (5-16%) and ARC (25%; Poling *et al*., 2013). However, pubertal timing was not altered in *GPR147* KO mice (Leon *et al*., 2014) and the action of RFRP-3 on gonadotropin secretion seemed to be highly dependent on species, photoperiod, age, sex, and stage of cycle (Henningsen *et al*., 2016). It is noteworthy that RFRP-3 is sometimes inhibitory and sometimes stimulatory on LH secretion. In addition, Kp may act on GPR147, based on affinity of Kp10 for GPR147 (Roumeas *et al*., 2015).

In sheep, data were inconsistent, with an apparent inhibitory effect on LH pulse amplitude, total LH secretion, and the estrogen-induced LH surge after continuous iv infusion of RFRP-3 in ovariectomized ewes (Clarke *et al*., 2008), and a reduction of LH pulsatility during the follicular phase in intact ewes (Clarke *et al*., 2012). However, there is no association, either positive or negative, between endogenous RFRP-3 in portal blood and LH in peripheral blood (Smith *et al*., 2012). Similarly, others reported no effects (Decourt *et al*., 2016a). Further work will be necessary to establish the role, if any, of RFRP-3 in controlling sheep reproduction.

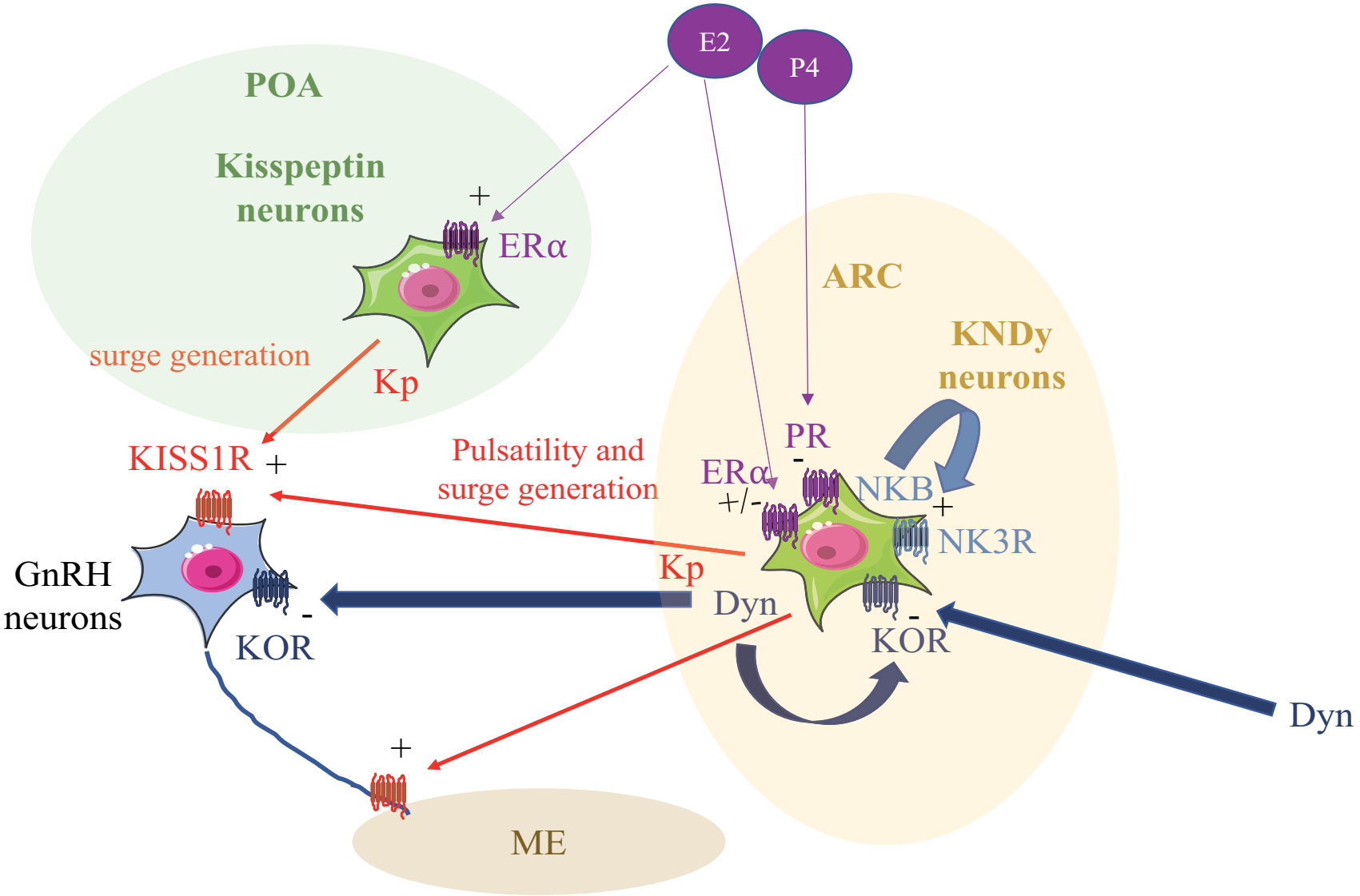

Figure1. Schematic representation of Kp (Kisspeptin) and KNDy (Kisspeptin, Neurokinin B, Dynorphin) neurons regulation in adult ewes. POA (Pre Optic Area), ARC (Arcuate nucleus), ME (Median Eminence), GnRH (Gonadotropin Releasing Hormone), NKB (Neurokinin B), Dyn (Dynorphin), E2 (17 β Estradiol), P4 (progesterone), KISS1R (Kp Receptor), NK3R (NKB Receptor), KOR (Dyn Receptor), ERα (Estrogen Receptor α), PR (Progesteron Receptor).

Regulation of steroids

GnRH neurons do not express estrogen receptor alpha (ERα Herbison and Pape, 2001) but are under estrogen positive and negative feedback. Kp neurons represent the link underlying feedback effects of steroids on GnRH secretion. The majority of Kp neurons express ERα (~90%; Smith *et al*., 2005a, b; Franceschini *et al*., 2006), but also androgen receptor (~65%; Smith *et al*., 2005b), and progesterone receptor (~86%; Smith *et al*., 2007).

The ARC and AVPV populations of Kp neurons respond to sex steroids but in an opposite manner (Smith *et al*., 2005a, b). In rodents, it was proposed that Kp neurons in the AVPV integrate E2 positive feedback and therefore would be involved in LH surge generation, whereas KNDy neurons in the ARC integrate E2 negative feedback and consequently are involved in LH pulsatility.

In prebubertal lambs, Kp immunoreactive cells of the ARC region increase after ovariectomy (Nestor *et al*., 2012). Because the ovary is the main source of estrogen, this implies a negative effect of E2 on this Kp population. These data are consistent with the hypothesis that puberty is the result of a reduction in steroid negative feedback, leading to an increase in Kp secretion in ARC in prepubertal ewes. However, the recent discovery that ERα mRNA expression on Kp ARC neurons increase approaching puberty does not explain this escape (Bedenbaugh *et al*., 2018). In postpubertal ewes, E2 enhanced Kp expression in POA and concentrations were high during the late follicular phase compared to the luteal phase (Smith *et al*., 2009a). Moreover, C-Fos was induced in POA during GnRH/LH surge (Hoffman *et al*., 2011). Collectively, these data clearly demonstrated the positive feedback of E2 on Kp neurons located in POA. However, the role of ARC NKDy neurons in integrating E2 feedback is more complex, at least in ewes. Kiss1 expression in the ARC is elevated during the late follicular phase compared to the luteal phase (Estrada *et al*., 2006; Smith *et al*., 2009a), and there is C-Fos induction in Kp neurons in ARC during GnRH/LH surge (Merkley *et al*., 2012). Conversely, it was suggested (Hoffman *et al*., 2011) that ARC neurons should integrate both negative and positive E2 feedback and therefore be involved in GnRH/LH surge generation. However, caution should be exercised when making conclusions, due to potential species differences.

Kisspeptin and puberty

The role of Kps in reproductive function has been suggested to start early in life. Kp and its receptor are present from embryonic day 13.5 in mice (Kumar *et al*., 2014). In sheep, perturbations by administration of testosterone propionate (TP) from 30 to 58 day of gestation (GD) reduced Kiss1 mRNA expression and decreased serum LH concentrations in GD59 fetuses. Cessation of maternal TP exposure restored normal endocrine secretion after 2 week. However, even after treatment cessation, differences emerged in gene expression of GnRH, estrogen receptor-β, and Kiss1R in GD75 fetuses, suggesting that normal HPG development was disrupted (Roselli *et al*., 2016). However, no changes in Kp-ir cell numbers in the POA and ARC were observed at the adult stage in a similar study (Cheng *et al*., 2010). It remains to be determined whether changes in gene expression persist in older animals and ultimately affects timing of puberty and/or alters adult fertility. During early stages of juvenile development, the number of Kiss1-expressing cells increase in both POA and ARC. This increase in the POA was unrelated to changes in the frequency of episodic LH release. However the increase in the ARC is associated with an acceleration of pulsatile LH release during maturation of the reproductive neuroendocrine axis in ovariectomized and E2-replaced lambs (Redmond *et al*., 2011a). In addition, number of immunoreactive Kp fibers in the ARC and ME increase gradually from 5 to 16 week of age (Polkowska *et al*., 2017), concomitant with increases in plasma LH concentrations and pulse frequency (Foster *et al*., 1975; Fig. 2).

Experiments have been performed to mimic patterns of Kp release occurring during puberty using repeated Kp administration to advance puberty onset. Icv administration of 1 nmol of Kp10 every 12 h from postnatal days 26 to 31 clearly advanced onset of puberty in female rats (Navarro *et al*., 2004). In addition, repeated injections of Kp10 sustain LH and FSH pulsatility in prepubertal cattle (Ezzat Ahmed *et al*., 2009) and LH pulsatility in lambs (Redmond *et al*., 2011b). In prepubertal (28 week) Suffolk ewes, intravenous injections of 20 µg Kp10 every hour for 24 h stimulated LH pulsatility and induced an LH surge and ovulation. However, luteal activity was of short duration, with a rapid decrease in progesterone concentrations within 2 days after its initial rise, and no change in timing of puberty onset (Redmond *et al*., 2011b). Perhaps after termination of Kp treatment, spontaneous LH release was insufficient to support normal luteal function and the reproductive axis at this age is not sufficiently mature to establish regular cycles.

Negative energy balance or energy excess have profound impacts on the Kp system (Manfredi-Lozano *et al*., 2018). Therefore, altering metabolic level may change the pattern of Kp secretion. This was attempted in prepubertal Tibetan ewes by supplementing either concentrates or minerals. Kiss-1, Kiss1R and ERα mRNA expression were higher in the AVPV of animals receiving concentrates and to a lesser extent in those receiving mineral supplementation compared to those eating only oat hay (Jing *et al*., 2017). In addition,

follicular development was enhanced in supplemented prepubertal animals. This study supported the hypothesis that Kiss1/Kiss1R system was modulated by feed intake, and that reproductive performance was improved by this treatment.

Conversely, a study was performed to inhibit reproduction by blocking puberty onset by acting at the level of Kp. Male lambs (8 wk) were imminized against Kiss1 on weeks 0, 3 and 6 of the experiment. This treatment induced a strong anti-Kiss1 antibody titer and suppressed gonadal function and sexual behaviour. Therefore, it could be consider using *Kiss1* as a novel target for developing an immunocastration vaccine in sheep (Han *et al*., 2015).

Impact of seasonality on kisspeptin system

In adult ewes, Kiss1 mRNA expression in ARC is higher during the breeding season compared to the non-breeding season (Wagner *et al*., 2008), with number of Kp neurons following a similar trend (Smith *et al*., 2007) suggesting that melatonin secretion influences Kiss1 expression. This effect is likely indirect, as Kp neurons do not express melatonin receptors (Li *et al*., 2011).

In addition, the inhibitory effect of E2 on Kiss1 expression in ARC is greater during the non-breeding season compared to the breeding season (Smith *et al*., 2008a). These data provide evidence that a seasonal change in estrogen sensitivity occurs at the level of Kp neurons in the ARC, leading to the switch from breeding to non-breeding seasons. In contrast, Kiss1 mARN expression in POA did not differ between breeding and non-breeding seasons and did not seem to be influenced by estrogen (Smith *et al*., 2008a). Therefore, in ewes, Kp neurons of the POA are implicated only in a positive feedback inducing an LH surge, but not in control of seasonality.

Kp induces a larger GnRH and LH increase during the non-breeding season compared to the luteal phase of the cycle (Smith *et al*., 2009b; Li *et al*., 2012). Perhaps lower pulsatility that occurs during the non-breeding season allows accumulation of a larger releasable pool of GnRH and LH compared to the luteal phase. Kiss1R expression on GnRH cells was greater during the non-breeding season than in luteal phase (Li *et al*., 2012) suggesting that low Kp concentrations during the non-breeding season induced a greater Kiss1R expression compared to the luteal phase.

Altogether, these data suggest that an increase in Kiss1R expression on GnRH neurons and the greater releasable pool of GnRH/LH contribute to the higher response of Kp in terms of GnRH/LH release during the nonbreeding season. This situation would reflect the ability of HPG to respond to an increase in GnRH pulsatility during the transition to the breeding season.

The sensitivity of HPG to Kp varies not only across seasons but also during the cycle and was correlated with Kiss1 mRNA expression. Indeed, LH response to Kp was greater during the late follicular phase in humans (Dhillo *et al*., 2007), sheep (Smith *et al*., 2009b) and rats (Roa *et al*., 2006).

Modulation of the kisspeptin system to induce ovulation in sheep

Given the involvement of Kp in the control of reproduction in sexually mature animals, manipulation of the HPG axis using Kp treatment to promote ovulation have been attempted. However, the short half-life of this peptide (30 min for hKp54 and 1 min for hKp10 in human blood (Dhillo *et al*., 2005; Chan *et al*., 2011) requires repeated injections or continuous administration to obtain a sustained gonadotropin release. Studies conducted in human were mainly performed using Kp54, whereas for domestic animals, Kp10 represents a better compromise between efficacy and cost.

During the non-breeding season, repeated injections of Kp10 sustain LH and FSH pulse frequency in adult ewes (Caraty *et al*., 2007). However, this stimulation was insufficient to induce an LH surge. Conversely, infusion of Kp10 for 48 h (12.4 nmol/h) induced ovulation in 80% of treated animals, compared to less than 20% of control animals. A later study indicated that during the non-breeding season, a minimum of 24 h of infusion was necessary to obtain at least an ovulatory rate $\geq$75% (Sebert *et al*., 2010). During the breeding season, 8 h of Kp10 infusion (0.48 µmol/h) administered 30 h after withdrawal of a progesterone priming period, induced a preovulatory LH surge followed by ovulation (Caraty *et al*., 2007).

Although there is potential to induce ovulation with Kp10 treatment, these protocols are impractical in the field. To overcome this problem, Kp10 analogues with improved pharmacological features were developed (Table 1).

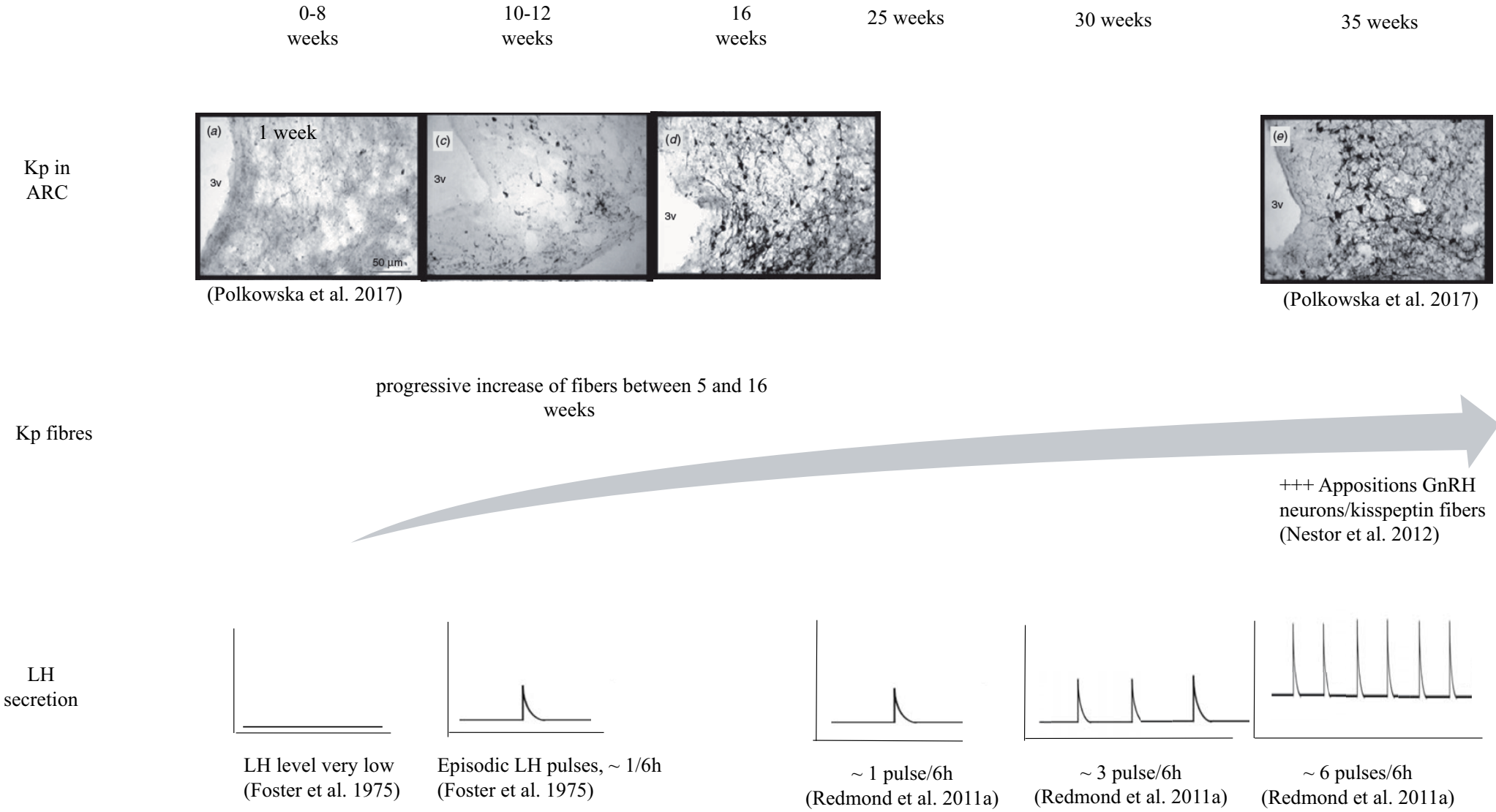

Figure 2. Evolution of Kp (Kisspeptin) expression in ARC (Arcuate nucleus) and LH (Luteinizing Hormone) secretion in peripheral blood, from birth to puberty onset in female lambs.

Table 1. Summary of effects observed on gonadotropin and/or steroid secretion and/or ovulation after kisspeptin-10 (Kp-10) or Kp-10 analog (FT080, Compound 17 or C6) administration in ewes. LH (Luteinizing Hormone), FSH (Follicle-Stimulating Hormone), E2 (17 β Estradiol), iv (intravenous), im (intramuscular).

Molecule	Ewes status	Dose and route of administration	Effect on gonadotropin and/or steroid secretion and/or ovulation	Reference
Kp-10	Prepubertal (28 weeks)	20 µg/h during 24 hours, iv	Increase LH pulsatility Induce ovulation	(Redmond et al. 2011b)
Kp-10	Adult non cyclic	6 nmol, iv	Increase LH and FSH after each injection	(Caraty et al. 2007)
KP-10	Adult non cyclic	15.2 nmol/h during 24h, iv	Increase LH and E2 Induce ovulation	(Sebert et al. 2010)
Kp-10	Adult, follicular phase	0.48 µmol/h during 8h, iv	Induce LH surge and ovulation	(Caraty et al. 2007)
FT080	Adult non cyclic	0.5, 2.5 or 5 nmol/kg, iv	Short lasting increase of LH (at all doses)	(Whitlock et al. 2015)
Compound 17	Adult non-cyclic	15 nmol, iv	Increase LH and FSH during approximatively 9 and 5 hours respectively	(Beltramo et al. 2015)
C6	Adult, follicular phase	15 nmol, im	Increase LH and FSH during approximatively 12 hours Induce ovulation	(Decourt et al. 2016)
C6	Adult, non-cyclic	15 nmol, im	Increase LH and FSH during approximatively 12 hours Induce ovulation	(Decourt et al. 2016)

Treatment with analogues

FTM080, a peptidomimetic containing a Gly-Leu dipeptide isostere (4-fluorobenzoyl-Phe-Gly-Leu-Arg-Trp-NH_2) was designed to avoid hydrolysis by metalloproteinase. This analogue has an extended half-life in murine serum compared to Kp10, with comparable binding affinity and efficacy to Kp10 *in vitro* (Tomita *et al*., 2008). Effects of intravenous injection of FTM080 (0.5, 2.5, and 5.0 nmol/kg) were evaluated in Katahdin female sheep during the non-breeding season. The increase of LH was very short in amplitude and duration compared to 0.5 nmol/kg of hKp10 (Whitlock *et al*., 2015). Despite the *in vitro* improved features of FTM080 compared to Kp10, this analogue seems have a modest activity in ewes, probably due to faster renal clearance due to its small size.

We generated a series of Kp10 analogues with improved resistance to degradation. The first compounds had an enhanced *in vitro* pharmacological profile compared to Kp10, but increase in gonadotropin secretions lasted only several hours and were insufficient to induce ovulation (Beltramo *et al*., 2015). Further modifications led to the creation of the analogue named C6. This analogue combined the introduction of a triazole peptidomimetic to reduce proteolytic degradation, incorporation of an albumin-binding motif on the N-terminal amine to delay renal clearance, and methylation of arginine to enhance proteolytic stability of Kp10 (Decourt *et al*., 2016b). The C6 effect on LH secretion was tested during the breeding season by a single intramuscular injection of 15 nmol/ewe, at 24 h after the withdrawal of a 14-days progesterone pre-treatment (intravaginal sponges containing fluogestone acetate). The treatment induced synchronized LH surges 5 h after C6 injection, followed by fertile ovulations, as demonstrated by 60% pregnancy rate and birth of full-term lambs. The same protocol was performed during the non-breeding season, resulting in a synchronized LH surge 4-6 h after C6 injection, that was followed by ovulation. This treatment also triggered estrus behaviour, with ewes standing to be bred by a ram. However, pregnancy rate (40%) was lower than in the breeding season (Decourt *et al*, 2018; Centre for Neuroendocrinology and Department of Anatomy, University of Otago, Dunedin, New Zealand; unpublished data). During the non-breeding season, ovaries are not fully ready to respond to an acute stimulation and the LH surge probably induced ovulation of immature follicles, reducing fertility. This protocol was also tested in goats during breeding and non-breeding season with similar results (Decourt *et al*, 2018; Centre for Neuroendocrinology and Department of Anatomy, University of Otago, Dunedin, New Zealand; unpublished data), and highlight the necessity to further refine the protocol to improve the pregnancy rate during the non-breeding season. Perhaps a low level constant stimulation of the gonadotropic axis in order to induce follicular growth and ovulation would be preferable to pronounced, acute stimulation.

In a preliminary study, we tested the ability of C6 to advance puberty onset in prepubertal female mice. Repeated daily injections of C6 (0.15 nmol/mouse/day), from postnatal days 26 to 30, significantly advanced puberty, with vaginal opening present in all animals by day 29 *vs*. day 32 for control, and first estrus also detected much earlier in animals receiving C6 treatment (Decourt *et al*., 2016b). These results suggest a potential interest to test this treatment in livestock species, ideally with a refinement of the protocol to avoid repeated daily injections.

Takeda Pharmaceuticals have developed a nonapeptide analog, TAK-683, based on substitution of natural L-aminoacids with D-aminoacids. As mentioned earlier, this strategy is widely used to improve biological potency of peptides by increasing resistance to enzymatic degradation, although it may decrease activity due to conformational properties alteration. In cyclic goats, intravenous administration of 35 nmol of TAK-683 during the follicular phase induced an LH surge but the stimulation of LH release induced early ovulation or atresia of follicles (Goto *et al*., 2014). During an artificial luteal phase, this analogue induced a small increase of LH pulsatility within 6 h after injection, associated with an increase in E2 concentration, and followed by a surge-like release of LH with a peak at 12.5 ± 1.0 h (Endo *et al*., 2015). During pre-synchronized follicular phase, intravenous or subcutaneous administration of 3.5 nmol of TAK-683, 12 h after withdrawal of progestogen pretreatment, induced a LH surge in the same manner, at 4.2 +/- 0.6 h and 4.6 +/- 0.4 h after iv and sc injection respectively, with ovulations detected within 3 days after injection (Kanai *et al*., 2017). However, data on fertility after these treatments are missing, and despite its good water solubility, gelation was observed within 3 h (Nishizawa *et al*., 2016). To solve this problem, they modified the analogue and created TAK-448, with no evidence of gelation within 5 days. Approximately a one-third dose of TAK-448 had similar efficacy to that of TAK-683 in rats, but efficacy on livestock species remains to be evaluated.

Conclusions

Kp is probably the most exiting discovery of neuropeptide implicated in reproductive function since identification of GnRH. Following this discovery, we have improved our knowledge regarding mechanisms controlling this function. Furthermore, manipulating Kp signalling may provide novel potential strategies to manage livestock reproduction by controlling ovulation in adult and modulating the time of puberty onset. However, further optimization of available analogues and of experimental procedures are still needed.

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DOI: 10.21451/1984-3143-AR2018-0013

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Neuroendocrine signaling pathways and the nutritional control of puberty in heifers

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Abstract

Puberty is a complex physiological process in females that requires maturation of the reproductive neuroendocrine system and subsequent initiation of high-frequency, episodic release of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH). Genetics and nutrition are two major factors controlling the timing of puberty in heifers. While nutrient restriction during the juvenile period delays puberty, accelerated rates of body weight gain during this period have been shown to facilitate pubertal development by programming hypothalamic centers that underlie the pubertal process. Among the different metabolic factors, leptin plays a critical role in conveying nutritional information to the neuroendocrine axis and controlling pubertal progression. Because GnRH neurons are devoid of the leptin receptor, leptin's effects on GnRH neurons must be relayed via an afferent neuronal network. Two neuronal populations located in the arcuate nucleus (ARC) that express the orexigenic peptide neuropeptide Y (NPY), and the anorexigenic peptide alpha melanocyte-stimulating hormone (αMSH), are key components of afferent pathways that convey inhibitory (NPY) and excitatory (αMSH) inputs to GnRH neurons. In addition, ARC neurons expressing kisspeptin, a potent stimulator of GnRH release, are also involved in the nutritional regulation of puberty. Our studies have demonstrated that increased planes of nutrition during juvenile development result in morphological and functional changes in hypothalamic pathways comprising NPY, proopiomelanocortin (POMC), and kisspeptin neurons. Changes included differential expression of NPY, POMC, and Kiss1 in the ARC, and plasticity in the axonal projections to GnRH and kisspeptin neurons. Additionally, increased rates of body weight gain also promoted changes in the pattern of DNA methylation, a key epigenetic mechanism for regulation of gene expression. Finally, our most recent findings suggest that maternal nutrition during gestation can also induce structural and functional changes in hypothalamic neurocircuitries that are likely to persist long after pubertal maturation and influence reproductive performance throughout adulthood in cattle.

Keywords: heifers, hypothalamus, leptin, nutrition, puberty.

Introduction

Pubertal maturation in female mammals is an intricate physiological process that involves physical and behavioral changes associated with activation of the hypothalamic-pituitary-ovarian axis and subsequent establishment of reproductive cyclicity (Sisk and Foster, 2004). Reproductive maturation is initiated primarily at the hypothalamic level by the acceleration of gonadotropin-releasing hormone (GnRH) secretion from GnRH neurons. The increase in pulsatile release of GnRH and subsequent rise in luteinizing hormone (LH) pulse frequency support the final maturation of ovarian follicles and steroidogenesis that are required for first ovulation (Ryan and Foster, 1980). The process of pubertal development is largely controlled by genetic and environmental factors, among which nutrition plays a prominent role. Data in humans and animals unequivocally demonstrate that increased nutrient intake during peripubertal development facilitates reproductive maturation in females (Ryan and Foster, 1980; Amstalden *et al*., 2011).

The timing of pubertal development has important implications for livestock production. In beef heifers, lifetime productivity is heavily dependent upon their ability to reach reproductive competence, to conceive early during their first breeding season, and to calve the first time by approximately 24 months of age (Lesmeister *et al*., 1973). Moreover, the incidence of multiple estrous cycles before the first breeding positively influences yearling fertility (Short and Bellows, 1971). However, a significant proportion of beef heifers within existing U.S. production systems fail to reach the developmental end-points necessary to achieve these objectives (Hughes, 2013). This is particularly true for later-maturing breeds (*e.g*., *Bos indicus*-influenced) in which the skeletal size required to support a healthy and safe pregnancy is frequently attained well before the establishment of regular estrous cycles. Therefore, a better understanding of the neuroendocrine mechanisms controlling puberty can assist in the development of novel managerial strategies that exploit brain plasticity during critical windows of development and lead to strategies for successfully programming the onset of puberty around 12 to 14 months of age.

The objectives of this review are to present an overview of the neuroendocrine processes controlling puberty in heifers, discuss the effects of nutrition on these processes, and summarize recent research findings regarding the programming effects of nutrition during early development on hypothalamic pathways that control reproduction. While this review focuses primarily on pubertal development in heifers, supportive data from other species including sheep and rodents are also discussed.

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Received: January 31, 2018
Accepted: May 7, 2018

Neuroendocrine control of reproductive maturation

Estradiol negative feedback on gonadotropin secretion

The onset of puberty in the heifer is characterized by a marked increase in the frequency of LH pulses that first becomes apparent at approximately 50 days before first ovulation. This characteristic increase serves as the most accurate predictor of pubertal onset (Day *et al*., 1984) and is resultant of coincident increases in pulsatile secretion of GnRH (Cardoso *et al*., 2014a). As frequency of LH pulses increases, their amplitude declines. However, despite the absence of consistent changes in pulse frequency or amplitude prior to 50 days, a trend for mean circulating concentrations of LH to increase has been detected in the heifer as early as 4 months preceding puberty (Swanson *et al*., 1972; Schams *et al*., 1981; Day *et al*., 1984). The heightened frequency of LH pulses reflects maturational changes of hypothalamic centers responsible for the pulsatile mode of GnRH release (Sizonenko and Aubert, 1986). These changes are highlighted by a marked decline in negative feedback responsiveness to estradiol-17β and are similar to those observed in the ewe lamb (Foster and Ryan, 1979). Although the synthesis and secretion of gonadotropins appear to be largely gonadal steroid-independent during the early postnatal period, a gonad-dependent suppression of GnRH/LH develops during the juvenile period and reflects an increase in responsiveness to estradiol negative feedback (Day *et al*., 1984, 1986; Foster and Ryan, 1979). Ovariectomy at this time, without estradiol replacement, results in establishment of high-frequency pulses of GnRH/LH, typical of those at puberty. Conversely, estradiol replacement prevents the castration-induced rise in GnRH/LH by restoring the inhibitory tone characteristic of the prepubertal state. Estradiol-mediated inhibition persists until hypothalamic changes associated with maturation of the reproductive neuroendocrine axis occur, at which time the GnRH pulse generator escapes from estradiol negative feedback in sheep (Foster and Ryan, 1979). Importantly, GnRH neurons do not contain estrogen receptor-alpha (ESR1; Lehman *et al*., 1993). Therefore, modifications in responsiveness to estradiol negative feedback are not mediated directly by estradiol at the level of the GnRH neuron. Moreover, although both follicle-stimulating hormone (FSH) and LH are ultimately controlled by GnRH from the hypothalamus, the change in negative feedback responsiveness to estradiol does not appear to result in a measurable modification in secretion patterns of FSH preceding puberty in heifers (Schams *et al*., 1981) or ewe lambs (Foster *et al*., 1975). Thus, a limitation in availability of FSH is not a primary factor regulating the timing of puberty in ruminants. Once pulses of LH occur at an interval of every 40 to 50 min, circulating concentrations of LH increase markedly and result in heightened stimulation of ovarian follicles, an increase in circulating concentrations of estradiol, and initiation of a LH surge that induces first ovulation or luteinization of a large follicle (Kinder *et al*., 1987). The estradiol-induced surge of LH occurs through a parallel positive feedback effect of estradiol at hypothalamic components that mediate a prolonged surge of GnRH. However, the ability of estradiol to induce a surge release of LH becomes functional between 3 and 5 months of age in heifers (Staigmiller *et al*., 1979) and thus is operable well before puberty.

Neuronal processes underlying the change in negative feedback responsiveness to estradiol

The neuronal network in the hypothalamus has the inherent ability to produce a pulsatile pattern of GnRH release and depends upon synchronous firing of GnRH neurons (Funabashi *et al*., 2000). However, since GnRH neurons do not contain ESR1, the role of changing responsiveness to estradiol negative feedback in modulating the GnRH pulse generator as puberty approaches has not been clearly delineated. Nonetheless, studies in mice have clearly shown that ESR1 is the major estrogen receptor mediating estradiol negative feedback effects on GnRH secretion (Dorling *et al*., 2003). Moreover, based on work in rodents, neurons located in the arcuate nucleus (ARC) that contain ESR1 are essential for communicating estradiol negative feedback (Bronson, 1981). In this context, it has been proposed that kisspeptin neurons are responsible for mediating the synchronized firing of GnRH neurons (Navarro *et al*., 2009; Qiu *et al*., 2016), underlie the estradiol feedback regulation of GnRH secretion (Dubois *et al*., 2016), and thus greatly influence pubertal maturation (Mayer *et al*., 2010; Redmond *et al*., 2011a, b) in both rodents and ruminants. Kisspeptin, a member of the RF-amide related peptide (RFRP) superfamily, is controlled by the *Kiss1* gene and its receptor (KISS1R), and is expressed in a variety of tissues, including the hypothalamus. Moreover, KISS1R colocalizes with GnRH neurons and is responsible for regulating the release of GnRH (Mayer *et al*., 2010). Gene mutations resulting in loss of this signaling pathway result in the failure to attain puberty in primates (Terasawa *et al*., 2013). Additional variants of Kiss1-containing cells have been localized specifically within the ARC and are termed KNDy neurons. Kisspeptin cells localized to the ARC co-express two other neuronal peptides, neurokinin B (NKB) and dynorphin. KNDy neurons express receptors for both NKB and dynorphin, but do not contain kisspeptin receptors. Thus, KNDy cells function as a signaling network, secreting kisspeptin in response to their own release of NKB which stimulates release of GnRH by its direct action on both cell bodies and nerve terminals of GnRH neurons (Navarro *et al*., 2009; Lehman *et al*., 2010). Dynorphin is then released which terminates KNDy neuron activity. It has been proposed that the repeating nature of this paradigm provides, for the first time, a plausible explanation of cellular activity that represents the physical source of the GnRH pulse generator (Lehman *et al*., 2010; Goodman *et al*., 2013). Deletion of ESR1 in all kisspeptin-expressing neurons in a rodent model advances the onset of puberty, supporting the idea that ESR1 has the inherent ability to

suppress GnRH/LH secretion and pubertal onset by its actions on kisspeptin neurons (Cheong *et al*., 2015). The interrelationship between ESR1 and kisspeptin neurons provides a potential basis through which changes in negative feedback responsiveness to estradiol could regulate the timing of puberty in heifers and ewe lambs. However, the exact mechanism remains elusive. Day *et al*. (1987) observed a reduction in the overall number of estradiol receptors in the mediobasal hypothalamus in intact heifers approaching puberty, similar to findings reported during juvenile development in rats (Kato *et al*., 1974). In contrast, Bedenbaugh *et al*. (2018) reported that the peripubertal increase in LH pulsatility in ovariectomized, estradiol-treated ewe lambs was associated with enhanced ESR1 mRNA expression in kisspeptin neurons in the ARC. Moreover, the absence of estradiol in ovariectomized ewe lambs was associated with the greatest ESR1 mRNA abundance and percentage of kisspeptin neurons containing ESR1 protein in the ARC. Therefore, changes in the expression of ESR1, particularly in kisspeptin neurons in the ARC, fail to explain the pubertal escape from estradiol negative feedback in the ruminant model.

Impact of nutrition on pubertal development

Nutritional acceleration of puberty

The important effects of nutrition controlling the reproductive neuroendocrine system and pubertal progression in heifers have been well established. Previous experiments conducted by our group (Cardoso *et al*., 2014a, b; Allen *et al*., 2017) and others (Gasser *et al*., 2006a) have demonstrated that increasing nutrient intake during the juvenile period can markedly advance the timing of puberty in beef heifers. In studies performed by Gasser *et al*. (2006a), the majority of heifers weaned at approximately 3.5 months of age and fed high-concentrate diets to achieve accelerated rates of body weight gain exhibited puberty before 300 days of age (precocious puberty). Although those studies were performed in *Bos taurus* breeds (Angus and Hereford), similar findings were observed in our studies using *Bos indicus*-influenced heifers, which are later maturing. In these studies, heifers (1/2 Angus × 1/4 Hereford × 1/4 Brahman) were weaned between 3.5 and 4 months of age and fed a high-concentrate diet to promote a rate of body weight gain of approximately 1 kg/day (Cardoso *et al*., 2014a, b). This dietary regimen was shown to significantly advance puberty, with a high percentage (~85%) of heifers reaching puberty before 12 months of age.

To identify the developmental window in which heifers are most responsive to the nutritional programming of puberty, we employed a stair-step nutritional regimen involving alternate periods of dietary energy-restriction and re-feeding during juvenile development. Interestingly, we observed that heifers that gained body weight at high rates between 4 and 6.5 months of age, and were subsequently subjected to a marked restriction in feed intake between 6.5 and 9 months of age, still attained early puberty (<12 months of age) at rates comparable to heifers fed a high-concentrate diet continuously (Cardoso *et al*., 2014b). Similarly, *Bos taurus* heifers that were fed to gain body weight at a relatively high rate between 126 and 196 days of age exhibited a high incidence of precocious puberty (Gasser *et al*., 2006a). However, puberty was not advanced to the same extent when heifers were fed a similar diet later during juvenile development. Collectively, these results demonstrate that during early development, plausibly between 4 and 9 months of age, heifers are more sensitive to the pubertal acceleration effects of nutrition (Fig. 1).

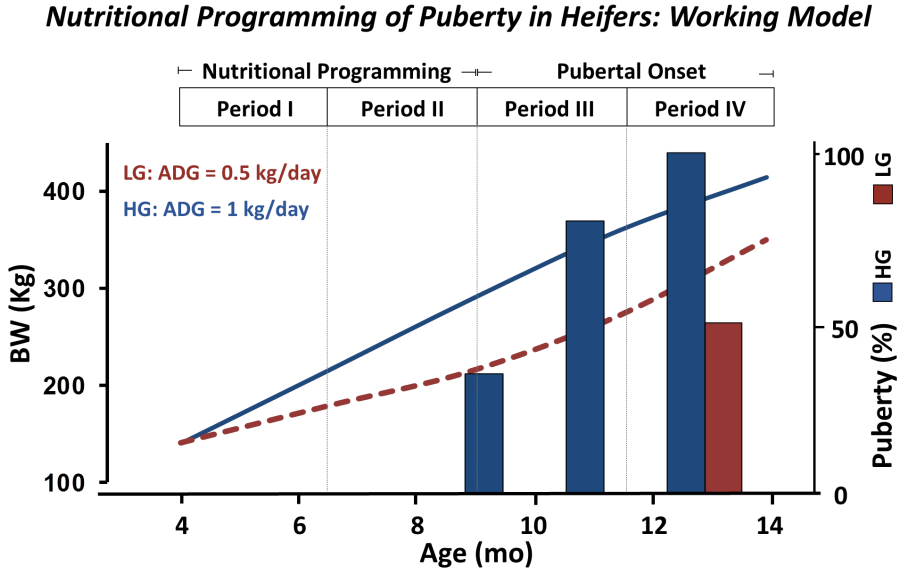

Figure 1. Schematic diagram of the working model for the nutritional programming of puberty in heifers. Heifers weaned at approximately 3.5 months of age and fed a high-concentrate diet to promote a relative high rate of body weight gain (1 kg/day; blue line) attain puberty significantly earlier (blue bars) when compared to heifers gaining body weight at lower rates (0.5 kg/day; red line and bar). ADG: Average daily gain; BW: body weight; LG: low-gain; HG: high-gain.

Metabolic signals and neuroendocrine maturation

Nutritional regimens that promote a relative high rate of body weight gain (1 kg/day) are accompanied by greater adiposity and increased circulating concentrations of the metabolic hormones leptin, insulin, and insulin-like growth factor 1 (IGF1) when compared to regimens that promote growth at a slower rate (0.5 kg/day; Allen *et al*., 2012; Cardoso *et al*., 2014b; Alves *et al*., 2015). We hypothesized that this positive metabolic profile induced by increased body weight gain would promote modifications in the reproductive neuroendocrine system, ultimately resulting in increased pulsatile release of GnRH and LH, which are required for first ovulation. Using the same research model of accelerated growth during the juvenile period, we characterized the secretion of GnRH and LH in prepubertal heifers by measuring the concentrations of GnRH in third-ventricle cerebrospinal fluid (IIIV-CSF) and LH in the peripheral blood, respectively (Cardoso *et al*., 2014a). As expected, we observed that all pulses of LH in blood plasma were preceded by a GnRH pulse in the IIIV-CSF and, more importantly, pulse frequency of GnRH and LH were greater in heifers gaining 1 kg/day when compared to heifers gaining 0.5 kg/day between 4 and 8 months of age. These observations further indicate that increased nutrition during the juvenile period advances puberty by promoting the maturation of the reproductive neuroendocrine axis, thus hastening the pubertal increase in the pulsatile release of GnRH/LH.

Postnatal programming of hypothalamic signalling pathways

A growing body of evidence indicates that metabolic factors associated with the individual's nutritional status can influence the activity of GnRH neurons. Among metabolic factors, leptin plays a critical role in conveying nutritional information to the neuroendocrine axis and controlling pubertal progression in heifers (Zieba *et al*., 2005). However, the long form of the leptin receptor (ObRb), the main receptor isoform involved in activation of intracellular signaling, is not present on GnRH neurons (Quennell *et al*., 2009). Therefore, it has been postulated that leptin signaling influences GnRH neuronal activity via an upstream neuronal network that ultimately targets GnRH neurons (Barb and Kraeling, 2004; Ratra and Elias, 2014). Two different neuronal populations located in the ARC that contain ObRb (Cheung *et al*., 1997; Elmquist *et al*., 1998) and directly regulate the function of GnRH neurons (Roa and Herbison, 2012) have been established as main components of this network: the neuropeptide Y/agouti-related peptide (NPY/AgRP) and the proopiomelanocortin/cocaine- and amphetamine-regulated transcript (POMC/CART) neurons. In addition, because the ARC population of kisspeptin neurons is involved in the control of GnRH pulsatile release (Li *et al*., 2009) and kisspeptin synthesis is responsive to metabolic cues (Castellano *et al*., 2011), kisspeptin neurons in the ARC are also likely to be involved in the nutritional regulation of puberty.

Neuropeptide Y/AgRP pathway

The NPY/AgRP neuronal population secretes two main orexigenic neuropeptides: neuropeptide Y (NPY) and agouti-related peptide (AgRP), both secreted predominantly in conditions of low energy balance (McShane *et al*., 1992; Hahn *et al*., 1998). Experiments in mature cows have demonstrated that NPY has an inhibitory effect on GnRH release (Gazal *et al*., 1998) and, although not tested in cattle, AgRP has been shown to suppress GnRH release in sheep (Miller *et al*., 2007) and monkeys (Vulliémoz *et al*., 2005). Both neuropeptides, NPY and AgRP, are important inhibitory signals to GnRH secretion during prepubertal development and play a key role in controlling the timing of pubertal maturation in females (Pierroz *et al*., 1995; Egan *et al*., 2017). Our studies in prepubertal heifers have shown that increased body weight gain between 4 and 8 months of age (juvenile period) reduces *AgRP* (Allen *et al*., 2012) and *NPY* (Allen *et al*., 2012; Alves *et al*., 2015) mRNA expression in the ARC, decreases the concentrations of NPY in the cerebrospinal fluid collected from the third ventricle of the brain (Cardoso *et al*., 2014a), and reduces the magnitude of NPY neuronal inputs to GnRH neurons (Alves *et al*., 2015). Altogether, our findings in the bovine female support the notion that NPY and AgRP inhibit GnRH pulsatile release during the prepubertal period and nutritional regimens that promote accelerated rates of body weight gain during juvenile development can attenuate the NPY/AgRP inhibitory tone, thus facilitating pubertal maturation (Fig. 2).

Proopiomelanocortin pathway

The POMC gene expressed in POMC/CART neurons encodes several peptides, including the anorexigenic alpha-melanocyte stimulating hormone (α-MSH), which is produced primarily during periods of positive energy balance (Cone, 1999). In rodents, α-MSH was shown to elicit a direct stimulatory effect on GnRH neurons (Leranth *et al*., 1988; Roa and Herbison, 2012) and administration of a melanocortin agonist stimulated LH secretion in the female sheep (Backholer *et al*., 2009). Notably, the melanocortin 4 receptor (MC4R) is antagonized by AgRP (Ollmann *et al*., 1997), indicating that AgRP may inhibit GnRH secretion not only directly but also indirectly via the melanocortin system. In our studies, the expression of *POMC* mRNA and α-MSH immunostaining in the ARC were both increased in heifers that gained body weight at accelerated rates during juvenile development (Allen *et al*., 2012; Cardoso *et al*., 2015). However, the number of α-MSH-immunopositive contacts on GnRH neurons was moderately low and was not affected by the nutritional status during early development (Cardoso *et al*., 2015). Collectively, these results suggest that the nutritional acceleration of puberty in heifers may require increased signaling of α-MSH in the hypothalamus, but not necessarily involving an increase

in direct α-MSH stimulation of GnRH neurons.

Kisspeptin pathway

There is emerging evidence indicating that the ARC population of kisspeptin neurons is involved in the nutritional control of reproductive function in females (Castellano *et al*., 2011). While a subset of kisspeptin neurons in the ARC contain ObRb (Backholer *et al*., 2010), leptin induction of STAT3 phosphorylation, a major intracellular signaling mechanism induced by leptin, is absent in kisspeptin neurons in sheep (Louis *et al*., 2011). Thus, the effects of leptin on kisspeptin expression and neuronal activity appear to be mediated indirectly via an upstream network of neurons (Donato *et al*., 2011; Manfredi-Lozano *et al*., 2016). In our studies in heifers, we tested if the number of close contacts of NPY- or α-MSH-containing fibers on kisspeptin neurons in the ARC would be associated with the nutritional regulation of puberty in heifers. While the intensity of NPY axonal contacts on kisspeptin neurons was not affected by nutritional status (Alves *et al*., 2015), accelerated rates of body weight gain resulted in an increased number of α-MSH immunopositive contacts on kisspeptin neurons and a greater percentage of kisspeptin neurons innervated by α-MSH fibers (Cardoso *et al*., 2015). Prepubertal heifers subjected to accelerated rates of body weight gain also exhibited reduced *Kiss1* mRNA content in the ARC (Alves *et al*., 2015; Cardoso *et al*., 2015), suggesting a possible influence of α-MSH on *Kiss1* gene expression during the juvenile period. Notably, a MTII melanocortin agonist has been shown to reduce *Kiss1* mRNA in the ARC of sheep (Backholer *et al*., 2009). In intact female rats, the developmental pattern of *Kiss1* expression in the ARC follows a U-like pattern, with expression declining from intermediate (infantile period), to minimal (juvenile period), followed by a postpubertal increase to maximum expression during adulthood (Cao and Patisaul, 2011). Therefore, reduced *Kiss1* expression in the ARC may indicate a more advanced stage of development of this neuronal population in heifers fed a higher plane of nutrition. Additionally, changes in *Kiss1* mRNA abundance in the ARC may result from the negative effects of estradiol on *Kiss1* expression (Smith, 2009), since steroidogenic capacity and circulating levels of estradiol increase as heifers approach puberty (Gasser *et al*., 2006b). Despite the observed changes in *Kiss1* mRNA expression, the number of kisspeptin-immunopositive neurons in the ARC was not affected by nutritional status in our studies (Alves *et al*., 2015; Cardoso *et al*., 2015). Based on those findings, the role of ARC kisspeptin neurons in the nutritional programming of puberty in heifers is still not fully understood. However, interactions between POMC and kisspeptin neurons appear to be relevant during this process (Fig. 2).

Other pathways

In addition to changes in neuronal populations and neuropeptide signaling as discussed above, the ARC encompasses other cell types and molecules impacting a variety of cellular processes that regulate GnRH secretion, including receptors and transcription factors. In two of our studies in prepubertal heifers, we obtained a comprehensive survey of the different cellular populations of the ARC by isolating ARC tissue from within hypothalamic sections. In one experiment (Allen *et al*., 2012), using microarray analyses to assess mRNA abundance, we observed that different nutritional regimens applied during the juvenile period promoted differential expression of a large number of genes, including those encoding for prolactin-releasing hormone receptor (*PRLHR*) and growth hormone receptor (*GHR*). In addition, we observed differential expression of genes associated with control of feed intake and metabolism (*NPY*, *AgRP*, and *POMC*), as well as genes involved in synaptic vesicle transport, axonal growth and neuronal plasticity. These findings, in conjunction with observed changes in neuronal projections (Alves *et al*., 2015; Cardoso *et al*., 2015), support the premise that neuronal remodeling plays a significant role in programming the timing of puberty in heifers. The well-established neurotropic actions of leptin are likely to be involved in this process (Bouret *et al*., 2004).

In a follow-up study (Alves *et al*., 2016), we investigated if DNA methylation (key epigenetic mechanism for regulation of gene expression) in the ARC was influenced by nutritional status during the juvenile period. Differential methylation was found in several genes, including *GHR* and *HMGA2* (a gene that encodes a chromatin-associated protein that modulates transcription), which were hypermethylated in heifers that gained body weight at higher rates. Importantly, the hypermethylation of these genes was associated with a reduced abundance of their mRNA expression levels. Growth hormone has been previously reported to play a role in pubertal development in heifers (Simpson *et al*., 1991) and its action in the ARC appears to be linked to the control of NPY synthesis (Chan *et al*., 1996). Moreover, a genome-wide association study (GWAS) has indicated that *HMGA2* is one prominent gene involved in the process of pubertal maturation in heifers (Fortes *et al*., 2011), therefore, further investigation of its role in the nutritional programming of puberty is warranted. While the exact mechanisms remain unknown, this initial investigation of DNA methylation supports the premise that epigenetic alterations promoted by increased body weight gain during the juvenile period may represent a suitable mechanism by which nutrition can change functionality of the ARC cellular machinery, thus facilitating the activation of the reproductive neuroendocrine axis.

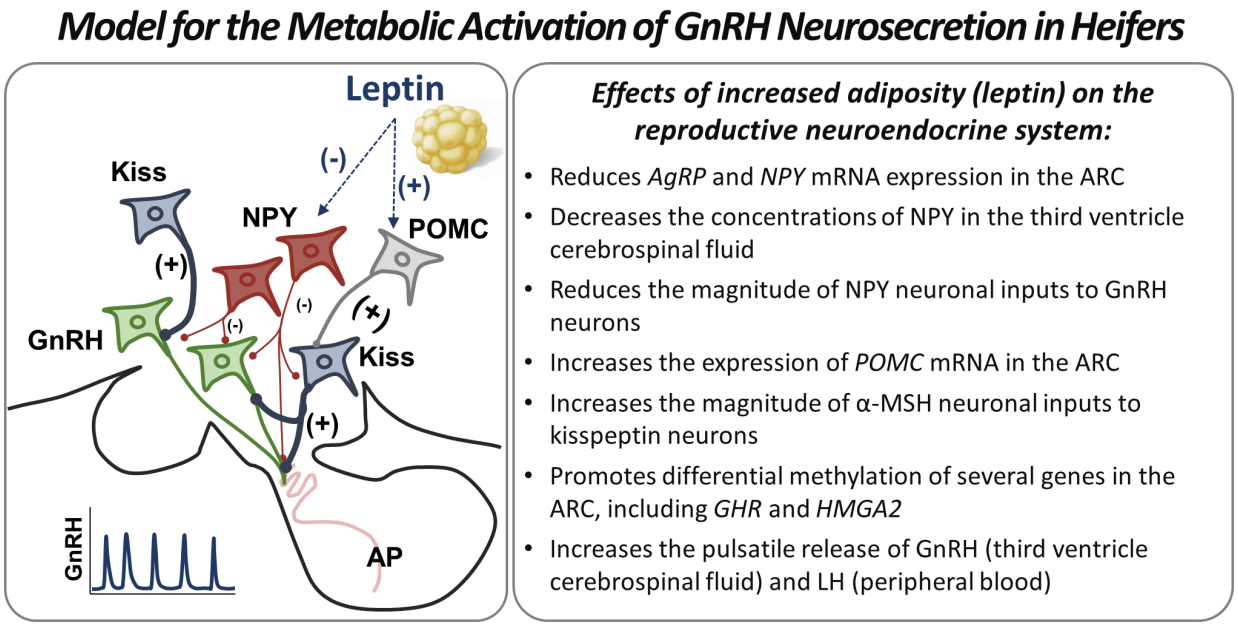

Figure 2. Neuroendocrine model for the metabolic activation of GnRH secretion during pubertal maturation in heifers. Left Panel: Representative scheme of neuronal pathways in the arcuate nucleus (ARC) that mediate the effects of leptin on GnRH pulsatile secretion. The adipocyte-derived hormone leptin inhibits (-) neuropeptide Y (NPY) and stimulates (+) proopiomelanocortin (POMC) neurons in the ARC. Consequently, the inhibitory (-) effects of NPY on GnRH neurons are diminished (thickness of lines relates to the intensity of the stimulus). POMC neurons project to kisspeptin (Kiss) neurons in the ARC, which stimulate (+) the pulsatile release of GnRH. The suppression of inhibitory (NPY) and increase in stimulatory (POMC and Kiss) pathways promote an increase in the frequency of GnRH pulses. GnRH neurons project to the hypophyseal portal circulation, allowing the access of this neurohormone to the anterior pituitary (AP), where it stimulates the secretion of gonadotropins. **Right Panel:** Summary of the effects of increased rates of body weight gain during the juvenile period on the reproductive neuroendocrine system in heifers.

Prenatal programming of hypothalamic signalling pathways

During the past decade, the developmental origins of health and disease (DOHaD) hypothesis by Barker and colleagues has attracted considerable attention to the concept of fetal programming (Barker, 2007). The DOHaD hypothesis gained momentum particularly after the emergence of epidemiological data from the 1944-1945 Dutch famine cohort demonstrating that maternal malnutrition during gestation is associated with a marked increase in the risks of the offspring for developing cardiovascular and metabolic diseases (Ravelli *et al*., 1976). These findings, in conjunction with subsequent clinical and animal studies (Barker, 2004; Gluckman and Hanson, 2004; Nijland *et al*., 2008), demonstrate that the perinatal period, a period in which organogenesis and tissue differentiation occur through a tightly controlled and timed process, is a critical window of opportunity for programming the offspring's phenotype.

In recent years, it has become evident that the mechanisms underlying the developmental origins of the adult phenotype require reprogramming of the epigenome by environmental factors (Ganu *et al*., 2012; Trevino *et al*., 2015). This is possible during fetal life due to the plasticity that allows the developing organism to adopt a phenotype that best suits the environment. From a neuroendocrine standpoint, studies in laboratory rodents have shown that maternal nutrition during gestation modulates the hypothalamic neurocircuitries controlling GnRH pulsatile release, thus programing pubertal maturation in the female offspring (Léonhardt *et al*., 2003; Iwasa *et al*., 2010; Sanchez-Garrido *et al*., 2013). In cattle, however, the concept of prenatal programing of the reproductive neuroendocrine axis remains virtually unexplored. For the past several years, our research group has developed a bovine model to study the interactive effects of prenatal and early postnatal nutrition on reproductive function in the female offspring. To accomplish this, *Bos indicus*-influenced (Brahman × Hereford; Brangus) cows bearing female pregnancies were fed to achieve thin, moderate, or obese body condition (BC) by approximately 6 months of gestation (second trimester) and maintained at the target BC until calving. Heifer offspring from each maternal group were then weaned at approximately 3.5 months of age and assigned randomly to be fed to achieve a relatively low (0.5 kg/day) or high (1 kg/day) rate of body weight gain until 8 months of age. While results of most of our studies using this animal model remain pending, initial findings are summarized in the following sections.

Leptin transport across the blood-brain barrier

As discussed previously, leptin is a critical metabolic hormone conveying nutritional information to the neuroendocrine axis and controlling pubertal development in females. Previous studies in sheep have shown that changes in nutritional status can modulate the transport of leptin across the blood-brain barrier. For example, in a study in which obese and lean adult sheep were fed to either gain or lose body weight, it was observed that obese animals had impaired transport of leptin across the blood-brain barrier (Adam and Findlay, 2010). Moreover, the transport of peripherally-administered leptin across the blood-brain barrier in obese sheep was not reversed following significant body weight loss. These and other data suggest that exposure of animals to a hyperleptinemic environment, such as that expected to occur in the fetus of a dam on a high nutritional plane, can result in irreversible physiological changes within the blood-brain barrier. Thus, we hypothesized that heifer offspring of dams with broadly varying degrees of BC may develop a leptin resistant state due to structural alterations in the blood-brain barrier.

To test this hypothesis, we investigated the expression of different isoforms of the leptin receptor in the choroid plexus of heifers subjected to the different maternal (thin, moderate, and obese) and postnatal (low and high) nutritional treatments. Importantly, the transport of leptin across the blood-brain barrier has been shown to depend on expression of the short form of the leptin receptor, which acts as a leptin transporter in endothelial cells (Banks, 2001). We found that prenatal nutrient restriction significantly reduced the mRNA abundance of the short form of the leptin receptor ObRc in the choroid plexus of heifers at 8 months of age (Zhang *et al*., 2017). Moreover, the expression of total leptin receptor (ObRt) was also reduced in the choroid plexus of heifers subjected to prenatal undernutrition. Interestingly, postnatal nutrient restriction increased the expression of ObRb, the long form of the leptin receptor, in the choroid plexus of heifers subjected to prenatal undernutrition but not in heifers subjected to other prenatal nutritional treatments (moderate or obese). Collectively, these results indicate that undernutrition during pregnancy interacts with postnatal nutrition to modulate the expression of the different isoforms of the leptin receptor in the choroid plexus of prepubertal heifers. The significance of changes in leptin receptor expression in regard to leptin transport across the blood-brain barrier is currently being investigated.

Neuropeptide y pathway

Using hypothalamic tissue from the same group of heifers discussed above, we investigated the interactive effects of prenatal and postnatal nutrition on the extent of NPY (inhibitory) projections toward GnRH neurons. While none of the treatment combinations altered the number of GnRH neurons, reduced rates of body weight gain during postnatal development increased the proportion of GnRH neurons in close apposition to NPY-containing fibers (Zhang *et al*., 2017). Notably, these effects were significantly greater in heifers from nutritionally-restricted dams, suggesting that prenatal undernutrition may amplify the effects of postnatal nutrition modulating the extent of NPY neuronal projections to GnRH neurons. While the functional relevance of this phenomenon remains to be determined, it is likely that the effects of prenatal undernutrition on NPY projections to GnRH neurons will hinder the process of pubertal maturation in heifers.

Ongoing and future studies

In our ongoing and future studies, we will further examine the interactive effects of prenatal nutrition with nutritional treatments imposed postnatally during the early juvenile period on hypothalamic processes controlling puberty. These include changes in expression of key genes (*e.g., Kiss1*, *POMC*, and *NPY*) in specific hypothalamic nuclei, epigenetic modification (*e.g.,* DNA methylation patterns), morphological development of neuronal pathways that modulate activity of the GnRH pulse generator, leptin signaling in the hypothalamus, among others. Importantly, we currently have available a large group of nutritionally-programmed heifers that will allow us to characterize the interactive effects of prenatal and early postnatal nutrition on multiple physiological processes, such as leptin transport across the blood-brain barrier, central release of neuropeptides, pulsatile secretion of GnRH and LH, responsiveness to estradiol negative and positive feedback, and estrous cycle events associated with postpubertal maturation. Moreover, because organizational changes brought about through perinatal nutritional programming are believed to be imprinted in the genome, they are likely to be associated with consistent physiological events that may, in some cases, be manifested only later in life. Thus, these nutritionally-programmed females represent a valuable resource for evaluating the long-term consequences of perinatal nutrition on reproductive function of sexually mature animals. We believe that the perinatal nutritional environment can program how the reproductive neuroendocrine axis of sexually mature heifers responds to leptin and other metabolic/hormonal factors, particularly under unfavorable metabolic conditions.

Finally, it will be important in the future to determine the transgenerational effects of perinatal nutrition in the bovine female. Studies in rodents have shown that experimental manipulation of the nutritional plane during the perinatal period can significantly impact the reproductive and metabolic phenotypes of subsequent generations (Pinheiro *et al*., 2008; Pentinat *et al*., 2010; Burdge *et al*., 2011). While these studies are difficult to perform in cattle due to the long generation interval, a better understanding of this process can have important implications for lifetime animal health and productivity.

Acknowledgments

This work was supported by the USDA

National Institute of Food and Agriculture, Hatch project 1012280 (RCC) and AFRI project 2013-67015-20960 (GLW).

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DOI: 10.21451/1984-3143-AR2018-0023

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Pathogenesis of uterine diseases in dairy cattle and implications for fertility

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Abstract

Uterine diseases in cattle occur at all stages of the reproduction cycle but the majority of cases is found in the postpartum period. The inflammation of the uterus is generally defined as metritis or endometritis, with several graduations, e.g. puerperal metritis, clinical metritis, clinical or subclinical endometritis. Whether uterine diseases have a negligible, moderate or detrimental effect on fertility is still under discussion and depends on definitions and classification. In the past, it was assumed that the pregnant uterus is free of pathogens, but recent studies found several species including pathogens in the uterus and endometrium of pregnant cows. After parturition, a broad diversity of bacteria with >200 different species has been found in the early postpartum period. Not all of these bacteria, however, are considered as pathogens. Furthermore, bacteriological findings provide only evidence for infection but not for inflammation. For some bacteria, particularly *Escherichia coli* and *Trueperella pyogenes* pathogenic mechanism resulting in metritis and endometritis have been elucidated in detail. The role of bacteria that can be regarded as opportunistic or potential pathogens, e.g. *Bacillus pumilus*, is still under investigation. The understanding of the uterine microbiota and its interactions is increasing with the use of modern high-resolution techniques such as Fourier-transform infrared spectroscopy. Endometrial cytology provides additional information about alterations in the endometrium. Knowledge of innate uterine defense mechanism in cattle has increased a lot in the recent past. It can be speculated that improving or modulating uterine defense mechanism will be part of future prevention and treatment approaches beyond the use of antimicrobials. In this context, cellular and molecular defense mechanisms have been in the focus of interest, e.g. the role of interleukins or mucins. This review gives a short overview on some aspects of recent research on uterine diseases in cattle.

Keywords: endometritis, metritis, microbiota, uterine defense mechanism, uterine disease.

Introduction

Reproductive performance of cows and heifers is one of the key parameters reflecting the economic success of dairy farming. Several fertility parameters can be used to describe negative effects of uterine diseases on reproductive performance, e.g. days to first service, estrus detection rate, and probability of pregnancy per insemination. Days to first service and estrus detection rate are mainly affected by management (time for estrus detection, accuracy of estrus detection, claw trimming, and several other aspects related to management) and to lesser extent by cows' diseases, e.g. cysts, conception risk is strongly related to uterine health. Thus, the prevention of uterine diseases, sufficient detection of affected cows and efficacious treatment strategies are important aspects of the fertility management of dairy cows.

Inflammation of the uterus, defined as metritis or endometritis is one of the most common disorders in the postpartum period of dairy cattle. Puerperal metritis is characterized by abnormal discharge, enlarged uterus, dullness, and a rectal temperature >39.5°C (Sheldon *et al*., 2006). Puerperal metritis is also referred to as acute metritis, toxic puerperal metritis or septic metritis and occurs usually within the first 10 days after parturition.

Clinical or chronic endometritis is defined by the occurrence of purulent or mucopurulent vulvar discharge detected more than 3 weeks postpartum. In contrast to acute metritis, chronic endometritis is not associated with elevated temperature and the animals do not show general signs of illness or a depressed attitude. It has been suggested to perform a clinical examination for endometritis later than 21 or 26 days postpartum. An earlier examination results in a greater proportion of false-positive diagnoses (LeBlanc *et al*., 2002). Although the term endometritis is well established for cows with vaginal discharge, there is clear evidence that the origin of pus in the vagina is not always the endometrium but could also be the cervix or vagina. Furthermore, Dubuc *et al*. (2010) found a poor agreement between cytological endometritis diagnosed by uterine cytology and vaginal discharge. Thus, Dubuc *et al*. (2010) suggested the term purulent vaginal discharge (PVD) as a more accurate description than clinical endometritis. The authors of this paper, however, prefer to follow the classical rules of medical terminology for a disease and not to use symptoms as synonymous for a diagnosis.

For the definition and diagnosis of subclinical or cytological endometritis, cytological samples are taken from the endometrium to determine the percentage of polymorphonuclear cells (PMN) in the smears (Kasimanickam *et al*., 2004). A proportion of 5% PMN can be regarded as diagnostic for subclinical endometritis (Madoz *et al*., 2013). Research on subclinical endometritis and diagnostic techniques has been recently reviewed by De Boer *et al*. (2014) and Wagener *et al*. (2017a).

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Received: April 25, 2018
Accepted: February5, 2018

Uterine infection, uterine microbiota

The contamination of the uterus after parturition with a broad diversity of bacteria is regarded as inevitable. This process with contamination, elimination and subsequent re-contamination is complex and not fully understood (Földi *et al*., 2006; Wagener *et al*., 2015). This concept becomes even more complex with the fact that the pregnant uterus is not sterile, as thought for many years, but may be colonized with several bacterial species including pathogens such as *Trueperella pyogenes* (Karstrup *et al*. 2017; Moore *et al*. 2017). These findings open a new discussion about long-lasting effects of uterine infections but also on pathogenic mechanism of bacteria, i.e. under which conditions an infection has detrimental effects - or not - on conception and pregnancy. Furthermore, there is some evidence that bacteria may also invade the uterus via blood, e.g from the gut (Jeon *et al*., 2017). Whether natural defense mechanisms eliminate the majority of invaded bacteria or clinical diseases manifest depends on the bacterial load and pathogenicity of bacteria as well as on the immune status of the cow (Sheldon *et al*., 2002; LeBlanc *et al*., 2011; Jeon *et al*., 2016). Thus, the occurrence of metritis and endometritis is not only a question of the invading bacterial species. Understanding these interactions and the changes in bacterial composition of the uterine microbiota prior to the appearance of clinical signs of a disease may play a crucial role for the development of optimal prevention and intervention strategies in the future (Madoz *et al*., 2014).

Persistent bacterial infection of the uterus may cause metritis and endometritis. *Escherichia coli, T. pyogenes*, *Fusobacterium necrophorum* and *Prevotalla spp*. are common intrauterine pathogens (Földi *et al*., 2006; Sheldon *et al*., 2006), although the bacterial virulence factors involved in uterine pathology and the exact mechanism of bacterial pathogenicity are largely unknown (Bicalho *et al*., 2012). In the recent past, however, the effects of uterine infections with *E. co*li and *E. coli*-derived endotoxins lipopolysaccharides (LPS) have been studied and described in detail (Williams *et al*., 2008; Sheldon *et al*., 2009, 2010; Herath *et al*., 2009; Goldstone *et al*., 2014). It has been shown that LPS, a component of the bacterial membrane, has detrimental effects on the endometrium, disrupts uterine and also ovarian function, but also plays role in the innate immune response. One mechanism is that Toll-like receptors (TLR) on the endometrial cells bind LPS, leading to secretion of cytokines and chemokines. Chemokines attract neutrophils and macrophages to eliminate bacteria. There is evidence that a specific strain of *E. coli*, EnPEC, is the main pathogen for metritis (Sheldon *et al*., 2009).

Whereas *E. coli* can be regarded as one of the major pathogens associated with early postpartum metritis, *T. pyogenes* is supposed to be responsible for endometritis later in the puerperium. In the past, preceding infections with *E. coli* were assumed to facilitate subsequent persistence of *T. pyogenes* infections (Dohmen *et al*., 1995, 2000). Recent studies, however, did not support this hypothesis (Prunner *et al*., 2014; Wagener *et al*., 2014b). In contrast, a positive correlation between occurrence of *Streptococcus uberis* on day 3 postpartum and subsequent *T. pyogenes* infections was found, which is in line with previous studies reporting an increased risk of purulent vaginal discharge related to the presence of α-hemolytic Streptococci (Werner *et al*., 2012). Furthermore, it was shown that specific subtypes of *S. uberis* were associated with the uterine health status of postpartum dairy cows (Wagener *et al*., 2014b).

A study from Amos *et al*. (2014) provided evidence that the exotoxin pyolysin (PLO) is the major virulence factor of *T. pyogenes*. Interestingly, endometrial stroma cells were more sensitive to PLO mediated cytolysis than epithelial cells. Thus, it seems that detrimental effects of *T. pyogenes* at the endometrium occur once the epithelium layer is disrupted after parturition. However, the PLO gene is present in all *T. pyogenes* strains and recombinant PLO alone did not stimulate a host inflammation response. Thus, further research should elucidate if the cellular response, that is commonly associated with bacterial infections, is triggered by other virulence factors of *T. pyogenes* (Bicalho *et al*., 2012) or by co-occurring other intrauterine bacterial species.

Beside *E.coli* and *T. pyogenes*, a broad variety of other bacterial species can be found in the bovine postpartum uterus (Földi *et al*., 2006; Prunner *at al*., 2014; Wagener *et al*., 2015). Particularly, the application of high-resolution techniques brought deep insights into the uterine microbiota. Metagenomic studies (Santos *et al*., 2011; Santos and Bicalho, 2012) and analyses performed with the aid of chemometric-assisted Fourier-transform infrared (FTIR) spectroscopy (Prunner *et al*., 2014; Wagener *et al*., 2015) indicated that the bovine uterine microbial community is diverse, highly dynamic and even much more complex than previously thought. In one of our studies, we could show that the aerobic uterine microflora comprised a huge diversity of bacteria belonging to 202 different species, representing 76 genera (Wagener *et al*., 2015). Members of the genus Bacillus, Streptococcus, Enterococcus and coagulase negative staphylococci (CNS) were the most frequently isolated intrauterine bacteria, and have been discussed as potential pathogens or opportunistic contaminants earlier (Westermann *et al*., 2010; Werner *et al*., 2012). On species level, the uterine microflora was dominated by *T. pyogenes*, *E. coli*, *Staphylococcus xylosus*, *B. pumilus* and *S. uberis*. It is interesting to see that the microbiota did not change only over time but showed also differences between cows with different uterine health status (Wagener *et al*., 2015). Additionally, known pathogens, e.g. *T. pyogenes* were identified not only in cows with endometritis but also in clinically healthy cows. Further attention should focus on identifying and characterizing opportunistic pathogens that may synergistically interact with *T. pyogenes.* Detailed information on potential interactions and the interpretation of these findings needs some more investigation.

To gain a deeper insight into mechanism of

intrauterine infections it is essential to elucidate the role of bacteria that may act as a mediator of inflammatory host response. Recent studies indicate that *S. uberis* and *B. pumilus* (Wagener *et al*., 2014b, 2015) might represent such bacterial candidates with a hitherto unknown role in uterine pathology. Both bacteria are prevalent in postpartum dairy cows. *S. uberis* is known as an emerging pathogen for mastitis, but has not been described as uterine pathogen. The rarely described *B. pumilus* was found to be associated with endometritis, but it is still unclear if this species is a causing agent for endometritis or is only an opportunistic contaminant in the inflamed endometrium. Results from *in vitro* cell culture studies pointed towards a pro-inflammatory potential of *B. pumilus* on endometrial cells (Gärtner *et al*., 2016).

As shown in some of the above mentioned studies (Santos *et al*., 2011; Santos and Bicalho, 2012; Wagener *et al*., 2015), it has to be underlined that in several cows with clinical signs of metritis and endometritis, neither *E.coli* nor *T. pyogenes* can be detected. In this context, the role of anaerobic bacteria, e.g. *Porphorymonas spp*., as a causing factor as well as a factor for cure rates after antibiotic treatment of metritis needs to be elucidated more in detail (Jeon *et al*., 2017, 2018). Additionally, further research is required to understand the association between uterine diseases and hitherto unknown species (Santos *et al*., 2011; Wagener *et al*., 2014a).

Molecular mechanism associated with subfertility

Uterine infections as well as cytological endometritis, which are not necessarily congruent, play a crucial role in subfertility in cows. It seems likely that underlying mechanisms for subfertility can be found on molecular level, as reviewed in detail by Sheldon *et al*. (2014).

Several studies found differences in endometrial gene expression of pro-inflammatory mediators, such as cytokines, antimicrobial peptides, acute phase proteins (APP) and prostaglandins between healthy cows and subfertile cows with e.g. subclinical or clinical endometritis (Fischer *et al*., 2010; Drillich *et al*., 2012; Hoelker *et al*., 2012; Peter *et al*., 2015; Ibrahim *et al*., 2016). Upregulated chemokine mRNA expression is required to mediate and direct the PMN influx into the uterine lumen (Zerbe *et al*., 2003). This chemoattractive effect has been found e.g. for Interleukin (IL) 8 and CXCL5 (Fischer *et al*., 2010; Galvão *et al*., 2011). Cytokines IL1A, IL1B, IL6 and TNFα can be regarded as mediators of nonspecific inflammatory processes but are also physiologically upregulated during the early puerperal period (Gabler *et al*., 2010). Highest cytokine and acute phase proteins mRNA expression was observed during the third week postpartum regardless of their health status (Gabler *et al*., 2010; Chapwanya *et al*., 2012). This supports the hypothesis that a certain immune response is essential for postpartum clearance of the uterus. Thus, it would be helpful for research as well as in practice to use pro-inflammatory markers as a diagnostic tool for uterine inflammation.

Other inflammatory mediators associated with uterine diseases are prostaglandins (PG; Arosh *et al*., 2002). In cows with subclinical endometritis 21 to 28 days postpartum concentrations of PGF2α were significantly lower whereas concentrations of PGE2 were higher compared with healthy cow (Baranski *et al*., 2013). It can be hypothesized that this may relate to a switch from PGF2α to PGE2 induced by *E.coli*-derived LPS (Herath *et al*., 2009). Additionally, dysregulated expression of enzymes for PG synthesis (PGES) was found in cows with uterine disorders and reduced fertility (Gabler *et al*., 2009, 2010; Peter *et al*., 2015), e.g. endometrial PGE2 synthase cPGES, which catalyzes the conversion from PGH2 into PGE2, was lower and PGD2 synthase was higher compared with healthy cows. Dys-regulation of PGE synthases might contribute to lower conception rates (Gabler *et al*., 2010). Detailed research is needed to elucidate long term effects of uterine inflammation in the early postpartum period on fertility that manifests later in lactation. In that context, Peter *et al*. (2015) provided some evidence that endometrial inflammation has such a long term effect on PGD2 synthase PTGDS. In this study, endometrial mRNA expression of several pro-inflammatory factors was measured in weekly intervals between 24 and 44 days postpartum. Cows initially diagnosed with subclinical endometritis showed a 3-fold increase in PTGDS expression at the end of the observation period compared with healthy cows. Low levels of PGD_2 are required for the maintenance of pregnancy (Saito *et al*., 2002). This, however, is only one example of several factors involved in fertility that need further investigation. Furthermore, the difference between the changes in gene expression and the occurrence of clinical signs of disease and future fertility should be elucidated.

In a recent study, we observed differences in gene expression of endometrial epithelial cells between subfertile (repeat breeder) cows and healthy heifers (Wagener *et al*., 2017b). Interestingly, the most pronounced differences were observed for mucins, molecules that are an integral component of the local uterine immune defense. Mucins are anti-adhesive glycoproteins covering epithelial surfaces to protect from bacterial infection and proteolytic attacks. In the uterus, high mucin levels may be desirable after parturition to prevent the establishment of bacterial infections (Sheldon, 2015). In contrary, before embryo implantation, mucin down-regulation of endometrial epithelial cells is required for blastocyst attachment in many species (Braga and Gendler, 1993; Johnson *et al*., 2001). The results of our previous study support the assumption that dys-regulated MUC4 and MUC12 mRNA expression in the uterus may contribute to subfertility in cows (Wagener *et al*., 2017b). Also Kasimanickam *et al*. (2014) suggested a potential relevance of endometrial MUC in subfertile cows.

The effects of an inflamed endometrium on embryo's quality and survival have been show in several studies (Hill and Gilbert, 2008; Drillich *et al*., 2012; Hoelker *et al*., 2012). From a study with superovulated cows and flushed embryos, we suggested

that a certain inflammatory endometrial reaction in terms of PMN influx is beneficial for the number of flushed embryos and, thus, for embryo survival (Drillich *et al*., 2012). Furthermore, changes in endometrial gene expression patterns in cows with and without (subclinical) endometritis included genes involved in cell adhesion and immune modulation (Hoelker *et al*., 2012). There is evidence for an effect of endometrial PMN infiltration on the early stage embryos. In the first week after fertilisation, altered embryonic gene expression profiles between healthy and subclinical endometritic cows were related to membrane stability, the cell cycle and apoptosis (Hoelker *et al*., 2012). Underlying mechanisms for dysregulated gene expression in cows with uterine diseases might be elucidated by expression analyses of microRNAs (miRNAs), regulators of post-transcriptional gene expression. Hailemariam *et al*. (2014) found miRNAs that were differentially expressed in cows with subclinical endometritis, and that are involved in inflammatory responses, cellular proliferation, cell movement, the cell cycle and apoptosis.

Link between negative energy balance and subfertility

Beside bacterial and viral infections (reviewed by Chastant-Maillard, 2015), several risk factors for endometritis are known, for example calving assistance (Prunner *et al*., 2014), negative energy balance pre- and post-partum (Potter *et al*., 2010), hypocalcaemia, and others (Dubuc *et al*., 2010; Giuliodori *et al*., 2013).

The *in vivo* situation of the reproductive tract environment in ruminants is not only influenced by physiological processes of regeneration, inflammation and infection but is also highly dependent on the metabolic situation of the cow (LeBlanc, 2012).

Because of tremendous needs for energy for milk production and decreasing dry matter intake around calving almost all dairy cows undergo a period of negative energy balance (NEB) in the first 100 days of lactation (LeBlanc, 2010). Cows compensate NEB by lipid mobilization, resulting in increased circulating levels of non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB) and reduced levels of insulin and insulin-like-growth-factor 1 (IGF-1). It is well known that metabolic imbalances during the transition period impair fertility (LeBlanc, 2012). Previous studies have investigated effects of NEB on ovarian function, oocyte and embryo quality (Leroy *et al*., 2008) and uterine health (Chapinal *et al*., 2011). A previous study observed altered gene expression of *IGF* binding protein in the oviduct of cows with NEB; however, the influence of gene expression changes on embryo development were not analyzed (Fenwick *et al*., 2008). It seems that feed restriction during the dry and post-partum period in general influences global gene expression in the oviduct (Valour *et al*., 2013). Thus it is important to consider the metabolic situation of the cow in studies investigating underlying reasons for subfertility. Furthermore, the role of the oviduct in cows with uterine diseases needs further evaluation.

Summary

In the past decades, our knowledge on uterine infections has increased enormously. Particularly the role and mechanisms of pathogenicity of bacteria have been elucidated, as reviewed by Sheldon *et al*. (2014). Current challenges are the understanding and interpretation of bacterial interactions in the uterine microbiota. Uterine infections cause a cascade of immune reactions in the endometrium which is at least in some ways different from healthy cows that also undergo cellular and molecular changes in the postpartum period. A detailed understanding of these pathogenic mechanisms leading to subfertility might be a key for future prevention and treatment options. Whereas uterine infection and inflammation has been the topic of many studies that elucidated several relations, deeper insights regarding the relationship between uterine diseases and alterations in the oviduct and its functionality is needed to get a broader picture of subfertility in cattle.

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DOI: 10.21451/1984-3143-AR2018-0056

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Impact of thermal stress on placental function and fetal physiology

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Abstract

In ruminants, prolonged exposure to high ambient temperatures negatively affects placental development and function. The pursuing limitations in placental oxygen and nutrient supply between the mother and fetus slow fetal growth lowering birth weights and postnatal performance. The pregnant ewe is a long-standing animal model for the study of maternal-fetal interactions and is susceptible to naturally occurring heat stress, which causes fetal growth restriction. In the pregnant ewe, studies show that the fetus adapts to hyperthermia-induced placental insufficiency to preserve placental transport capacity of oxygen and nutrients. These adaptive responses are at the expense of normal fetal development and growth. Enlarged transplacental gradient for oxygen and glucose facilitates diffusion across the placenta, but develops by lowering fetal blood oxygen and glucose concentrations. Fetal hypoxemia and hypoglycemia slow growth and alter their metabolic and endocrine profiles. Deficits in amino acids transport across the placenta are present but are overcome by reduced fetal clearance rates, likely due to fetal hypoxemia or endocrine responses to hypoxic stress. Here, we provide an overview of the performance limitations observed in ruminants exposed to heat stress during pregnancy, but we focus our presentation on the sheep fetus in pregnancies complicated by hyperthermia-induced placental insufficiency. We define the characteristics of placental dysfunction observed in the fetus of heat stressed ewes during pregnancy and present developmental adaptations in organogenesis, metabolism, and endocrinology that are proposed to establish maladaptive situations reaching far beyond the perinatal period.

Keywords: heat stress, intrauterine growth restriction, placental insufficiency, sheep fetus.

Introduction

Environmental heat stress diminishes revenue for livestock producers by negatively impacting nutrient utilization, growth, and reproductive performance. Consequences of heat stress on early embryonic survival have been well documented, both scientifically and economically (Hansen *et al*., 2001). However, financial losses from warm environmental conditions are not limited to embryonic wastage. Maternal exposure to prolonged high ambient temperatures during gestation has been associated with lighter birth weights, greater incidence of morbidity before weaning, lower survival rates, and less desirable carcass traits (Shelton, 1964; Monteiro *et al*., 2016). As we will explain, these latter complications likely are products of developmental adaptations to nutrient and oxygen deprivation caused by hyperthermia-induced placental insufficiency. During maternal heat stress, fetal growth restriction may be considered beneficial for the dam, as a smaller conceptus yields less metabolically active tissue, greater maternal surface area to mass ratio, and less nutritional strain on the mother (Wells and Cole, 2002). Although fetal growth restriction is advantageous for the dam, fetal growth restriction and the accompanying adaptations to placental restriction are associated with a myriad of metabolic complications that negatively affect future performance.

Because offspring from a heat stressed dam are growth restricted during gestation, we begin with a closer look at the characteristics of placental dysfunction that restrict fetal growth and cause metabolic adaptations. We provide an overview of the performance limitations observed with maternal heat stress in ruminants, but focus our presentation on work conducted in sheep that are experimentally heat stressed during mid gestation, a time when the placenta is established and placental growth is at maximum (Regnault *et al*., 2002a). The pregnant sheep has been used extensively over the past 50 years to investigate placental and fetal physiology due to the ability to surgically place and maintain catheters in the maternal and fetal vasculature that allow for repetitive blood sampling from non-anesthetized ewes (Meschia *et al*., 1965; Barry *et al*., 2008). The substantial groundwork on maternal-fetal interactions in sheep provides ample knowledge for normal pregnancy, as well as information on models of pregnancies complicated by experimentally or naturally produced placental restriction, which includes a model of hyperthermia-induced placental insufficiency.

Pregnant ewes exposed chronically to high ambient temperatures in the laboratory from early to late gestation have fetuses that are significantly growth restricted close to term (Bell *et al*., 1987; Thureen *et al*., 1992). Ultrasonographic measurements indicate that biometric parameters for determining fetal growth restriction, for example abdominal circumference begins to diverge from normal as early as mid-gestation. This is a developmental point, prior to rapid fetal growth and at the apex of placental growth (Galan *et al*., 1999). Terminal studies indicate that significant reduction of placental mass precedes declines in fetal weight (Fig. 1A and B). Before 110 days of gestation (term 149 days), placental weights were significantly less in heat stress ewes than controls (280 ± 32 g vs. 443 ± 32 g).

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Received: April 9, 2018
Accepted: May 16, 2018

However, fetal weights were not affect at this younger age (0.9 ± 0.2 kg versus 1.0 ± 0.2 kg). After 130 days of gestation, both fetal and placental weights were significantly less in heat stressed ewes compared to controls (49% for fetus and 56% for placenta). These data indicate that the majority of fetal growth restriction occurs during the final stages of gestation after placental growth restriction (de Vrijer *et al*., 2006; Macko *et al*., 2013). Limitations are caused by reduced placental mass and function, which leads to the development and progressive decline in fetal glucose (21 to 33% less) and oxygen (25 to 46%) concentrations over the final third of gestation, when fetal growth rate is at maximum (Limesand *et al*., 2013; Rozance *et al*., 2018; Fig. 1C and D). This evidence supports the hypothesis that placental deficiencies are responsible for fetal growth restriction in late gestation. Abnormal placental growth, vascular organization, and angiogenesis were described as possible causes of placental insufficiency due to aberrant expression patterns of angiogenic growth factors and their receptors (Vatnick *et al*., 1991; Regnault *et al*., 2002b; Galan *et al*., 2005; Hagen *et al*., 2005). Together, the pregnant ewe and this model of hyperthermia-induced placental insufficiency provides a unique opportunity to investigate fetal adaptive responses and growth restriction caused by a naturally induced placental restriction, which negatively effects their future health and performance. We review the outcomes in the placenta and fetus that are associated with adaptive responses to hyperthermia-induced placental insufficiency and discuss how they relate to future deficiencies in production.

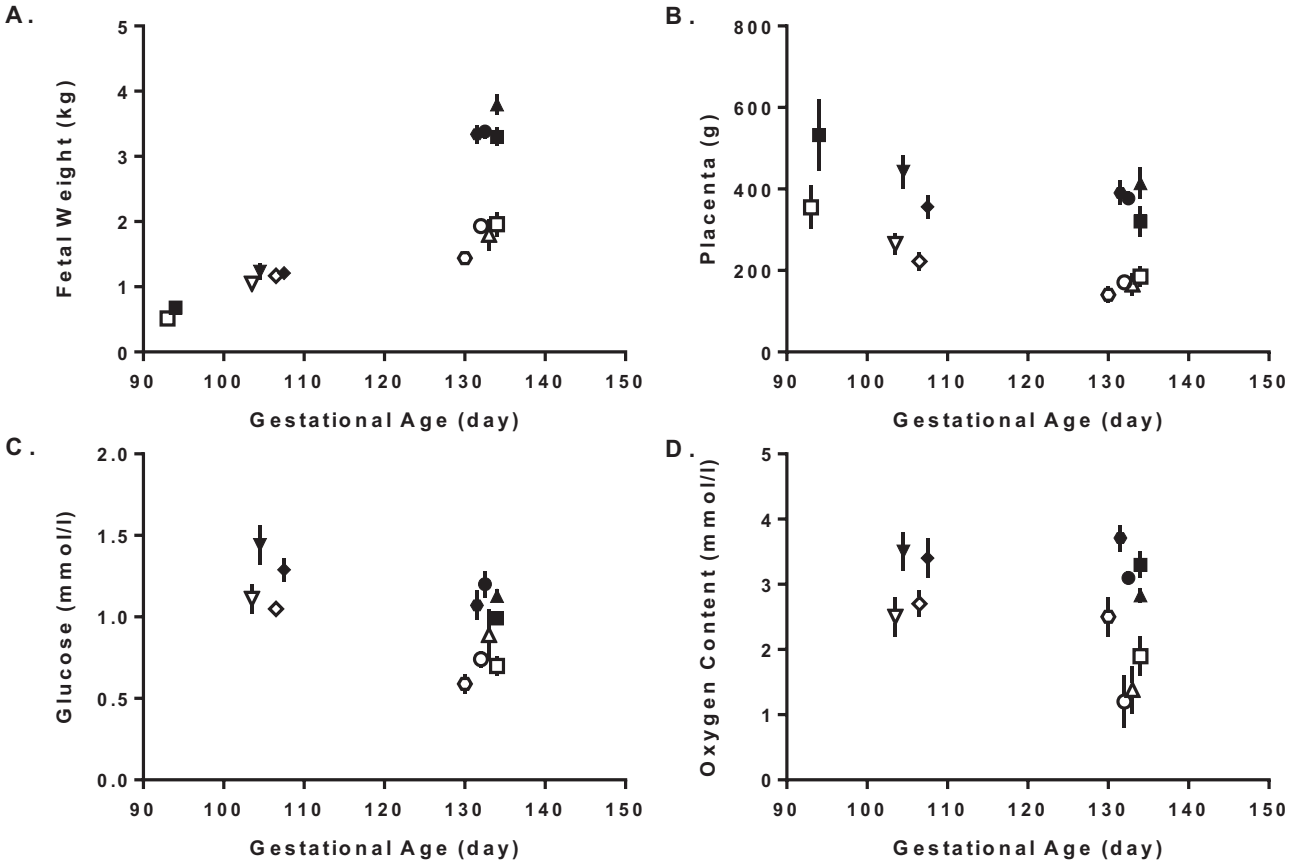

Figure 1. Progression of hyperthermia-induced placental insufficiency. Fetal weights (A), placental weights (B), fetal arterial plasma glucose concentrations (C), and fetal arterial blood oxygen contents (D) are presented as means reported in Ross *et al*., 1996 (hexagon); Brown *et al*., 2012 (circle); de Vrijer *et al*., 2006 (square <100 days); Limesand *et al*., 2013 (downward triangle), Macko *et al*., 2013 (diamond); and Rozance *et al*., 2018 (square >130 days). Each individual point represents a group mean for thermoneutral control ewes (fill shapes) and heat stress ewes (open shapes) for that specific report (symbol). At younger gestational ages (<110 days), all group means were significantly different within a report, except for fetal weight. At older gestational ages (>130 days), group means within the report were significantly different. Conclusions from these data indicate that advancing gestational age leads to greater differences in all parameters measured, which includes slower rates of fetal growth.

Effects of heat stress in ruminants during gestation and lactation

Environmental heat stress imposes significant limitations on fetal growth and milk production in several species of ruminants, but some species, usually those with higher production rates, are more susceptible to heat stress. For this reason, lactating dairy cows have a low tolerance to heat stress because as milk production per cow is increased there is a concurrent increase in metabolic heat production (Collier *et al*., 2006). Similarly, substantial economic consequences are incurred during gestation and lactation due to high metabolic heat output (West, 2003; Collier *et al*., 2006).

Mechanisms that explain placental and fetal growth restriction include the redistribution of blood flow to the skin and nasal mucosa at the expense of other internal organs including the pregnant uterus (Reynolds *et al*., 1985; McCrabb *et al*., 1993). Reductions in placental function, along with maternal factors caused by heat stress, restrict mammary gland development and lower the potential yield for the subsequent lactation. In addition to losses in milk yield and quality following the immediate pregnancy, heat stress in late pregnancy effects immune processes associated with poor transition to lactation including: lower phagocytic activity and decreased hepatic prolactin signaling (PRL-R, SOCS-3, and CAV-1 mRNA) during the dry period (do Amaral *et al*., 2011). Milk yield and milk protein are influenced by calving season, as both are lowest during the warmest months compared to cows that calved in the winter (Barash *et al*., 2001). Lactating sheep are also susceptible to heat stress, which leads to differences in milk composition, specifically lower fat and protein content (Abdalla *et al*., 1993). In addition to lighter birth weights, calves from heat stress cows exhibited both immediate and prolonged effects on their passive immunity, growth, activity patterns, and thermotolerance (Ahmed *et al*., 2017; Laporta *et al*., 2017). Furthermore, the effects of heat stress on offspring persist, resulting in lower yearling weights and less heifers reaching their first lactation. Cows exposed to heat stress during fetal life produced less milk compared to cows that received heat abatement strategies (Monteiro *et al*., 2016). This evidence shows that heat stress during gestation affects fetal development and also creates lasting complications that lower the future productivity of the offspring, which may be caused by developmental adaptations to placental insufficiency.

Maternal heat stress limits placental transport capacity

The capacity of the placenta to transfer oxygen and nutrients must increase throughout pregnancy to meet metabolic demands of the growing fetus. In sheep, placental transport capacity continues to increase by expanding the surface area of the maternal-fetal interface and by thinning of the placental barrier to promote the exchange and permeability of metabolic substrates. Amino acids, oxygen, and glucose are transported across the placenta by active transport, passive diffusion, and facilitated diffusion (Battaglia and Meschia, 1978). For diffusion mechanisms, the rate of transplacental transport is dependent on uterine and umbilical blood flow, substrate permeability, and substrate concentration difference across the placenta.

Placental clearance is diminished with heat stress due to lower permeability for metabolic substrates. Oxygen and glucose permeability is reduced by a smaller placenta with less surface area and transport capacity, which combine to lower uterine extraction efficiency (Fig. 2). Evidence for this conclusion is that the transplacental gradients of oxygen and glucose increase in ewes exposed to environmental heat stress during pregnancy compared to pregnant ewes maintained under thermoneutral conditions (Fig. 3). Unlike placental transfer rates, placental clearance is independent of concentration gradients, but dependent on the properties of the exchanger (membrane) permeability or perfusion. Studies with inert molecules that have flow-limited placental transport show equivalent transplacental clearance rates in heat stressed and thermoneutral ewes. For example, there is no difference in ethanol clearance across the placenta between thermoneutral and heat stressed ewes when expressed relative to placental mass (Bell *et al*., 1987; Thureen *et al*., 1992; Regnault *et al*., 2007). This observation excludes shunting or uneven perfusion of uterine and umbilical blood flow as a cause for decreased transplacental clearance in heat stressed ewes. In addition to lower placental permeability, placental transport capacity of metabolic substrates is hindered by alterations in uteroplacental consumption of the substrate itself. In heat stressed ewes, uteroplacental oxygen consumption normalized to placental weight is unaffected, and glucose utilization by uteroplacental tissue is less (Bell *et al*., 1987; Thureen *et al*., 1992; Regnault *et al*., 2007). Therefore, the enlargement in the transplacental concentration difference for oxygen and glucose are due to lower placental permeability, which for glucose may be explained partially by lower abundance of facilitated glucose transporters (Limesand *et al*., 2004; Wallace *et al*., 2005).

Transplacental gradients and uterine-umbilical blood flow ratios adapt under heat stress to preserve the net umbilical uptake of glucose and oxygen, but as discussed later, this compensatory mechanism causes reductions in substrate concentrations in the fetus that become detrimental to development and growth (Bell *et al*., 1987; Thureen *et al*., 1992; Regnault *et al*., 2007, 2013). Simple concepts for diffusion indicate that larger transplacental concentration gradients (Fig. 3A and B) will increase the net movement of oxygen and glucose across the placenta to the fetus. Another compensatory mechanism is greater uterine-to-umbilical blood flow ratio, which further demonstrates impaired placental diffusion capacity in heat stressed ewes (Fig. 3C). Normally the uterine-to-umbilical blood flow ratio is ~2, which is predicted to fulfill the delivery requirements for the placenta and fetus because maximum fetal clearance occurs when flows are equivalent (Wilkening *et al*., 1982). Increases in the blood flow ratio lowers uterine arteriovenous differences for oxygen and aids in expanding the transplacental gradient to facilitate uterine uptake (Bell *et al*., 1987; Regnault *et al*., 2003). This adaptation may be advantageous to the fetus because it lowers cardiac output to the placenta to increase umbilical uptake, but the increase vascular resistance in the placenta is postulated to negatively affect the cardiovascular development (Galan *et al*., 2005). Together, the enlarged transplacental concentration gradients of oxygen and glucose and increased uterine-to-umbilical blood flow ratio are sufficient to minimize reductions in net umbilical oxygen and glucose uptake per fetal mass. However, comparisons for means across several

reported cohorts indicate that there were modest but significant reductions of 9 and 14% in net umbilical oxygen and glucose uptakes, respectively (Fig. 4).

Placenta delivery of amino acids to the fetus is also lower in heat stressed ewes even though fetal plasma amino acid concentrations are not necessarily reduced (Thorn *et al*., 2009; Regnault *et al*., 2013). For most amino acids, concentrations in fetal circulation are normally greater than in maternal circulation and therefore are transported actively across the placenta against their concentration gradient. In heat stressed ewes, the absolute placental flux for essential amino acids from mother to fetus is reduced ~80%, whereas the flux relative to placental mass is ~40% less (Ross *et al*., 1996; Anderson *et al*., 1997; de Vrijer *et al*., 2004). Similar reductions in transplacental uptake of essential amino acids are seen when expressed relative to fetal mass, with the exception of lysine (Regnault *et al*., 2013). Two important points become evident from these independent studies on amino acid placental transport in heat stress ewes. First, fetal uptakes of essential amino acids are reduced to similar magnitudes. Second, the impaired transport of amino acids is due to a reduction in placental size as well as decreased transport capacity per unit mass. Therefore, amino acid transfer depends on surface area of the maternal-fetal interface, which is reduced, as well as on the concentrations of amino acid transporters (Regnault *et al*., 2005). Interestingly, decreased transplacental flux of amino acid does not always lower their concentration in fetal plasma, implicating adaptive mechanisms in fetal metabolism or clearance of amino acids, which were associated with low oxygen concentrations (Regnault *et al*., 2013). Again, these data show that placental insufficiencies produced by heat stress depend on decreased placental mass and function, even though compensatory mechanism by the fetus are in place to assist with the placental deficiencies.

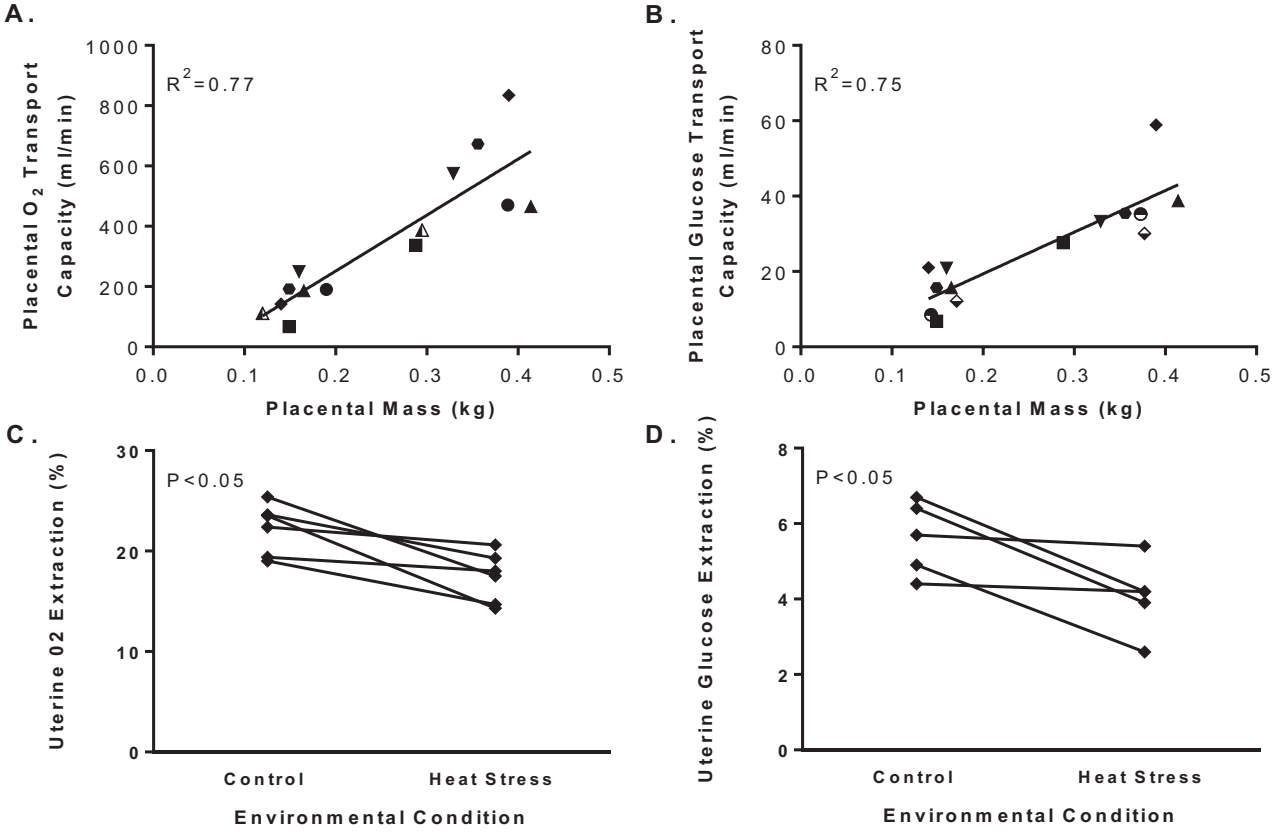

Figure 2. Placental transport capacity and uterine extraction efficiency for oxygen and glucose. Placental transport capacity for oxygen (A) and glucose (B) are presented by placental weights for independent group means from previously reported cohorts of thermoneutral and heat stressed ewes during gestation. Placental transport capacity is the net umbilical (fetal) uptake (µmol/min) divided by the maternal arterial-fetal arterial plasma concentration difference (µmol/ml) according to the equation: transport capacity = uptake/concentration difference. Linear regression analysis shows a positive association for placental transport capacity and placental mass (R2). Uterine extraction of oxygen was calculated by expressing the whole blood arterial-venous oxygen concentration difference across the uterine circulation as a percent of the arterial concentration (C). Uterine extraction of glucose was expressed as the plasma arterial-venous concentration difference as a percent of the arterial glucose concentration (D). Group means for thermoneutral control (fill symbols) and heat stressed animals (open symbols) were reported in Bell *et al*., 1987 (downward triangle); Thureen *et al*., 1992 (hexagon); Ross *et al*., 1996 (diamond); Anderson *et al*., 1997 (small circle, panel A); Regnault *et al*., 2003 (large circle, panel A); Limesand *et al*., 2004 (circle, panel B); de Vrijer *et al*., 2004 (triangle); Limesand *et al*., 2007 (large square, panel B) and Brown *et al*., 2012 (small square, panel B). An ANOVA with reported study as the repeated measure identify group (control and heat stress) differences (P-values) for panels C and D.

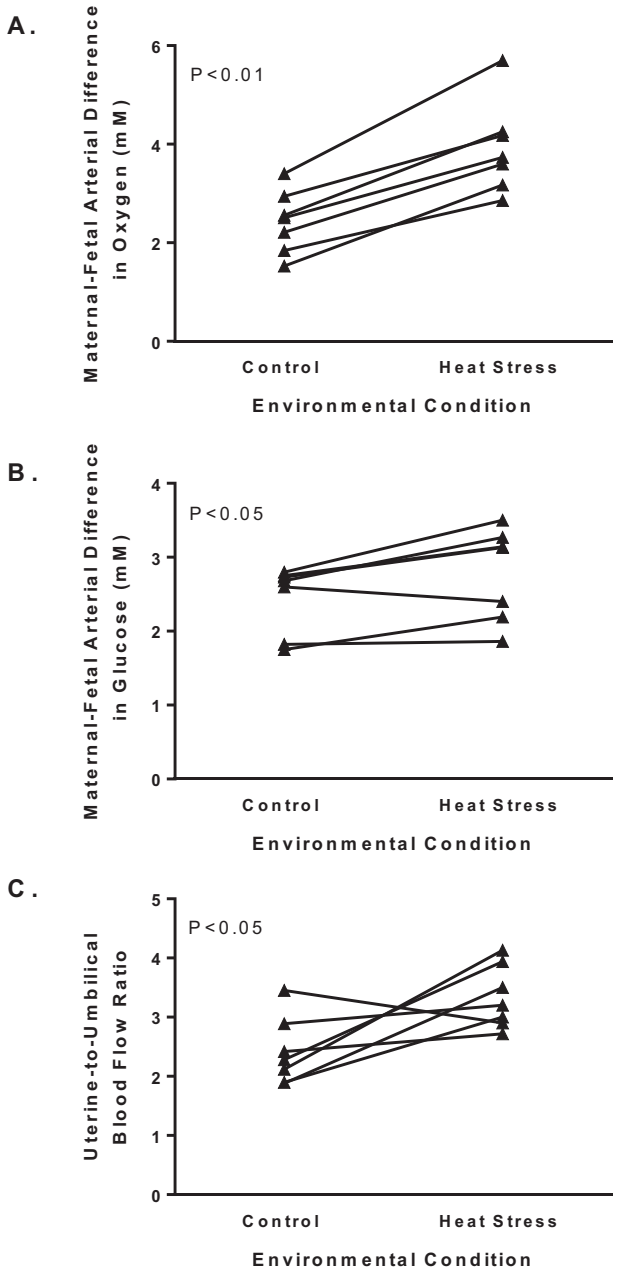

Figure 3. Transplacental gradients and uterine-umbilical blood flow ratio. Maternal-fetal arterial difference for oxygen (A) and glucose (B) concentrations are presented for pregnant ewes at approximately 90% of gestation. Uterine-to-umbilical blood flow rates are presented in panel C. Group means for thermoneutral control and heat stressed ewes were reported in Bell *et al*., 1987; Thureen *et al*., 1992; Ross *et al*., 1996; Anderson *et al*., 1997; Regnault *et al*., 2003; Limesand *et al*., 2004, 2007; de Vrijer *et al*., 2004. An ANOVA with report as the repeated measure identifies group (control and heat stress) differences (P-values).

Fetal responses to hyperthermia-induced placental insufficiency

Maternal hyperthermia is natural in sheep that, uncharacteristically, carry pregnancies in summer months producing a smaller placenta with lower transport capacity for glucose, amino acids, and oxygen. Therefore, hyperthermia-induced placental insufficiency under-nourishes the fetus and leads to asymmetric intrauterine growth restriction (IUGR) that spares brain and heart growth relative to overall body weight (Fig. 5). In heat stressed sheep with placental insufficiency, we and others have characterized fetal adaptations in metabolism, endocrinology, and selected organ functions to specify mechanisms responsible for developmental programming. These studies have focused on fetal metabolism and endocrinology, pancreatic insulin secretion, hepatic glucose production, skeletal muscle growth and metabolism, and cardiac metabolism. In combination with oxygen and nutrient deficits, elevations in norepinephrine and epinephrine impinge on nearly all adaptive fetal responses measured including growth, glucose metabolism, and insulin secretion (Davis *et al*., 2015; Macko *et al*., 2016). How these fetal responses allow normal cellular oxidation to continue, maintain viability at the expense of growth, but ultimately become maladaptive for future performance are described herein.

Fetal metabolism and endocrinology

The fetus uses the umbilical uptake of nutrients to fulfill two major requirements: oxidation to fuel energy metabolism and accretion for growth and storage of substrates. Energy metabolism can be estimated from rates of oxygen consumption, which based on net umbilical oxygen uptake per fetal mass was only marginally less in IUGR fetuses compared to control

fetuses (Fig. 4). For metabolic studies, the rate of oxygen consumption is one of the most useful standards of reference because the metabolic quotient defines the quantity of substrate needed to satisfy energy requirements of the fetus or fetal organs. This allows us to judge whether the quantity of nutrients being supplied to the fetus by the placenta is larger or smaller than the energy demands of the fetus, thus indicating whether the placental nutrient supply is sufficient to support fetal growth through accretion. The metabolic quotient for individual substrates are calculated as the ratio of the substrate oxygen equivalents to oxygen uptake. Oxygen equivalents are the quantity of oxygen molecules required for complete oxidation of that substrate to carbon dioxide and water. For example, when calculating the glucose/oxygen quotient, the glucose concentration difference (mmol/l) is multiplied by six and then divided by the oxygen concentration difference (mmol/l). In the fetus, the major sources for oxidative substrates are glucose, lactate, and amino acids, which explain the emphasis for studying their placental transport capacity and the resulting consequences to fetal growth when their delivery is restricted. Strikingly, the sum of the nutrient/oxygen quotients for glucose, lactate, and amino acids in IUGR fetus barely exceeds the umbilical oxygen quotient, which indicates that fetal uptake of nutrients is just sufficient to meet the oxidative requirements with no surplus for accretion (Regnault *et al*., 2013). A similar limitation in substrate availability was identified for the hindlimb quotients in IUGR fetuses (Rozance *et al*., 2018). Numerous studies have been conducted to explain how IUGR fetuses adapt to placental insufficiency by altering organ metabolism for these primary substrates as well as describing the potential endocrine regulation (Fig. 6).

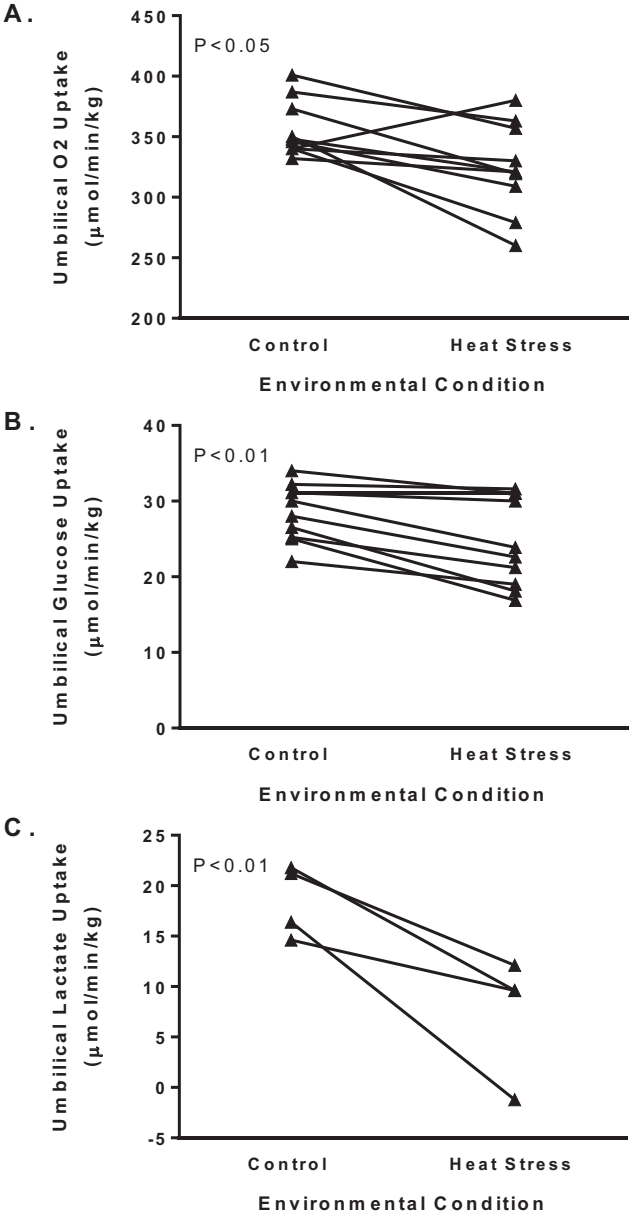

Figure 4. Umbilical uptakes for oxygen, glucose, and lactate. Fetal weight-specific net umbilical uptakes for oxygen (A) and glucose (B) are presented for pregnant ewes at 90% of gestation. Group means for thermoneutral control and heat stressed ewes were reported in Bell *et al*., 1987; Thureen *et al*., 1992; Ross *et al*., 1996; Anderson *et al*., 1997; Regnault *et al*., 2003; Limesand *et al*., 2004, 2007; de Vrijer *et al*., 2004; Brown *et al*., 2012, 2015; and Thorn *et al*., 2013. An ANOVA with reported study as the repeated measure identifies group (control and heat stress) differences (P-values) for each of the uptakes.

Although net umbilical (fetal) glucose uptakes were not significantly lower for all reports individually, the meta-analysis performed herein for ten reports that measured umbilical glucose uptake show that glucose uptake was lower in IUGR fetuses (Fig. 4). Experiments with glucose tracers to determine rates of fetal glucose

utilization and oxidation show significant alterations in glucose metabolism in IUGR fetuses with placental insufficiency (Limesand *et al*., 2007; Thorn *et al*., 2013; Brown *et al*., 2015). Body-weight specific glucose utilization rates are not different between IUGR and control fetuses, despite IUGR fetuses having markedly lower plasma insulin and glucose concentrations. In control fetuses, umbilical glucose uptake is normally equivalent to the glucose utilization rate, which demonstrates that placental glucose uptake is sufficient and glucose production is negligible. In IUGR fetuses with hyperthermia-induced placental insufficiency, glucose utilization rates exceed umbilical glucose uptake, thus the IUGR fetus has endogenous glucose production (Limesand *et al*., 2007; Thorn *et al*., 2013). The fraction of glucose oxidized to carbon dioxide is also less in IUGR fetuses, which suggests peripheral tissues may have increased glycolysis to supply lactate for hepatic glucose production (Limesand *et al*., 2007; Brown *et al*., 2015). Together, these findings identify important metabolic responses in glucose metabolism that are predicted to caused alterations in tissues such as liver and muscle, changes in endocrine factors, or a combination of both.

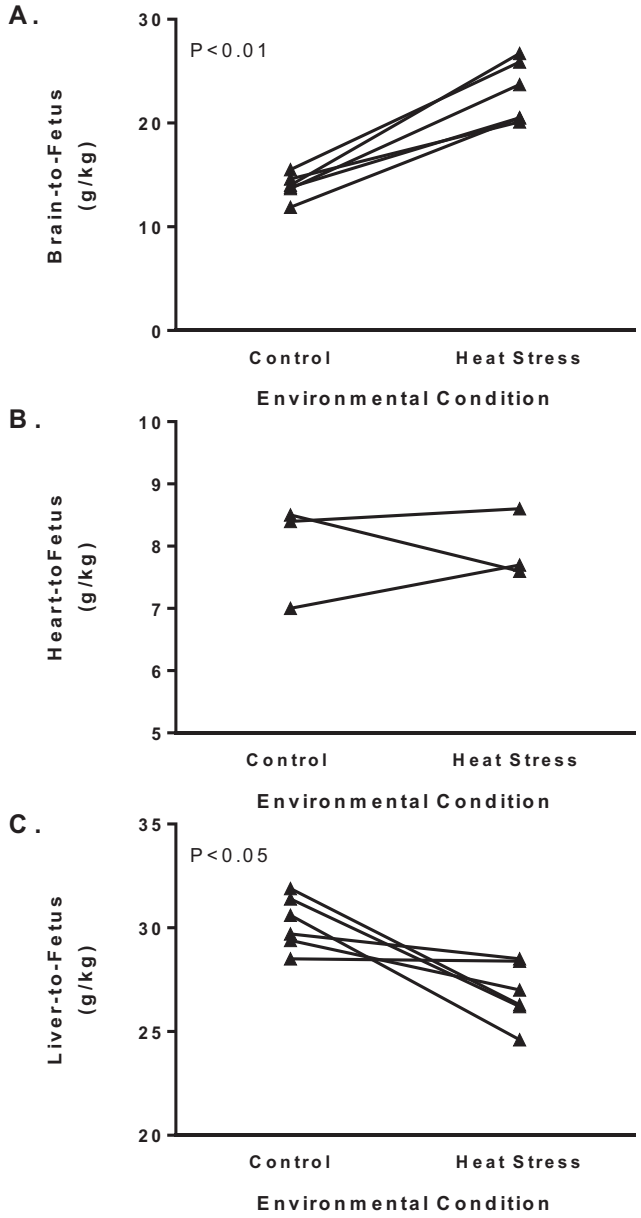

Figure 5. Asymmetric growth restriction with hyperthermia-induced placental insufficiency. Brain (A), heart (B), and liver (C) weights in grams are expressed relative to fetal weight (kg) for fetuses necropsied at 90% of gestation. Group means for thermoneutral control and heat stressed ewes were reported in Thureen *et al*., 1992; Anderson *et al*., 1997; de Vrijer *et al*., 2004; Brown *et al*., 2012; Davis *et al*., 2015; and Barry *et al*., 2016. An ANOVA with reported study as the repeated measure identifies group (control and heat stress) differences (P-values) for organ to fetal weight ratios.

For glucose metabolism, three adaptations are apparent in IUGR fetuses with placental insufficiency. First, there is greater avidity for glucose uptake and utilization by fetal tissues in the presence of low glucose and insulin concentrations, indicating that there is greater insulin sensitivity in the IUGR fetus. Explanations for the greater glucose uptake capacity include a larger proportion of neuronal tissue to body weight (Fig. 5) and is supported by upregulation of glucose transporter 1 concentrations in the brain (Limesand *et al*., 2007). Furthermore, the glucose extraction efficiency and glucose uptake into both the hindlimb and myocardium are similar between control and IUGR fetuses, despite low glucose and insulin concentrations in the IUGR fetus (Barry *et al*., 2016; Rozance *et al*., 2018). Because glucose transporter expression was unaffected in IUGR muscle, adaptations in proximal insulin signaling were apparent and appear

to enhance insulin sensitivity due to increased insulin receptor concentrations and decreased phosphoinositide-3 kinase (p85) with no change in the p110 catalytic subunit (Limesand *et al*., 2007; Thorn *et al*., 2009). Second, IUGR fetuses exhibit hepatic glucose production, which is normally uncommon, but augmented by significant increases in gluconeogenic enzymes, PEPCK and glucose-6-phosphatase, perhaps in response to cAMP-response element-binding protein activation due to increases in cortisol, glucagon, and norepinephrine (Fig. 6; Limesand *et al*., 2007; Thorn *et al*., 2013). Third, enzymes that regulate the tricarboxylic acid cycle are altered in skeletal muscle and liver of IUGR fetuses. For example, pyruvate dehydrogenase kinase 4 mRNA expression is increased 5-fold in IUGR skeletal muscle. Pyruvate dehydrogenase kinase 4, when phosphorylated, inhibits pyruvate dehydrogenase, which converts pyruvate to acetyl CoA for use in the tricarboxylic acid cycle (Brown *et al*., 2015). Pyruvate carboxylase and lactate dehydrogenases expression also were depressed in the skeletal muscle from IUGR fetuses, and the former may also play a role in sparing pyruvate via glycolysis from oxidative metabolism. Interestingly, lactate output from the hindlimb was not increased in IUGR fetuses. However, the lactate/oxygen quotient was greater, which shows greater lactate output per mole of oxygen consumed by the hindlimb of the IUGR fetus (Rozance *et al*., 2018). The sum of glucose and lactate quotient was similar between control and IUGR fetuses, and the amino acid/oxygen quotient was lower. This indicates that alterations in substrate utilization are more dependent on amino acid metabolism and protein synthesis and growth are expendable.

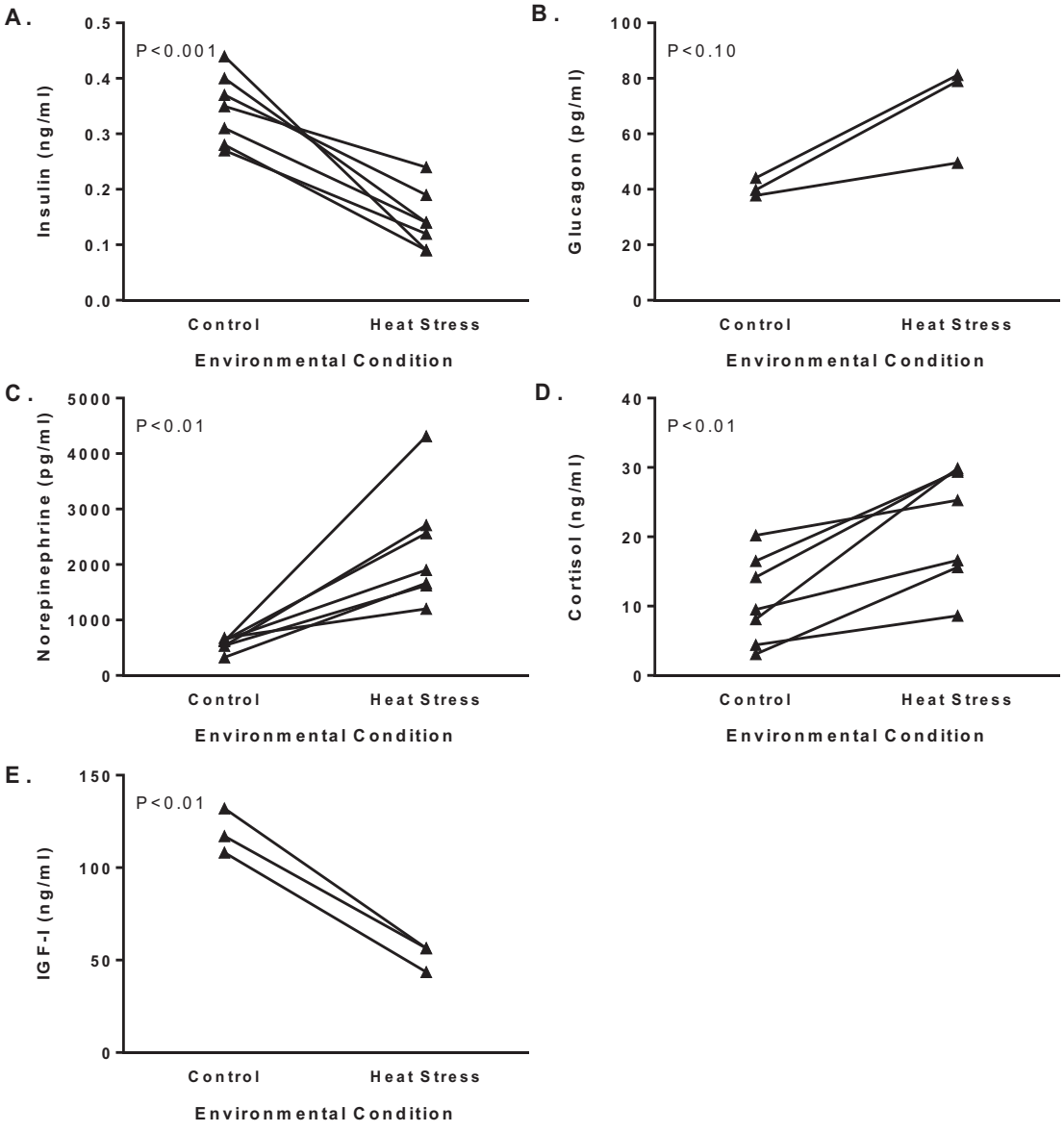

Figure 6. Endocrine profile in fetuses with hyperthermia-induced placental insufficiency. Mean plasma insulin (A), glucagon (B), norepinephrine (C), cortisol (D), and insulin-like growth factor I (IGF-I, E) concentrations are presented for fetuses at 90% of gestation. Group means for thermoneutral control and heat stressed ewes were reported in Limesand *et al*., 2006; Brown *et al*., 2012, 2016; Thorn *et al*., 2013; Macko *et al*., 2016; and Rozance *et al*., 2018. An ANOVA with reported study as the repeated measure identifies group (control and heat stress) differences (P-values) for hormone concentrations.

We have followed IUGR lambs from heat stressed ewes and identified persistent augmentations in insulin sensitivity at least until two weeks of age (Camacho *et al*., 2017). These experiments demonstrate that in utero adaptations could have negative consequences during the postnatal growth period, and ultimately progress into insulin resistance because IUGR lambs with less muscle mass are adapting to their postnatal environment while also undergoing age related declines in insulin sensitivity (Gatford *et al*., 2004). Low birth weight and less muscle mass may cause metabolic limitation that decrease subsequent performance (Wu *et al*., 2006). As postnatal growth is largely dependent upon fetal growth and development, it is important to determine how management decisions could impact the growth trajectory of the fetus; however, it is not always feasible to prevent IUGR in production settings. Thus, identifying the mechanism that regulate skeletal muscle metabolism in IUGR during pre- and postnatal development is necessary.

Pancreatic insulin secretion

Pancreatic β-cells secrete insulin in response to elevated plasma glucose concentrations to stimulate glucose uptake into fetal tissues and promote growth. In fetal sheep, β-cells become responsive to glucose after mid-gestation (Aldoretta *et al*., 1998). Since insulin cannot cross the placenta, insulin secretion from fetal pancreatic β-cells is critical for coordinating fetal growth rates with the placental glucose transport. Insulin secretion parallels changes in glucose concentrations, and both glucose and insulin concentrations are lower in IUGR fetuses (Boehmer *et al*., 2017; Fig. 6). In IUGR fetuses, glucose concentrations are chronically low (Fig. 1), which negatively affects β-cell mass and insulin secretory capacity. The IUGR fetus has impaired insulin secretion responsiveness due to two primary deficits: less β-cell mass as a consequence of slower rates of β-cell proliferation and less insulin content per β-cell (Limesand *et al*., 2005, 2006).

Another factor contributing to the suppression of insulin secretion in IUGR fetuses is persistent elevations of plasma norepinephrine and epinephrine, which inhibit insulin secretion through α2-adrenergic receptors on β-cells (Jackson *et al*., 2000). In the IUGR fetus, glucose-stimulated insulin concentrations during an adrenergic receptor blockade are equivalent to maximal insulin concentrations in control fetuses (Leos *et al*., 2010; Macko *et al*., 2013). This response occurs despite IUGR fetuses having fewer β-cells that contain less insulin. Therefore, chronic adrenergic stimulation inhibits insulin secretion from fetal β-cells, but the chronic suppression in IUGR fetuses causes developmental changes in β-cells insulin secretion responsiveness. Following chronically high norepinephrine fetal infusions, a subsequent hyper-secretory response of insulin has been confirmed in normally grown fetuses (Chen *et al*., 2014). Strikingly, in fetuses with a chronic norepinephrine infusion, the enhanced insulin secretion responsiveness to glucose and arginine persisted for five days after termination of the infusion. This is consistent with the observation of insulin hyper-secretion in week old IUGR lambs because norepinephrine concentrations are high during gestation but expected to decrease after birth when oxygen and nutrients are sufficient (Camacho *et al*., 2017; Chen *et al*., 2017; Limesand and Rozance, 2017). Surgical ablation of the fetal adrenal medulla prevents acute hypoxia-induced norepinephrine secretion and partially explains the lower glucose stimulated insulin concentrations in IUGR fetuses (Macko *et al*., 2016). While mechanisms for adrenergic inhibition of insulin secretion include distal steps in exocytosis, acute adrenergic stimulation also inhibits oxidative metabolism in β-cells and islets, which supports a role for norepinephrine to lower oxidation rates of glucose and to inhibit islet metabolism in IUGR fetuses (Kelly *et al*., 2018).

When islets from IUGR and control fetuses were analyzed for molecular changes using high throughput RNA sequencing (RNAseq), more than 1000 genes were differentially expressed and explained decreased cell proliferation (Kelly *et al*., 2017). This unbiased approach also revealed novel mechanisms underlying IUGR islet dysfunction including down regulation of immune function, suppressed Wnt signaling, adaptive stress responses, and impaired proteolysis. These transcriptional changes define adaptive responses of β-cells during IUGR and may provide the framework for understanding programming mechanisms that lead to metabolic complications in later life following hyperthermia-induced placental insufficiency.

Skeletal muscle growth and metabolism

Lambs with fetal growth restriction are lighter at birth and grow less efficiently, yielding carcasses with insufficient muscle growth (Greenwood *et al*., 1998, 2000). In sheep, the formation of new fibers (myogenesis) is complete around 110 days of gestation after which the myofibers grow by hypertrophy, which is when declines in fiber size are detected in fetuses with placental insufficiency-induced IUGR (Maier *et al*., 1992; Wilson *et al*., 1992; Hay *et al*., 2016). Conserved myonuclear domains during early muscle growth increase protein synthesis, such that myonuclear accumulation drives growth in young animals (Pavlath *et al*., 1989). Fetal myoblast incorporation (via differentiation) into myofibers is required to increase nuclei content because nuclei within these myofibers are post-mitotic (Allen *et al*., 1979). Myoblasts proliferate and differentiate in response to an activation signal, followed by a cascade of regulatory transcription factors (e.g. Pax7, MyoD, myogenin, and others). IUGR fetuses from hyperthermia-induced placental insufficiency have similar numbers of myoblasts compared to controls but have impaired myoblast differentiation due to slower rates of myoblast proliferation. (Yates *et al*., 2014, 2016; Brown *et al*., 2017). When myoblasts are isolated and cultured from IUGR skeletal muscle, they replicate slower than controls. This is likely an intrinsic defect in

the IUGR myoblast because it occurs in culture and independent of nutrient availability (Yates *et al*., 2014). Furthermore, IUGR fetuses have decreased fiber size regardless of the fiber type (Yates *et al*., 2016). These complications explain smaller muscle fibers, as well as identify programing effects that could limit growth later in life (Brown, 2014).

Skeletal muscle is a primary target for metabolic complications because it represents approximately 40% of total body weight and greater than 50% of energy expenditure (Brown, 2014). Metabolic energy requirements in skeletal muscle are met through oxidative phosphorylation. Thus, the number and efficiency of mitochondria determines metabolic capacity of muscle fibers. Traditionally, fibers are classified by oxidative capacity including Type Ia (slow oxidative), Type IIb (fast glycolytic), and Type IIa and IIx (fast oxidative; Dunlop *et al*., 2015). In the developing sheep the percentage of slow oxidative fibers (Type I) increases in number from less than 10% at 110 days gestation to greater than 25% near term and continues to increase postnatally (Maier *et al*., 1992). Myofiber area for both Type I and Type IIa fibers are decreased in hindlimb muscles from IUGR fetuses (Yates *et al*., 2016; Rozance *et al*., 2018). While there are less oxidative fibers in hindlimb muscles from IUGR fetuses, the proportion of glycolytic fibers is similar to control fetuses (Yates *et al*., 2016). We expect this developmental adaptation will lower oxidative-to-glycolytic fiber ratios and explains impaired glucose oxidation rates in the IUGR fetus (Limesand *et al*., 2007; Brown *et al*., 2015).

Long-term exposure to elevated catecholamines down regulates adrenergic receptor concentrations, lowers sensitivity, and impairs skeletal muscle metabolism (Yates *et al*., 2014). Chronic adrenergic stimulation is associated with adaptive programming responses in fetal metabolic tissues: pancreatic islets (Chen *et al*., 2014; Camacho *et al*., 2017), skeletal muscle (Yates *et al*., 2012), and adipose (Chen *et al*., 2010). Moreover, adaptations to chronic adrenergic stimulation persist after the insult, creating the potential for life-long metabolic programming. Specifically, adrenergic receptor β2 mRNA concentrations are reduced >60% in IUGR fetuses and lambs but adrenergic receptors β1 and β3 are not different (Chen *et al*., 2010). Therefore, potential desensitization of adrenergic receptor β2, but not other β-adrenergic receptors, might impair insulin responsiveness in skeletal muscle because it persists in lambs after birth.

Cardiac metabolism

The effects of maternal heat stress on fetal development and maturation are also evident in the heart. Blood flow to essential fetal organs (the brain, heart, and adrenal glands) is increased preferentially in response to acute bouts of hypoxia. As a result, blood flow decreases to the gastrointestinal, renal, and peripheral vasculature. This pattern for redistribution of cardiac output is also maintained during chronic periods of hypoxia such as those found in hyperthermia-induced placental insufficiency, which may result in the asymmetric fetal growth (Fig. 5).

IUGR fetal myocardium responds through unique metabolic and cardiovascular adaptations that support myocardial growth and function. The reduced circulating anabolic factors (Fig. 6) present in IUGR would be expected to suppress cardiac growth, as the fetal heart is sensitive to insulin and IGF-1, and reliant on glucose and lactate as major carbon sources for metabolism (Bartelds *et al*., 1998, 2000). The myocardium of the IUGR fetus responds by increasing plasma membrane concentrations of the insulin-stimulated glucose transporter 4 and insulin receptor β protein (Barry *et al*., 2006). In contrast, myocardial membrane protein concentrations of glucose transporter 1 are unchanged in the IUGR fetus. Additionally, blood flow to, and glucose delivery/uptake by, the left ventricle is significantly increased by insulin stimulation (Barry *et al*., 2016). These adaptations appear to promote myocardial energy supply and utilization by increasing its sensitivity to insulin, which supports cardiac growth despite the significant nutrient deprivation.

Fetal cardiac function appears to be relatively unaffected by placental insufficiency because heart rates are similar between IUGR and control fetuses (Galan *et al*., 2005; Barry *et al*., 2016). However, IUGR fetuses have increased indices of umbilical artery resistance and a significant reduction in umbilical blood flow, which did not always cause greater mean aortic blood pressure (Galan *et al*., 2005; Barry *et al*., 2016). The increased placental vascular resistance might reflect a mechanism by which the fetus is able to increase extraction of nutrients from the placenta as discussed above. A similar model that induces IUGR by removing the majority of the uterine caruncles in the sheep also found no difference in fetal blood pressure under baseline conditions; however, there was a greater hypotensive effect in IUGR fetuses following administration of phentolamine, an α-adrenergic antagonist, and captopril, an angiotensin-converting enzyme inhibitor (Edwards *et al*., 1999; Danielson *et al*., 2005). These findings indicate that the α-adrenergic and renin-angiotensin systems have a greater role in blood pressure maintenance in IUGR fetuses, which are likely involved in mediating some of the organ sparing phenomena present in this model (McMillen *et al*., 2001; Danielson *et al*., 2005).

Conclusions

We have presented evidence for prolonged exposure to heat stress causing placental insufficiency in ruminants. Maladaptive responses during development, which include fetal growth restriction, persist as lifelong deficiencies lowering the performance and health of the animal. We discuss how the enlarged transplacental gradient for oxygen and glucose facilitates umbilical uptakes but results in low blood oxygen and plasma glucose concentration in the fetus. These conditions slow growth by altering glucose metabolism, decreasing amino acid clearance, and decreasing anabolic hormones while increasing catabolic hormones.

Acknowledgements

Funding for this work was from the National Institutes of Health R01 DK084842 (S.W. Limesand, Principal Investigator). L.E. Camacho was supported by Award 2015-03545 (L.E. Camacho, Principal Investigator) from the National Institute of Food and Agriculture, USDA. A.T. Antolic was supported by T32 HL7249 (J. Burt, Principal Investigator). We thank all our colleagues who contributed to the seminal studies reported in this review. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies.

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DOI: 10.21451/1984-3143-AR2018-0017

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Influences of nutrition and metabolism on reproduction of the female ruminant

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Abstract

Beef cows and ewes grazing native pastures are exposed to cycles of undernutrition that reflect the seasonal variations of biomass production. In grazing dairy cows, the physiological undernutrition during early lactation due to increased demands for lactation and low dry matter intake is exacerbated by the need to get sufficient intake from pasture and the extra grazing energy costs. Undernutrition has profound impacts on reproduction by affecting multiple reproductive processes at different levels of the reproductive axis. The objective of this paper is to review the influence of undernutrition on reproductive events of the adult female ruminant, with emphasis on both grassland and mixed rain-fed grazing farming systems. The comparative endocrinology and reproductive biology among ewes, beef and dairy cows may provide a comprehensive knowledge of the metabolic and reproductive adaptation to feed restriction. Understanding the critical underlying physiological mechanisms by which nutrition affects reproduction is the base of focus feeding strategy to improve the reproductive performance of the female ruminant.

Keywords: metabolism, reproduction, ruminant.

Introduction

The world's population numbered nearly 7.6 billion in 2017, a large proportion of its increase has been in developing countries where livestock production is a major factor of agriculture growth (Food and Agriculture Organization - FAOSTATS, 2017). Despite the ability of herbivores to convert human-inedible fibrous biomass into human-edible food, the sustainability of this food production is under discussion (Gill *et al*., 2010). The innovations in livestock practices in the last decades resulted in increased animal production. However, the increase in the concentration of greenhouse gases and of minerals in surface and ground water in high-density livestock operations, illustrates that current livestock practices do not meet the definitions of environmental sustainability (Van Vuuren and Chilibroste, 2013). In this scenario, grasslands and mixed rain-fed systems (i.e., farming practices that rely on rainfall for water availability) constitute an alternative for sustainable livestock production. Moreover, maximizing the proportion of pastures in the diet is a pivotal factor for minimising production costs.

Grasslands (25% earth surface; FAOSTATS, 2017) are found primarily in marginal areas unfit for cropping. Native pastures are exposed to high variability in biomass production throughout the year due to normal seasonal variation and increasingly abnormal, extreme climate events. These seasonal variations, also influenced by the stocking rate, are normally absorbed by body weight loss during the winter or dry periods, and gain during the summer or rainy periods. Thus, cows and ewes managed under extensive grassland systems are exposed to a structural underfeeding system (i.e. annual periods of feed restriction due to low herbage mass production by native pastures). Reproductive function is aligned closely with food supply, which is easily exemplified by the seasonality of sheep to ensure birth during seasons that are favourable for lamb survival. While the initial events of the reproductive process (ovulation/fertilization/early gestation) do not demand relevant amounts of energy, the requirements during late gestation and lactation are considerable, and disruptive events may be life-threatening to the mother and the offspring. However, since the initial reproductive events are sensitive to fluctuations of nutrient availability, the efficient use of energy of the adult female in the reproductive cycle is maximized. Although meat production has increased during the last decades (FAOSTATS, 2017), reproductive efficiency remains low, with calving rates around 60 to 65 % (Pallarés *et al*., 2005). In beef cows, poor nutritional status (reflected in low body condition scores, BCS) together with calf presence/suckling, determines long postpartum anestrus, early embryonic death, reduced pregnancy and weaning rates (Stagg *et al*., 1998; Hess *et al*., 2005; Diskin and Morris, 2008).

In mixed rain-fed dairy systems in which herbage is the primary diet component, in contrast with indoor systems (total mixed ration, TMR), prediction of nutrient availability is complex since it includes uncertainties associated with grazing. Moreover, grazing dairy cows do not take in sufficient dry matter intake (DMI) to sustain the high milk production that could be achieved with their actual genetic potential (Kolver and Muller, 1998). Maximizing DMI is crucial to achieve the desired milk production as well as to minimize the magnitude and duration of the negative energy balance (NEB). The NEB is known to decouple energy requirements of lactation and nutrient supply that takes place during the transition period (Drackley, 1999). The biological processes affected after decades

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Received: February 1, 2018
Accepted: July 13, 2018

of selection for milk production resulted in physiological undernutrition during early lactation (Chilliard, 1999). Although several reports have showed the abrupt decline in fertility in dairy cows worldwide (Butler, 2000; Lucy, 2001), the improved management in nutrition, health and reproduction, and the genetic selection for health and fertility traits, have changed this trend in the last years in some countries (Berry *et al*., 2014; Thatcher, 2017).

The present review will focus on the effects of NEB on reproductive performance of adult female ruminants, with emphasis on both grassland and mixed rain-fed grazing farming systems. Whilst early investigations focused on the effects of nutrition on the hypothalamic-pituitary axis, studies of the last decade have tested the hypothesis that nutritional signals (metabolic hormones) also exert direct effects at peripheral levels. The potential causes of reproductive failure include impaired folliculogenesis, abnormalities of the ovum/embryo, luteal inadequacy and/or failure of the supply of progesterone to the uterus, abnormal functionality of the reproductive tract and disruption of maternal recognition of pregnancy.

Ruminant metabolic adaptation to undernutrition: endocrine signals

The metabolic adaptation to undernutrition implies adjustments in nutrient partitioning that are regulated by complex signalling pathways among organs and tissues, and depends on multiple intrinsic and extrinsic factors (see Fig. 1). Faced with nutritional deficiency, and depending on its severity, the natural physiological response is to renounce reproduction. As an immediate response in the adult female, cyclicity would be compromised; if this limitation is overcome, the next challenge is pregnancy success and/or the number of offspring (Scaramuzzi *et al*., 2006). Even among sequential reproductive events, undernutrition influences may tip the balance towards success or failure in certain steps of the reproductive cycle. Although biological processes are similar among sheep and cows, ruminant females may show different adaptive mechanisms depending on the species and their environments. For instance, the reproductive responses of the adult female to NEB include the increase in the duration of seasonal anestrus (ewes), postpartum anestrus (cows), decreased fertilization rate and/or increased embryo mortality (Butler, 2000; Hess *et al*., 2005; Forcada and Abecia, 2006; Diskin and Morris, 2008; see Fig. 2). The outcome will vary depending on the energy status and the presence of the offspring (Short *et al*., 1990; Stagg *et al*., 1998). Moreover, the metabolic and reproductive responses to undernutrition depend on recent (feeding level) or more longer term metabolic history (reflected in the body reserves, Chilliard *et al*., 2005). Thus, to understand the influences of undernutrition and metabolism on female reproduction, these aspects have to be considered, as changes in nutrient flux will affect reproductive processes.

Undernutrition and/or NEB in the female ruminant is characterized by decreased blood glucose, insulin and insulin-like growth factor-I (IGF-I), and increased concentrations of non-esterified fatty acids (NEFA; Ciccioli *et al*., 2003; Meikle *et al*., 2004a; Lake *et al*., 2006; Sosa *et al*., 2006a). These metabolic adaptations are coordinated not only by changes in the plasma concentration of key hormones, but by tissue-specific variations in hormonal sensitivity and responsiveness (Bauman, 2000; see Fig. 1). During NEB, the growth hormone (GH)-IGF axis is uncoupled in the liver, resulting in a reduction of circulating IGF-I, despite high GH concentrations (Kobayashi *et al*., 1999). This uncoupling may be the result of a state of hepatic resistance to GH due to decreased mRNA expression of the GH receptor (GHR), especially its isoform 1A, which occurs during NEB in dairy cows (Kobayashi *et al*., 1999). The increased GH concentration in early lactation promotes mobilization of NEFA from adipose tissue and their oxidative use by the rest of the body (Bauman, 2000; Block *et al*., 2001). Thus, anabolism is inhibited directly by the decrease in insulin concentrations and increased insulin resistance of tissues, and indirectly by the lack of insulin stimulation of GHR that limits hepatic synthesis of IGF-I (Butler *et al*., 2003; Rhoads *et al*., 2004; Fig. 1). Information about the effect of undernutrition/NEB on the hepatic molecular mechanism that explains the uncoupling of GH-IGF axis may differ according to species and/or management. Indeed, although blood IGF-I concentrations were decreased during NEB, no reduction in hepatic *GHR-1A* and *IGF-I* mRNA were observed in beef cattle and sheep (Jiang *et al*., 2005; Sosa *et al*., 2006a; Astessiano *et al*., 2014; Laporta *et al*., 2014). In addition, the activity of IGF-I is modulated by complex interactions with specific binding proteins (IGFBPs) that alter the availability of the active growth factor and its cellular receptors (Jones and Clemmons, 1995). Most IGF-I is bound to IGFBP3 and the acid-labile subunit in a ternary complex. The NEB is associated with decreased IGFBP3 and increased IGFBP2 shifting this binding to IGF-I/IGFBP2 complex and reducing the half-life of IGF-I (Jones and Clemmons, 1995; Laporta *et al*., 2014). In our experiments, plasma IGF-I turned out to be the best marker integrating the static (body reserves) and acute effects of nutrition in sheep (Fernández-Foren *et al*., 2011), dairy (Meikle *et al*., 2004a; Adrien *et al*., 2012) and beef cows (Soca *et al*., 2013a; Laporta *et al*., 2014). Indeed, Adrien *et al*. (2012) suggested that insulin profiles were associated more to day-to-day nutritional inputs, while IGF-I profiles more likely reflected the changes in energy balance. Both, insulin and IGF-I, affect the reproductive axis at central (hypothalamus-pituitary gland) and peripheral (gonads, reproductive tract and embryo) levels (Fig. 1).

The adipose tissue plays a role not only in energy storage, but is also an active endocrine tissue sensing metabolic status. Leptin and adiponectin concentrations have been proposed as indexes of metabolic status, as well as metabolic signals to the reproductive system (Blache *et al*., 2000; Kim *et al*., 2011). Plasma concentrations of leptin decrease with undernutrition in sheep and cows (Delavaud *et al*.,

2000; Ciccioli *et al*., 2003; Meikle *et al*., 2004a; Sosa *et al*., 2006a; Fig. 1), which facilitates a decrease in metabolic rate and enhances voluntary feed intake (Ingvartsen and Boisclair, 2001). As leptin is mainly synthetized by adipocytes, the decrease in leptin concentrations after restriction in dairy and beef cows and ewes is greater in females with more adipose tissue that also have higher initial concentrations of leptin (Meikle *et al*., 2004a; Fernández-Foren *et al*., 2011; Astessiano *et al*., 2014). Data on adiponectin in undernourished female ruminants are limited. A negative association between adiponectin concentrations and BCS is reported in dairy cows during the dry period (De Koster *et al*., 2017) and during the postpartum period in beef cows (Astessiano *et al*., 2014). Interestingly, in dairy cows, both leptin and adiponectin concentrations decrease around calving, and increase thereafter (Giesy *et al*., 2012; Singh *et al*., 2014; Astessiano *et al*., 2015). Since adiponectin suppresses gluconeogenesis (Zhou *et al*., 2005), its reduction may imply a physiological mechanism to increase glucose supply to the mammary gland for milk production (Saremi *et al*., 2014).

The regulation of these hormones is interdependent (e.g., each hormone affects the synthesis/secretion of the other) to facilitate crosstalk among tissues regulating metabolism (liver, pancreas, adipose tissue, Fig. 1), as reported for these species and rodents (Wabitsch *et al*., 1996; Rhoads *et al*., 2004; Yoshida *et al*., 2007). Thus, the different metabolic responses to undernutrition found among studies can be attributed, among other factors, to differences in body reserves. Indeed, differential BCS responses induced experimentally in sheep (Fernández-Foren *et al*., 2011), beef (Ciccioli *et al*., 2003) and dairy cows (Chagas *et al*., 2006; Adrien *et al*., 2012) were associated with different metabolic responses. Even if underfed ewes had decreased glucose concentrations, insulin concentrations decreased abruptly immediately after undernutrition in lean BCS (<2.25, scale 0-5) ewes, while in moderate BCS (>2.75) ewes the decrease in insulin concentrations was observed two weeks after the start of the treatments (Fernández-Foren *et al*., 2011). Thus, metabolic adaptation to feed restriction depends on body reserves: females with very low energy stores respond rapidly (in terms of metabolites/hormones) to DMI, while the response of animals with greater energy stores seems to be somewhat delayed.

In grazing dairy cows, pre-partum IGF-I and/or leptin concentrations were associated with the level of body reserves in both naturally occurring or induced BCS (Meikle *et al*., 2004a; Chagas *et al*., 2006; Adrien *et al*., 2012). In contrast, subtle or no associations were detected between pre-partum BCS and leptin/IGF-I concentrations after calving in the latter studies. Increased leptin and IGF-I concentrations after calving were reported in response to greater concentrate intake (Reist *et al*., 2003). Indeed, greater plasma insulin and IGF-I concentrations were found in dairy cows fed a TMR compared to grazing dairy cows, even if offered more than 35 kg DM/cow/day of herbage and also supplemented to cover maintenance energy requirements plus 10 liters of milk (Meikle *et al*., 2013). This was consistent with the higher nutrient density of TMR diets and no extra requirements to cover grazing activities or walking. When beef cows were classified according to their BCS at calving (<3.5≥, scale 1-8), IGF-I concentrations during prepartum were greater in moderate than low-BCS cows, with no differences during the postpartum (Astessiano *et al*., 2014). In addition, beef cows grazing high herbage allowance of native pastures during the annual gestation-lactation cycle presented greater BCS and higher IGF-I concentrations than cows grazing low herbage allowance, but also serum IGF-I increased in early spring in response to pasture availability and energy balance only in the high allowance group (Laporta *et al*., 2014). Similarly, when temporary suckling restriction was applied to primiparous beef cows two months after calving, the increase in IGF-I concentrations was greater in cows with moderate (≥4, scale 1-8) *vs*. low (≤3.5) BCS at calving (Soca *et al*., 2013a). Moreover, the endocrine response of these cows to flushing (supplementation for 22 days immediately after suckling restriction) was dependent on BCS at calving, as IGF-I and insulin concentrations increased in moderate BCS cows but did not change in low BCS cows. Overall, the endocrine response to nutritional management depends, at least partially, on body reserves.

In addition, metabolic adaptation to lactation is affected by age (parity): primiparous cows, which have not reached their adult body size and continue growing during pregnancy and lactation, present metabolic differences respect to older cows (Wathes *et al*., 2007). The competing demands of the mammary gland are superimposed on the requirements for growth, and both insulin and IGF-I stimulate growth. The profiles of these hormones and metabolites, such as NEFA, during the transition period according to parity have been inconsistent (Vandehaar *et al*., 1999; Meikle *et al*. 2004a). Wathes *et al*. (2007) modelled metabolic traits, milk yield and BCS, and reported greater IGF-I concentrations in primiparous cows, which may limit nutrient partitioning into milk. In contrast to multiparous cows, there was no relationship between insulin concentration and milk production in primiparous cows. The authors suggested that insulin is less important in controlling the relative partitioning of nutrients between body tissue and milk synthesis, possibly due to the prevailing higher IGF-I concentration in primiparous cows still growing. Moreover, body reserves are usually related to parity, as primiparous dairy and beef cows under pasture-based systems present better BCS than multiparous cows probably for not having the energy demands of a previous lactation. Thus, prepartum leptin concentrations are generally higher in primiparous cows (Meikle *et al*., 2004a; Wathes *et al*., 2007). On the other hand, a steeper decrease in body reserves and leptin concentrations was observed during early lactation in primiparous cows when compared to multiparous cows (Meikle *et al*., 2004a). As both categories are usually managed together under grazing conditions, this may be also explained by a dominance effect for food availability (Grant and Albright, 2001). These confounding factors should be considered since they are at the basis of the poor reproductive performance.

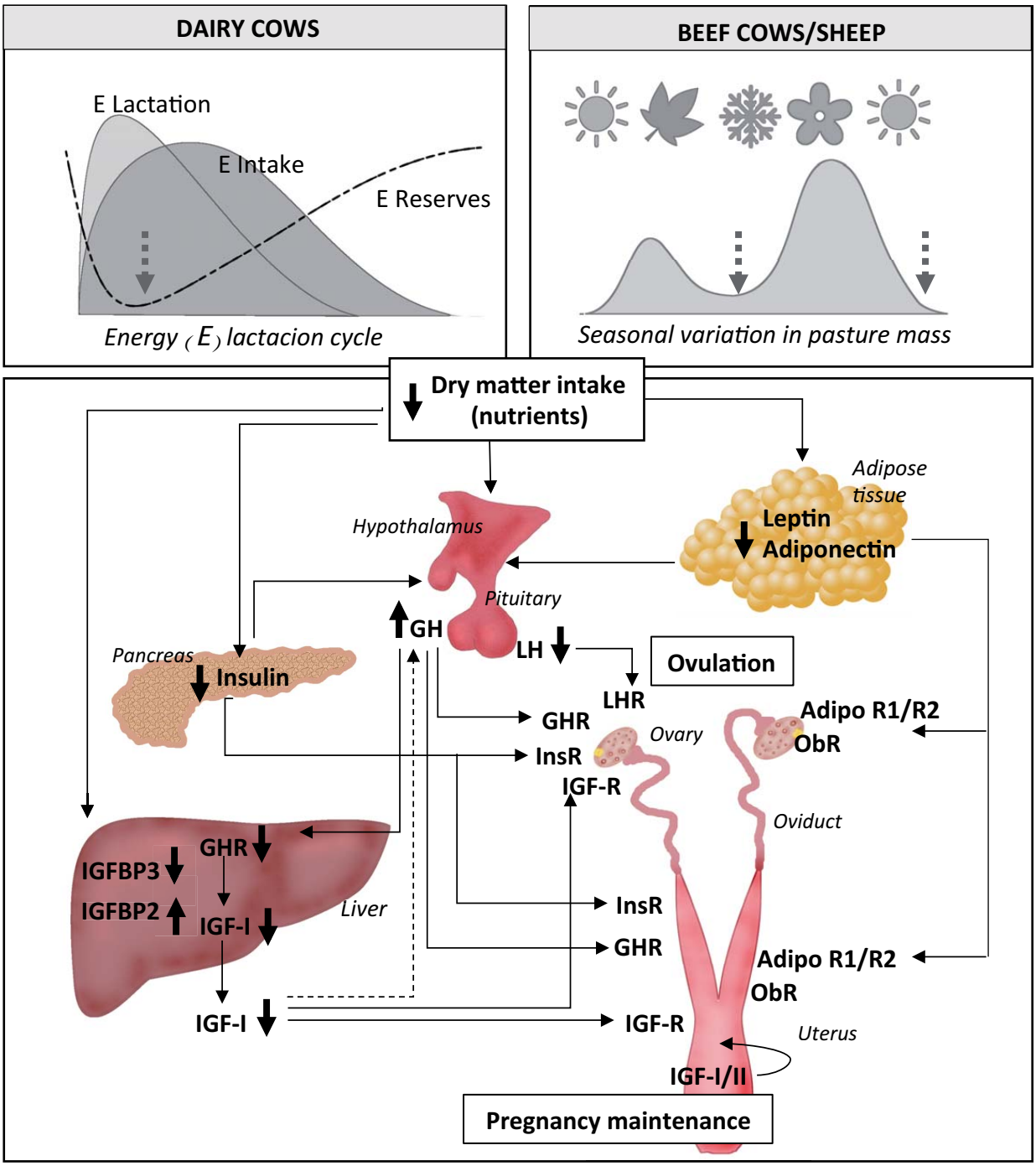

Figure 1. Simplified model of metabolic influences on reproduction of the female adult ruminant. While undernutrition in grazing beef cows and ewes is the result of energy intake below their requirements associated with the seasonal variation in pasture mass, in grazing dairy cows the negative energy balance (NEB) is the consequence of the increased demands for lactation and the low dry matter intake exacerbated by the need to get sufficient intake from pasture and the extra grazing energy costs. Grazing management interacts with parity in the metabolic adaptation to restricted dry matter intake or NEB that also depends on body reserves (metabolic memory). Reproductive events are affected by how and when this adaptation takes place. The nutrient flux affects all tissues and nutrient partitioning is modulated by the endocrine signals. Peripheral tissues (liver, adipose tissue, pancreas and others) secrete signals that not only regulate metabolic pathways, but also inform of the energy status. Hormones interact with each other by regulating their synthesis/secretion to ensure a coordinated regulation of energy partitioning. A complex system in the hypothalamus senses nutrient restriction and regulates voluntary feed intake, nutrient partitioning and reproductive function accordingly. The reproductive axis will respond both hierarchically by regulation of hypothalamus-pituitary gland and at follicular level (i.e., hormone receptors of the metabolic hormones) sensing the metabolic status to overcome the first reproductive limitation in the adult female, which is the pospartum/seasonal anestrus. The endocrine signals have also direct effects on the reproductive tract where they may tip the balance towards pregnancy success or failure. Broken lines: decrease, i.e., negative feedback IGF-I/GH, which is diminished and contributes to higher GH concentrations. Solid lines: increase (positive effect).

Follicular and luteal function and physiology of the reproductive tract

Follicular diameter has been associated positively with acquisition of oocyte competence (Arlotto *et al*., 1996) and with enhanced embryo growth and development since the corpus luteum (CL) originated from these follicles secretes more progesterone (P4) concentrations to improve maintenance of pregnancy (Vasconcelos *et al*., 2001; Perry *et al*., 2007). Reduced feed intake has been associated with decreased follicle size in cows (Burns *et al*., 1997). Undernourished ewes presented a reduced number of large follicles compared to control ewes (McNeilly *et al*., 1987). Moreover, undernourished ewes presented larger follicles in the static phase that were functionally altered because there was no inhibition in development of subordinate follicles (Sosa *et al*., 2010).

The ability of the dominant follicle to grow and ovulate depends on LH pulsatility as well as concentration of many growth factors (e.g, IGF family) and nutrients (Fortune *et al*., 2004; Fig. 2). The changes that take place in the dominant follicle (e.g, LH sensitivity and content of steroidogenic enzymes) allow 17-ß estradiol (E2) secretion and subsequent ovulation (Crowe *et al*., 2014). Thus, undernutrition and/or NEB delays ovulation by inhibition of LH frequency and diminished concentrations of IGF-I/insulin and other nutrients, which reduce E2 production by the dominant follicles (Butler, 2000; Fig. 2). Follicular fluid plays a critical role whereby the microenvironment impacts on oocyte development and future embryo quality (Fortune *et al*., 2004; Revelli *et al*., 2009). Increased concentrations of E2 and E2/P4 ratio in follicular fluid have been associated with improved follicular growth and follicle dominance, oocyte quality and pregnancy outcome (Revelli *et al*., 2009). Differences in metabolomic profiles of follicular fluid in cows with different energy balances have been reported (Forde *et al*., 2015) and some components have been associated (positively: alanine and linolenic acid; negatively: total fatty acids and urea) with oocyte competence (Matoba *et al*., 2014).

Beef cows with moderate BCS at calving or grazing high herbage allowance had greater maximum diameter of dominant follicles during the postpartum when compared with low BCS cows or low herbage allowance, respectively (Quintans *et al*., 2010; Carriquiry *et al*., 2011). The E2/P4 ratio did not differ in the preovulatory follicle of beef cows grazing high vs. low herbage allowance. However, intrafollicular IGF-I concentrations were greater in cows grazing high herbage allowance of native pastures consistent with an earlier postpartum ovulation in these cows (Carriquiry *et al*., 2011). Likewise, there is an inverse relationship between plasma IGF-I/insulin and duration of postpartum anestrus in beef and dairy cows (Lucy, 2001; Meikle *et al*., 2004; Quintans *et al*., 2010; Soca *et al*., 2013a; Laporta *et al*., 2014).

The magnitude of NEB and its association with the different types of anestrus have been reviewed before (Peter *et al*., 2009). The first postpartum follicular wave takes place within 10-14 days after calving and the fate of the dominant follicle will depend on LH pulsatilty and the availability of many growth factors within the follicle. Thus, the first pospartum ovulation in well-managed dairy cows will take place within the first month after calving (Crowe *et al*., 2014; Fig. 2). In grazing dairy cows (n = 824 in 7 commercial herds) with none, one or two previous calvings, milk P4 was determined twice weekly for 90 days after calving to monitor luteal activity: 53% of the cows ovulated within 30 days after calving, 14% from 30 to 40 days, and lower percentages were found until 90 days after calving, while 13% of the cows did not ovulate during this period (Meikle, 2018; Facultad de Veterinaria, Udelar, Montevideo, Uruguay; unpublished data). Although dairy cattle usually present a more profound NEB due to lactation than beef cattle grazing pastures, the latter suffer a prolonged restricted nutrient availability in addition to suckling (Fig. 2). The major physiological difference between dairy and beef cows during early postpartum is the lower frequency of LH pulses in beef cows due to suckling inhibition and presence of calf (see Fig. 2). While dairy cows may ovulate in the first follicular wave, in beef cows if nutrition is adequate, the first postpartum ovulation takes place on the third follicular wave (~30 days), but if beef cows present poor body condition, ovulation is delayed (~70-100 days, reviewed by Crowe *et al*., 2014).

The resumption of ovarian cyclicity after calving associated with body reserves is well documented. Indeed, in grazing production systems dairy and beef cows with better BCS during the pre and postpartum periods, or with better BCS at calving, had an earlier first postpartum ovulation (Meikle *et al*., 2004a; Quintans *et al*., 2010; Soca *et al*., 2013b). Soca *et al*. (2013b) determined that the length from calving to first ovulation in beef cows decreased by 49 days for each incremental unit of improvement of BCS at calving (scale 1 to 8). Stagg *et al*. (1995) reported a prolonged period of anestrus in cows with poor body condition. Likewise, increased BCS advanced first ovulation by 59 days in high vs. low herbage allowance cows (Laporta *et al*., 2014). Although less data on postpartum resumption of ovarian cyclicity is available in sheep, it was shown that the first postpartum ovulation in the breeding season was delayed in undernourished ewes with low BCS (<2.75, scale 0 to 5) at lambing in comparison to undernourished ewes with moderate BCS (>2.75) or control ewes (Sosa *et al*., 2006b). The relevance of BCS also was documented in its effect on the length of seasonal anestrus and ovulation rate, as ewes with a moderate BCS (>2.75) had a shorter seasonal anestrus (64 days) than ewes with a low BCS (<2.75; 113 days) and a higher rate of double ovulation as observed by the greater mean number of CLs (1.67 *vs*. 1.08 CL, respectively), especially in the transition between seasonal anestrus and the breeding season (Forcada and Abecia, 2006). These data show that the resumption of postpartum/seasonal ovarian cyclicity depends on female body reserves.

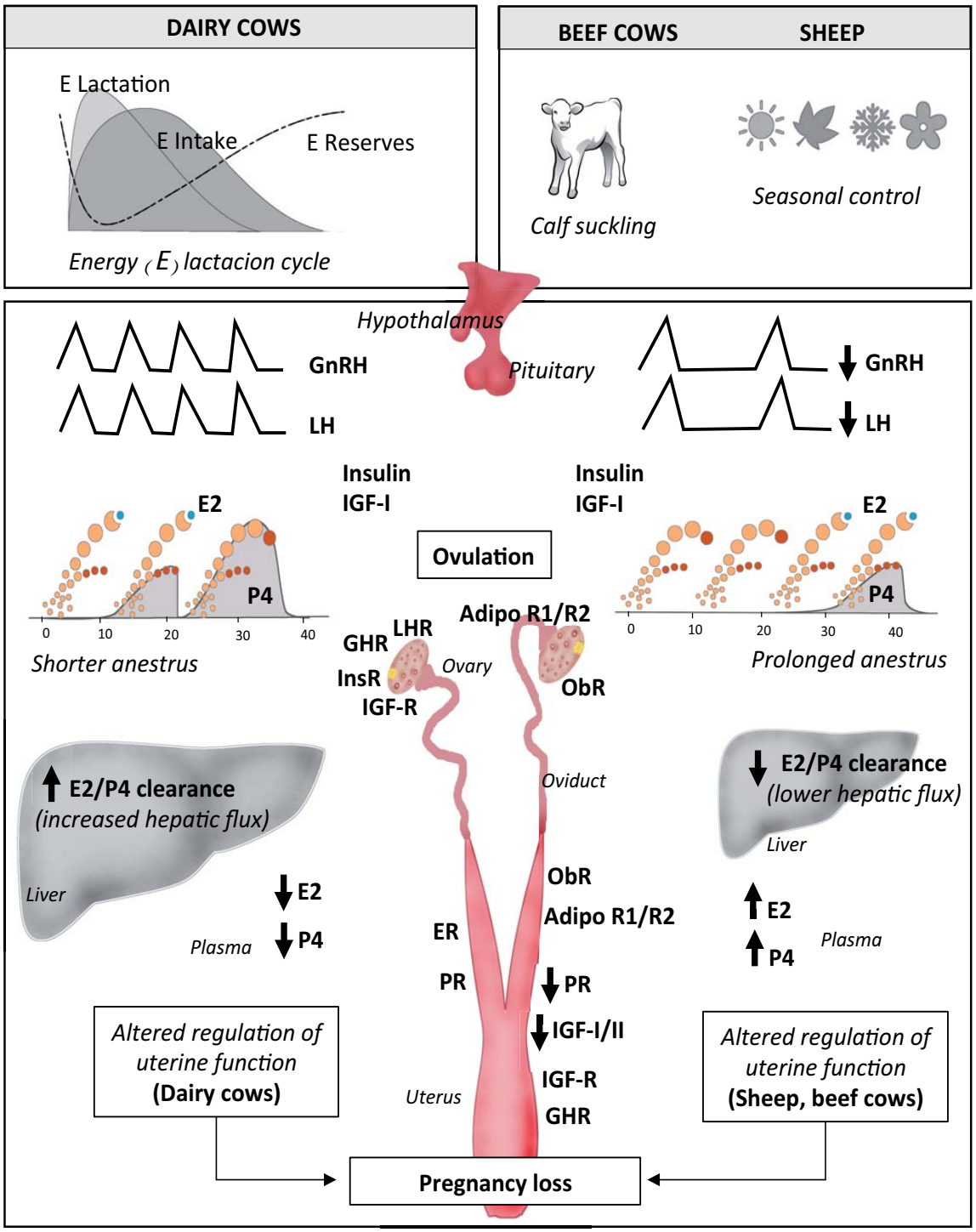

Figure 2. Proposed model of reproductive responses to negative energy balance (NEB) and environment in dairy and beef cows and ewes. Although dairy cows usually present a more profound NEB due to lactation requirements than beef cows and ewes grazing pastures, the later may suffer a more prolonged restricted nutrient availability. Beef cows and ewes present low frequency of LH pulses (suckling or seasonal inhibition, respectively) and undernutrition prolongs pospartum or seasonal anestrus, respectively. Although severe NEB also prolongs pospartum anestrus in dairy cows, is comparatively shorter. Body reserves affect the length of pospartum (dairy and beef cows) and seasonal anestrus (ewes). Once ovulation has occurred, the sequential preparation of the oviducts and uterus by E2 and P4 sustains embryo growth. Clearance of E2 and P4 is affected by liver metabolic flux: in dairy cows the high hepatic flux (intake-lactation) is related to low steroid plasmatic concentrations, whereas in ewes undernutrition is associated with high steroid circulating concentrations (no data as such was found in beef cows). In both dairy cows and sheep, data indicate that uterine sensitivity to P4 (PR) is diminished in NEB, as well as uterine sensitivity to other metabolic hormones. These findings may explain the embryo losses. Embryo mortality seems to be more important in the high producing dairy cow than in beef cows and ewes. The schematic representation of follicular waves in dairy and beef cows has been taken from Crowe *et al*. (2014).

Parity is a relevant factor when considering postpartum resumption of ovarian cyclicity, which occurred 16-23 days later in primiparous than multiparous grazing dairy cows (Meikle *et al*., 2004a; Adrien *et al*., 2012). This is consistent with the lower percentage of inseminated primiparous cows in the first 80 days after calving compared to multiparous cows (39.8 *vs*. 50.4%, 3772 dairy cows from 12 commercial farms; Cruz, 2018; Facultad de Veterinaria, Udelar, Montevideo, Uruguay; unpublished data). On the other hand, conception rate at first service in the later study was higher in primiparous cows (49.4 *vs*. 38.1%). Similar data was reported in pasture-based dairy systems (for reviews, see Rhodes *et al*., 2003 and Roche *et al*., 2011). In high-producing confined dairy herds, Santos *et al*. (2009) reported that multiparous cows were more likely to start ovarian cyclicity earlier than primiparous cows, but conception rate on day 30 after AI (~100 days after calving) was higher in primiparous cows (41.1 *vs*. 36.1%). Thus, although NEB heavily influences postpartum resumption of ovarian cyclicity, it only explains partially the decreased reproductive efficiency due to nutrition.

Once ovulation and fertilization has occurred, an adequate functionality of the oviduct and uterine horn is essential for the maintenance of pregnancy. The morphological, histological and biochemical changes of the reproductive tract are modulated by the fluctuating concentrations of E2 and P4 acting through their specific receptors in the target tissues, which also vary during the estrous cycle and early pregnancy (Meikle *et al*. 2001, 2004b; Robinson *et al*. 2001; Binelli *et al*., 2018). Thus, their function can be modified by manipulating the growth of the preovulatory follicle and its associated E2 secretion, as well as the consequent CL formation and associated P4 production (Mesquita *et al*., 2015; Ramos *et al*., 2015). Indeed, a positive association between preovulatory concentrations of E2 and the duration of proestrus regarding the uterine environment and fertility was found (Bridges *et al*., 2010, 2012). Pugliese *et al*. (2016) showed that the diameter of the preovulatory follicle and its blood flow and size, vascularization of CL and pregnancy rates were greater in beef cows ovulating large follicles. Moreover, Ramos *et al*. (2015) reported decreased uterine redox capacity in cows that ovulated a smaller follicle during early diestrus, and the authors suggested that it might be one of the causes of the reduced fertility found in these animals. Recently, de la Mata *et al*. (2018) demonstrated that extending the proestrus interval increased the rate of the dominant follicle growth from the time of P4 device removal to ovulation, the luteal area and serum P4 concentrations, which were associated with endometrial differences in PR immunostaining, *PR* and *IGF-I* mRNA expression, and pregnancy rates.

Progesterone supports the secretory function of the endometrium that sustains conceptus elongation and further implantation (for a review see Spencer *et al*., 2007). Moreover, there is a positive relationship between P4 concentrations during the early luteal phase and the synthesis of the embryo signal, interferon tau (IFN-τ). The IFN-τ, by altering uterine gene expression, modifies the episodic $PGF_{2\alpha}$ release that is responsible for the luteal regression (Mann and Lamming, 2001). Different authors have managed to modify endometrial function and improve embryo growth by injecting P4 during the early luteal phase in the female ruminant (Robinson *et al*., 1989; Forde *et al*., 2009). However, results are not consistent as effects depend on many factors, e.g., the timing of start and the duration of P4 supplementation affect CL and embryo development in beef heifers (Parr *et al*., 2017). Moreover, Bisinotto *et al*. (2015) reported that P4 supplementation during timed AI programs in cows without a CL at the start of the treatment improved pregnancy per timed AI, but P4 supplementation had no positive effect in cows that had a CL at time of supplementation.

In sheep, P4 concentrations are 800-fold higher in the ovarian than in the jugular vein, but did not differ between high and low feeding levels (reviewed by Abecia *et al*., 2006). On the other hand, plasma P4 concentrations were negatively associated with the level of feed intake, with higher P4 concentrations in underfed ewes likely due to lower liver steroid metabolism, thus a lower clearance rate (Rhind *et al*., 1989b; Lozano *et al*., 1998; see Fig. 2). In dairy cows, increased feed intake has been associated with the increase of E2 and P4 clearance (Sangsritavong *et al*., 2002), which can contribute to inadequate endometrial functionality. On the other hand, plasma P4 circulating concentrations may not reflect actual P4 concentrations in the reproductive tract. Indeed, in the ruminant, P4 is transferred locally from the ovarian/oviductal veins to the uterine arterial segments adjacent to the ovary (Weems *et al*., 1989). Although receptors specifically bind hormones with high specificity, few studies have analysed P4 and its receptor (PR) concentrations where transfer systems are in place in the fed restricted female ruminant. The region and side of the uterus affect transcript abundance in bovine endometrium (Sponchiado *et al*., 2017). Also, a differential oviductal gene expression associated with CL location was reported (de Brun *et al*., 2013) in agreement with greater P4 concentrations in the ipsilateral oviduct of recipient ewes (Graña *et al*., 2018; Facultad de Veterinaria, Udelar, Montevideo, Uruguay; unpublished data). These specific molecular differences according to location are of functional importance: when sheep IVF zygotes were transferred to oviducts ipsilateral and contralateral to the CL on day 1, a greater recovery rate and a lower proportion of degenerated embryos were found in the ipsilateral uterine horn on day 6 (de Brun *et al*., 2016a).

In cyclic undernourished ewes, higher plasma but lower endometrial P4 concentrations were found on day 5 (Lozano *et al*., 1998). This was later explained by the lower expression of *PR* mRNA, and lower PR protein abundance and binding capacity in uterus/oviduct of undernourished ewes (Sosa *et al*., 2006a; Fig. 2). As P4 regulates endometrial gene expression, differences in uterine P4 sensitivity (PR) were associated with different functionality and early embryo growth, as reported for different sheep breeds (Sequeira *et al*., 2016). Indeed, undernutrition in sheep

was also associated with a lower expression of *IGF-I* mRNA in uterus and *IGF-II* mRNA in oviduct at day 5 of pregnancy (de Brun *et al*., 2013; Fig. 2). Overall, these findings support the concept that local P4 concentrations (i.e. oviducts and uterine horns), and the consequent differential regulation of the reproductive tract physiology, may explain the higher embryo losses found in undernourished ewes (Rhind *et al*., 1989a, b).

The effect of underfeeding and/or NEB on gene expression in the reproductive tract of cyclic and/or postpartum cows has also been investigated. Endometrial and oviductal gene expression of IGF signaling pathways and several metalloproteinases were altered in dairy cows in severe NEB two weeks after calving (Wathes *et al*., 2011). It was suggested that altered postpartum functionality of the tract might affect tissue repair with consequent lower fertility. Less data are available regarding NEB on uterine gene expression in lactating cows at breeding time. Rhoads *et al*. (2008) reported no changes in mRNA endometrial expression of *GHR* and *IGFBP2* in lactating cows on 40, 80, 120 and 160 days after calving, although higher *IGF-I* mRNA levels were found on day 160; it was suggested that days in milk had a small effect on uterine gene expression. In a recent study in lactating dairy cows, Astessiano *et al*. (2017) reported greater intercaruncular endometrial mRNA expression of *IGF-I*, *IGFBP3*, *PR* and adiponectin receptors on day 7 of the estrous cycle at the end of the voluntary waiting period when cows fed a TMR and high herbage allowance were compared to medium and/or low herbage allowance cows (Fig. 2). Thus, the ruminant endometrium seems to be able to sense the metabolic status and to adapt its physiology accordingly, thereby determining possible success or failure of pregnancy.

Uterine functionality during early pregnancy

Although the preimplantation embryo is to a certain extent metabolically autonomous, the fate of the embryo is affected by the nutritional status of the maternal unit. Maternal undernutrition in sheep results in retarded embryonic development at 8 to 11 days after mating and reduced pregnancy rates after 2 weeks of pregnancy (Rhind *et al*., 1989a; Abecia *et al*., 2006). Although relatively high fertilization rates are found in dairy cows, conception rates are low (Lucy, 2001; Diskin and Morris, 2008). The proportion of embryos recovered from lactating cows was lower than from dry cows, consistent with the lower embryo quality reported for high-producing dairy cows (Sartori *et al*., 2010). Data suggest an effect of the energy balance on the functionality of the reproductive tract as reviewed previously (Sartori *et al*., 2010; Lonergan *et al*., 2016).

Much focus has been put on the effects of NEB around the moment of maternal recognition of pregnancy (i.e., day 14 in sheep, and day 17 in cows). In dairy cows, at day 17-intercaruncular endometrium, genes were differentially expressed according to pregnancy and lactation (Cerri *et al*., 2012; Thompson *et al*., 2012). Lactation altered the metabolic status (lower glucose and IGF-I plasma concentrations) and decreased P4 plasma concentrations (Thompson *et al*., 2012). The presence of the embryo had profound effects on endometrial expression of genes involved in immune pathways, but lactation also upregulated genes related to immunoglobulins, so that it was suggested that lactation could cause an immune imbalance with potential negative effects on conceptus survival (Cerri *et al*., 2012). Moreover, lactation affected the expression of genes involved in glucose homeostasis suggesting that it could be deleterious for the embryo. Lesage-Padilla *et al*. (2017) demonstrated that day 19-intercaruncular endometrium of pregnant lactating cows presented greater mRNA expression of oxidative stress-related genes when compared to pregnant dry cows, suggesting that lactation is associated with an increase in reactive oxygen species in the endometrium. As endometrial expression of conceptus-regulated genes was not affected by the metabolic status, it was suggested that the endometrial ability to respond to embryonic signals when implantation occurs seems not to be affected by maternal metabolism (Lesage-Padilla *et al*., 2017). No reports on the effect of NEB on local P4 endometrial concentrations and its association with uterine gene expression in pregnant or cyclic dairy cows have been found.

Lower fertilization (27%) and transferability (30%) rates were observed in undernourished superovulated donor ewes when compared to well-fed ewes (Abecia *et al*., 2015). When only good quality embryos (n = 2) from undernourished and control superovulated donor ewes were transferred to undernourished and control recipient ewes, no differences in pregnancy rates were found at day 18, but recipient undernourished ewes had a higher occurrence of late embryonic mortality (from days 18-40; de Brun *et al*., 2016b). These ewes had lower plasma insulin and P4 concentrations during the early luteal phase, which may have affected conceptus development leading to pregnancy loss after day 18. Overall, data suggest that when morphologically good quality embryos are transferred, maintenance of pregnancy relies on the nutrition of the mother regardless of embryo origin.

Embryos collected on day 15 of pregnancy from underfed ewes (0.5 maintenance requirements) secreted lower amounts of IFNτ in vitro, and the endometrial tissue collected from those ewes secreted higher amounts of PGF2α than control ewes (Abecia *et al*., 2006). Since this was accompanied by a reduction in embryo survival, it was suggested that the lower fertility observed in underfed ewes could be mediated through altered signals of maternal recognition of pregnancy. Further studies showed no effects of undernutrition on the intercaruncular endometrial expression of candidate genes involved in mechanisms of maternal recognition of pregnancy in pregnant ewes (such as PR, estrogen and oxytocin receptors, cyclooxygenase 2 and members of the IGF family) on day 14 (Sosa *et al*., 2009). It was suggested that conceptuses present in the uterus of undernourished mothers managed to elicit effects similar to those in well-fed pregnant ewes. In order to gain in-depth knowledge of the underlying mechanisms, we have studied the uterine transcriptome of the same

ewes (de Brun *et al*., 2016c). Interestingly, pregnancy in control ewes upregulated the gene expression of the citrate cycle and oxidative phosphorylation pathways, suggesting increased energy use by the endometrium to maintain pregnancy, as was previously reported in pregnant dairy cows (Cerri *et al*., 2012). In contrast, pregnancy in undernourished ewes did not affect these pathways, but upregulation of catabolic pathways for fatty acids and amino acids was observed (de Brun *et al*., 2016c). Moreover, undernutrition in pregnant ewes induced a 9-fold change in acyl-CoA synthetase short-chain family member 2 (an enzyme that converts acetate to acetyl-CoA) mRNA expression compared to control pregnant ewes. This differential response is indicative that endometrial cells utilize energy from these pathways, probably sparing glucose for utilization by the embryo. The immune system is also critical for proper embryo development and is activated in pregnant animals from both nutritional groups. Intercaruncular endometrium from pregnant ewes had a 3.4-fold or 2.0-fold greater expression of interferon-induced helicase C domain1 (*IFIH1*) than cyclic ewes in control or undernourished ewes respectively (de Brun, 2018; Facultad de Veterinaria, Udelar, Montevideo, Uruguay; unpublished data). The IFIH1 gene modulates local immune cells in the endometrium during pregnancy (Song *et al*., 2007). In the same study, the chemokine CXCL10, a promoter of trophoblast growth migration and adhesion (Nagaoka *et al*., 2003) was upregulated more than 6 and 3-fold in pregnant vs. cyclic control and undernourished ewes respectively (de Brun, 2018; Facultad de Veterinaria, Udelar, Montevideo, Uruguay; unpublished data), which could be associated with less efficient growth or adhesion of the embryo in undernourished ewes. Genes participating in the biosynthesis of unsaturated fatty acids were highly downregulated in undernourished pregnant compared with control pregnant ewes, which could be consistent with an energy-sparing mechanism. On the other hand, as unsaturated fatty acids are precursors for various eicosanoids and prostaglandins involved in the adhesion of the ovine trophoblast to the endometrium and in vascular permeability (Salleh, 2014), the downregulation observed in pregnant underfed ewes may be related with the greater late embryo mortality detected in undernourished animals (de Brun *et al*., 2016b). Overall, the endometrial machinery appears to have an adaptive ability to respond to adverse changes in metabolic status due to feed restriction that is dependent on presence of the embryo.

Concluding remarks

The ruminant adult female reproductive physiology is highly influenced by the environment and energy intake is a pivotal factor. Depending on the degree of energy deficit, the reproductive adaptation to metabolic distress may range from an increased duration of postpartum or seasonal anestrus to impairment of pregnancy establishment. The metabolic adaptation to NEB depends on body reserves that also modulate reproductive responses. The challenge to improve sustainable livestock production resides in maintaining reproductive efficiency of the female ruminant while facing periods of undernutrition, both in grassland (beef and sheep) or mixed rain-fed farming systems (dairy cows). The goals are to identify the biological reproductive processes that are most affected by NEB, and to determine when and how they can be improved by management complementary to cultural, economic and environmental sustainable systems.

Acknowledgments

The authors are very grateful to Professors William W. Thatcher and Mats Forsberg for their critical correction of the manuscript and invaluable contributions.

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DOI: 10.21451/1984-3143-AR2018-0050

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Effect of maternal diet on placental development, uteroplacental blood flow, and offspring development in beef cattle

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Abstract

Considerable effort has been made to understand how nutrition influences livestock health and productivity during the postnatal period. Moreover, while efforts have been made to understand how nutrition impacts many different aspects of female reproduction, there is a growing body of literature that focuses on how maternal nutrition impacts the developing fetus. Providing adequate nutrition during pregnancy is important for maternal health and well-being, as well as conceptus development. Proper establishment of the placenta is important for fetal survival. However, placental adaptations to inadequate maternal nutrition, or other stressors, are imperative for fetal growth to be optimal. By understanding how the maternal environment impacts uterine and umbilical blood flows and other uteroplacental hemodynamic parameters, we can better implement supplementation strategies to protect the developing offspring. This review focuses on how maternal nutrition affects conceptus growth in sheep and beef cattle and offspring performance after birth.

Keywords: maternal nutrition, placenta, umbilical blood flow, uterine blood flow.

Introduction

The maternal system can be influenced by many different extrinsic factors, including nutritional status, which can program nutrient partitioning and ultimately growth, development and function of the major fetal organ systems (Wallace, 1948; Godfrey and Barker, 2000; Wu *et al*., 2006). The trajectory of prenatal growth is sensitive to direct and indirect effects of maternal environment, particularly during early stages of embryonic life (Robinson *et al*., 1995), the time when placental growth is exponential. Understanding the impacts of the maternal environment on placental growth and development is especially relevant as the majority of mammalian livestock spend 35-40% of their life within the uterus, being nourished solely by the placenta. Moreover, pre-term delivery and fetal growth restriction are associated with greater risk of neonatal mortality and morbidity in livestock and humans. Offspring born at an above average weight have an increased chance of survival compared with those born at a below average weight in all domestic livestock species, including the cow and ewe. Just as growth restricted human infants are at risk of immediate postnatal complications and diseases later in life (Barker *et al*., 1993; Godfrey and Barker, 2000), there is increasing evidence that production characteristics in our domestic livestock may also be impacted by maternal diet (Wu *et al*., 2006). Some of the complications reported in livestock include increased neonatal morbidities and mortalities (Hammer *et al*., 2011), intestinal and respiratory dysfunctions, slow postnatal growth, increased fat deposition, differing muscle fiber diameters and reduced meat quality (reviewed in Wu *et al*., 2006).

The objective of this review is to highlight maternal adaptation to pregnancy, some of our laboratories investigations on how maternal nutrition can impact uterine and/or umbilical blood flow in cattle and sheep, as well as to highlight beef cattle studies that have investigated different supplementation strategies that may influence carcass development or fertility of the offspring.

Cardiovascular responses of the pregnant dam

Maternal cardiovascular capacity changes dramatically during pregnancy, with decreases in systemic arterial blood pressure and vascular resistance and increases in cardiac output, heart rate, heart stroke volume, and blood volume (Magness, 1998). Mean arterial pressure decreases in early pregnancy and persists throughout gestation in several mammalian species. The decrease in arterial pressure (~5 to 10% decrease) is minor compared to the approximate 20 to 30% decrease in total peripheral vascular resistance. Maternal cardiac output has been shown to increase as much as 30 to 40% in pregnant *vs.* non-pregnant ruminants (Magness, 1998).

An adequate blood volume increase is necessary in order to protect the mother and fetus from the deleterious effects of a reduced venous blood return and cardiac output (Pritchard, 1965; Torgersen and Curran, 2006). While it is well established why maternal blood volume needs to increase, understanding how blood volume increases is still under study. There are currently two theories that attempt to explain blood volume expansion: the decreased vascular resistance theory and the endocrine theory.

The decreased vascular resistance theory (Schrier and Briner, 1991; Duvekot *et al*., 1993) states that when the female becomes pregnant, a new vascular system is added to her pre-existing vascular system, which decreases the total vascular resistance of the mother's cardiovascular system. A decrease in total vascular resistance in turn increases maternal heart rate,

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Received: July 13, 2018
Accepted: March 21, 2018

activating the plasma volume regulating mechanisms in the liver, kidneys, and adrenal glands (Schrier and Briner, 1991; Duvekot *et al*., 1993). As plasma volume increases, blood volume increases as well.

The endocrine control theory (Longo, 1983) suggests a fetal-placental influence on blood volume in the pregnant female. As gestation advances, the fetal adrenal glands increase in size and their production of dehydroepiandrosterone, stimulates estradiol production in the mother (Longo, 1983). Estradiol stimulates the renin-angiotensin system, ultimately increasing plasma volume (Longo, 1983). Moreover, as placental size increases, there is increased somatomammotropin (i.e. placental lactogen) and progesterone production (Longo, 1983). These two hormones stimulate the production of erythropoietin in the mother, ultimately stimulating erythrocyte production (Longo, 1983).

In women, failure to adequately increase blood volume during pregnancy has been linked to pregnancy-induced toxemia (preeclampsia), fetal growth restriction, and premature labor (Goodlin *et al*., 1981). An inadequate function of the mechanisms necessary to increase blood volume in a state of decreased vascular resistance could consequently increase heart rate and produce vasoconstriction (Lund and Donovan, 1967; Goodlin *et al*., 1981). To our knowledge, there is little information available on how maternal blood volume is affected in our livestock species, let alone how nutritional or other environmental factors may be altering plasma volume expansion. In sheep, maternal nutrient restriction resulted in a decreased plasma volume, and reduced concentration of angiotensinogen, compared to well-fed ewes (Dandrea *et al*., 2002). Upon realimentation, previously restricted ewes had greater plasma volume compared to the ewes that were well-fed continuously, and authors concluded that these adaptations may have assisted in placental growth and function (Dandrea *et al*., 2002). More work is needed to determine if placental insufficiencies observed in various nutritional models are associated with the inability for the maternal blood volume to expand adequately in times of suboptimal nutritional resources.

The ruminant placenta

Unlike most eutherians, ruminant livestock have non-invasive placentas. Gross morphology of the ruminant placenta is termed cotyledonary, where the fetal placenta attaches to discrete site on the uterine wall called caruncles (Ford, 1999). The placental membranes attach at these sites via chorionic villi in areas termed cotyledons. The caruncular-cotyledonary unit is called a placentome and is the primary functional area of physiological exchanges between mother and fetus. Microscopically, livestock species have epitheliochorial placentation, with six cellular layers separating maternal and fetal blood. Some argue that ruminant placentas are better classified as syndesmochorial due to their formation of giant trophoblast cells by chorionic and uterine epithelia (Wooding and Wathes, 1980; Hoffman *et al*., 1986). The binucleate cells migrate during implantation and fusion with the caruncular epithelium to cause a shift from cellular to maternofetal syncytial plaques (Wooding, 1984; Wango *et al*., 1990). Binuclear cells have two principal functions: 1) to create the maternofetal syncytium necessary for proper implantation and 2) to promote placentomal growth, as well as to produce and deliver a variety of steroid and protein hormones (Wooding, 1992).

In the ewe, the growth of the cotyledonary mass is exponential during the first 70 to 80 days of pregnancy, thereafter slowing markedly until term (Stegeman, 1974). In contrast, the placental growth in the cow progressively increases throughout gestation (Vonnahme *et al*. 2007; Funston *et al*., 2010). Perhaps, these alterations in growth patterns in the sheep and cow placenta help explain the change of capillary area density (*i.e.*, a blood flow related measure; Borowicz *et al*., 2007) that exist from mid- to late-gestation (Reynolds *et al*., 2005). While sheep placentas remain relatively similar in weight from mid- to late-gestation, their caruncular and cotyledonary capillary area density increase ~200 and 400%, respectively (Reynolds *et al*., 2005; Borowicz *et al*., 2007). Bovine placentas exhibit relatively modest changes in capillary area density (compared to sheep) from mid- to late-gestation with capillary area density in caruncular tissue decreasing ~30% and cotyledonary tissue increasing ~190%, with caruncular and cotyledonary tissue weights increasing ~530 and ~650%, respectively (Vonnahme and Lemley, 2011).

Prenatal dietary impacts on conceptus development

This idea that improper maternal nutrition in late pregnancy decreases offspring performance had already been suggested by Wallace (1948) and because the majority (75%) of ovine fetal growth occurs over the last two months of gestation (Robinson *et al*., 1977) it is logical to understand why adequate maternal nutrition during late gestation is critical for maintaining fetal growth. These deleterious effects observed in offspring of nutrient restricted dams are not limited to neonatal growth and health, rather this programmed effect has a profound effect on lifelong growth and increases the likelihood of developing non-communicable diseases later in life (Barker *et al*., 1993; Godfrey and Barker, 2000). In addition to the evident need for developmental programming research in biomedical science, improper maternal nutrition during pregnancy also has profound consequences on livestock offspring health and performance (Wu *et al*., 2006). Compounding on offspring altered growth trajectories, improper maternal nutrition also leads to a decrease in fertility as well as the carcass quality of the offspring, including altered fat deposition, muscle fiber type and reduced meat quality (Wu *et al*., 2006). To understand how maternal diet can influence offspring fertility, fetal ovaries collected from late pregnant ewes that experienced a 40% nutrient restriction from mid to late gestation, had primordial follicles with a decreased cellular proliferation rate compared to ovaries from fetuses of adequately fed ewes (Grazul-Bilska *et al*., 2009). This decreased proliferation in the primordial follicle may impact future follicular activity, fertility, and reproductive longevity of the female offspring. Unfortunately, data do not

indicate the reproductive success of these offspring. It has been previously proposed maternal protein supplementation may affect oocyte quality or early embryonic formation, resulting in fewer calves born during the first 21 days of the calving season (Martin *et al*., 2007). Furthermore, heifers born from dams protein supplemented during the last third of pregnancy had an increased pregnancy rate compared to heifers from non-supplemented dams (Martin *et al*., 2007). Fewer heifers from non-supplemented dams attained puberty before the first breeding season compared with heifers from supplemented cows in a subsequent study (Funston *et al*., 2010). Additionally, in rats where dams were protein restricted during pregnancy, female pups had a delay to vaginal opening (i.e. puberty) and time to first estrus compared to control dams (Guzman *et al*., 2006).

Several authors have established that many of the models of placental insufficiency are, in part, due to reduced placental vascularity and uterine or umbilical blood flows (reviewed in Owens *et al*., 1986; Fowden *et al*., 2006; Vonnahme and Lemley, 2011; Burton and Fowden, 2012). When placental growth is restricted in ewes, umbilical and uterine blood flows are reduced, limiting fetal growth (Owens *et al*., 1986). Recently, in our laboratories, we have investigated if placental vascularity and uterine/umbilical blood flows are impacted by different maternal dietary treatments. In sheep, while we have observed reductions in umbilical blood flow (Lemley *et al*., 2012) and increases in arterial indices of resistance (Lekatz *et al*., 2013), we have not observed alterations in placental capillary densities (Lekatz *et al*., 2010; Eifert *et al*., 2015). Umbilical blood flow of singleton fetuses was reduced after 30 days of receiving a 40% global nutrient restriction compared to adequately fed control ewes (Lemley *et al*., 2012). This reduction in umbilical blood flow remained through late gestation (day 130). In beef cattle, we initially hypothesized that, similar to sheep, reductions in intake would lead to reductions in uterine blood flow. In contrast, we observed that during a 110 day nutrient restriction (i.e., 40% of the control diet), uterine (Camacho *et al*., 2014) and umbilical (Camacho *et al*., 2018) blood flows were similar. Interestingly, upon realimentation, blood flow to the ipsilateral horn increases (Camacho *et al*., 2014). While our work has been done with global nutrient restriction and realimentation, Perry *et al*. (1999) reported that protein restriction during the first trimester of pregnancy followed by increased protein concentration during the second trimester enhances placental development and fetal growth. Increased dry matter intake has been linked to enhanced maternal insulin-like growth factor-1 during late pregnancy (Lemley *et al*., 2014). If exogenous insulin-like growth factor-1 is administered to the dam, there is increased glucose and amino acid uptake by both fetal and maternal tissues (Sferruzzi-Perri *et al*., 2007). Uteroplacental blood flow is undoubtedly associated with fetal growth and development; however, specific nutrient transport across the feto-placental unit rely not only on adequate blood flow but also on adequate nutrient transporter densities.

The timing and duration of inadequate maternal nutrition

If there is a nutritional inadequacy during pregnancy, the timing, duration, and severity of that restriction greatly impacts fetal development. While most fetal growth occurs during late gestation (Robinson, 1977), inadequate nutrition during early gestation can have profound effects on placental development, vascularization, and fetal organogenesis (Funston *et al*., 2010). Adult sheep that are nutrient restricted during late gestation experience a 17 to 32% decrease in uterine blood flow, decreased caruncular capillary area density as well as reduced fetal weight (Anthony *et al*., 2003; reviewed in Reynolds *et al*., 2006). Additionally, underfed multiparous cows that were nutrient restricted during mid and late gestation experienced significant reductions in calf birth weights (reviewed in Greenwood and Café, 2007).

Underfed adolescent animals often respond differently than mature animals. Heifers that were nutrient restricted during gestation experienced more extreme birth weight reductions in their calves than mature beef cows (reviewed in Greenwood and Café, 2007). Additionally, placental alterations can occur due to altered maternal nutrition during early-to mid-gestation without impacting fetal weights (Rasby *et al*., 1990). The bovine placenta also appears to be sensitive to protein supplementation during early- to mid-gestation but calf birth weight was not impacted (Perry *et al*., 1999; Perry *et al*., 2002). However, because the bovine placenta continues to grow throughout gestation (Prior and Laster, 1979; Ferrell, 1989) it has been suggested, that the bovine placenta is not as sensitive to nutritional deficiencies as the ovine placenta (Ferrell, 1989; Greenwood and Café, 2007). While there are many brilliant reviews written about the impacts of maternal nutrition on ovine conceptus development, the focus of this review will be how maternal nutrient restriction and supplementation strategies impact calf development.

Nutrient restriction in the beef cow

Global nutrient restriction on the dam during various stages of gestation can impair placental function and calf growth depending on the severity of the restriction, timing of nutritional insult, parity, and pregnancy type (Tables 1 and 2). In comparison to sheep, the developing bovine fetus is more susceptible to alterations in myogenesis and adipogenesis when subjected to maternal nutrient restriction during mid- to late-gestation (Greenwood and Café, 2007) whereas nutritional restrictions during early pregnancy can have subtle effects on organogenesis, causing long-term health complications (Greenwood and Bell, 2003; Bell *et al*., 2005). Pre-breeding management of heifers influenced uterine hemodynamics as low input (fed to 45 to 55% of mature body weight at breeding) females had increased uterine blood flow (adjusted for maternal body weight) compared to conventionally fed heifers, with authors hypothesizing the increased uterine blood flow compensated for inadequate heifer body reserves (Cain *et al*., 2017; Table 2).

When heifers are not provided adequate metabolizable energy or crude protein during early- to mid-gestation, fetal weights are reduced (Micke *et al*., 2010). Interestingly, fetuses from restricted heifers had increased umbilical diameters compared to fetuses from adequately-fed heifers (Sullivan *et al*., 2009; Micke *et al*., 2010; Table 3). If an increased umbilical diameter equates to the potential for increased umbilical blood flow, this may serve as a compensatory mechanism to support fetal growth, as evidenced by greater fetal abdominal circumference and crown-nose length and thoracic diameter (Sullivan *et al*., 2009; Micke *et al*., 2010). Our laboratory has also demonstrated that nutrient restriction during early pregnancy (day 30 to 85) in multiparous cows results in calves being larger, which is probably due to the larger placentome mass observed in those restricted cows (Camacho *et al*., 2018; Table 2). Others have reported that heifers nutrient restricted to 60% of their NRC requirements in early gestation (conception to day 60 gestation) had normal calf birth weights and placental weights (Spiegler *et al*., 2014).

When global nutrient restriction occurs during mid- to late-pregnancy (day 118 to term), dams progressively lost body condition and had reduced calf birth weights (Freetly *et al*., 2000). Similarly, Corah *et al*. (1975) reported that heifers nutrient restricted to 57% of their NRC requirements during late gestation had a reduction in fetal weights. In contrast, no birth weight differences were detected when mature cows were nutrient restricted to 55% of their NRC requirements during a similar time period (Hough *et al*., 1990). Perhaps, multiparous cows have a compensatory mechanism for maintaining proper fetal growth that first time dams do not possess.

While skeletal muscle development *in utero* sets the foundation for postnatal growth performance, nutrient delivery within the fetus is shuttled to more important organs, such as the brain and heart (Bauman *et al*., 1982; Close and Pettigrew, 1990). The fetal period is crucial for skeletal muscle development, because no net increase in the number of muscle fibers occurs after birth (Glore and Layman, 1983; Greenwood *et al*., 2000; Nissen *et al*., 2003). Therefore it should not be surprising that skeletal muscle development is altered by maternal diet (Table 4). Calves from nutrient restricted beef cows have decreased average daily gain in the feed yard, fatter carcasses at 30 months of age (Café *et al*., 2006; 2009), and had reduced carcass weights (Greenwood *et al*., 2004). Gonzales *et al*. (2013) could rescue muscle fiber size and muscle progenitor cell numbers through realimentation of late-gestating beef cows that were previously nutrient restricted during early pregnancy. Heifers that only received 55% of their NRC requirements during early gestation (day 32 to 115) had calves with increased muscle fiber diameter, faster glucose clearance, but similar feed efficiency as calves from continuously well-fed dams (Long *et al*., 2010a; Long et al., 2010b). Interestingly, weaning weights, carcass composition, and carcass quality of those cattle were unaffected (Long *et al*., 2010b) possibly due to the realimentation.

By increasing the forage quality of late gestating beef cows, Underwood *et al*. (2010) improved average daily gain, increased 12th rib subcutaneous fat, and hot carcass weights of calves, although earlier differences in neonatal performance were not detected. Similarly, Mohrhauser *et al*. (2013) reported that cows fed diets exceeding their energy requirements during mid-gestation, had calves that were fatter and had lower yielding carcasses compared to offspring from dams fed a negative energy diet.

Maternal supplementation strategies in the beef cow

Because there are many times during pregnancy when the dam only has access to low quality forages, supplementation of dietary protein and energy supplements have been examined (Tables 3, 4, and 5). Cows supplemented with dried distiller's grains plus solubles (DDGS) during late gestation (Radunz *et al*., 2010; Gunn *et al*., 2014, 2017) as well as into early lactation (Winterholler *et al*., 2012) had increased calf birth weights, however, this effect disappeared by weaning. Conversely, Kennedy *et al*. (2016) observed increased birth weights coupled with heavier weaning weights of calves whose dams received DDGS supplementation at 0.3% body weight during late gestation (day 200 to 270). This difference is likely due to the improved roughage intakes of DDGS supplemented beef cows and their increased uterine blood flow (Kennedy *et al*., 2016). Perhaps *ad libitum* access to forage is a critical component to the response of DDGS supplementation. When cows that were fed low quality hay at 2% of body weight and supplemented with DDGS at 1.7 g/ kg of body weight, uterine blood flow was reduced (Mordhorst *et al*., 2017). Despite the decreased uterine blood flow, calf birth weights were similar (Mordhorst *et al*., 2017).

To examine effective timing of dam protein supplementation and improved forage strategies, multiparous beef cows were provided with either a protein supplement at 0.45 kg/day or no supplement during late gestation and grazed on either subirrigated meadow or cool-season grass hay during lactation (Stalker *et al*., 2006; Martin *et al*., 2007). While no differences in calf birth weight or maternal milk production were detected, calves from protein supplemented dams who grazed subirrigated meadow during lactation had heavier calves at weaning (Stalker *et al*., 2006; Martin *et al*., 2007). While no differences in carcass composition or carcass quality were detected amongst the steers (Stalker *et al*., 2006), the heifers of protein supplemented dams had improved pregnancy rates compared to heifers whose dams did not receive the supplement (Martin *et al*., 2007). In a subsequent study, steers from protein supplemented dams who grazed winter range had heavier weaning weights and improved hot carcass weight and USDA quality grades (Larson *et al*., 2009). Supplementation during late pregnancy also benefits postnatal performance if early weaning strategies are implemented (Shoup *et al*., 2015a, b), where weaning weights, average daily gain, and marbling are increased. In spite of these interesting findings, there are other investigators that report no impacts of protein supplementation, which may be due to the stage of gestation, severity of basal nutritional insult, and components of the supplement (Greenwood and Café, 2007).

Table 1. The effects of bovine maternal nutrient restriction on calf and placental parameters.

Parity and stage[1]	Diet[2]	Offspring sex	Change in dam body weight	Fetal/ birth weights	Placental weights[3]	Placental vascularity[4]	Reference
N; day 183- term	CON (100% NRC) or RES (55% NRC)	♂ + ♀	RES ↓	RES ↓	---	---	Corah *et al*., 1975
M; day 193- term	CON (100% NRC) or RES (57% NRC)	♂ + ♀	RES ↓	NSE	---	---	Hough *et al*., 1990
M; Mid-gestation - rebreeding	Maintain BCS (HHH); ↓ BCS 2nd trimester then ↑ (LHH); or ↓ BCS until 28 day of lactation (LLH)	♂ + ♀	HHH > LLH at calving	LLH ↓	---	---	Freetly *et al*., 2000
M; day 30-125	100 *vs*. 50% NRC from day 30-125 then 100% NRC to day 250	♂	---	NSE	NR COT and CAR ↓ @ day 125 and 250	NR ↑ Akt & ERK1/2 phosphorylation @ day 125	Zhu *et al*., 2006
M; day 30-125	100 *vs*. 50% NRC from day 30-125 then 100% NRC to day 250	♀	RES ↓ then realimented BCS 5.75	NSE	---	Realimentation ↑ CAR CSD; ↓ COT CAD and CSD	Vonnahme *et al*., 2007
M; day 30-125	100 *vs*. 50% NRC from day 30-125 then 100% NRC to day 250	♀	RES ↓ then realimented BCS 5.75	NR- IUGR ↓ day 125	NR-IUGR ↓ COT wt	---	Long *et al*., 2009

[1]Parity: M, multiparous; N, nulliparous; Stage: stage of pregnancy. [2]Description of diets; NRC, National Research Council; BCS, body condition score. [3]CAR, caruncle; COT, cotyledon; IUGR, intrauterine growth restricted. [4]CSD, capillary surface density; CAD, capillary area density.

Table 2. The effects of bovine maternal nutrient restriction on uterine hemodynamics and birth weights.

Parity and stage[1]	Diet[2]	Offspring sex	Change in dam body weight	Uterine blood flow	Placental weights	Fetal/ birth weights	Reference
M; early through late (day 30 to 85, early; day 85 to 140, mid; day 140 to 254, late)	Early: C (100% NRC), R (60% NRC); Mid: CC, RR, RC; Late: CCC, RRR, RCC	♂ + ♀	day 85 = R ↑BW; day 140 = RR ↓BW; day 254 = RCC ↑BW	RCC > RRC=CCC	R and RR ↑ placentome number and weight compared to C, CC	d 85 = R ↑ *vs*. C	Camacho *et al*., 2018
M; early through mid	CON (100% NRC); RES (60% NRC) until day 140 then realimented	♂ + ♀	RES ↓	NSE during restriction; RES after realimentation ↑ ipsi UBF *vs*. CON	---	---	Camacho *et al*., 2014
P; Pre-breeding management until day 45	Spring (S) or Fall (F) fed LOW input or CONventional	♂ + ♀	CON|S > CON|F > LOW|F > LOW|S	Spring ↑UBF; LOW ↑ adjusted UBF	---	Spring ↑ *vs*. Fall	Cain *et al*., 2017

[1]Parity: M, multiparous; P, primiparous; Stage: stage of pregnancy. [2]Description of diets; NRC, National Research Council; BCS, body condition score. [3]UBF, uterine blood flow; Ipsi, ipsilateral uterine horn. NSE, no significant effect; ---, not reported.

Table 3. The effects of bovine maternal nutrient supplementation on uterine hemodynamics, placental parameters, and neonatal performance.

Parity and stage[1]	Diet[2]	Offspring sex	Supplement	Offspring weights	Uterine/ umbilical blood flow[3]	Calf weaning weight	Reference
P; Early and mid-gestation; realimentated late gestation	HIGH (High ME and CP) or LOW (Low ME and CP) fed early and/or mid gestation (2 x 2 factorial)	♂ + ♀	---	HIGH-HIGH > LOW-LOW	Umbilical cord diameter ↑ high protein (first trimester); ↓ high protein (2nd trimester)	---	Micke *et al*., 2010
M; day 177 - 13 day lactation	5 supplementation diets: DDGSLow, DDGSIntermediate, DDGSHigh, positive status (POS) and negative (NEG)	♂ + ♀	DDGSL (0.77kg/day); DDGSI (1.54 kg/day); DDGSH (2.31kd/day)	DGSH > NEG	---	NSE	Winterholler *et al*., 2012
M; day 200-270	CON = 90% corn stover, 10% corn silage basal diet fed ad libitum; SUP = CON diet + DDGS at 0.3% body weight	♂ + ♀	DDGS at 0.3% body weight	SUP ↑ *vs*. CON	SUP ↑UBF and HR, ↓PI *vs*. CON	SUP ↑ *vs*. CON	Kennedy *et al*., 2016
M; day 170-234	Hay at 2% BW (CON), DDGS+ hay (SUP)	♂ + ♀	DDGS at 1.7 g/kg BW	NSE	SUP ↓ UBF *vs*. CON	NSE	Mordhorst *et al*., 2017

[1]Parity: M, multiparous; P, primiparous; Stage: stage of pregnancy. [2]Description of diets; ME, metabolizable energy; CP, crude protein; DDGS, dried distillers grains plus solubles; [3]UBF, uterine blood flow. NSE, no significant effect; ---, not reported.

Table 4. The effects of bovine maternal nutrient restriction during gestation on offspring postnatal performance and carcass parameters.

Parity & Stage[1]	Diet	Offspring sex	Fetal/ Birth weights	Weaning weights	Feed efficiency	HCW	USDA QG	USDA YG	12th rib fat	Study
M; day 80- term	High (H) or Low (L) plane of nutrition during gestation. Cross-over at birth	♂ + ♀	H ↑	NSE	H ↑ ADG and DMI	---	NSE	NSE	NSE	Café *et al*., 2006, 2009
M; day 120 - 180	Native pasture (protein restricted) vs. improved pasture (IP)	♂	NSE	IP ↑ vs. native pasture	IP ↑ ADG vs. native pasture	IP ↑ vs. native pasture	NSE	NSE	IP ↑ *vs*. native pasture	Underwood *et al*., 2010
M; day 84 -175	Dormant range = positive energy status (PES) vs. 80% of NRC = negative energy status (NES)	♂ + ♀	---	---	---	NSE	NSE	PES ↓ vs. NES	PES ↑ *vs*. NES	Mohrhauser *et al*., 2013

[1]Parity: M, multiparous; Stage: stage of pregnancy. NSE, no significant effect; ---, not reported.

Table 5. The effects of bovine maternal supplementation during gestation on offspring postnatal performance and carcass parameters.

Parity & Stage[1]	Basal diet	Supplement	Experimental design	Weaning weight	Feed efficiency	Hot carcass weight	USDA quality grade	USDA yield grade	12th rib fat	Reference
M; Late gestation - early lactation	Meadow or Hay forage	0.45 kg/day with 42% CP (PS) or no supplement (NS)	2 x 2 factorial; forage x supplement	M-PS ↑	NSE	NSE	NSE	NSE	NSE	Stalker *et al*., 2006
M; Nov- Mar	Winter range (WR) vs. corn residue (CR)	Protein supplement (PS) or none (NS)	2 x 2 factorial; forage x supplement	WR-PS > WR-NS	NSE	WR-PS ↑	PS ↑ vs. NS	NSE	NSE	Larson *et al*., 2009
M; day 160 - 275	Hay; or 4.1 kg DDGS; 5.3 kg corn	1 kg pelleted supplement for DDGS and Corn		Corn > hay	NSE	NSE	Corn ↓	NSE	NSE	Radunz *et al*., 2010, 2012
M; day 45- 185	CON (100% NRC), NR (70% NRC); NRP (70% NRC + essential amino acids)	NRP treatment only = essential amino acids supplement to equal CON		NSE	---	NSE	NR ↑ adipocyte	NR ↓	NSE	Long *et al*., 2012
M; day 180- term; early	Pasture fed; normal or early wean	Low supplement = 2.16 kg/day, high supplement = 8.61 kg/day or no supplement (NS)	2 x 3 factorial; time of weaning x supplement	Low supplement > no supplement when early weaned	Low supplement in normal wean ↑	NSE	High supplement > No supplement	NSE	NSE	Shoup *et al*., 2015a, b
P; day 142- term	CON = hay; HI = hay + DDGS: LO = hay + corn-gluten	HI- DDGS (0.83 kg/day); LO- corn gluten (0.83 kg/day)		NSE	CON ↑DMI and RFI	NSE	CON ↑	CON < LO	CON > LO	Summers *et al*., 2015 a, b

[1]Parity: M, multiparous; P, primiparous; Stage: stage of pregnancy. NSE, no significant effect; ---, not reported.

Conclusions

It is important that we continue to understand the capabilities of the maternal system to various stressors that occur during pregnancy. With increased knowledge of how the dam responds to stressful nutritional paradigms (i.e., inappropriate nutrient supply, conditional increased nutrient demand, specific nutrient imbalances, etc.), we may have the chance to increase the welfare for the dam as well as the offspring throughout their productive life. It appears that the multiparous beef cow is quite resilient to many different nutritional stressors compared to the first calf heifer. While this may be due to age, previous pregnancies, and the increased hormonal profiles associated with being pregnant, surely alters the uterine vasculature and its ability to nourish subsequent calves. Moreover, it appears that the placenta is responsive to inadequate nutrition, so that in times when nutrients are suboptimal, it simply grows to increase its surface area of attachment. Knowing the placental adaptations that the multiparous beef cow is capable of may explain why in many models of nutrient restriction, calves do not have negative impacts on their carcass phenotypes.

Protein supplementation, when forage is not limiting, appears to enhance uterine blood flow, which in turn may allow greater nutrient delivery to the calf. While increased birth weights are not always reported when a protein supplement is provided during late gestation, oftentimes increased weaning weights, and increased carcass outcomes are noted. Continued efforts to understand how maternal diet impact uteroplacental blood flow, placental vascularity, and other factors associated with nutrient absorption may be key for enhancement of nutrient transfer within the reproduction tissues.

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DOI: 10.21451/1984-3143-AR2018-0018

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Identification of genes associated with reproductive function in dairy cattle

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Abstract

The use of genomics has improved response to selection for functional traits with low heritability such as fertility traits. Much of the work on fertility traits has been performed through use of genome-wide association studies (GWAS) to identify genetic loci associated with reproductive traits. Under a GWAS approach, the assumption is that the markers on the panel are in linkage disequilibrium with causative mutations. In many cases, identification of the causative mutation is difficult because an associated genetic marker can be in intergenic regions and can be in linkage disequilibrium with variants in several nearby genes. Another approach is to identify candidate genes using knowledge of the biological pathways controlling a trait to search for single nucleotide polymorphism (SNP) in genes in those pathways. This should reveal putative causative markers responsible for genetic variation in biological function, and it is expected that the marker will be more strongly associated with a trait than one in linkage disequilibrium. An example of how a series of candidate gene studies demonstrate that identification of markers in genes involved in reproductive processes can lead to discovery of additional markers associated with genetic variation in reproductive traits is presented. In addition, the inclusion of candidate markers for fertility can improve reliability of genetic estimates for fertility traits, and the repeatability of the effects across a separate population of animals gives confidence that association elucidated by this set of markers is likely to be real. More importantly, the use of candidate genes can provide insights into the biology underpinning genetic variation in fertility, and that this understanding can lead to physiological interventions to improve reproductive function.

Keywords: candidate genes, fertility, genomic selection, reproductive function.

Introduction

Fertility is a complex trait and, it is regulated in part by genetics. In the dairy cow, genetic merit for fertility and production are negatively correlated ranging from 0.35 - 0.60 (Boichard and Manfredi, 1994; VanRaden *et al*., 2004; Pritchard *et al*., 2013) and the intense selection for milk production during the last five decades has been one of the causes of a decrease in the genetic merit for fertility in dairy breeds (Butler, 2003). Nevertheless, improvements in reproductive performance of dairy cows has been made during the last decade because of advancements in reproductive management (Royal *et al*., 2000; Petersson *et al*., 2008), increased emphasis on genetic selection of reproductive traits (Norman *et al*., 2014), and incorporation of genomics into genetic selection schemes (García-Ruiz *et al*., 2016).

Most reproductive traits are controlled by many genes, each of which has a small effect. This is evident from genome wide association studies (GWAS) in which genetic variation in a trait is partitioned into associations with individual single nucleotide polymorphism (SNP). The low heritability characteristic of reproductive traits is indicative that only a small proportion of phenotypic variance is due to additive actions of individual genes and that reliability estimates of breeding values are prone to be low. It does not, however, mean that reproductive traits are not under genetic control, many specific genes have been identified that contain mutations that are associated with reproductive function. Furthermore, clear differences in fertility have been found between genetic lines of animals. In Holstein, for example, cows with higher genetic merit for fertility had fewer services per conception, and shorter intervals from calving to conception compared with cows with low genetic merit for fertility (Cummins *et al*., 2012a; Ortega *et al*., 2017a).

Reproductive traits in dairy cattle

In the United States, three main female fertility traits are used in official genetic evaluations of dairy cattle: daughter pregnancy rate (DPR), cow conception rate (CCR) and heifer conception rate (HCR). DPR is defined as the percent of cows eligible for breeding that become pregnant in each 21-day period (i.e., over one estrous cycle). Conceptually, DPR is the product of estrous detection rate (the percent of cows in estrus that are detected in estrus) and pregnancy rate per insemination (the percent of inseminated cows that become pregnant). Practically, DPR is calculated from the term days open, which is the interval from calving to conception. Predicted transmitting ability (PTA) for DPR and days open are nearly linear function of each other. An increase of 1% in PTA for DPR equals a decrease of 4 days in the PTA for days open (VanRaden *et al*., 2003). Cow conception rate is defined as the percent of lactating cows that become pregnant after each service while HCR is the same variable for heifers (VanRaden *et al*., 2004). Heritability for these traits in Holsteins range from 0.001 - 0.016 (Pryce *et al*., 2004; VanRaden *et al*., 2004; Kuhn *et al*., 2006). Nonetheless, low heritability has not prevented progress in genetic selection for fertility. During the last 15 years, breeding values for DPR have improved, in part as a result of

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Received: February 1, 2018
Accepted: May 14, 2018

including fertility traits into economic indexes such as net merit and by inclusion of genomic information for breeding value calculations.

Types of mutations responsible for genetic variation in reproduction

Genetic variation is the basis of biological diversity in a population. In the bovine genome, the total sequence length is 2,670,123,310 bp (UMD 3.1.1). As of December 2017, there were 102,499,615 SNP and 10,462 other genetic structural variations (>50 base pairs) including deletion/insertions, copy number variant, duplications, inversions, translocations and complex chromosomal arrangements (Aken *et al*., 2017). Genetic mutations ultimately affect the proteome of the organism either by affecting the structural properties of a protein or by modifying amount of protein in specific tissues. Most of the genetic studies are based in the association of SNP genotypes with a specific phenotype. How the physical location of a mutation relative to the coding and regulatory regions of specific genes can cause variation in phenotype is illustrated in Fig 1.

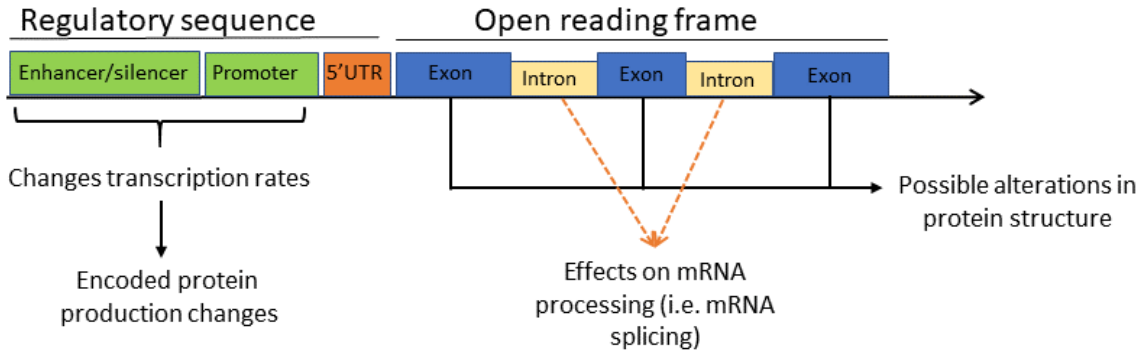

Figure 1. Possible effects of mutations relative to its location within a gene structure.

Approaches for gene discovery and genetic selection for fertility

Genome-wide association studies

GWAS are used to localize genomic regions that contribute to genetic variation of a trait. This approach is based on linkage disequilibrium, which refers to the association of any pair of alleles at different loci. Linkage disequilibrium exists because genes located closely together on a chromosome are more likely to be inherited together; i.e., cross-over events in meiosis are less likely to occur between two loci close together than two loci further apart or on different chromosomes. In a typical GWAS, thousands of SNP are interrogated for association with phenotypic variation in a trait, based on the assumption that SNP studied are in linkage disequilibrium with the causative mutation (Weller and Ron, 2011).

There are advantages and disadvantages of GWAS (Stringer *et al*., 2011; Riancho, 2012; Frąszczak and Szyda, 2016; Zondervan *et al*., 2016). The approach is unbiased with respect to previous knowledge of the trait of interest. Moreover, interrogation of a dense number of SNP across the genome can reveal novel markers associated with a trait. On the other hand, the large number of statistical tests performed during the analysis can lead to false positives so that stringent significance thresholds are necessary. One result is that markers with large effects are more likely to be detected and some important markers having a smaller effect may not reach significance. To get around these problems, large sample sizes are required to detect associations, particularly when multiple genes are involved in a trait. A limitation of GWAS is that often markers with significant associations with a trait are located in intergenic regions, and even when they are in linkage with the causative mutation, it is difficult to use this information to understand the basis of the genetic variance of the trait in question (Stringer *et al*., 2011; Riancho, 2012; Frąszczak and Szyda, 2016; Zondervan *et al*., 2016). Another limitation of GWAS is poor repeatability. Ioannidis *et al*. (2011) compiled results from a series of GWAS for human disease and found that replication of markers found by GWAS was around 1%. In cattle, the percent of significant SNP found in one population that were repeated in independent populations ranged from 0 (Littlejohn *et al*., 2012) to 18% (Höglund *et al*., 2014). Nevertheless, with appropriate sample sizes and statistical testing, GWAS can be very successful at identifying genes and genomic regions associated with specific traits. As an example, Cole *et al*. (2011) used a population of 1654 animals to identify 1586 SNP distributed in 486 genes that were associated with 31 production, reproduction, health and body conformation traits in Holstein cows. As more data becomes available, opportunities to validate these studies across populations become feasible, Liu *et al*. (2017) identified SNP in a Chinese Holstein population and those were later validated in a separate population of Nordic Holsteins. A detailed description of GWAS studies for female fertility can be found in the by Fortes *et al*. (2013), and the meta-assembly by Khatkar *et al*. (2014).

In dairy cattle, VanRaden *et al*. (2008) showed that incorporating data from GWAS into genetic estimates can improve reliability of genetic estimates over those based on parent averages. However, the

amount of improvement depended on the trait, being higher for production traits (increases in reliabilities of 23-43%) than for DPR (17%; Wiggans *et al*., 2011). The lower increase for DPR probably reflects low heritability and the high degree of polygenicity. The inclusion of genomic information in dairy cattle improvement programs has been of particular importance in the artificial insemination industry, allowing more accurate selection of young bulls and increasing the rate of genetic gain (García-Ruiz *et al*., 2016). In Holstein cattle, during the last 7 years the inclusion of genomic information derived from GWAS has helped shorten generation interval in sires of bulls from 6.8 to ~2.5 years, boosted genetic gains for milk yield of from 50 to 109 kg per year, and for daughter pregnancy rate from negative values close to 0 to ~0.3 (García-Ruiz *et al*., 2016).

Candidate gene approach

Another scheme to gene discovery is the use of candidate genes. A candidate gene is any gene thought to contain mutations responsible for a specific phenotype. Identification can be based on several approaches. The first is to search for genes located near genetic markers identified by GWAS. Kirkpatrick and Morris (2015) searched for candidate genes associated with ovulation rate in cattle. A GWAS was followed by Sanger sequencing of the target region in chromosome 10 which included *SMAD3*, *SMAD6* and *IQCH.* A total of 30 SNP in these genes were identified, and a haplotype comprising three SNP (two in *SMAD6* and one in *IQCH*) was associated with increased ovulation rate in daughters of bulls carrying the haplotype. After identification of a deficit of homozygotes for a JH1 haplotype associated with reduced fertility in Jersey using GWAS, sequencing performed in Jersey bulls revealed a nonsense mutation in *CWC15* which is embryonic lethal, as no homozygous individuals are present in the population (Sonstegard *et al*., 2013). Another approach was presented by Moore *et al*. (2016), where 58 candidate genes for regulation of fertility were identified by searching for genetic variants in differentially expressed genes in the endometrium and corpus luteum of cows with good or poor genetic merit for fertility.

Alternatively, candidate genes can be identified by using existing knowledge of the biological pathways controlling a trait, and search for SNP in genes in those pathways. Work at University of Wisconsin from the Khatib group has focused on using the candidate gene approach to identify genes associated with embryonic development. In one study, SNP were identified in eight genes in the POU1F1 pathway: *POUF1F1*, *GH*, *GHR*, *PRL*, *OPN*, *PRLR*, *STAT5A*, and *UTMP* (Khatib *et al*., 2009). There were significant associations for a SNP in *OPN* and *STAT5A* with fertilization rate, and for SNP in *GHR*, *STAT5A*, *PRLR* and *UTMP* with development of the embryo to the blastocyst stage. Likewise, Li *et al*. (2012), evaluated 25 genes of the TGFB signaling system. SNP were identified in *IBD3* associated with fertilization rate, and for a SNP in *BMP4* associated with development of the embryo to the blastocyst stage. Khatib *et al*. (2008a), studied the involvement of SNP in *FGF2* on embryonic survival because of the role of the FGF2 in regulation of *IFNT* expression in the trophectoderm (Michael *et al*., 2006). One SNP in the intron of *FGF2* was identified that was significantly associated with development of the embryo to the blastocyst stage (Khatib *et al*., 2008a). Likewise, and intronic SNP in *PGR* associated with fertilization rate and embryonic development to the blastocyst stage (Driver *et al*., 2009).

Tests of association for candidate genes have relatively high statistical power since the number of independent statistical tests is lower than for GWAS (Amos *et al*., 2011). Unlike GWAS, where genetic markers can change over time or between breeds because of crossover events during meiosis, the allelic association between a functional mutation and a genetically-controlled trait would be stable over time and more likely to extend across breeds. Furthermore, knowledge gained about the role of the gene in control of the trait could lead to improved understanding of the gene's functionality (Zhu and Zhao, 2007; Weller and Ron, 2011). There are limitations to the candidate gene approach. First, it is not easy to determine whether the association of a SNP in a candidate gene is causative or is in linkage disequilibrium with a nearby functional SNP. Increased confidence that a SNP is causative if the same genetic variants have similar effects in an independent population. The best way to verify the functionality of a candidate SNP is often impractical for livestock, namely the use gene editing technology to produce animals with the mutation and evaluate effect on the phenotype of interest. Another problem with the candidate gene approach is that it is most useful for identifying causative mutations in the coding region of genes. However, much genetic variation is located outside the coding region – in the regulatory region of the gene and at distantly located loci involved in epigenetic regulation.

Whole genome sequencing

Whole genome sequencing surveys the entire genetic code of an individual. The advantage of use whole genome sequencing is that it allows identification of complex forms of genetic variation besides SNP, including for example copy number variations. Moreover, by using whole sequencing the reliance on linkage disequilibrium disappears, as the causative mutation is on the generated data (Daetwyler *et al*., 2014). Haplotypes affecting fertility in dairy breeds previously identified with SNP50 chip (VanRaden *et al*., 2011), were further studied using whole genome sequence data by Fritz *et al*. (2013); and three novel mutations with damaged protein structure were identified in *GART*, *SHBG* and *SLC37A2* genes. Kadri *et al*. (2014), combining first SNP50 chip genotyping and whole genome sequencing identified a 660-kb deletion in chromosome 12 including four genes which is embryo-lethal in Nordic Red cattle. Using whole genome sequence data on 234 bulls, a mutation in

SMC2 was identified as causative for embryonic loss in cattle (Daetwyler *et al*., 2014). Given the rapidly decreasing cost of sequencing and the increase in number of animals in which whole genome sequences are available, it is likely that whole genome approaches to gene discovery are likely to predominate in the future.

From genotype to function: a fertility story

Identification of genetic variants associated with reproduction can provide clues to understand fertility regulation. Cochran *et al*. (2013a), used a candidate gene approach to identify genes associated with genetic variation in female fertility in Holstein bulls. Genes were identified by searching the literature for two kinds of genes. The first were genes well known to be involved in reproductive processes such as steroidogenesis, follicular development and embryonic development. The second kind, were genes differentially expressed between various physiological conditions in tissues involved in reproductive function. Examples include genes differentially expressed in the endometrium of lactating vs non-lactating cows, and genes differentially expressed between embryos produced *in vitro* compared to embryos produced *in vivo*. In each candidate gene, SNP where identified and only those present in the coding region or regulatory region where selected.

The final list of SNP for analysis included 422 novel candidate SNP (1 SNP per gene) and 12 SNP previously associated with fertility in the literature including *CAST* (Garcia *et al*., 2006), *FGF2* (Khatib *et al*., 2010), *FSHR* (Yang *et al*., 2010), *GHR* (Waters *et al*., 2011), *HSPA1L* (Rosenkrans Jr. *et al*., 2010), *ITGB5* (Feugang *et al*., 2009), *LEP* (Brickell *et al*., 2010), *NLRP9* (Ponsuksili *et al*., 2006), *PAPPA2* (Luna-Nevarez *et al*., 2011), *PGR* (Driver *et al*., 2009), *SERPINA14* (Khatib *et al*., 2007), and *STAT5A* (Khatib *et al*., 2008b). A population of 550 Holstein bulls with divergent genetic merit for DPR, where bulls of low DPR were those with a PTA of -2 or lower, and bulls of high DPR had a PTA of +1.7 or higher was used to test association of SNP with fertility traits (DPR, CCR, and HCR). Significant association were found for 40 SNP with DPR, 22 with HCR, and 33 with CCR. The function of the genes associated with fertility included steroid biosynthesis, genes regulated by estradiol and progesterone and immune function. In a second study, the same SNP were tested in 93 bulls for association with sperm fertilization ability, and subsequent *in vitro* embryonic development (Cochran *et al*., 2013b). There were SNP in 12 genes associated with the percent of cleaved embryos that became blastocysts. From the genes containing SNP associated with percent of cleaved embryos that became blastocyst, *C1QB*, *MON1B*, *PARM1*, *PCCB*, *PMM2*, and *TBC1D24* were associated with DPR, *C1QB* and *PARM1* were associated with HCR, and *C1QB*, *MON1B*, *PARM1*, *PMM2*, *SLC18A2*, *TBC1D24* were associated with CCR.

More recently, SNP with significant associations with fertility found by Cochran *et al*. (2013a) were tested and validated in a separate population of Holstein cows with divergent genetic merit for fertility, cows were selected to have a high (≥1.5) or low PTA for DPR (≤-1.0). Of 51 genes previously associated with one or more estimates of fertility in bulls, 22 were associated with genotypic estimates of fertility in the cow population (Ortega *et al*., 2016a). In addition, SNP effects were associated with phenotypic measures of fertility, where animals carrying allelic variants associated with higher genetic merit for fertility also exhibited more favorable phenotypic measurements of fertility, having in general higher conception rates, fewer services per conception, and fewer days open (Ortega *et al*., 2017a). Thus, selection for those markers is likely to change actual reproductive performance. The list of SNP found associated with fertility in these studies can be found in Table 1.

There was a modest increase in reliability of genetic estimate for DPR (0.2%) when the SNP were included in the markers currently used for the national genetic evaluation system (Ortega *et al*., 2016a). This increase compares favorably to the 0.5% increase in reliability caused by adding up to 300,000 markers to the 50K bovine SNP chip (VanRaden *et al*., 2013). These findings indicate that the SNP under study here explain genetic variation not fully captured by GWAS and that the SNP are either causative or in higher linkage disequilibrium with the causal mutations than markers distributed across the genome.

The functions that were most represented by those genes containing SNP repeatedly associated with reproductive traits provides an indication of physiological processes important for variation among cows in reproductive function. There were 14 genes containing SNP associated with fertility that were regulated by estradiol and 6 by progesterone (Ortega *et al*., 2017a). Both steroids are essential for reproduction in mammals and there are compelling data indicating the importance of circulating concentrations of steroid hormones for cow fertility. Progesterone concentrations on days 4-7 after AI have been positively associated with pregnancy rate in Holstein heifers (Parr *et al*., 2012), and when follicular development occurs under low progesterone concentrations there is subsequent reduced fertility (Bisinotto *et al*., 2010). Circulating concentrations of steroids may be particularly important in high producing dairy cows, because steroid catabolism is increased and circulating concentrations of estradiol and progesterone are decreased (Wiltbank *et al*., 2006, 2014). It has been shown that cows with high genetic merit for fertility have larger corpora lutea and greater circulating concentrations of progesterone, and improved phenotypic fertility than cows with lower genetic merit for fertility (Cummins *et al*. 2012a, b; Moore *et al*., 2014).

The other function represented by genes with SNP associated with reproduction was immune function. Six genes associated with immune function were associated with genetic and phenotypic measures of fertility (Ortega *et al*., 2017a). Immune function is an important determinant of fertility. Cows that experience

diseases postpartum have reduced reproductive function, are more likely to remain anovular, have decreased pregnancy rates and higher pregnancy losses than healthy cows (Santos *et al*., 2011, 2016; Ribeiro *et al*., 2016). In other studies, several of the genes differentially expressed in endometrium, liver, and muscle of Holstein cows with divergent genetic merit for fertility are involved in inflammatory processes (Moran *et al*., 2015, 2016). There is also evidence that cows can be identified by their immune response (high or low immune responders) and this is associated with the risk of developing diseases including retained placenta and metritis, which directly impact reproductive function (Thompson-Crispi *et al*., 2012).

Further research on the SNP in *COQ9* provided indirect evidence that function of the protein varied with genotype (Ortega *et al*., 2017b). *COQ9* was subjected to additional study because the SNP in this gene explained 3% of genetic variation in DPR in Holstein cows (Ortega *et al*., 2016a). COQ9 is involved in the biosynthesis of COQ10 (Tran and Clarke, 2007; Ben-Meir *et al*., 2015), which is a critical component of the mitochondrial electron transport system and which is required for mitochondrial ATP synthesis. The missense mutation studied causes a change in the predicted protein structure and was associated with a change in oxidative phosphorylation as reflected in changes in mitochondrial respiratory function. The allele associated with improved fertility was also associated with lower substrate requirements to maintain basal cellular function and reduced proton leaks from the electron transport system. *COQ9* is expressed in reproductive tissues, and these alterations could affect the function of these tissues by improving energy utilization of the cells. Additionally, because of reduced proton leak, the SNP could affect production of reactive oxygen species (Murphy, 2009; Jastroch *et al*., 2010). Further experimental work in the oocyte revealed that the variant associated with higher fertility was also associated with increased mitochondrial DNA copy number, which is associated with oocyte ATP production, successful oocyte maturation and fertilization (Reynier *et al*., 2001; May-Panloup *et al*., 2005; Tsai and St. John, 2016). Therefore, one of the reasons for differences in fertility among *COQ9* genotypes could reside in the allele associated with improved fertility, affects the competence of the oocyte due to higher mitochondrial content.

Another study was performed to understand the possible role of 12 genes containing SNP previously related to embryo competence to become a blastocyst by Cochran *et al*. (2013b). From the 12 genes, only two: *WBP1* and *PARM1* had increased expression at the moment of genome activation. Since the previous associations were based on the paternal SNP genotype, these were the genes most likely to represent actual effects of the SNP on embryonic development. Further evaluation showed that the SNP in *WBP1* caused changes in predicted protein structure. By reducing transcript abundance of this gene using Gapmer antisense oligonucleotides, it was revealed that *WBP1* plays a critical in trophectoderm formation. WBP1 is a single transmembrane adaptor protein (Pei and Grishin, 2012) that functions to bind a variety of signaling proteins containing the WW1 or WW2 domains. Among these are the proteins KIBRA, SAV1, and YAP involved in the Hippo signaling pathway (Zhao *et al*., 2010). Hippo signaling has been implicated in differentiation of the blastocyst in the mouse (Nishioka *et al*., 2009; Lorthongpanich *et al*., 2013). The transcription factor YAP interacts with TEAD4 to induce transcription of *CDX2* which in turn causes differentiation of the outer cells of the developing blastocyst into trophectoderm (Nishioka *et al*., 2009). Perhaps the effects of the SNP in *WBP1* modify the interactions of WBP1 with proteins of the hippo signaling pathway.

Evidence was also provided that the SNP in the promoter region of *HSPA1L* improves thermotolerance in the embryo (Ortega *et al*., 2016b). Previous work has associated this same mutation with increased calf crop in Brahman cattle (Rosenkrans Jr. *et al*., 2010), and with increased transcription of *HSPA1A/HSPA1L* (primers do not distinguish between the genes) in cells when exposed to high temperatures (Basiricò *et al*., 2011). Heat stress is known to affect fertility, particularly in dairy cattle, where cows in heat stress conditions show reduced pregnancy rates and pregnancies per AI (Gwazdauskas *et al*., 1973; Hansen and Aréchiga, 1999; Flamenbaum and Galon, 2010). In this study, expression of *HSPA1A/HSPA1L* was high at the 2-cell stage in the bovine embryo, and when putative zygotes were exposed to heat shock or high oxygen conditions, those embryos inheriting the deletion mutation in *HSPA1L* had greater survival after being exposed to adverse conditions. Perhaps embryonic survival during heat stress could be improved by selecting for thermotolerant genotypes.

Taking all together, this series of studies demonstrated that identification of SNP in genes involved in reproductive processes can lead to discovery of additional markers associated with genetic variation in reproductive traits. Inclusion of these markers in current genomic evaluations also can increase reliability of genetic estimates for fertility. The fact that SNP effects were frequently repeated among two independent populations of animals and that phenotype as well as genotype was affected provides confidence that selection of these markers will improve genetic merit for fertility. As shown for the SNP in *COQ9*, the use of candidate genes can provide insights of the biology underpinning genetic variation in fertility, and that this understanding can lead to physiological interventions to improve reproductive function.

Table 1. SNP associated with fertility traits in more than one candidate gene study[1].

		Cow phenotype[2]			Cow genotype[2]			Bull genotype[3]		
SNP id	Gene	PR	SPC	DO	DPR	HCR	CCR	DPR	HCR	CCR
rs109967779	*ACAT2*				C		C	C		C
rs41766835	*APBB1*				G			G	G	G
rs133700190	*AP3B1*				T	T	T	T	T	T
rs109669573	*BCAS1*			C	C				C	
rs110217852	*BSP3*			A	A		A	A		
rs109332658	*C7H19orf60*				C		C	C		
rs135744058	*CACNA1D*					G		G	G	
rs137601357	*CAST*		T	T	T		T	T		T
rs109621328	*CD14*		C	C				C	C	
rs41711496	*CD40*					G	G	G		
rs133449166	*CSNK1E*				C	C		C	C	C
rs109137982	*FCER1G*	A	A	A	A					
rs43745234	*FSHR*	C							C	
rs41893756	*FUT1*		A	A	A		A	A		A
rs109262355	*FYB*		A	A					A	
rs109830880	*GCNT3*		T			T				
rs109711583	*HSD17B12*				G	G	G	G		
rs110828053	*HSD17B7*		C	C	C	C	C	C	C	C
rs110789098	*IBSP*	T				T	T			
rs111015912	*LDB3*		T					T	T	T
rs41256848	*LHCGR*		G		G					
rs134264563	*OCLN*		G	G	G		G	G		G
rs109813896	*PCCB*		C	C	C		C	C		
rs109629628	*PMM2*	G	G	G	G		G	G		G
rs133729105	*RABEP2*			G				G		G
rs110660625	*TBC1D24*	A	A	A	A			A		A

[1]Shown are genes containing SNP in which a significant association between the SNP and one or more reproductive traits was observed in at least two studies. The letter represents the allele associated with superior reproduction. SNP significant in more than one study but where different alleles were associated with superior reproduction are not included in the table; [2]Based on the population of 2273 Holstein cows. [3]Based on a population of 550 Holstein bulls from Cochran *et al*. (2013a). The table is reproduced from the Journal of Dairy Science (Ortega *et al*., 2017a).

Concluding remarks

The introduction of genomic selection in dairy cattle has increased rates of genetic gain, particularly for low heritability traits such as fertility. The use of GWAS as a tool for genomic selection has been very successful in improving accuracy of genetic selection in dairy cattle. The pathway to choose for gene discovery will depend on several variants: available information of the phenotype or trait of interest, population size and overall goal of the work. Without previous knowledge of genes involved in the phenotype of interest, GWAS are a powerful tool to identify regions associated with the trait. This also could elucidate candidate genes for further study as GWAS by themselves are not designed primarily to illuminate the underlying biology of the studied phenotype. The use of candidate genes in turn, allow also to improve the SNP panels used for genetic evaluations, by finding markers with stronger associations with the traits of interest that can be included in genomic evaluation schemes. Furthermore, with the identification of candidate genes, functional studies involving gene editing or gene knockout modifications can be developed to understand the tight regulation of reproductive function in cattle. As genotyping cost decrease, more datasets and whole genome sequence data becomes available that can be used to validate markers in different populations; and in the case of sequencing, identifying causal mutations of the phenotypes of interest.

Acknowledgments

Author research on this topic was funded by Agriculture and Food Research Initiative Competitive Grant no. 2013-68004-20365 from the USDA National Institute of Food and Agriculture and a grant from the Southeast Milk Inc. Milk Check-off Program.

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DOI: 10.21451/1984-3143-AR2018-0054

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Genetic control of reproduction in dairy cows under grazing conditions

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Abstract

Fertility performance is a key driver of the efficiency and profitability of seasonal-calving pasture-based systems of milk production. Since the 1990's and early 2000's, most countries have placed varying levels of emphasis on fertility and survivability traits, and phenotypic performance has started to improve. In recent years, the underlying physiological mechanisms responsible for good or poor phenotypic fertility have started to be unravelled. It is apparent that poor genetic merit for fertility traits is associated with multiple defects across a range of organs and tissues that are antagonistic to achieving satisfactory fertility performance. The principal defects include excessive mobilisation of body condition score (BCS), unfavourable metabolic status, delayed resumption of cyclicity, increased incidence of endometritis, dysfunctional estrous expression, and inadequate luteal phase progesterone concentrations. At a tissue level, coordinated changes in gene expression in different tissues have been observed to orchestrate more favourable BCS, uterine environment and corpus luteum function. Interestingly, cows with poor genetic merit for fertility traits have up-regulated inflammation and immune response pathways in multiple tissues. Sire genetic merit for daughter fertility traits is improving rapidly in the dairy breeds, especially in the predominant Holstein and Friesian breeds. With advances in animal breeding, especially genomic technologies to identify superior sires, genetic merit for fertility traits can be improved much more quickly than they initially declined.

Keywords: fertility phenotypes, genetic merit, pasture.

Introduction

"The finest trick of the devil is to persuade you that he does not exist"

Charles Baudelaire (9 April 1821 – 31 August 1867).

Genetic gain in dairy cow milk production during the last century has been impressive, highlighting the success that can be achieved through intensive selection on traits of interest. In large part, the genetic gain was achieved by selecting exclusively on milk production traits, and ignoring other functional traits (health, fertility). The foregoing thinking was that selection for daily milk production would be more successful if intensively selected (true), and that improvements in management could adequately compensate for any deterioration in genetic merit for fertility traits (in hindsight, not true). Eventually, this led to a marked decline in both genetic merit for fertility traits and phenotypic fertility performance (Pryce *et al*., 2014). The belief that a genetic influence on phenotypic fertility does not exist is clearly no longer valid. We now know that genetic background has a strong influence on phenotypic fertility performance, but as a complex trait, there are many genes that have an influence, each with very small effect (Berry *et al*., 2014; Pryce *et al*., 2014).

A compact calving pattern, with most animals calving within six weeks after the planned start of calving, is a cornerstone of efficient seasonal pasture-based milk production (Butler, 2014). Achieving this compact calving pattern necessitates excellent fertility performance during a compact breeding period. The deterioration in phenotypic fertility reached its zenith in the late 1990's and early 2000's in many countries that operate seasonal-calving pasture-based systems, resulting in longer breeding periods, spread out calving patterns and greater culling due to fertility failure (Evans *et al*., 2006). During the last twenty years, there has been renewed focus on selecting for improved fertility traits, with recent trends indicating marked improvements in both genetic merit for fertility traits and phenotypic fertility performance in many countries (Pryce *et al*., 2014). Between 1990 and 2000, average calving interval increased by 1.25 days per year, but since the mid-2000s calving intervals have plateaued or decreased in many countries (Pryce *et al*., 2014). Major gene effects on cow fertility have been previously reviewed (Butler, 2013). This review will describe the cow fertility phenotypes that are under genetic control in pasture-based lactating dairy cows.

Selecting for Improved Fertility

The initial selection indices in most dairy countries focused primarily on milk production traits (Cole and VanRaden, 2018). In addition to selecting for cows that produced more milk, there was also a focus on 'dairy type', meaning that greater angularity or sharpness was also considered favourable (i.e., cows also *looked* like they produced more milk). Many studies in different systems of production have indicated that body condition score (BCS) is a key driver of cow health and fertility (Berry *et al*., 2003; Buckley *et al*., 2003; Lucy, 2003; Weigel, 2006; Roche *et al*., 2009; Cummins *et al*., 2012c; Fenlon *et al*., 2017). Favourable BCS, however, is the opposite of favourable angularity. It is likely that selecting for angularity directly contributed to the decline in phenotypic fertility and

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Received: April 6, 2018
Accepted: June 20, 2018

increased the incidence of metabolic disorders (Hansen, 2000). Selecting for improved BCS has been identified as a strategy to improve health and fertility (Berry *et al*., 2003; Weigel, 2006). The advent of automated technologies to facilitate frequent low-cost collection of BCS records from large numbers of cows could facilitate incorporation of this phenotype into selection indexes.

For many decades, fertility and health traits (most notably mastitis) have been incorporated into the breeding index of the Scandinavian breeds. While fertility globally declined between 1985 and 2005, non-return rates, culling rates due to infertility and calving interval remained relatively constant in the Norwegian Red breed (Refsdal, 2007). The incidence of veterinary treatments for reproductive disorders in 503,683 first-lactation daughters of 1,058 Norwegian Red sires was 3.1% for silent heats, 0.9% for metritis, 0.5% for cystic ovaries, and 1.5% for retained placenta (Heringstad, 2010). The low incidence of fertility disorders and maintenance of high phenotypic fertility performance provide support for the objective of selecting for improved fertility. In the US, a specific index for grazing herds was developed in 2014 (Gay *et al*., 2014), with relative index weights on productive life (7%) and daughter pregnancy rate (20%) that were a reversal of the weights on these traits in the net merit index at that time (19% and 7%, respectively) (VanRaden, 2017). The greater emphasis on daughter pregnancy rate reflects the increased importance of phenotypic fertility in seasonal-calving grazing systems than in year-round calving systems, whereas less emphasis on productive life is required in grazing systems because less production per cow generally results in longer survival.

In Ireland, liberalisation of semen importation regulations and intense selection for milk production traits lead to the introgression of North American Holstein genes into the predominantly British Friesian national herd. During the period from 1990 to 2001, genetic merit for milk yield increased by 25 kg per year, the proportion of Holstein genes increased from 8% to 63% and the calving rate to first service declined from 55% in 1990 to 44% in 2001 (Evans *et al*., 2006). High milk production North American Holstein cows were bred for a confinement based system, where energy dense Total Mixed Ration diets were the standard feeding practice. In a grass based system, the energetic demands associated with milk production could not be met solely by grass dry matter intake (DMI), rendering the cows susceptible to excessive tissue mobilisation, negative energy balance, poor BCS and reproductive failure (Buckley *et al*., 2000a; Horan *et al*., 2004).

To address the problem of declining fertility, the Irish national breeding programme introduced a multi-trait selection index called the Economic Breeding Index (EBI) in 2001 (Veerkamp *et al*., 2002). This index included production and non-production traits, thus identifying sires of superior genetic merit for delivering on-farm profit. Since its introduction, the EBI has evolved to include 6 sub-indexes, with the fertility sub-index accounting for 35% of the relative index weight (ICBF, 2018). The fertility sub-index is comprised of 2 traits; calving interval (23.5%) and survival (11.6%).

North American and New Zealand lactating cow strain-comparison studies

Several strain-comparison studies in Ireland and New Zealand compared lactating cows with North American (NA) and New Zealand (NZ) ancestry, and the main outcomes were discussed in a recent review (Butler, 2013). In general, these studies highlighted lower milk volume but similar milk solids production, greater BCS and reduced BCS loss, similar DMI per unit of metabolic body weight, similar or earlier commencement of luteal activity, greater insulin responsiveness, greater circulating insulin-like growth factor 1 (IGF1), similar or greater hepatic IGF1 expression, greater endometrial expression of genes associated with (i) immune tolerance to the embryo, (ii) prevention of luteolysis, and (iii) embryo support and development, and superior reproductive performance for the NZ Holstein–Friesian compared with NA Holstein–Friesian (Harris and Kolver 2001; Horan *et al*., 2004, 2005; McCarthy *et al*., 2007; Patton *et al*., 2008; Lucy *et al*., 2009; McCarthy *et al*., 2009; Walker *et al*., 2012). Data from these studies collectively suggest that early lactation adaptations may have more adverse effects in the NA compared with the NZ strain of dairy cow, and that this is likely related to their greater genetic potential for milk volume.

Fert+ and Fert- lactating cow genetic model of fertility

Cows with high genetic merit for milk production have generally been reported to have poorer fertility than cows with average genetic merit for milk production (Lucy and Crooker, 1999; Buckley *et al*., 2000; Horan *et al*., 2004). It is unlikely; however, that high phenotypic milk production *per se* is directly responsible for poor fertility. A number of studies have indicated similar or even superior fertility in high yielding cows compared to lower yielding cows (Nebel and McGilliard, 1993; Gröhn and Rajala-Schultz, 2000; Bello *et al*., 2013). As a result, it is difficult to identify specific mechanisms under genetic control responsible for poor fertility using animal models that differ in phenotypic milk production potential in addition to a wide range of associated phenotypes (milk composition, body weight, feed intake capacity, etc.).

To address this issue, a lactating cow model with similar genetic merit for milk production, but either good (Fert+) or poor (Fert-) genetic merit for fertility traits was recently developed and validated at Moorepark (Cummins *et al*., 2012a). These cows have similar proportions of Holstein genetics, and similar body weight, milk yield and milk composition. Fertility performance, however, is markedly poorer in the Fert- cows compared to the Fert+ cows (Cummins *et al*., 2012a). A series of experiments was performed to characterise uterine health during uterine involution, follicular and luteal growth, reproductive hormone concentrations, behavioural estrus during the estrous cycle, metabolic status and control of nutrient partitioning during lactation in Fert+ and Fert- cows.

The research conducted to date with this animal model has clearly demonstrated that the causes of reduced fertility in the Fert- cows are multifactorial.

Uterine health

The reproductive tract of all cows becomes exposed to microbial pathogens while the cervix remains open after delivery of the fetal-placental unit. The development of uterine disease depends on the type of bacteria involved and on the immune response of the cow, and is associated with reduced subsequent fertility (Sheldon *et al*., 2009). We recorded vaginal discharge scores weekly after calving to assess the temporal changes in clinical endometritis, and also examined uterine cytology at three and six weeks postpartum to assess subclinical endometritis (Moore *et al*., 2014a). The vaginal discharge scores and uterine cytology results indicated greater incidence of clinical and subclinical endometritis in the Fert- cows, respectively. These findings indicate that the Fert+ cows were capable of mounting a stronger and/or timelier immune response following exposure to microbial pathogens. Endometritis adversely affects the local uterine environment, but also indirectly affects fertility through altered follicle development and function (Sheldon *et al*., 2002), and post-ovulatory effects on corpus luteum development (Williams *et al*., 2007).

The estrous cycle

The estrous cycle of lactating cows was synchronized when cows were approximately 80 to 100 days postpartum. The estrous synchronization protocol lasted 10 days [day 0: i.m. GnRH and insertion of P4 device; day 7: i.m. PGF2α; day 8 removal of P4 insert; day 10: expected day of estrus]. Ultrasound exams and blood sample collection were conducted daily beginning on the expected day of estrus (See Cummins *et al*. (2012b) for details). The estrous cycle was 4.1 days longer in Fert- cows compared with Fert+ cows (25.1 vs. 21.0 days; $P = 0.01$), and this was associated with Fert- cows tending to have more follicular waves (2.7 vs. 2.2; $P = 0.07$). Circulating progesterone (P4) concentrations were similar during the first five days of the estrous cycle, but from day 5 to day 13, circulating P4 concentrations were 34% greater in Fert+ cows (5.15 vs. 3.84 ng/mL; $P < 0.001$). The difference in circulating P4 was associated with a 16% larger CL volume in Fert+ cows. A follow-up study also detected greater circulating P4 concentrations in Fert+ cows, but failed to detect differences in metabolic clearance rate of P4 or hepatic mRNA abundance of genes responsible for P4 catabolism (*CYP2C*, *CYP3A*, *AKR1C* family; Moore *et al*. (2014b). This suggests that the greater circulating P4 concentrations in Fert+ cows is primarily a result of greater luteal P4 synthetic capacity (larger CL size and greater P4 output per unit of CL tissue). The effects of circulating P4 may be manifest pre- and post-ovulation. A large volume of literature supports the pivotal role of P4 on the preovulatory oocyte and follicle (Inskeep, 2004), from day 5 to 13 of the estrous cycle to influence functional changes in histotroph composition (Green *et al*., 2005), structural changes in endometrial glandular duct density (Wang *et al*., 2007), endometrial gene expression (Forde *et al*., 2009), maternal recognition of pregnancy (Mann and Lamming, 2001) and likelihood of subsequent pregnancy establishment (Herlihy *et al*., 2013). Inherent differences in circulating P4 concentrations likely represent a key phenotype responsible for fertility differences between these two strains.

Estrous behaviour

Estrous behaviour (measured using automated activity meters and electronic mount detectors) and the timing of ovulation (measured using transrectal ultrasound) were recorded at a synchronised estrus and the subsequent spontaneous estrus (Cummins *et al*., 2012b). Fert- cows had a greater incidence of silent heats (i.e., ovulation in the absence of behavioural estrus) at the end of the synchronised estrous cycle. A greater proportion of Fert- cows also displayed behavioural signs of estrus, but subsequently failed to ovulate. Of the estrus events recorded, 36% fell into the combined categories of silent heats and heats without ovulation in Fert- cows, whereas only 2% fell into the combined categories in Fert+ cows. As the Fert+ cows have been repeatedly observed to have greater luteal phase P4 concentrations (Cummins *et al*., 2012b; Moran *et al*., 2015; Moore *et al*., 2016), differences in luteal-phase P4 priming of the neural mechanisms involved in estrous behaviour and GnRH release could explain some of the differences in estrus behaviour between Fert+ and Fert- cows. It is possible that sub-optimal P4 concentrations in the estrous cycle pre-breeding interferes with the normal endocrine feedback mechanisms that are required to facilitate appropriately timed estrous behaviour and ovulation.

Endometrium - corpus luteum interaction

The differences in circulating P4 concentrations between Fert+ and Fert- cows during the luteal phase prompted an investigation into the simultaneous gene expression profile in the corpus and the endometrium (Moore *et al*., 2016). Cows were synchronised, blood samples were collected daily, periodic ultrasound exams were conducted to assess the corpus luteum development, and biopsies of the corpus luteum and endometrium were collected on day 13 post-estrus. Once again, CL volume and circulating progesterone concentrations were greater in Fert+ cows compared with Fert- cows. Global transcriptomics of the endometrium indicated greater inflammation, less favourable cellular energy status and greater synthesis and secretion of prostaglandin F2α in Fert- cows. Global transcriptomics of the corpus luteum indicated greater PGF2α response, and lesser steroidogenesis, and mRNA processing in Fert- cows. Hence, coordinated communication between the corpus luteum and the endometrium was evident, highlighting the exquisite regulation necessary to facilitate pregnancy establishment.

Metabolic status and BCS

Fert+ cows maintain greater postpartum BCS, which is facilitated by greater DMI (Moore *et al*., 2014a). Differences in metabolites and metabolic hormones are broadly reflective of better metabolic status. Circulating concentrations of IGF1 are greater in Fert+ cows throughout lactation (Cummins *et al*., 2012a). Despite Fert+ cows having greater circulating IGF1 concentrations, hepatic *IGF1* gene expression is greater only in mid to late-lactation (Cummins *et al*., 2012c). The half-life of IGF1 in circulation is ~10 minutes as a free peptide, ~30 to 90 minutes when bound to a low molecular weight binding protein (IGFBP2, IGFBP4, IGFBP5 and IGFBP6), and 12 to 15 hours in the ternary complex of IGF1, IGFBP3 and insulin-like growth factor binding protein, acid labile subunit (Jones and Clemmons, 1995). Fert+ cows had reduced expression of low molecular weight binding proteins during early lactation (Cummins *et al*., 2012c), allowing longer IGF1 half-life in the ternary complex. Fert+ cows have greater circulating concentrations of insulin and glucose during the immediate postpartum period (Cummins *et al*., 2012a; Moore *et al*., 2014a). Elevated circulating concentrations of glucose in the peripartum period increased the likelihood of early ovulation (Butler *et al*., 2006) and conception at breeding (Garverick *et al*., 2013).

Cellular Control of Nutrient partitioning

Hepatic and muscle transcriptomics were examined in Fert+ and Fert- cows during late pregnancy (LP), early lactation (EL) and mid-lactation (ML) to examine the molecular mechanisms that underpin the observed differences in BCS (Moran *et al*., 2016). We found 807 and 815 unique genes to be differentially expressed in at least one time-point in liver and muscle respectively, of which 79% and 83% were only found in a single time-point; 40 and 41 genes were found differentially expressed at every time-point, possibly indicating chronic dysregulation. We found 402, 338 and 282 genes differentially expressed in liver and 262, 527 and 212 genes differentially expressed in muscle at LP, EL and ML, respectively. Across all three time points, the differentially expressed genes pointed to the biological theme '*metabolism, lipid and carbohydrate'*, and specific functional annotation groups that were detected included '*gluconeogenesis*' and '*extra-cellular growth factor*' during late pregnancy, '*biosynthetic process*', '*lipid lipoprotein*' and '*metabolic process*' during early lactation, and '*lipid*' and '*lipoprotein particle*' during mid-lactation. The collective findings indicated key differences at each stage of lactation: (1) Fert+ cows were less reliant on mobilised muscle tissue as a source of glucose precursors and mobilised fat for cellular energy requirements during LP; (2) in EL, Fert+ cows had greater hepatic gluconeogenic capacity; and (3) in ML Fert+ cows had greater hepatic *IGF1* expression as well as up-regulation of fatty acid synthesis pathways. Clearly, the ability of Fert+ cows to maintain superior BCS and similar milk energy output compared with the Fert- cows is dependent on orchestrated changes involving multiple tissues, including liver and muscle, indicating better homeorhetic adaptation to lactation.

Inflammation

A notable observation from multiple studies that examined global transcriptomics in biopsy samples of liver, muscle, endometrium and corpus luteum collected from Fert+ and Fert- cows was differences in immune and inflammation pathways. In liver and muscle biopsies, differentially expressed genes at LP, EL and ML time points pointed toward the biological theme *'immune and inflammatory'* processes, and were generally up-regulated in Fert- cows. Specific annotation terms identified included 'chemokine' and 'MHC complex' in LP, 'defense response' and 'immunoglobuiln' in EL, and 'acute phase response' in ML (Moran *et al*., 2016). In endometrium tissue samples collected on day 7 (Moran *et al*., 2015) or on day 13 (Moore *et al*., 2016) post-estrus, the acute-phase protein serum amyloid A3 (*SAA3*) was up-regulated in Fert- cows, which has been reported to be highly induced in bovine endometrium in response to Escherichia coli infection (Chapwanya *et al*., 2013). This finding was consistent with greater incidence of clinical and sub-clinical endometritis in Fert- cows (Moore *et al*., 2014a). *SAA3* expression was also up-regulated in Fert- cows in corpus luteum tissue samples collected on day 13 post-estrus (Moore *et al*., 2016).

Conclusions

The main phenotypes that are different between cows with good and poor genetic merit for fertility traits are summarized in Table 1. Their contribution and relative importance to overall reproductive importance is likely collaborative rather than independent. Numerous genome-wide association studies consistently illustrate the multifactorial nature of bovine fertility (Berry *et al*., 2014). The impact of genetic selection programmes on improved dairy cow fertility over the past decade is supported by an abundance of scientific literature demonstrating only minor and inconsistent effects from nutritional supplementation (Roche *et al*., 2011; Butler, 2014) and hormonal manipulation (Bisinotto *et al*., 2015). Importantly, well-established phenotypes (BCS, estrous behaviour, hormone concentrations) associated with dairy cow fertility (Walsh *et al*., 2011) are under genetic control and may become useful in fertility genetic evaluations if sufficient records become available. The prospect of automated monitoring of animal health, body condition score, ovarian activity, estrous behaviour, and milk hormone concentrations is quickly becoming a reality due to developments in milking automation, camera technology, activity monitors and in-line milk analysis. Access to large datasets of fertility phenotypes collected from diverse cow populations with genotype information may further enhance our ability to accurately identify QTL's associated with reproductive efficiency and increase the rate of genetic gain. This approach was

recently utilised to elucidate the genetic control of stature in cattle (Bouwman *et al*., 2018), and could also be successful for female fertility traits despite the low heritability. Considering the differences in reproductive management between confinement (reliance on hormonal treatment) and pasture-based (AI after spontaneous estrus) and the greater selection pressure placed on fertility in pasture-based systems, further investigation is warranted to determine if the genetic and physiological differences between fertility genotypes are conserved across production environments. Nevertheless, after many decades of declining fertility, genetic merit for fertility and phenotypic reproductive performance now appears to be on the opposite trajectory.

Table 1. Summary of the principal physiological mechanisms responsible for greater fertility in Fert+ cows compared with Fert- cows.

Early postpartum	Pregnancy establishment
Greater DMI	Stronger expression of estrus
Shorter postpartum anestrous interval	Fewer silent heats, and less incidence of ovulation failure after expression of estrus
Reduced incidence of clinical and subclinical endometritis	Greater luteal phase circulating P4
More favourable systemic indicators of metabolic status	Better coordination of corpus luteum and endometrium gene expression to support luteal P4 synthesis and endometrial receptivity
Better coordination of hepatic and peripheral tissue gene expression in support lactation and BCS maintenance	Better coordination of hepatic and peripheral tissue gene expression in support lactation and BCS maintenance
Less inflammation in liver and muscle	Less inflammation in liver, muscle, endometrium and corpus luteum

Acknowledgements

The authors acknowledge financial support from the Department of Agriculture, Food and the Marine (Grant 13S528), Dairy Research Ireland, and the Teagasc Walsh Fellowship scheme.

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DOI: 10.21451/1984-3143-AR2018-0020

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Evolution of fixed-time AI in dairy cattle in Brazil

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Abstract

Various programs have been used to synchronize ovulation of a fertile oocyte, accompanied by fixed-time artificial insemination (FTAI). These programs involve a series of hormonal treatments to achieve four physiologic outcomes: 1) synchronize an ovarian follicular wave; 2) optimize conditions for ovulatory follicle development; 3) synchronize corpus luteum (CL) regression; and 4) synchronize ovulation. This manuscript summarizes studies conducted in Brazil with lactating dairy cows that aimed to increase pregnancy rates to E2/P4-based programs.

Keywords: FTAI, pregnancy rates.

Introduction

Recent reviews have considered the history, physiological basis, and practical use of FTAI for reproductive management of dairy cattle (Baruselli *et al*., 2012; Binelli *et al*., 2014; Wiltbank and Pursley, 2014). This review will examine some specific published research manuscripts that were done with ample statistical power (>200 cows/treatment) and, in our opinion, have been critical for the development of the programs that are in current use for FTAI in dairy cattle in Brazil.

Successful synchronization of ovulation and timed AI involves 4 essential physiological processes: 1) synchronize emergence of a new ovarian follicular wave; 2) optimize the environment for follicular wave development and selection of a single dominant follicle; 3) induce complete CL regression, resulting in low circulating progesterone (P4) near the time of AI; and 4) induce synchronized ovulation, combined with an optimal schedule for FTAI. These programs use various hormones, including: gonadotropin releasing hormone (GnRH), estradiol (E2) derivatives including estradiol benzoate (EB) and estradiol cypionate (ECP), intravaginal (P4) implants, and prostaglandin $F_{2\alpha}$ analogues (PGF). Achieving these physiologic goals using various combinations of hormones has generally resulted in two types of programs, one based primarily on GnRH (Pursley *et al*., 1995, 1997), used in the USA and several other countries, and E2/P4-based programs (Vasconcelos *et al*., 2011b; Wiltbank *et al*., 2011a), used in Brazil and elsewhere. Recent research has altered timing, dosages, and sequences of hormonal treatments, including merging these two types of programs striving to optimize the four principal processes of a synchronization program and thus improve fertility. This manuscript will focus on research done in Brazil to optimize FTAI programs in lactating dairy cattle. Information will be presented in the context of the four physiological processes essential for a successful FTAI program.

Physiology I: Synchronization of emergence of a new ovarian follicular wave

Understanding follicular waves was essential for initial development of FTAI protocols and their subsequent improvement (Thatcher and Santos, 2007; Wiltbank *et al*., 2011a; Wiltbank and Pursley, 2014). Synchronization of a new follicular wave near the beginning of a FTAI protocol has generally been done by two methods: 1) ovulation of the dominant follicle, usually with GnRH; or 2) inhibition of gonadotropin secretion, usually with EB, leading to regression of the current follicular wave and emergence of a new follicular wave. Neither of these treatments are completely efficient in lactating dairy cows, with only 50-65% of cows ovulating in response to GnRH treatment given at a random stage of the estrous cycle (Vasconcelos *et al*., 1999; Thatcher *et al*., 2002; Giordano *et al*., 2013; Wiltbank and Pursley, 2014) and 25 to 30% of dairy cows ovulating a persistent follicle, due to lack of regression of the previous follicular wave following EB treatment (Monteiro *et al*., 2015; Melo *et al*., 2016). Since it is likely that certain physiological processes, such as synchronized emergence of a new follicular wave, may be more critical in FTAI than during ET programs and therefore results of AI and ET have been compared in some of these studies.

An experiment (Vasconcelos *et al*., 2011b) was done to compare effects of two protocols for synchronization of ovulation (GnRH- *vs*. E2-based protocols) on P4 concentrations and fertility in lactating dairy cows subjected to either FTAI or fixed-time embryo transfer (FTET). A total of 883 lactating Holstein cows (166.2 ± 3.3 days postpartum, yielding 36.8 ± 0.34 kg of milk/day) from eight commercial dairy farms were used. Within each farm, cows were randomly assigned to receive one of the two following treatments for synchronization of ovulation: 1) GnRH Group: day-10 P4 insert (1.9 g of P4; CIDR®) + GnRH, day-3 P4 withdrawal + PGF, day-2: ECP, day 0 FTAI or day 7 FTET (nFTAI = 180; nFTET = 260); and 2) EB Group: Same as above, except EB on day-10 in lieu of GnRH

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Received: February 2, 2018
Accepted: April 20, 2018

(nFTAI = 174; nFTET = 269). Circulating P4 on day-3 was greater in GnRH than EB treatment (2.89 ± 0.15 *vs*. 2.29 ± 0.15 ng/ml; P < 0.01), but there were no effects of treatments on P4 on day 7 (3.15 ± 0.13 *vs*. 3.03 ± 0.14 ng/ml; P > 0.10). Pregnancy rate at 60 days was higher for FTET compared to FTAI (37.6% [199/529] *vs*. 26.5% [94/354]; P < 0.001). However, there were no effects of GnRH *vs*. EB on synchronized ovulation (87.0% [383/440] *vs*. 85.3% [378/443]), P/AI at 60 days (27.2% [49/80] *vs*. 25.9% [45/174]) or P/ET (38.8% [101/260] *vs*. 36.4% [98/269]), or when only synchronized cows are considered for P/AI at 60 days (35.3% [55/156] *vs*. 33.8% [50/148]) or P/ET (50.7% [115/227] *vs*. 51.3% [118/230]). Thus, either GnRH or EB could be used at the start of the protocol; although GnRH-treated cows had higher P4 during the protocol than those given EB, reproductive performance was similar.

Another study (Pereira *et al*., 2015) evaluated fertility in a FTAI protocol that compared EB versus EB + GnRH at the start of the protocol. For this study a GnRH treatment at the beginning of the protocol was added to a standard Brazilian protocol that was initiated with EB in order to evaluate whether ovulation of a dominant follicle in some cows could improve fertility due to greater synchronization of the follicular wave and greater circulating P4 during growth of the ovulatory follicle. Due to ovulation of a new follicle and consequently a CL, this study also evaluated using two PGF treatments at the end of the protocol to optimize CL regression (discussed in next section). Lactating Holstein cows (n = 1808) were randomly assigned during cool or hot seasons to receive FTAI (day 0) after one of three treatments: Control: CIDR + 2 mg of EB on day-11, PGF on day-4, CIDR withdrawal + 1.0 mg of ECP on day-2, and FTAI on day 0; 2PGF: Identical to the Control protocol, with addition of a second PGF treatment on day-2; and GnRH: Identical to the 2PGF protocol, with addition of 100 µg GnRH on day-11. Pregnancy diagnoses were done 32 and 60 days after FTAI.

Season had major effects on many reproductive measures, with more cool *vs*. hot season cows having a CL at PGF (62.9 *vs*. 56.2%), expressing estrus (86.7 *vs*. 79.9%), ovulating following the protocol (89.7 *vs*. 84.3%), becoming pregnant following the protocol (45.4 *vs*. 21.4%), and having larger ovulatory follicle diameter at AI (15.7 *vs*. 14.8 mm). The GnRH protocol increased percentage of cows with a CL (Control = 56.9%; 2PGF = 55.8%; GnRH = 70.5%) and circulating P4 concentrations on day-4 (Control = 3.28 ± 0.22; 2PGF = 3.35 ± 0.22; GnRH = 3.70 ± 0.21 ng/ml). GnRH also increased P/AI at 32 days (37.3% [219/595]) and 60 days (31% [179/595]) after TAI, compared to Control (30.0% [177/604] and 25.1% [145/604]) with intermediate results for the 2PGF protocol (33.2% [196/609] and 28.0% [164/609]). Positive effects of GnRH treatment on P/AI were only detected during the cool season (Control = 41.0%; 2PGF = 44.2%; GnRH = 50.9%) but not during the hot season (Table 1). In addition, there was only a significant effect of GnRH in cows with low P4 (<3 ng/ml) at the start of the protocol, with no significant effect in cows that had high P4 at the outset. Further, there was an interaction for presence of CL at PGF with follicle diameter; cows with a CL at PGF had greater P/AI if they ovulated larger *vs*. smaller follicles near TAI.

In conclusion, combining GnRH with EB increased fertility, as compared to EB alone, when used at the start of an E2/P4-based protocol, particularly during the cool season and in cows with low P4 at the outset.

Table 1. Effects of various treatment protocols on pregnancies per AI (P/AI) during hot *vs*. cool seasons.

	Protocol			
Item*	Control	2PGF	GnRH	P Value
P/AI 32d for all cows[1]				
Cool	41.0 (116/283)^{b}	44.2 (125/283)^{y}	50.9 (148/291)^{ax}	0.05
Hot	19.0 (61/321)	21.8 (71/326)	23.4 (71/304)	0.40
P Value	<0.01	<0.01	<0.01	
Combined	30.0 (177/604)^{b}	33.2 (196/609)^{b,y}	37.3 (219/595)^{a,x}	0.02
P/AI D60 for all cows[1]				
Cool	32.9 (93/283)^{b}	36.4 (103/283)^{ab}	41.6 (121/291)^{a}	0.09
Hot	16.2 (52/321)	18.7 (61/326)	19.1 (58/304)	0.59
P Value	<0.01	<0.01	<0.01	
Combined	25.1 (145/604)^{b}	28.0 (164/609)^{a,b}	31.0 (179/595)^{a}	0.06
P/AI D32 for synchronized cows[2]				
Cool	46.6 (110/236)^{b}	49.0 (120/245)^{ab}	55.7 (142/255)^{a}	0.11
Hot	22.5 (52/231)	26.0 (60/231)	27.4 (61/223)	0.47
P Value	<0.01	<0.01	<0.01	
Combined	34.7 (162/467)^{b}	37.8 (180/476)^{y}	42.5 (203/478)^{ax}	0.05
P/AI D60 for synchronized cows[2]				
Cool	37.7 (89/236)^{y}	40.4 (99/245)^{xy}	45.5 (116/255)^{x}	0.20
Hot	19.5 (45/231)	21.7 (50/231)	21.5 (48/223)	0.81
P Value	<0.01	<0.01	<0.01	
Combined	28.7 (134/467)^{y}	31.3 (149/476)^{xy}	34.3 (164/478)^{x}	0.18

*Least square means (n./n.); [1]All inseminated cows; [2]Includes only cows that ovulated to ECP (visible CL at day 7); a,b Within a row = P ≤ 0.05; x, y Within a row = P > 0.05 and P ≤ 0.1. From Pereira *et al*. (2015).

Physiology II. Optimization of environment for follicular wave development and dominant follicle selection

In dairy cows, a variety of methods have been evaluated to increase fertility in synchronization of ovulation programs, including: increasing P4 concentration during ovulatory follicle development (Bisinotto *et al*., 2010; Martins *et al*., 2011; Wiltbank *et al*., 2011b), increasing length of proestrus (Peters and Pursley, 2003; Pereira *et al*., 2013b), reducing follicle age (Cerri *et al*., 2009; Santos *et al*., 2010), and supplementing estrogen (E2) during proestrus (Cerri *et al*., 2004; Brusveen *et al*., 2009; Souza *et al*., 2011). Enhanced steroid metabolism in lactating dairy cows (Sangsritavong *et al*., 2002; Vasconcelos *et al*., 2003) alters reproductive physiology in dairy cattle (Wiltbank *et al*., 2006), including changes in the preovulatory follicle and ovulated oocyte. For example, there is a decrease in fertility following ovulation of larger follicles, persistent follicles, or follicles that grew when P4 concentrations are low, apparently by reducing embryo quality 1 week after AI (Wiltbank *et al*., 2014). In addition, greater P4 concentrations during the TAI protocol and greater E2 concentrations near time of AI may optimize oviductal and uterine environments, thus improving fertility in high-producing dairy cows (Miller *et al*., 1977; Buhi, 2002).

Cows without a CL at initiation of FTAI protocols have lower circulating P4 concentrations during development of the preovulatory follicular wave and reduced P/AI after FTAI. An experiment (Pereira *et al*., 2017a) was designed to evaluate effects of increased circulating P4 during preovulatory follicle growth prior to FTAI or FTET, in lactating dairy cows without a CL. Lactating dairy cows with no CL and low circulating P4 (≤1.0 ng/ml) were assigned to a protocol using one or two intravaginal P4 implants (CIDRs) and subjected to FTAI or FTET. The low P4 cows for this experiment were identified on nine farms, of which four utilized FTAI (n = 326 of 1,160 cows examined) and five utilized FTET (n = 445 of 1,396). All cows were synchronized by insertion of one or two P4 implant(s) (CIDR[s]) at start of protocol (day-11) and simultaneous treatment with 2 mg of EB. Seven days later, cows were treated with PGF (day-4) and 2 d later treated with 1.0 mg ECP and CIDR(s) were removed (day-2). Cows received FTAI on day 0 or FTET on day 7. Cows were also randomly assigned to receive either one or two CIDRs from day-11 until day-2 (1CIDR *vs*. 2CIDR). The 2CIDR treatment increased circulating P4 at day-4 (2.18 ± 0.24 *vs*. 1.77 ± 0.23 ng/ml) but had no effect on ovulation at end of protocol (83.6 *vs*. 82.6%) or ovulatory follicle diameter (15.6 ± 0.3 *vs*. 15.3 ± 0.3 mm). If only cows that ovulated to the protocol were included, 1CIDR tended to have lower P/AI than 2CIDR at 32 days (42.8 *vs*. 52.6%; P = 0.10) and 60 days (37.7 *vs*. 48.1%; P = 0.08), with no effect on pregnancy loss. There was an interaction (P = 0.05) between ovulatory follicle diameter and CIDR treatment (Fig. 1) on P/AI (day 60). In cows ovulating larger follicles≥(14 mm) , 2CIDR treatment increased P/AI compared to 1CIDR (53.3 *vs*. 34.9%; P = 0.02) but not in cows ovulating small follicles (<14 mm). There was no effect of treatment on P/ET at 32 days (30.0 *vs*. 32.0%) or 60 days (24.7 *vs*. 25.6%). Thus, these results add evidence to the concept that increased circulating P4 concentrations during preovulatory follicle development may improve P/AI, most likely due to improved oocyte quality in cows that ovulate larger follicles, since there was improved fertility only in cows ovulating larger follicles, with no significant effect of preovulatory P4 concentrations in cows that ovulated smaller follicles or that received FTET.

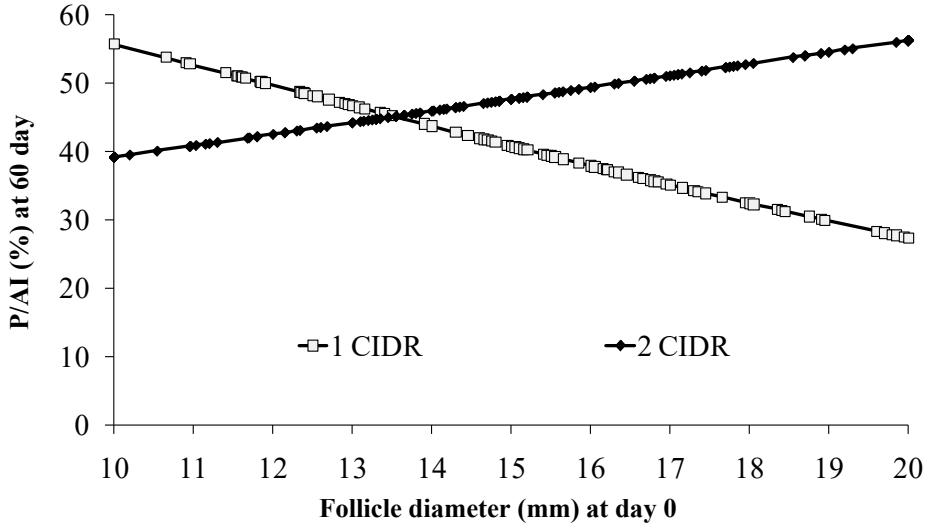

Figure 1. Effects of follicle diameter at time of AI (day 0) on P/AI at day 60 pregnancy diagnosis in dairy cows that ovulated to the protocol (CL on day 7). There was an interaction (P = 0.05) between treatment and follicle diameter on P/AI at day 60 apparently due to P/AI decreasing in cows with larger follicles in cows with only 1 CIDR but increasing with follicle size in cows with 2 CIDRs. From Pereira *et al*. (2017a).

Length of an E2/P4 FTAI protocol has also been evaluated (Pereira *et al*., 2014). Lactating Holstein cows (n = 759) were given a CIDR for either 8 or 9 d with 2 mg EB treatment at beginning, PGF 2 days before CIDR removal, and CIDR removal with 1 mg ECP at 2 days before FTAI. Cows were considered to have their estrous cycle synchronized in response to the protocol by the absence of a CL at AI (D0) and presence of a CL on day 7. Pregnancy diagnoses were performed at 32 and 60 days. Ovulatory follicle diameter at FTAI did not differ, although the 9 d program tended (P = 0.06) to have greater P4 on day 7 in synchronized cows (3.14 ± 0.18 ng/ml) than the 8 days program (3.05 ± 0.18 ng/ml). Although P/AI at 32 days (8 days = 45% [175/385] *vs*. 9 days = 43.9% [166/374]; P = 0.77) and at 60 days (8 days = 38.1% [150/385] *vs*. 9 days = 40.4% [154/374]; P = 0.52) was not different, the 9 days program had lower (P = 0.04) pregnancy losses (7.6% [12/166]) than the 8 days program (14.7% [25/175]). Cows in the 9 days program were more likely (P < 0.01) to be in estrus (72.0% [269/374]) than those in the 8 days program (62% [240/385]). Expression of estrus improved estrous cycle synchronization (97.4% [489/501] *vs*. 81% [202/248]; P < 0.01), P4 concentrations at day 7 (3.22 ± 0.16 *vs*. 2.77 ± 0.17 ng/ml; P < 0.01), P/AI at 32 days (51.2% [252/489] *vs*. 39.4% [81/202]; P < 0.01) and at 60 days (46.3% [230/489] *vs*. 31.1% [66/202]; P < 0.01), and it decreased pregnancy loss (9.3% [22/252] *vs*. 19.8% [15/81]; P < 0.01), compared to cows not detected in estrus. Those not detected in estrus with small (<11 mm) or large follicles (>17 mm) had greater pregnancy loss (P = 0.01); however, in cows detected in estrus, there was no effect (P = 0.97) of follicle diameter on pregnancy loss (Fig. 2). In conclusion, increasing the length of the protocol for FTAI increased the percentage of cows detected in estrus and reduced pregnancy losses.

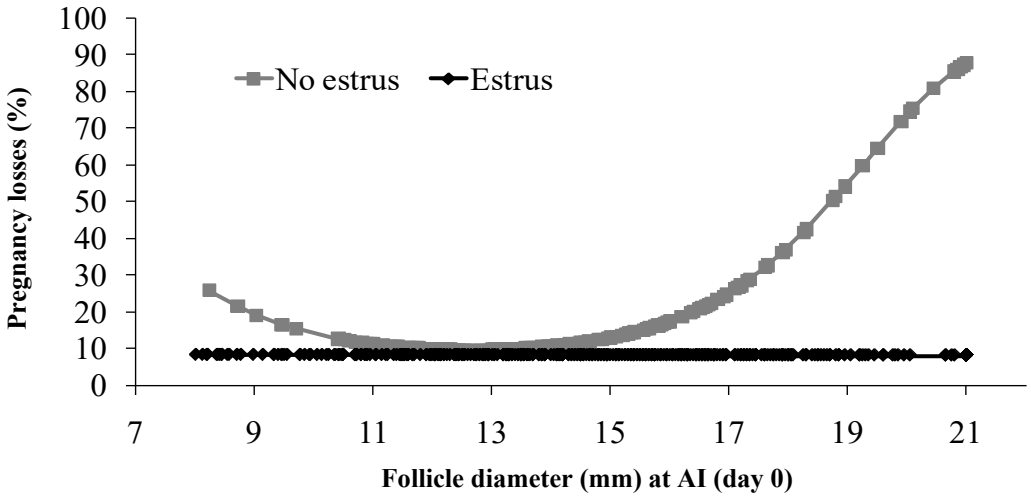

Figure 2. Effects of expression of estrus and follicle diameter at AI (day 0) on pregnancy losses between 32 and 60 days in synchronized dairy cows. No estrus P = 0.01, estrus P = 0.97. From Pereira *et al*. (2014).

A final experiment was done to compare two protocols that increase P4 during preovulatory follicle development (Pereira *et al*., 2017b). One treatment utilized two intravaginal P4 implants (CIDR), whereas the other utilized GnRH at start of the protocol. Lactating Holstein cows that had been diagnosed as non-pregnant were randomly assigned to receive FTAI following one of two treatments (n = 1,638 breedings): GnRH – CIDR + 2 mg EB + 100 µg GnRH on day-11, PGF on day-4, CIDR withdrawal + ECP + PGF on day-2, and TAI on day 0; or 2CIDR - Two CIDR + EB D-11, one CIDR withdrawn + PGF on day-4, second CIDR withdrawn + ECP + PGF on day-2, and FTAI on day 0. There was no effect of treatments (P > 0.10) on P/AI or pregnancy loss. Various physiological measurements associated with greater fertility were reduced (P < 0.01) in cows with an elevated body temperature (compared to those without), including: cows with CL at PGF (decreased 7.9%), ovulatory follicle diameter (decreased 0.51 mm), expression of estrus (decreased 5.1%), and ovulation near FTAI (decreased 2.8%). There were a greater proportion of cows (30.2%; P < 0.01) with a CL at PGF in the GnRH (74.1% [570/763]) than 2CIDR treatment (56.9% [434/763]). However, circulating P4 was greater (P = 0.05) at PGF treatment (day-4) for cows treated with 2CIDR (4.26 ± 0.13 ng/ml) than GnRH (3.99 ± 0.14 ng/ml). Thus, these two protocols yield similar fertility (Table 2), although that was likely due to somewhat different physiological alterations. Exogenous GnRH increased the proportion of cows with a CL at PGF; however, the 2CIDR protocol increased circulating P4 under all circumstances.

In conclusion, optimizing the length of the protocol and increasing circulating P4 during the protocol are strategies currently used to increase fertility during FTAI protocols in dairy cattle in Brazil. Similarly, with high-yielding dairy cattle in other countries, increasing P4 during the FTAI protocol can increase fertility (Wiltbank *et al*., 2014).

Table 2. Effects of two different protocols and rectal temperature on ovulation to ECP, P/AI at the 32 or 60 days pregnancy diagnosis, and pregnancy loss from 32 to 60 days. The two protocols were both designed to increase circulating P4 during the protocol using either GnRH at beginning (GnRH) or 2 CIDRs rather than only 1 CIDR during the protocol (2CIDR).

	Rectal temperature (ºC)		P value		
Item*	<39.1	≥39.1	Temp.	Prot.	Interaction
Synchronized cows[1]					
GnRH	88.1 (355/402)	83.3 (304/361)	0.09	0.93	0.34
2CIDR	86.2 (359/415)	84.8 (298/348)			
P/AI 32 days for all cows[2]					
GnRH	39.7 (162/402)	26.2 (98/361)	<0.01	0.64	0.42
2CIDR	38.9 (164/415)	29.3 (105/348)			
P/AI 60 days for all cows[2]					
GnRH	34.0 (138/402)	22.0 (81/361)	<0.01	0.91	0.79
2CIDR	33.6 (141/415)	22.9 (81/348)			
P/AI 60 days for synchronized cows[3]					
GnRH	38.9 (138/355)	26.6 (81/304)	<0.01	0.86	0.98
2CIDR	39.3 (141/359)	27.2 (81/298)			
Pregnancy loss (32-60 days)[3]					
GnRH	14.8 (24/162)	17.4 (17/98)	0.09	0.48	0.34
2CIDR	14.0 (23/164)	22.9 (24/105)			

*Least square means (n./n.); [1]Based on presence of CL on day 7, as determined by ultrasound; [2]Includes all inseminated cows; [3]Includes only cows that ovulated to ECP (visible CL on day 7 after FTAI). From Pereira *et al*. (2017b).

Physiology III. Inducing complete regression of CL and low P4 near TAI

Low P4 concentrations near the time of AI is essential for optimal fertility in both GnRH-based (Souza *et al*., 2005; Brusveen *et al*., 2009; Martins *et al*., 2011; Wiltbank *et al*., 2015) and E2/P4-based (Pereira *et al*., 2013b; Monteiro *et al*., 2015) protocols. Various methods have been used to ensure lower P4 near FTAI, including performing treatments with PGF prior to removal of the intravaginal P4 implant in E2/P4-based TAI programs (Meneghetti *et al*., 2009; Peres *et al*., 2009; Pereira *et al*., 2013b) and treatment with a second PGF, generally 24 h after the first PGF treatment (Brusveen *et al*., 2009; Pereira *et al*., 2015; Wiltbank *et al*., 2015; Melo *et al*., 2016). Earlier treatment with PGF should allow more time for CL regression and subsequent reductions in circulating P4 that could be critical for fertility in cows with a CL during an E2/P4-based program.

An experiment (Pereira *et al*., 2013b) investigated P4 concentrations and fertility comparing treatment with PGF at two times in an E2/P4-based FTAI and FTET program in lactating dairy cows. A total of 1,058 lactating Holstein cows, primiparous (n = 371) and multiparous (n = 687), yielding 34.1 ± 10.4 kg of milk/d were randomly assigned to receive treatment with PGF on either day-3 or day-2 of the following protocol: day-10: 2 mg EB+CIDR; day-2 CIDR removal + 1.0 mg ECP; day 0 - FTAI or day7 - FTET. Only cows with a CL on day 7 received an embryo and all cows received GnRH at time of FTET. Pregnancy diagnoses were performed at 28 and 60 days. Fertility (P/AI or P/ET) was affected by breeding technique (AI *vs*. ET) and time of PGF treatment (day-3 *vs*. day-2), for FTAI (32.9% [238] *vs*. 20.6% [168]) and FTET (47% [243] *vs*. 40.7% [244]) at 28 days, and 60 days for FTAI (30% [238] *vs*. 19.2% [168]) and FTET cows (37.9% [243] *vs*. 33.5% [244]). Circulating P4 on day 0 altered fertility in FTAI with greater P/AI in cows with P4 < 0.1 ng/ml compared to cows with P4 ≥ 0.1 ng/ml, and in FTET with greater P/ET in cows with P4 < 0.22 ng/ml compared to cows with P4 ≥ 0.22 ng/ml (Table 3). Treatment with PGF at day-3 increased percentage of cows with P4 < 0.1 ng/ml on day 0 (39.4 *vs*. 23.2%). Reducing the interval between PGF and FTAI from 72 to 48 h in dairy cows dramatically reduced fertility in cows bred by FTAI and had a subtle negative effect in cows that received FTET. Earlier PGF treatment benefits were most likely mediated through improvements in gamete transport, fertilization, or early embryo development, with other effects of earlier PGF manifest after ET on day 7.

It is critical that FTAI programs have low P4 concentrations near FTAI. This can be done in various ways: increasing the dose of PGF, when cloprostenol was used (Giordano *et al*., 2013), increasing number of PGF treatments (Pereira *et al*., 2015; Wiltbank *et al*., 2015), particularly in programs with GnRH at beginning of protocol, and an increased interval from PGF to FTAI (Pereira *et al*., 2013b) to allow sufficient time for circulating P4 to reach low concentrations.

Table 3. Effects of P4 concentrations on day 0 (at AI or 7 days before ET) at day 60 pregnancy diagnosis in lactating dairy cows after FTAI or FTET.

Item[1]	Progesterone (ng/ml) on day 10			P-value
	≤0.09	0.10-0.21	≥0.22	
TAI P/AI at Day 60				
PGF day-3	39.4 (36/85)	27.5 (8/26)	24.0 (12/45)	_
PGF day-2	23.2 (15/54)	15.1 (8/45)	14.6 (4/22)	_
Combined[2]	34.1 (51/139)[ax]	20.2 (16/71)[b]	21.4 (16/67)[y]	0.05
TET P/ET at day 60				
PGF day-3	46.8 (37/77)	44.2 (23/52)	25.3 (12/49)	_
PGF day-2	40.0 (24/58)	46.0 (33/73)	20.5 (9/50)	_
Combined[2]	43.8 (61/135)[a]	45.3 (55/125)[a]	22.9 (21/99)[b]	0.0006

[1]Each value includes least-squares means % (no./no.); [2]Combined values of treatments to determine the effect of P4 at day 0 on P/AI or P/ET; a, b Within a row = P < 0.05; x, y Within row = P > 0.05 and P ≤ 0.01. From Pereira *et al*. (2013b).

Physiology IV: Synchronizing time of ovulation and optimizing fertility to TAI

Serum E2 concentrations at FTAI were positively correlated with ovulatory follicle diameter (Vasconcelos *et al*., 2001; Perry *et al*., 2005). Furthermore, cows ovulating smaller follicles following GnRH treatment were more likely to have reproductive failure (Perry *et al*., 2005; Pereira *et al*., 2013a; Vasconcelos *et al*., 2013). There is likely an interaction among preovulatory follicle diameter, and optimal hormonal environment (manifested by expression of estrus) and establishment and maintenance of pregnancy (Jinks *et al*., 2013; Perry *et al*., 2014).

Studies comparing induction of ovulation using GnRH *vs.* ECP (Souza *et al*., 2009) or ECP *vs*. EB (Melo *et al*., 2016) were done in Brazil. All of these ovulation-inducing hormones yielded similar fertility. Therefore, despite differences in interval from treatment to ovulation, they were similarly effective in synchronizing ovulation and produced similar fertility.

In lactating dairy cows synchronized with E2/P4 protocols that used ECP for induction of ovulation, estrus expression dramatically affected fertility. A large study (Pereira *et al*., 2016) evaluated expression of estrus and fertility in FTAI (n = 5,430) or FTET (n = 2,003) programs. Ovarian ultrasonography (US) was performed on day0 (time of AI) and day 7 to determine ovulatory follicle diameter and ovulation. Only cows with a visible CL on day 7 were used. At CIDR removal, all cows received a tail-head device for detection of estrus and were considered in estrus when the paint of the device was completely removed by day 0. Circulating P4 concentrations were evaluated on day 7. At pregnancy diagnosis on day 32, cows with expression of estrus had increased (P < 0.01) P/AI (no estrus = 25.5% [222/846] *vs*. estrus = 38.9% [1785/4584]) and P/ET (no estrus = 32.7% [193/606] vs. estrus = 46.2% [645/1397]). Similarly, at pregnancy diagnosis on day 60, expression of estrus increased (P < 0.01) P/AI (no estrus = 20.1% [179/846] *vs*. estrus = 33.3% [1530/4584]) and P/ET (no estrus = 25.1% [150/606] *vs*. estrus = 37.5% [525/1397]). Pregnancy loss was lower (P = 0.01) in cows that expressed estrus in FTAI (no estrus = 20.1% [43/222] *vs*. estrus = 14.4% [255/1785]) and FTET (no estrus = 22.7% [43/193] *vs*. estrus = 18.6% [120/645]) compared to cows with no estrus. Independent of expression of estrus, P/AI was reduced in cows ovulating either too small or too large of follicles (Quadratic effect; P < 0.01; Fig. 3). There was no effect (P = 0.40) of ovulatory follicle diameter on P/ET in cows that expressed estrus; however, cows that did not express estrus tended to have lower (P = 0.08) P/ET if they ovulated larger follicles. In cows detected in estrus, follicle diameter did not affect pregnancy loss (AI: P = 0.46; ET: P = 0.45), but cows not detected in estrus and ovulating larger follicles tended to have greater pregnancy loss after FTAI (P = 0.13) and had greater pregnancy loss after FTET (P = 0.05; Fig. 4). There was a positive effect of day 7 circulating P4 concentrations on P/AI (P < 0.02), independent of estrus (Fig. 5). In contrast, there was no effect (P > 0.5) of circulating P4 concentration on day 7 on P/ET. Thus, expression of estrus during protocols for FTAI or FTET was associated with an increase in fertility and reduction in pregnancy loss. During FTAI programs, optimizing follicle diameter and increasing circulating P4 on day 7 after AI were also associated with increased fertility, independent of expression of estrus. However, in cows with FTET, the association of fertility with either ovulatory follicle diameter or P4 on day 7 was less dramatic and seemed to be related to whether cows expressed estrus.

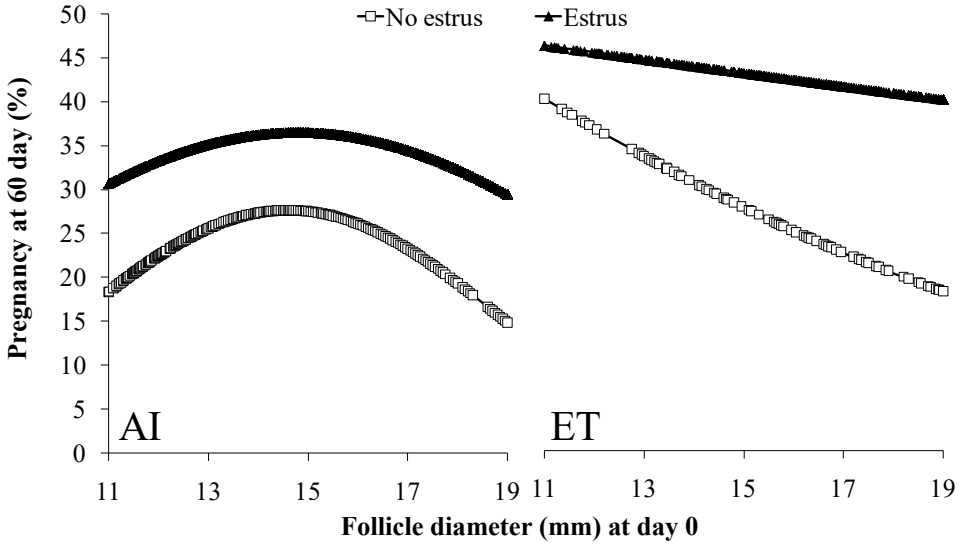

Figure 3. Effects of follicle diameter on day 0 on P/AI (AI) or P/ET (ET) at day 60 in cows that did or did not display estrus. P/AI: No Estrus P < 0.01; Estrus P < 0.01. P/ET: No Estrus P = 0.08; Estrus P = 0.40. From Pereira *et al*. (2016).

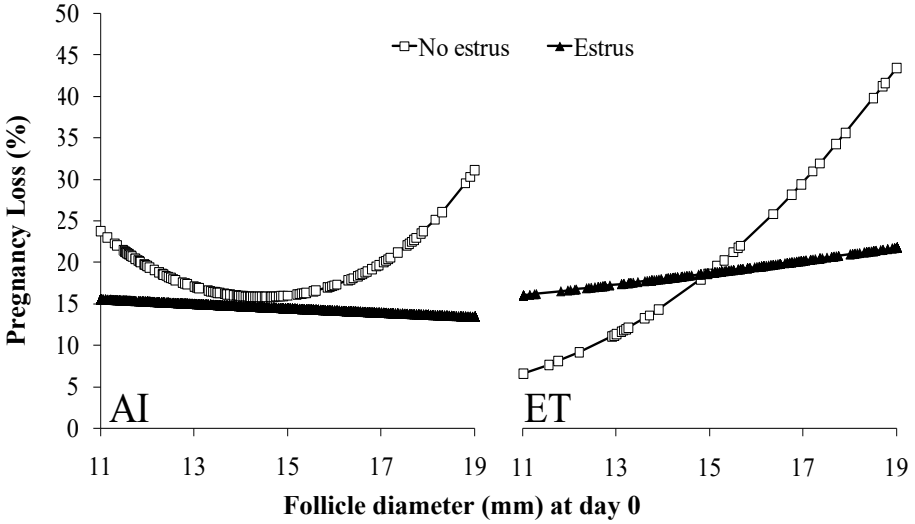

Figure 4. Effects of follicle diameter on day 0 on pregnancy losses (after AI or ET) between day 32 and day 60 in cows that did or did not display estrus or did not display estrus AI: No Estrus, P. 0.13; Estrus, P = 0.46. ET: No Estrus, P = 0.05; Estrus P = 0.45. From Pereira *et al*. (2016).

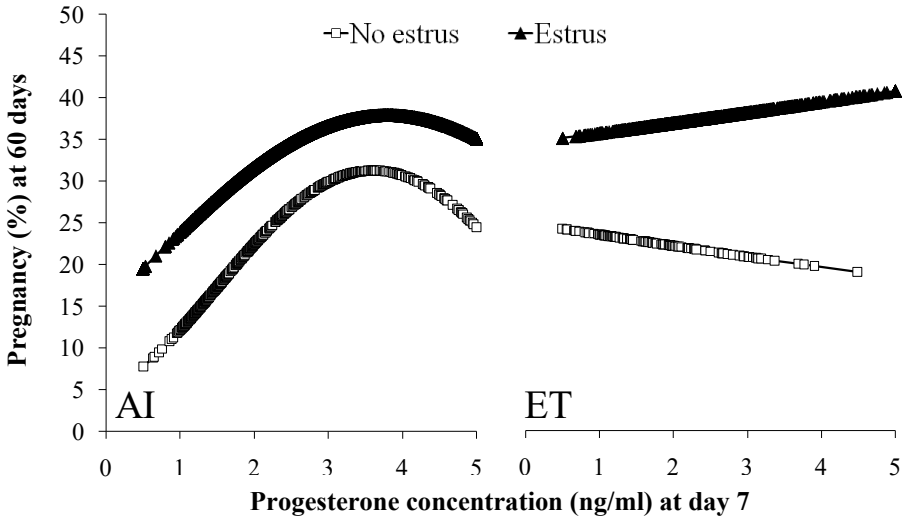

Figure 5. Effects of P4 concentration on day 7 on P/AI or P/ET at day 60 in cows that did or did not display estrus. AI: No Estrus, P = 0.02; Estrus, P = 0.01. ET: No Estrus, P = 0.76; Estrus P = 0.52. From Pereira *et al*. (2016).

Direct comparison of GnRH *vs*. E2/P4-based TAI programs

As discussed above, FTAI programs based on GnRH have a somewhat different physiology than E2/P4-based FTAI programs, despite similar physiologic and practical goals. One experiment (Pereira *et al*., 2013a) was designed to directly compare a GnRH-based to an E2/P4-based protocol for estrous cycle synchronization and FTAI. For this experiment, a 5 days GnRH protocol was compared to the standard E2/P4-based program, since both are designed for synchronization of ovulation and a reduction in the interval from follicular emergence to ovulation in cows with a synchronized follicular wave. A total of 1,190 lactating Holstein cows, primiparous (n = 685) and multiparous (n = 505), yielding 26.5 ± 0.30 kg of milk/day at 177 ± 5.02 DIM were randomly assigned to one of the following programs: 5-days Cosynch protocol (day-8: CIDR + GnRH, day-3: CIDR removal + PGF, day-2: PGF, day 0: FTAI+GnRH); or E2/P4 protocol (day-10: CIDR + EB, day-3: PGF, day-2: CIDR removal + ECP, day 0: FTAI). Rectal temperature and circulating P4 concentration were measured on the day–3, –2, 0 (FTAI) and day 7. The estrous cycle was considered synchronized when P4 was ≥1.0 ng/ml on day 7 in cow that had previously undergone luteolysis (P4 ≤ 0.4 ng/ml on day 0). To evaluate effects of heat stress, cows were classified by number of heat stress events, either 0, 1, or 2+ measurements of elevated body temperature (≥39.1ºC). Pregnancy success (P/AI) was determined at 32 and 60 days after FTAI. Cows in the 5-days Cosynch protocol had increased (P < 0.01) circulating P4 concentrations at PGF treatment (2.66 ± 0.13 *vs*. 1.66 ± 0.13 ng/ml). Cows in the E2/P4 protocol were more likely (P < 0.01) to be detected in estrus (62.8 *vs*. 43.4%) compared to cows in a 5-days Cosynch, and expression of estrus improved (P < 0.01) P/AI in both treatments. Cows in the 5-days Cosynch protocol had greater (P = 0.02) percentage of synchronized cycles (78.2%), compared to cows in the E2/P4 protocol (70.7%). On day 60, the E2/P4 protocol tended (P = 0.07) to improve P/AI (20.7 *vs*. 16.7%) and reduced (P = 0.05) pregnancy loss from 32 to 60 days (11.0 *vs*. 19.6%), compared to 5-days Cosynch protocol. In cows with a synchronized cycle, the E2/P4 protocol had greater (P = 0.03) P/AI (25.6 *vs*. 17.7%) on day 60 and lower pregnancy loss (P = 0.01) from day 32 to day 60 (6.7 *vs*. 21.7%) compared to cows in the 5-days Cosynch protocol. Follicle diameter affected (P = 0.04) pregnancy loss from 32 to 60 days only in cows in the 5-days Cosynch protocol, with smaller follicles resulting in greater pregnancy loss (Fig. 6). P/AI at day 60 was different (P = 0.01) between protocols in the cows with two or more measurements of heat stress (5-days Cosynch = 12.2% *vs*. E2/P4 = 22.8%), but not in cows without or with only one heat stress measurement (P = 0.6). In conclusion, the 5-days Cosynch protocol apparently produced better estrous cycle synchronization than the E2/P4 protocol but did not improve P/AI. The potential explanation for these results is that increased E2 concentrations during the periovulatory period can improve pregnancy success and pregnancy maintenance; furthermore, this effect appeared to be greatest in heat-stressed cows, apparently with lower circulating E2 concentrations.

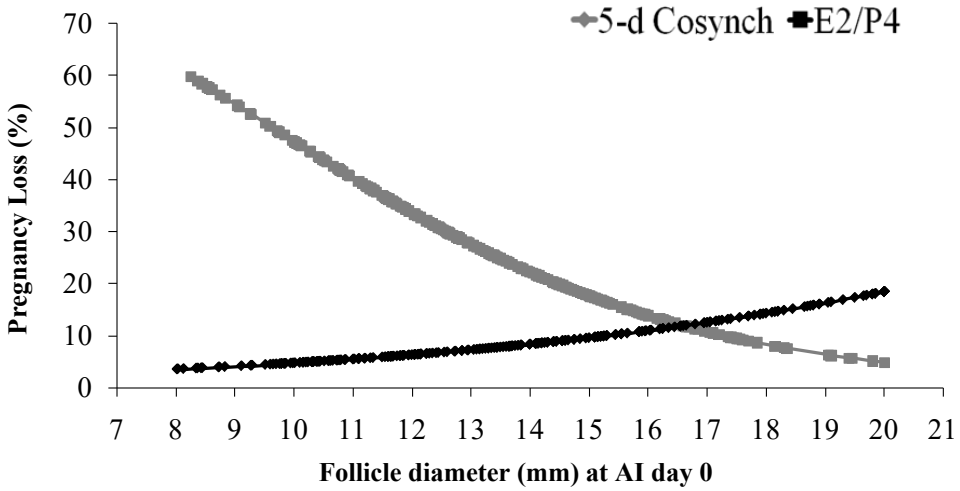

Figure 6. Effect of follicle diameter at AI on pregnancy loss between day 32 and day 60 in cows subjected to either E2/P4 or 5-days Cosynch protocols and had their cycle synchronized (P4 ≤ 0.4 ng/ml at day 0 and P ≥ 1.0 ng/ml at day 7) 5-days Cosynch (P = 0.04): y = -0.276x +2.4648; E2/P4 (*P* = 0.32): y = 0.01063x - 3.8241. From Pereira *et al*. (2013a).

Discussion

Research on FTAI in lactating dairy cows in Brazil during the last 20 years has highlighted some key physiological principles that are important for improving fertility to FTAI and FTET programs. First, slight elevations in P4 near AI can reduce fertility. This was illustrated by the finding that an earlier treatment with PGF (i.e. 1 day prior to removal of intravaginal P4 implant; day-3 *vs*. day-2) allowed greater time for regression of the CL, lower P4 at CIDR removal and at FTAI, and greater fertility. Greater P4 concentrations at FTAI (>0.1 ng/ml) were clearly associated with reduced fertility in FTAI cows. In ET cows, fertility

improvement after earlier regression of the CL was less dramatic than after AI, with no reduction in fertility to FTET until circulating P4 concentrations near ovulation reached >0.21 ng/ml. Thus, it appears that there is a dramatic effect on fertility of earlier PGF treatment that was likely mediated through more complete CL regression which likely improved gamete transport, fertilization, or early embryo development with more subtle effects of earlier PGF treatment in FTET programs likely mediated through changes in the uterine or hormonal environment after day 7 (time of ET).

Second, the absence of a CL and low circulating P4 at initiation of a protocol also appears to be a problem for dairy herds in Brazil. Two methods for increasing P4 during E2/P4-based FTAI protocols in lactating dairy cows that were discussed in this manuscript are: 1) combine GnRH with the EB at the beginning of the protocol (Pereira *et al*. 2015) or 2) utilize two intravaginal P4 implants rather than only one implant during the protocol (Pereira *et al*. 2017a). Both of these strategies have resulted in greater fertility in lactating cows that do not have a CL at the start of the protocol.

Third, follicle diameter was linked to P/AI in several studies. Ovulation of an undersized follicle was generally associated with reduced P/AI, reduced E2 concentrations, an increased incidence of short luteal phases (Vasconcelos *et al*., 2001), and sometimes increased pregnancy loss (Perry *et al*., 2005). In contrast, ovulation of an oversized follicle can also be associated with reduced P/AI, perhaps due to ovulation of a persistent dominant follicle (Townson *et al*., 2002, Bleach *et al*., 2004, Cerri *et al*., 2009). In a number of these studies, follicle size in cows that did not display estrus was related to P/AI and P/ET at either the 32 or 60 days pregnancy diagnoses.

Fourth, the critical effect of estrus as a predictor of fertility in E2/P4-based FTAI programs has been clearly illustrated. Expression of estrus requires low circulating P4 concentrations plus increased circulating E2 of a sufficient magnitude and duration (Allrich, 1994). We measured P4 near the time of AI in a subset of cows (n = 2372; Pereira *et al*., 2016); there was a difference ($P < 0.01$) in circulating P4 in cows that expressed (0.15 ± 0.03 ng/ml) versus cows that did not express estrus (0.19 ± 0.03 ng/ml). Although relatively small in magnitude, this may account for a lack of behavioral estrus in some cows. In cows that had their cycle synchronized, expression of estrus was associated with an increase in P/AI and P/ET at 32 and 60 days and reduced pregnancy losses between 32 and 60 days of pregnancy. Similar results were reported in previous studies in dairy cows using various FTAI protocols. For example, using Heatsynch protocols, cows that displayed estrus after the ECP had greater P/AI (42.5% [306]) than cows not in estrus (21.1% [71]) at FTAI (Cerri *et al*., 2004). Therefore, expression of estrus was a predictor of fertility and pregnancy loss in both FTAI and FTET programs.

Finally, one of the largest problems for reproductive management programs of lactating dairy cows in Brazil is the reduced P/AI that occurs due to heat stress (Vasconcelos *et al*., 2006, 2011a, b, c). Plasma E2 concentrations are reduced by heat stress in dairy cows (Wolfenson *et al*., 1995, 1997; Wilson *et al*., 1998) and E2 supplementation may improve fertilization, subsequent embryonic development, and pregnancy maintenance. Pregnancy loss was reduced or tended to be reduced in cows with expression of estrus (Pereira *et al*., 2013a, 2016), those with greater circulating E2 concentrations near FTAI (Souza *et al*., 2007, 2011; Hillegass *et al*., 2008), and cows that had increased length of proestrus (Ribeiro *et al*., 2012). In these studies, cows detected in estrus had decreased pregnancy losses, irrespective of preovulatory follicle diameter.

In addition, the P4 concentration at day 7 after AI was associated with P/AI (quadratic effect), independent of estrus expression, but was not related to P/ET (Demetrio *et al*., 2007). Higher P4 concentrations may be indicative of a better CL, due to enhanced follicle and oocyte health or physiological function.

Conclusions

Based on field experiences and the results reported in these studies, heat stress is one of the major factors that reduce fertility in dairy cows during FTAI protocols in Brazil as demonstated by cows with an increased body temperature (≥39.1ºC) having large reductions in P/AI. In addition, fertility can be substantially altered by the hormonal concentrations during the protocol, when circulating P4 needs to be elevated, and near the end of the protocol, when P4 needs to be basal and circulating E2 needs to be elevated. Evidence was provided that increasing circulating P4 during preovulatory follicle development improved P/AI, particularly in cows with low P4 at the start of the protocol. This was done by inducing ovulation (with exogenous GnRH) at the beginning of an E2/P4-based protocol, or by inserting a second CIDR at the beginning of the protocol. Near the end of the protocol, treatment with PGF one day before removal of the intravaginal P4 implant, reduced circulating P4 near FTAI and increased fertility following FTAI and FTET. It also appears that increasing E2 concentrations prior to AI can improve pregnancy success and pregnancy maintenance as evidenced by the dramatic effects of expression of estrus on P/AI, P/ET, and pregnancy loss. In addition, increasing the length of the protocol for FTAI increased the percentage of cows detected in estrus and decreased pregnancy loss. Following FTAI programs, increasing circulating P4 7 days after AI was associated with increased fertility, independent of expression of estrus. In cows with FTET, the association of fertility with either preovulatory follicle diameter or circulating P4 concentrations at time of ET (day 7) was less dramatic and seemed to be related to whether cows expressed estrus. Thus, during the last two decades FTAI protocols have been improved and are likely to continue to be improved for dairy cows in Brazil by focusing on increasing synchronization of follicular waves, optimizing hormonal concentrations during specific stages of the protocol, and by improving

percentage of cows ovulating in a synchronized time period at the end of the protocol.

Acknowledgments

We thank Brazilian granting agencies FAPESP, CAPES and CNPq for research grant support; Zoetis for pharmaceutical products; students and staff at São Paulo State University for technical support; owners and staff of collaborating dairies for use of cattle and facilities.

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DOI: 10.21451/1984-3143-AR2018-0025

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Programs for fixed-time artificial insemination in South American beef cattle

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Abstract

Fixed-time artificial insemination (FTAI) has been widely applied in South America within the last 20 years for the genetic improvement of commercial beef herds. Most FTAI treatments for beef cattle used in South America are based on the use of progesterone (P4) releasing devices and estradiol to synchronize follicle wave emergence, with pregnancies per AI (P/AI) ranging from 40 to 60%. More recent protocols focusing on extending the interval from device removal to FTAI (i.e. increasing the growing period of the ovulatory follicle) have been reported to improve P/AI in beef cattle. These new protocols and the more traditional FTAI protocols have also been adapted for use with sexed-sorted semen with acceptable P/AI in beef cattle. Finally, color-flow Doppler ultrasonography has been incorporated recently to determine the vascularity of the CL and thereby detect pregnancy as early as Day 22 after the first AI for re-synchronization of ovulation for a second FTAI in non-pregnant animals. In summary, FTAI protocols have facilitated the widespread application of AI in South American beef cattle by allowing for the insemination and re-insemination of herds during a defined breeding season, without the necessity of clean up bulls to achieve high pregnancy rates.

Keywords: color-flow Doppler ultrasonography, proestrus length, sexed-sorted semen.

Introduction

Artificial insemination (AI) is the most useful method for genetic improvement in cattle, and because estrus detection is difficult and inefficient, fixed-time artificial insemination (FTAI) is necessary. There are basically two types of FTAI protocols currently used in beef cattle; GnRH-based and estradiol-based protocols, both of which are combined with progesterone (P4) releasing devices and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Estradiol/P4-based protocols are most commonly used in South-American beef herds because of the availability of estradiol, its high effectiveness both in heifers and in cows in postpartum anestrus, and its relative low cost. With 20 years of experience with FTAI in South America, results are now more consistent with pregnancy per AI (P/AI) ranging between 40 to 60% (Bó *et al*., 2013). However, recently developed protocols that extend the period from P4 device removal to ovulation (defined as the proestrus period) have provided new opportunities for increasing P/AI (Bridges *et al*., 2008; Bó *et al*., 2016). Furthermore, treatments to re-synchronize ovulation have provided the opportunity to do sequential FTAI, without the necessity of using clean-up bulls (Baruselli *et al*., 2017a). The objective of this manuscript is to review protocols that are currently available and discuss their applications in beef herds.

Estradiol/P4-based treatments for FTAI

Estradiol and P4 treatments consist of insertion of a P4 releasing device and the administration of 2 mg of estradiol benzoate (EB) on random days of the cycle (Day 0; to induce follicle atresia and synchronize follicular wave emergence), $PGF_{2\alpha}$ at the time of P4 device removal on Days 7, 8 or 9 (to ensure luteolysis) and the subsequent application of 1 mg EB 24 h later, GnRH or LH 54 h later or 0.5 or 1 mg of estradiol cypionate (ECP) at the time of P4 device removal (Bó *et al*., 2013) to synchronize ovulation. Most practitioners prefer the use of ECP to synchronize ovulation because it reduces the need to handle animals for the administration of EB. Treatment protocols that are applied to suckling beef cows usually include the administration of equine chorionic gonadotropin (eCG) at the time of removal the P4 device (Baruselli *et al*., 2004; Bó *et al*., 2013), which has been reported to stimulate the growth of the dominant follicle, increased ovulation rate (Sá Filho *et al*., 2010a; Núñez-Olivera *et al*., 2014) and circulating P4 concentrations in the subsequent luteal phase in cows experiencing postpartum anestrus (Baruselli *et al*., 2012; Núñez-Olivera *et al*., 2014). Although the use of eCG has been widely used in *Bos indicus* herds with high incidence of postpartum anestrus (Baruselli *et al*., 2004; Sá Filho *et al*., 2010a) improvements in *Bos taurus* herds with high incidence of postpartum anestrus have been also reported (Menchaca *et al*., 2013; Núñez-Olivera *et al*., 2014), while no improvements in P/AI has been reported in herds in with high proportions of cycling cows or heifers at the time of treatment (reviewed in Bó and Baruselli, 2014).

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Received: February 7, 2018
Accepted: May 3, 2018

GnRH-based treatments

GnRH-based protocols are used widely for beef cattle in North-America and Europe, but GnRH use is limited in South-America because of cost and the availability of estradiol esters. The most commonly used protocol is called Co-Synch; GnRH is administered at the time of FTAI to synchronize ovulation (Geary *et al*., 2001). In general Co-Synch protocols have included the insertion of a P4 device to overcome poor ovulation rates after the first GnRH in heifers (Martinez *et al*., 2002) and in postpartum suckled beef cows experiencing anestrus (Lamb *et al*., 2001). Data on the addition of eCG to GnRH/P4-based treatment protocols have been more controversial, with reported improvements in P/AI in *Bos indicus* (Pincinato *et al*., 2012) and *Bos taurus* cows in postpartum anestrus (Huguenine *et al*., 2013) and in primiparous *Bos taurus* cows that had not been pre-synchronized (Small *et al*., 2009). However, no improvement in P/AI has been reported in *Bos taurus* cows with low incidence of postpartum anestrus and moderate to high body condition scores (BCS; Marquezini *et al*., 2013).

Protocols that prolong the proestrus period

Extending the proestrus period in GnRH/P4-based protocols

New protocols for FTAI were developed to prolong the period from P4 device removal to ovulation with the objective of incrementing the period of preovulatory estradiol exposure and improving uterine function and early embryo development (Bridges *et al*., 2008; 2012). The protocol was named 5-day Co-Synch+P4 and resulted in higher P/AI than with the 7-day Co-Synch+P4 in beef cows (Bridges *et al*., 2008; Whittier *et al*., 2013). The main changes in this protocol was a reduced period of insertion of the P4 releasing device from 7 to 5 days, to avoid the adverse effects of persistent follicles on fertility of the cows not ovulating to the first GnRH, and to prolong the period from P4 device removal to the GnRH treatment to increase the exposure to circulating estradiol concentrations prior to ovulation (Bridges *et al*., 2014). Higher estradiol concentrations in the preovulatory period have been associated to an increased ability of the uterus to support conceptus development (Bridges *et al*., 2013, Binelli *et al*., 2014) and were also related to lower embryonic losses in the time period between maternal recognition of pregnancy and placental attachment (Madsen *et al*., 2015).

Because of the shorter interval between the first GnRH and induction of luteolysis in the 5-day Co-Synch+P4 protocol, a single administration of $PGF_{2\alpha}$ was not effective in inducing luteolysis in beef cows that had ovulated to the GnRH (Souto *et al*., 2009); two doses of $PGF_{2\alpha}$ 8 to 12 hours apart resulted in higher P/AI (Kasimamickam *et al*., 2009). In a large field trial with 2,465 postpartum beef cows, P/AI was greater ($P < 0.05$) in cows receiving 2 $PGF_{2\alpha}$ 8 h apart (55%) than those receiving only one $PGF_{2\alpha}$ (48%), with those receiving 2 $PGF_{2\alpha}$ at the same time being intermediate (51%; Bridges *et al*., 2012). Hence, double $PGF_{2\alpha}$ given 8 to 24 h apart seemed necessary to maximize fertility with the 5-day protocol. If farm conditions do not permit the extra handling, a double dose of $PGF_{2\alpha}$ given at device removal would be an acceptable alternative.

The 5-day Co-Synch+P4 protocol has also been investigated in *Bos indicus* cows in South America, with lower P/AI in suckled Nelore cows than those treated with the conventional 8-day estradiol/P4-based protocol (Ferraz Jr *et al*., 2016). An important difference was that 400 IU eCG was used in the estradiol/P4-based protocol but it was not used in the 5-day Co-Synch+P4 protocol. To confirm this notion, we have reported no differences in P/AI in cycling cows treated with the 5-day Co-Synch+P4 and the estradiol/P4-based protocol, but P/AI was higher in cows in postpartum anestrus that received 400 IU eCG at P4 device removal (5-day Co-Synch+P4: 46.3%, 120/259; estradiol/P4-based: 54.5%, 151/277) than in cows treated with 5-day Co-Synch+P4 but without eCG (26.8%, 71/265; $P < 0.05$; Huguenine *et al*., 2013).

The 5-day Co-Synch+P4 protocol has also been tested in heifers (Day, 2015), with some modifications introduced; for example, Colazo and Ambrose (2011) and Cruppe *et al*. (2014) showed that P/AI did not differ in heifers that did not receive GnRH at the time of insertion of a P4 device. The important issue with not administering the first GnRH is that a single injection of $PGF_{2\alpha}$ is all that is required. The two alternative 5-day Co-Synch+P4 protocols are depicted in Fig. 1.

Controversy still exists concerning the necessity of using one or two doses of $PGF_{2\alpha}$, when the first GnRH is administered with no reported differences (Kasimanickam *et al*., 2014) and higher P/AI when two doses of $PGF_{2\alpha}$ were used with intervals between 6 to 24 h (Day, 2015; Peterson *et al*., 2011). In relation to the optimal timing of FTAI, Kasimanickam *et al*. (2012) reported higher P/AI with heifers inseminated at 56 h after device removal than those inseminated at 72 h and Day (2015) suggested FTAI 60 to 66 h after P4 device removal or insemination 12 h after estrus using tail-patches or tail-paint and FTAI/GnRH to all those not in heat by 72 h. Certainly, expression of estrus has been shown to influence P/AI in cows (Richarson *et al*., 2016) and Colazo *et al*. (2017) have reported similar findings in heifers inseminated with sexed-sorted semen; suggesting the possibility of splitting the insemination based on estrus expression (i.e., delaying the insemination in those animals not showing estrus by the time of FTAI).

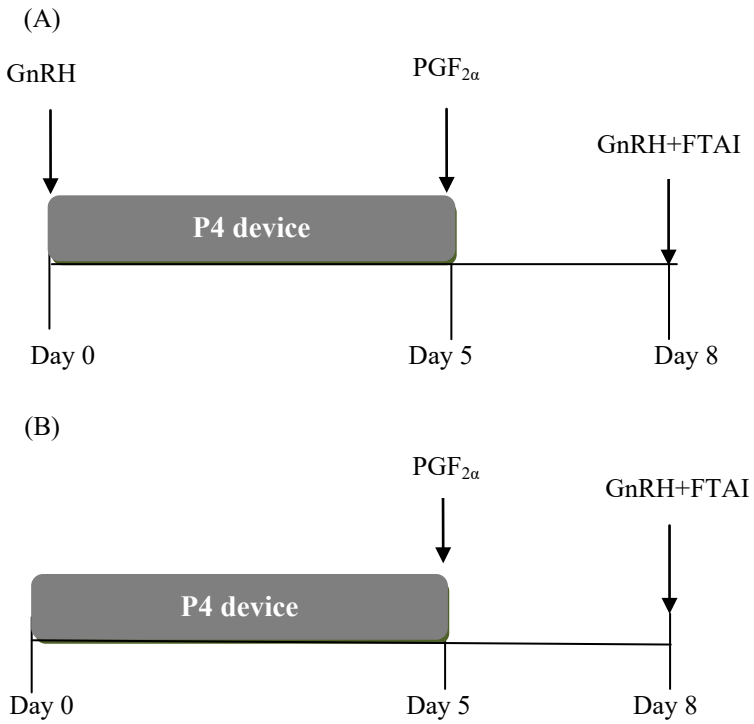

Figure 1. Two protocols for FTAI in beef cattle using GnRH. (A) 5-day Cosynch+P4 protocol. The interval from P4 device removal to FTAI is 66-72 h in heifers and 72 h in cows. If tail-paint or patches are used for estrus detection, FTAI begins at 60 h in all heifers with altered paint and those with the paint intact receive GnRH and are FTAI at 72 h. A second $PGF_2\alpha$ administered at the same time of the device removal or 6 to 12 h later is recommended in cows and 400 IU of eCG may also be given in cows in postpartum anestrus. (B) Modified 5-day GnRH+P4 protocol. GnRH is not administered at P4-device insertion and only one $PGF_2\alpha$ is required on Day 5. The recommended intervals from P4 device removal to FTAI are similar to those described previously.

Extending the proestrus period in estradiol/P4 based protocols

We have recently conducted a series of experiments to evaluate an estradiol/P4-based protocol with a prolonged proestrus interval, which has been named J-Synch (de la Mata and Bó, 2012). The treatment consists of the administration of 2 mg EB at the time of insertion of a P4 device that is removed 6 days later. A single dose of $PGF_{2\alpha}$ is given at device removal, and animals receive GnRH at the time of FTAI, 72 h later (Day 9).

A comparison of follicular and luteal dynamics between heifers treated with the 6-day J-Synch protocol and the conventional 7-day estradiol/P4-based protocol in which ECP was given at device removal (Day 7) showed that heifers in the J-Synch group ovulated 28 h later (93.7 ± 12.9 h after device removal; $P < 0.05$) than those in the conventional treatment (65.0 ± 13.7 h after device removal). Although the diameter of the preovulatory follicle did not differ between groups, the growth rate of the dominant follicle from P4 device removal to ovulation was greater in heifers in the J-Synch group (1.3 ± 0.4 mm/day) than those in the conventional group (1.0 ± 0.4; $P < 0.05$). Furthermore, serum P4 concentrations on Days 6 to 12 after ovulation were greater in heifers in the J-Synch group than in those in the conventional group ($P < 0.05$). Immunohistochemistry and real-time PCR of biopsies taken on Day 6 after ovulation suggested that heifers with the prolonged proestrus (J-Synch) had a more mature uterine environment for embryo development. This notion was based on lower ($P < 0.05$) staining intensity of the endometrial P4 receptors (PGR) in the intercaruncular uterine stroma and a tendency ($P < 0.08$) for lower PR mRNA and IGF_1 mRNA in heifers treated with the J-Synch protocol than in those treated with the conventional protocol (de la Mata *et al*., 2018).

Several field trials were conducted to compare P/AI in heifers treated with the J-Synch protocol or the conventional estradiol/P4-based protocol (reviewed in Bó *et al*., 2016). In this series of experiments heifers in the J-Synch group were FTAI at the time of GnRH administration (i.e. 72 h after P4 device removal), whereas those in the conventional group were FTAI 54 h after device removal and ECP treatment. Although in the first experiment performed during the winter with heifers losing weight, P/AI was lower in heifers in the J-Synch group, in two experiments performed in the spring, with heifers gaining weight, the cumulative P/AI were higher in those treated with the J-Synch protocol than in those treated with the conventional estradiol/P4-based protocol. The contradictory results in the previous experiments was attributed to lower estradiol concentrations in the heifers that were losing weight in the winter compared to those gaining weight in the spring (Perry, 2017a). As it was mentioned earlier, high estradiol concentrations in the proestrous period have

been associated with a more appropriate uterine environment, higher luteal phase P4 concentrations and a lower incidence of embryo loss (Perry, 2017b). Therefore, the administration of ECP in the heifers in the conventional protocol in the winter may have higher P/AI through higher exposure to estradiol and the ovulation of smaller follicles (Jinks *et al*., 2013). Conversely, the heifers treated in the spring while gaining weight may have had larger estradiol-producing follicles, thus no additional estradiol was needed to achieve high fertility (Perry, 2017a).

In order to avoid the confounding effects of nutrition on fertility in the J-Synch protocol, a study was designed to evaluate the effect of adding 300 IU of eCG at device removal to stimulate the growth of the dominant follicle on P/AI (Bó *et al*., 2016). The addition of 300 IU eCG at the time of P4 device removal resulted in higher P/AI (57.1%; 739/1295) as compared to those that did not receive eCG (53.1%; 692/1303). In another experiment, all heifers received 300 IU eCG at device removal but half were treated with the J-Synch protocol and the other half with the conventional estradiol/P4 based protocol with ECP at device removal. Again, P/AI was significantly higher in the J-Synch group (56.1%; 631/1,125) than in the conventional treatment group (50.7%; 620/1,224). The J-Synch protocol was also tested in 945 recipients receiving *in vitro*-produced embryos (Menchaca *et al*., 2015). Pregnancy rate following embryo transfer 7 or 8 d after GnRH (J-Synch + 400 IU eCG) or 9 or 10 d after ECP (conventional + 400 IU eCG) was higher ($P < 0.01$) in recipients synchronized with the J-Synch protocol (49.3%) than the conventional estradiol/P4-based protocol (40.9%). In conclusion, the strategy for extending proestrus significantly improved fertility in *Bos taurus* heifers. This improvement was considered to be due to a more competent CL that produced greater P4 concentrations in the ensuing luteal phase after AI and a uterine environment that may favor embryo development.

Although, more research is required in *Bos indicus* heifers, in one study, P/AI did not differ in Nelore and Nelore crossbred heifers treated with the J-Synch protocol or with the conventional protocol (both with 200 IU eCG at device removal; Motta *et al*., 2016). A similar approach of a prolonged proestrus and FTAI at 72 h after device removal, but by giving 1 mg EB 36 h after device removal instead of GnRH at the time of FTAI was evaluated in Brahman heifers (Edwards *et al*., 2015). P/AI was significantly higher in heifers treated with the prolonged proestrus protocol than those treated with the conventional 8-day protocol in one farm, but no differences were detected in two other farms.

Proestrus length, estrus expression and GnRH treatments

An experiment was designed to evaluate the effect of the length of proestrus (i.e. interval from P4 device removal to GnRH and FTAI) on fertility in heifers (Núñez-Olivera *et al*., 2016). Angus and Angus crossbred heifers (n = 911) received a P4 releasing device containing 0.5 g P4 (DIB 0.5, Zoetis, Argentina) and 2 mg of EB (Gonadiol, Zoetis) on Day 0. At the time of P4 device removal (Day 6), 500 µg of cloprostenol (Ciclase DL, Zoetis) and 300 IU of eCG (Novormon, Zoetis) were administered IM. Heifers were then allocated in three groups to receive GnRH (100 µg gonadorelin acetate; Gonasyn GDR, Zoetis) and FTAI at 48, 60 or 72 h later. The diameter of the largest follicle (measured by ultrasonography) and estrus expression using tail-paint were recorded in a subset of heifers (n = 525) at the time of FTAI. Results are shown in Table 1. The largest follicle was smaller ($P < 0.05$) when GnRH/FTAI was performed at 48 h compared with 60 or 72 h. In addition, more heifers tended to display estrus by 72 h ($P < 0.1$) than 48 or 60 h. Although the overall P/AI tended to be greater ($P < 0.1$) in heifers inseminated at 72 h than at 48 or 60 h, P/AI was significantly greater ($P < 0.05$) among cycling heifers (i.e. with a CL on Day 0) in those FTAI at 72 h than in those FTAI at 48 or 60. In non-cycling heifers, P/AI did not differ among groups (58.9%, 247/419). Among the heifers showing estrus at the time of FTAI, P/AI was higher ($P < 0.05$) in those FTAI at 72 h (70.1%, 96/137) than in those FTAI at 60 h (56.7%, 68/120; $P < 0.05$), while 48 h was intermediate (63.9%, 78/122).

Based on the findings of the previous studies, it was proposed that heifers that manifest estrus earlier could be inseminated earlier without affecting P/AI, but it was needed to determine the optimum time for FTAI in those not showing estrus. To answer this question, 1,283 Angus and Hereford crossbred heifers were treated with the J-Synch protocol as described above and all heifers were tail-painted at P4 device removal. Heifers received GnRH/FTAI at either 60 or 72 h, regardless of paint removal. P/AI was higher in those that showed estrus prior to FTAI than in those that did not regardless of insemination time (53.6%, 542/1,012 vs. 45.0%, 122/271, respectively, $P < 0.05$). The P/AI in heifers that were in estrus by 60 h was similar whether the FTAI/GnRH was performed at 60 or 72 h. However, in those not showing estrus, P/AI was higher when the FTAI/GnRH was performed at 72 h (52%, 45/143) than at 60 h (37%, 47/128). The practical implication of this result is that when large herds are synchronized (i.e. 400 to 500 head), the device could be removed in the afternoon of Day 6 and FTAI begins at 60 h (Day 9 AM) in all heifers with altered paint as they come through the chute; those with the paint intact could be separated off to receive GnRH/FTAI in the afternoon of Day 9 (i.e. around or after 72 h).

The second question that was raised was the necessity to administer GnRH in those heifers that had already shown estrus prior to FTAI. An experiment was performed with 1,879 Angus heifers that were treated similarly to the previous study. All heifers displaying estrus at 60 h (85%, 1594/1879) were FTAI at that time, but GnRH was administered to only half. The heifers not displaying estrus at 60 h received GnRH/FTAI at 72 h. P/AI in the heifers that had manifested estrus by 60 h did not differ whether they received or did not receive GnRH (56.2% 451/802, vs. 58.6% 464/792, respectively), but P/AI was higher than in those not showing estrus by 60 h

and receiving FTAI/GnRH at 72 h (40.4%, 15/285; P < 0.05). Therefore, with the use of tail-paint the cost of treatment could be reduced by giving GnRH to only the 25-30% of the heifers not showing estrus. The recommended protocol for FTAI is shown in Fig. 2.

Most of the experiments with the J-Synch protocol were performed in heifers and more information is needed about the performance of this protocol in lactating beef cows. Preliminary information using the J-Synch in non-lactating cows (i.e. after early weaning at 60 days postpartum) indicated P/AI were comparable to those reported in heifers (62.5%; 1,188/1,900), with higher P/AI in cows showing estrus by the time of FTAI (Menchaca *et al*., 2017). As with heifers, it is possible to avoid the use of GnRH at the time of the FTAI if tail-paint is used to determine the expression of estrus.

Only one study has been performed with the J-Synch protocol in suckled beef cows, with lower P/AI than in cows treated with the conventional estradiol/P4-based 7-day protocol (Bó *et al*., 2017), raising questions about the optimal period of P4 device insertion (6 or 7 days), the interval from device removal to ovulation (to determine the time for FTAI) and the requirement of ECP or GnRH to induce ovulation. More studies are underway to answer these questions.

Table 1. Diameter of the largest follicle, estrus expression and P/AI in beef heifers synchronized with the J-Synch protocol and received GnRH and were fixed-time AI at different intervals from the removal of the P4 releasing device.

	Follicular diameter at FTAI (mm)	Heifers in estrus at FTAI (%)	P/AI in cycling heifers* (%)	Overall P/AI (%)
GnRH/FTAI 48 h	12.2 ± 0.1[a] (n = 179)	68.2%[a] (122/179)	67.7%[a] (107/158)	63.6%[c] (196/308)
GnRH/FTAI 60 h	12.8 ± 0.1[b] (n = 168)	71.4%[a] (120/168)	68.3%[a] (110/161)	63.1%[c] (183/290)
GnRH/FTAI 72 h	12.9 ± 0.2[b] (n = 178)	77.0%[a] (137/178)	77.5%[b] (134/173)	70.0%[d] (219/313)

[ab] Denotes significant (P < 0.05) differences between treatment groups. [cd] Denotes a tendency (P < 0.1) for higher overall P/AI in heifers inseminated at 72 h. *Heifers with a CL detected by ultrasonography at the time of P4 device insertion.

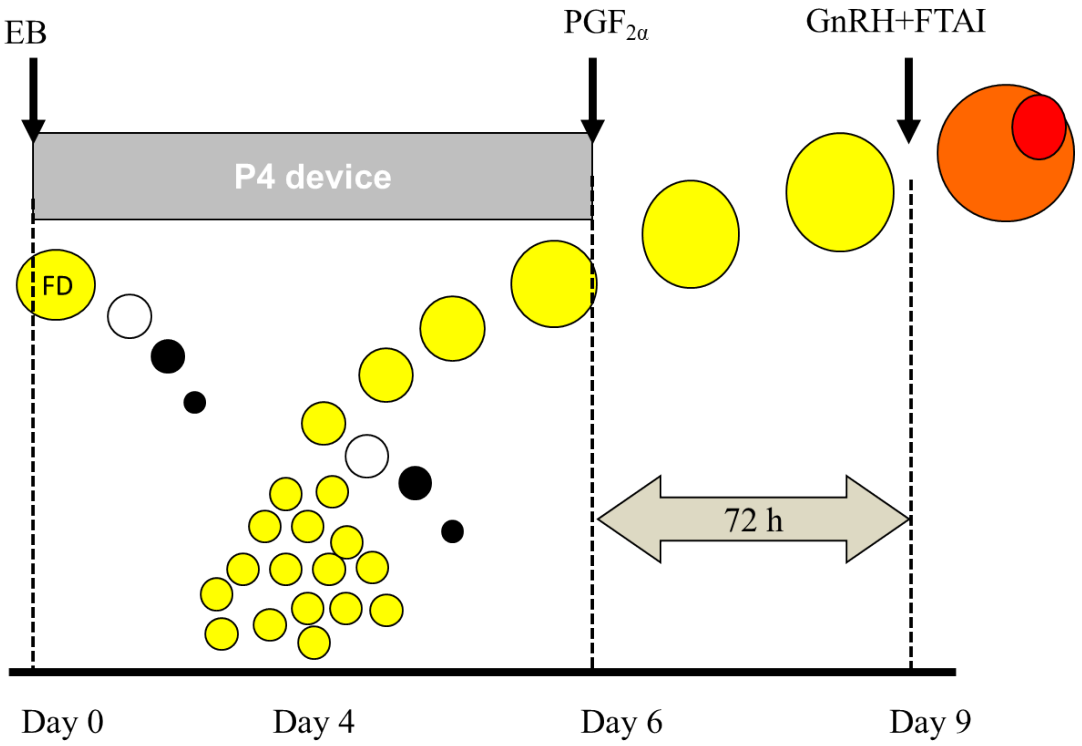

Figure 2. Estradiol/P4-based protocol with prolonged proestrus (J-Synch) in beef heifers. For FTAI without estrus detection, heifers receive GnRH and are inseminated 66-72 h after P4-device removal. If tail-paint or patches are used for estrus detection, FTAI begins at 60 h in all heifers with altered paint and those with the paint intact receive GnRH and are FTAI at 72 h. eCG (300 IU in heifers and 400 IU in cows) may also be given in those animals in anestrus.

Protocols for fixed-time AI using sexed-sorted semen

The use of sexed-sorted semen has created great interest in the insemination of dairy heifers over the past 20 years; however, the widespread use of sexed semen was somewhat limited because fertility was compromised, especially with FTAI (DeJarnette *et al*., 2011). However, the sorting procedure has been improved and a new product called SexedULTRA^{TM}, with the option of increasing the number of sperm from $2.1x10^6$ to $4x10^6$ sperm per straw has been launched (Vishwanath, 2015).

A series of experiments were designed to determine P/AI using modifications of the existing estradiol/P4-based protocols for sexed semen. Two experiments were carried out using suckled Nelore cows that received the conventional protocol for FTAI as follows: 2 mg EB + P4 device on Day 0, removal of the device and $PGF_{2\alpha}$ + 300 IU of eCG + 1 mg of ECP on Day 8 and FTAI 60 h after P4 device removal (Baruselli *et al*., 2017b). In the first experiment, 796 cows were inseminated with female sexed-selected semen and non-sexed (Control) semen from three different Nelore bulls. The experimental groups were: 1) non-sexed semen with $20x10^6$ sperm per dose; 2) Legacy (previous method of sexing), sexed semen with $2.1x10^6$ sperm per dose; 3) SexedULTRA with $2.1x10^6$ sperm per dose and 4) SexedULTRA 4.0, sexed semen with $4.0x10^6$ sperm per dose. No differences were detected between sires (P = 0.15). Although P/AI was highest (P < 0.05) in cows in the non-sexed semen group (56%, 112/199), cows in the SexedULTRA 4.0 group had higher P/AI (43%, 86/200) than those in the Legacy 2.1 group (28%, 58/206). In cows inseminated with the SexedULTRA 2.1 semen P/AI was intermediate (38%, 72/191) and not different than the other groups inseminated with sexed semen. In the second experiment, 613 cows were treated as those in the first experiment and inseminated with semen from three Angus sires. The experimental groups were 1) non-sexed semen ($20x10^6$ sperm); 2) SexedULTRA 4.0 and 3) SexedULTRA Pure 4.0 (dead sperm were removed). A subgroup of 431 cows was tail-painted for estrus determination at the time of FTAI. P/AI among sires did not differ (P = 0.12). Overall P/AI was not different among groups (non-sexed semen: 51.2%, 107/209; SexedULTRA 4.0: 42.0%; 84/200 and SexedULTRA Pure 43.1%; 88 / 204; P = 0.10; Baruselli *et al*., 2017b). Furthermore, in the subgroup of animals in which estrus was recorded there was a significant interaction between type of semen and expression of estrus (P < 0.01). In cows showing estrus, P/AI was not different between cows inseminated with sexed or non-sexed semen (Table 2). Conversely, in cows not showing estrus P/AI was higher (P < 0.05) for those inseminated with non-sexed semen and those inseminated with SexedULTRA Pure, whereas P/AI in those inseminated with SexedULTRA was intermediate and not different from the other two groups. The recommended protocol for FTAI with sexed-sorted semen is shown in Fig. 3.

Another study was carried out to evaluate P/AI in heifers treated with the J-Synch protocol and inseminated with SexedULTRA semen (Huguenine *et al*., 2017, unpublished). Angus and Angus crossbred beef heifers (n = 850) were treated with the J-Synch protocol with 300 IU eCG at P4 device removal (Day 6) and were tail-painted for estrus determination. The heifers with the paint-rubbed off at 60 or 72 h after device removal were randomly subdivided into two subgroups to be inseminated at 72 h with female SexedULTRA 4.0 semen or with non-sexed semen from the same four Angus sires. Heifers that did not showed estrus by 72 h received GnRH at that time and were inseminated at 84 h with the same two types of semen. The protocol is shown in Fig. 4 and the results are shown in Table 3. There were 72.7% (618/850) of heifers in estrus at 60 and 72 h and the overall P/AI was 54.0% (459/850) regardless of expression of estrus. There was a significant (P < 0.01) effect of semen type (Table 3), time of AI and sire on P/AI, but no interactions. P/AI were 64.1% (223/348) for heifers in estrus at 60 h and inseminated at 72 h, 51.8% (140/270) for those in estrus and inseminated at 72 h, and 40.9% (95/232) for those not in estrus by 72 h and inseminated at 84 h after P4 device removal. Regarding sires, P/AI ranged from 40.5% to 67.8% with non-sexed semen and 26.5% to 59.3% with SexedULTRA semen. In summary, protocols designed for FTAI in cows and heifers can be adapted for sexed-sorted semen. Although, P/AI are lower than those obtained with non-sexed semen, delaying the time of AI or limiting the AI to those animals showing estrus would result in P/AI between 40 and 50% or even higher.

Table 2. Pregnancy rates in lactating Nelore cows inseminated with sexed-sorted (SexedULTRA^{TM}) or non-sexed semen according to the expression estrus (reading of paint-stick) and time of insemination (AI) after the removal of the progesterone releasing device.

	n	Estrus 60 h AI 60 h	No estrus at 60 h AI 60 h	Total
SexedULTRA ($4x10^6$ sperm)	138	50/94 (53.1%)	14/44 (31.8%)^{ab}	64/138 (46.4%)
SexedULTRA Pure ($4x10^6$ sperm)	144	54/102 (52.9%)	9/42 (21.4%)^{bc}	63/144 (43.8%)
Non-sexed ($20x10^6$ sperm)	149	57/110 (51.8%)	18/39 (46.2%)^{c}	75/149 (50.3%)

^{abc} Denotes differences in P/AI between sexed-sorted or non-sexed semen in cows not showing estrus (P < 0.05).

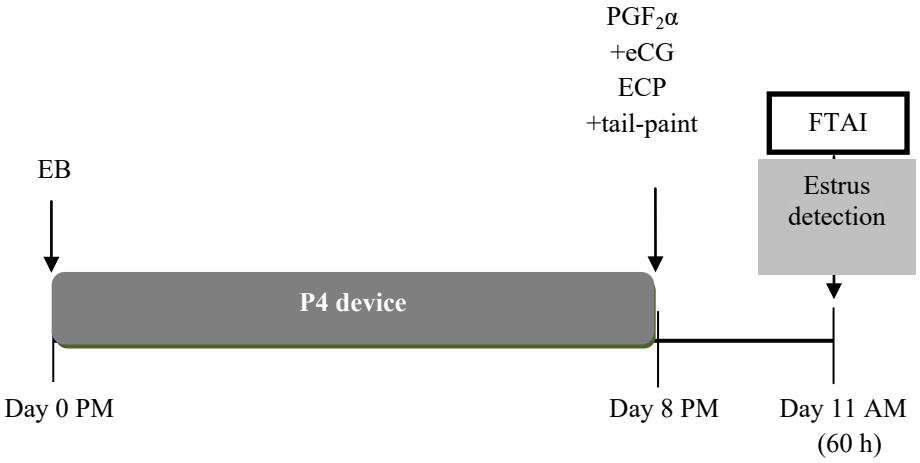

Figure 3. Conventional estradiol/P4-based protocol for FTAI with sexed-sorted semen. Tail-paint or patches are used to detect those animals in estrus 60 h after P4-device removal. Animals in estrus by 60 h are inseminated with sexed-sorted semen whereas those animals not in estrus by 60 h receive GnRH and are inseminated at the same time with sexed-sorted or non-sexed semen.

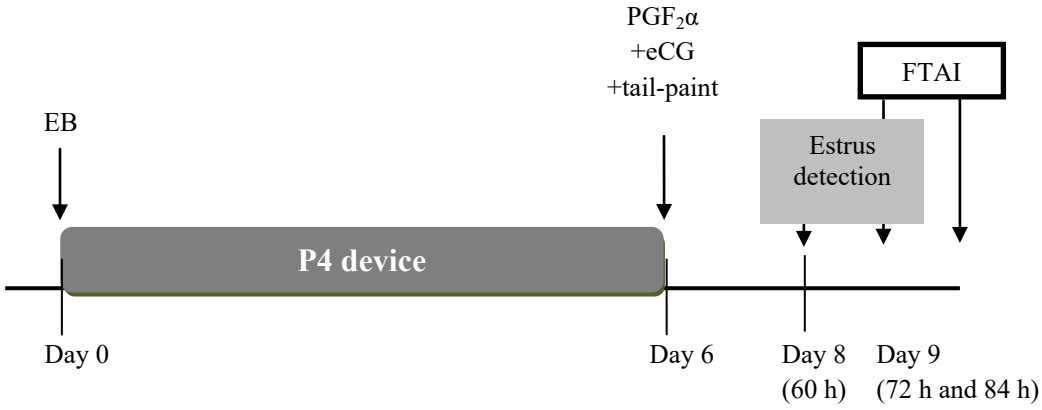

Figure 4. Alternative estradiol/P4 based protocol with prolonged proestrus for FTAI beef heifers with sexed-sorted semen. Tail-paint or patches are used to detect those heifers in estrus at 60 and 72 h after P4-device removal. Heifers in estrus at 60 and 72 h are inseminated at 72 h with sexed-sorted semen. Heifers not in estrus by 72 h receive GnRH and are inseminated at 84 h with sexed sorted or non-sexed semen.

Table 3. Pregnancy rates in Angus heifers inseminated with sexed-sorted (SexedULTRA^{TM}) or non-sexed semen according to the expression estrus (reading of tail-paint) and time of insemination (AI) after the removal of the progesterone releasing device.

	n	Estrus 60 h AI 72 h	Estrus 72 h AI 72 h	No estrus at 60 or 72 h AI 84 h	Total
SexedULTRA ($4x10^6$ sperm)	426	104/176 (59.0%)	61/134 (45.5%)[a]	45/116 (38.8%)	210/426 (49.3%)[a]
Non-sexed ($25x10^6$ sperm)	424	119/172 (69.2%)	79/136 (58.0%)[b]	49/116 (42.2%)	247/424 (58.3%)[b]

[ab] Denotes differences in P/AI between sexed-sorted or non-sexed semen ($P < 0.05$).

Re-synchronization treatments

With the advent of FTAI in beef herds, producers have been seeking strategies that can be implemented to obtain the highest possible number of cows pregnant through AI early in the breeding season. Several approaches have been developed over the years; however, most require estrus observations or an interval of approximately 40 days between the first and second FTAI (reviewed in Bó *et al*., 2016 and Baruselli *et al*., 2017a). In order to be able to inseminate non-pregnant cows as early as possible, re-synchronization treatments must start earlier than pregnancy diagnosis. One protocol was developed in which a re-used device impregnated with 1 g of P4 (i.e. previously used in the first synchronization) was re-inserted on Days 14-16

after the first AI and is removed on Day 23 at the time that GnRH was administered. Pregnancy diagnosis is performed on Day 30 and those that are found to be non-pregnant receive $PGF_{2\alpha}$ combined with either 0.5 mg ECP at that time or simply GnRH at the time of FTAI on Day 32. P/AI for the first and second FTAI and the overall P/AI in a field trial involving 6,431 beef cows and heifers were 57%, 51% and 79%, respectively (Bó *et al*., 2016).

Two new protocols for re-synchronization are called Resynch 22 and Resynch 14 (Baruselli *et al*., 2017a). In the Resynch 22, cows receive 2 mg EB (Pessoa *et al*., 2015) and heifers 1 mg EB (Sá Filho *et al*., 2014) at P4 device insertion on Day 22. Pregnancy diagnosis is performed at device removal (Day 30) and non-pregnant animals also receive $PGF_{2\alpha}$ and ECP and are inseminated on Day 32. The Resynch 14 protocol involves the use of color Doppler ultrasonography for the detection of pregnancy based on the vascularization and size of the CL on Day 22 after the first AI (Siqueira *et al*., 2013; Pugliesi *et al*., 2014, 2017). For Resynch 14 the initial treatment starts 14 days after FTAI with the re-insertion of a re-used device and the administration of 100 mg P4 IM (Rezende *et al*., 2016) to avoid the possible luteolytic effect of EB (Vieira *et al*., 2014). The new wave emerges 3.0 ± 0.7 days after P4 administration in Nelore cows (Rezende *et al*., 2016); thus cows are scanned with Doppler ultrasonography for pregnancy at device removal (Day 22) and non-pregnant animals receive $PGF_{2\alpha}$ and ECP and are inseminated on Day 24. In a recent study, similar P/AI were observed for Resynch 22 and Resynch 14 groups following the first FTAI (48% vs 53%; P = 0.57) and re-synchronization (56% vs 51%; P = 0.37), respectively. However, the Resynch 14 reduced the interval between FTAI, which resulted in a 21-day P/AI of 87.5% compared to 66% with the Resynch 22 (Penteado *et al*., 2016). In a more recent field trail, the use of three consecutive FTAI with the Resynch 22 protocol had a similar overall pregnancy rate (87.8%, 663/755) as that achieved using clean-up bulls after two FTAI using Resynch 22 (87.7%, 263/300) and greater pregnancy rate than one FTAI followed by bull exposure (77.1%, 347/450; Crepaldi *et al*., 2017). In conclusion, with the existing re-synchronization programs it is now possible to breed beef cows exclusively with FTAI, eliminating the need for estrus detection and clean-up bulls.

Summary and conclusions

Protocols for FTAI have allowed the widespread use AI in South-America, with the possibility of obtaining P/AI of 50% or more with a single insemination. The addition of eCG at P4-device removal to stimulate the growth of the ovulatory follicle has been especially useful in increasing P/AI in cows experiencing postpartum anestrus. New treatment protocols that do not require the use of estradiol or GnRH shortly after P4 device removal and allow for a longer proestrus period prior to insemination are alternatives for increasing fertility to FTAI; however, the animals must be in optimal nutritional conditions to achieve high P/AI. Furthermore, FTAI treatments combined with estrus detection using tail-paint or heat-detection patches permits the use of sexed-sorted semen with acceptable P/AI. Finally, early pregnancy diagnosis with ultrasonography can be easily implemented in beef herds in order to perform sequential FTAI without estrus detection, resulting in pregnancy rates that are similar or higher than obtained with clean-up bulls, maximizing the use of the improved genetics through AI.

Acknowledgments

Research was supported by FONCYT (PICT 2017-4550), Universidad Nacional de Villa María, Zoetis and Sexing Technologies from Argentina, and ANII, Syntex Uruguaya SA and Fundaciba from Uruguay.

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DOI: 10.21451/1984-3143-AR2018-0016

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

A historical perspective of embryo-related technologies in South America

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Abstract

Livestock production is of great importance for the economy of most South American countries, a region that accounts for 23.0% of the world cattle population (Food Agriculture Organization - FAO, 2017). Not surprisingly, the embryo industry is historically very active in this region, particularly in Argentina and Brazil. The field of bovine embryo transfer underwent a remarkable change in the past two decades in Brazil, mainly due to *in vitro* embryo production (IVEP). Total embryo production increased dramatically, along with constant changes in the main features of the embryo industry – from market niches to mass production, from beef to the dairy sector, from zebu to European breeds. Recently, IVEP has also emerged in other South American countries. This review summarizes and describes factors driving the changes in the Brazilian embryo industry and discusses some of the impacts upon other embryo-related technologies.

Keywords: cattle, cloning, *in vitro* fertilization, statistics, superovulation.

Introduction

The embryo industry has been very active in South America in the past 20 years, with several countries reporting data on embryo production and transfers to the International Embryo Technology Society (IETS), mainly in cattle and horses. Until 2008, however, the methodology used by the data retrieval committee pooled data from each region and presented results as totals. Therefore, before 2009 detailed records are only available from Brazil and Argentina. The IETS reports published afterwards show data for the production of bovine embryos, either *in vivo* or *in vitro*, from the following countries: Argentina, Brazil, Chile, Colombia, Ecuador, Peru, and Uruguay, as well as reports on the export of embryos to Ecuador and Paraguay (Stroud, 2011, 2012; and Perry, 2013, 2014, 2015, 2016). Thus, there are reports of cattle embryo transfer (ET) activity in the past eight years in most of the South American countries. These countries encompass 92.8% of the cattle herd within this region (FAO, 2017), so available data provides a good picture of the ET activity in South America.

Argentina and Brazil have both the largest cattle herds (51,646,544 and 212,366,132 heads in 2014, respectively) and the most active embryo industries in South America. They were consistently ranked among the top countries doing ET in the past 20 years. Brazilian embryo industry has undergone a dramatic increase between 2002 and 2012 (+642.7, or about 55% per year), primarily due to the commercial use of *in vitro* embryo production (IVEP; Viana *et al*., 2017). On the other hand, the use of IVEP in Argentina increased in the past five years and only in 2016, transfers of *in vitro* produced (IVP) embryos reached a 5-digit number (20,234), overtaking *in vivo* derived (IVD) embryos (15,586).

The earlier adoption of *in vitro* embryo technologies was the main reason for the divergence in embryo production trends between the two countries after 2001 (Fig. 1). Taking into account the ratio between embryo production and cattle population in 2014, Brazil ranks 11th in the world, whereas Argentina is the 22nd (Viana *et al.*, 2017). Nevertheless, in both countries the use of IVEP was associated with an increase in total embryo production in cattle.

Argentina and Brazil have also a very active horse embryo industry, reporting an average of 7,400 and 12,840 embryos collected per year, respectively, from 2006 to 2015. Note that there are some missing data for both countries in a couple of the years mentioned, which limits the characterization of the scenario and trends for this species. There are also occasional reports on ET activity in sheep, goats, and alpacas in South America (Perry, 2013, 2014, 2015, 2016). Gathering data from species other than cattle and horse has been a challenge over the years and the numbers reported undoubtedly underestimate the use of embryo technologies in small ruminants in this region.

In summary, bovine is the most important species for the South American embryo industry and Brazil is the main player for the development of embryo technologies in this region, considering both the total numbers of embryos and launching novel trends, such as the earlier use of IVEP in large scale. Thus, many of the aspects of ET development discussed in this paper are supported by data in cattle and from Brazil.

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Received: January 31, 20018
Accepted: April 5, 2018

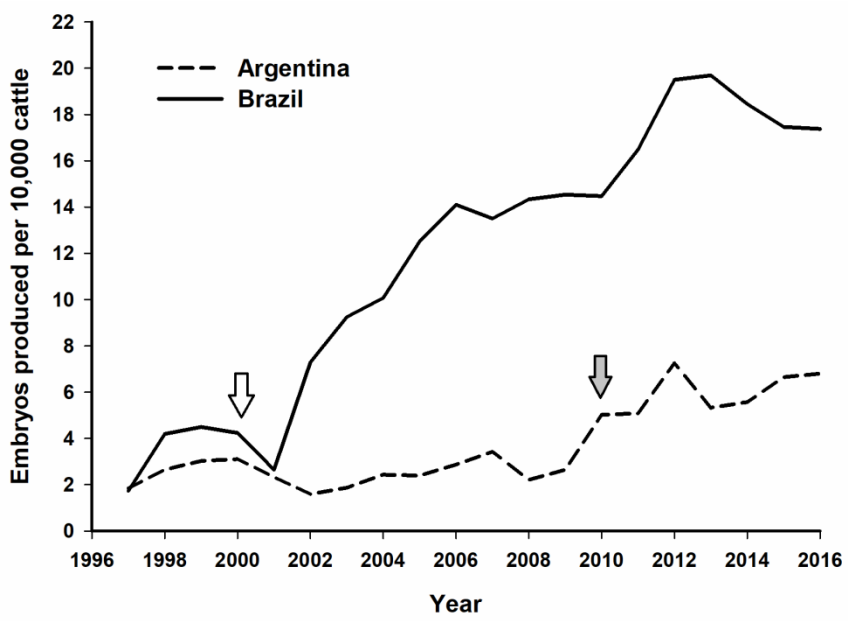

Figure 1. Embryo production relative to cattle population (embryos per 10,000 heads) in Argentina (dashed line) and Brazil (solid line) over the past 20 years. The arrows indicate when the commercial use of IVP embryos was first reported in Brazil (white) and Argentina (grey).

The effect of IVEP in South America embryo industry

Perhaps the most noticeable features of the embryo industry in South America were the changes introduced by the adoption of *in vitro* technologies. The history of IVEP in Brazil goes back to the early 1990`s, when different research groups started to focus on these technologies. The first calf produced *in vitro* in Brazil was born in 1993, followed by the birth of the first zebu IVP calf, and then the first calf from a cryopreserved IVP embryo (Rubin, 2005). However, there are no official reports about the commercial use of IVEP in Brazil until 1997, and less than 100 IVP embryos were transferred per year in 1998 and 1999.

Meanwhile, IVEP was already intensively used in other continents. By the middle of the 1980's, several commercial IVEP laboratories were developed in North America and Europe (Faber *et al.*, 2003) and, by 1997, IVP embryos corresponded to 12.6% of all transfers in Europe and 17.5% in Asia (Thibier, 1998). Europe was the region leading the use of IVEP, with 59.9% (18,380 of 30,569) of all IVP embryos transferred worldwide in 1997. In this scenario, although Brazil and Argentina usually ranked within the top five countries in the transfer of IVD embryos outside Europe and North America, there was still no clear sights on the significant shift in the embryo industry scenario that was about to come.

The whole picture started to change in 2000, when Brazil first reported the production of more than 10,000 IVP embryos. A consistent growth in commercial IVP boosted South American numbers. Only two years later, in 2002, this region already accounted for 58.4% (48,670 of 83,329) of the IVP embryos transferred worldwide (Thibier, 2003). In spite of the usual fluctuations in numbers among different regions through the years, since 2006 Brazil is responsible for >50% of the IVP embryos worldwide. In fact, the adoption of IVEP was responsible for the remarkable participation of Brazil in the world total embryo production after 2002 (Fig. 2). The growth in the use of IVEP in other regions, as currently observed in North America (Fig. 3), will probably increase world total numbers and, consequently, balance the participation of the main players in the world's embryo industry.

It is important to highlight that IVEP changed not only the magnitude of ET numbers, but also the *modus operandi* of the embryo industry in Brazil and, together with its emergence in other countries, is likely to cause similar effects elsewhere. Since 2005, IVP replaced superovulation as the technique of choice for bovine embryo production (Viana *et al.*, 2012) and the rapid expansion of commercial IVP laboratories pushed traditional ET companies to embrace the new technology. The embryo production process, previously centered in the veterinarian practitioner, now resembles a complex and multi-step production line, which requires technicians with a number of distinct skills.

With the use of transvaginal ultrasound-guided follicle aspiration (OPU), the availability of cumulus-oocyte complexes was no longer a bottleneck because multiple donors can be collected to achieve the number of oocytes predicted to be necessary to generate the required number of embryos. Conversely, the low cryotolerance of IVP embryos have made the availability of suitable recipients a critical factor within

ET programs. The increasing efficiency of embryo production *in vitro* (Watanabe *et al.*, 2017), as well as the use of IVEP in large scale (Pontes, 2010, 2011) affected the costs of IVP embryo-related services, the profit margins, and consequently the business model adopted by commercial companies.

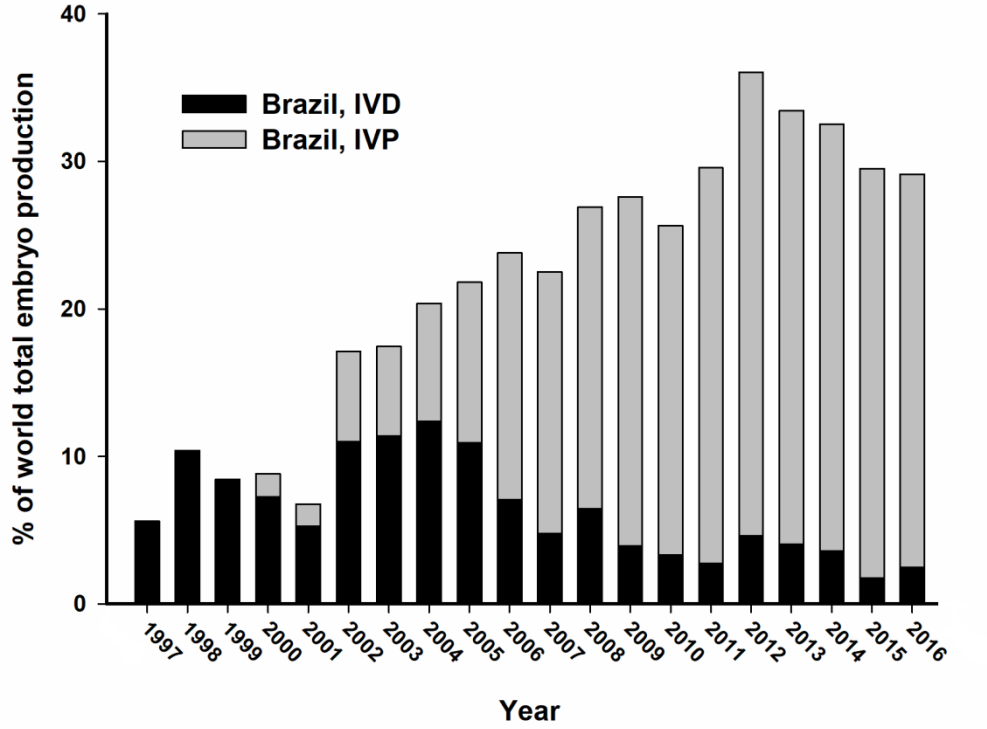

Figure 2. Brazilian contribution to cattle embryo production in the world, in the past 20 years. The columns for each year were subdivided in the percentages corresponding to embryos produced *in vivo* (IVD, black) or *in vitro* (IVP, grey).

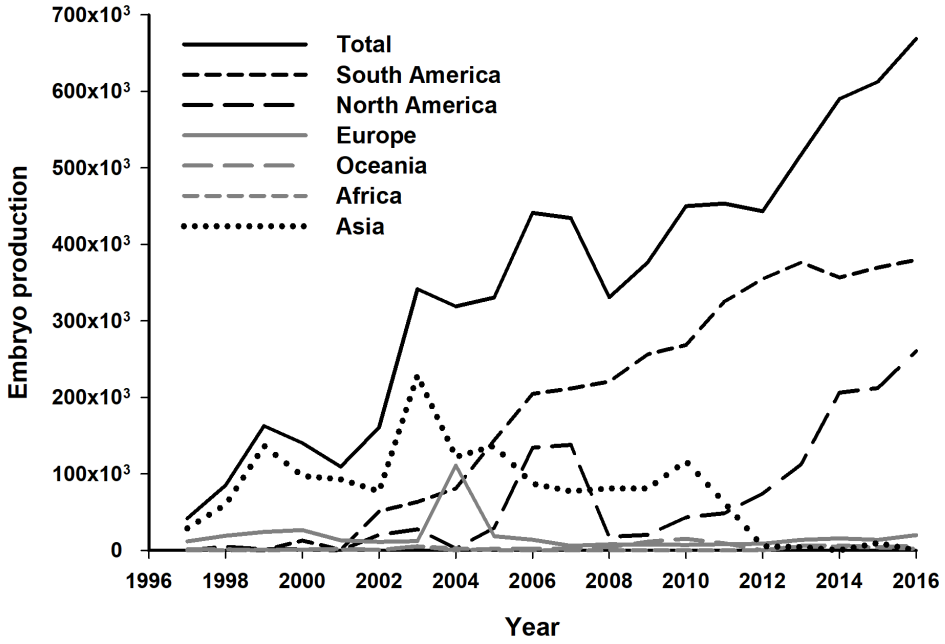

Figure 3. Total bovine embryo production (*in vivo* plus *in vitro*) in the world (solid black line) and divided into different regions (grey, dashed, and dotted lines) ordered according 2016 rank (higher to lower), in the period 1997-2016. Adapted from Perry (2017).

The factors driving the embryo market in Brazil

The reasons behind changes in the Brazilian embryo industry in the past two decades are complex and frequently misunderstood. Embryo technologies have been intensively studied over the past decades and a number of comprehensive review articles have addressed many practical aspects of IVEP. It has some advantages, when compared to embryo production *in vivo* by superovulation, such as the shorter interval between embryo production cycles, the possibility of collecting pre-pubertal or pregnant donors, and of generating contemporaneous calves from the same dam using multiple sires, among other benefits (Galli *et al.*, 2001; Hansen and Block, 2004; Thibier, 2005).

The efficiency of *in vitro* maturation, fertilization, and embryo culture, however, is generally low (Lonergan and Fair, 2008). Moreover, artificial *in vitro* culture conditions can alter epigenetic marks (Urrego *et al.*, 2014) and affect embryonic gene expression (Lonergan *et al.*, 2006). Altogether, these factors affect the quality and cryotolerance of IVP embryos (Lonergan *et al.*, 2006) and have also been associated with higher pregnancy losses, errors in fetal programming (Chen *et al*., 2015; Siqueira *et al*., 2017), and abnormal offspring phenotype, a condition frequently referred to as the large offspring syndrome (Farin *et al.*, 2006). Besides, IVEP requires a much more complex and expensive laboratory infrastructure, with direct impact upon production costs. In this scenario, it was reasonable to consider IVEP as an expensive and uneconomic technology (Gordon, 2003), with a perspective of use as a complement to multiple ovulation and embryo transfer (MOET) programs, particularly for donors that do not respond to superovulation or presenting abnormalities in the reproductive tract (Faber *et al.*, 2003). So, what made IVEP so successful in South America?

Despite the known flaws of the technology, commercial IVEP in Brazil was initially supported by the high demand and high prices of selected sires and dams, especially high-genetic merit oocyte donors of zebu breeds (Viana *et al*., 2012). The focus back then was to produce superior animals for breeding programs, as occurred in other parts of the world (Galli *et al.*, 2003; Smeaton *et al.*, 2003). Nevertheless, particularities of the Brazilian embryo industry at that time generated a *virtuous circle* for IVEP. The high number and quality of cumulus-oocyte complexes (COC) retrieved from zebu breeds (Pontes *et al.*, 2011), for example, resulted in a high embryo yield per session. This led to a reduction in pregnancy costs, which in turn stimulated an increase in the use of IVEP, and the scale effect to promoted further declines in the price of embryos and services. Thus, the technology progressively became economically viable for a greater number of breeds and farmers. Finally, development of IVEP promoted the parallel growth of a chain of suppliers of veterinary services, hormones, IVP media, equipment, disposables, recipients, etc; contributing to a cost reduction and improvements in logistics.

Three phases of IVEP development in Brazil have been previously described (Viana *et al.*, 2012). The initial period described in the paragraph above was followed by two distinct growth cycles (Fig. 4). First, beef breeds, mainly Nelore, accounted for 82.7% of all embryos transferred in Brazil in 2005 (Viana *et al.*, 2012). This growth cycle was probably associated with a repressed demand for young sires, if one takes into account that in 2008 only approximately 6% of beef cows and heifers were artificially inseminated in Brazil (Baruselli *et al.*, 2012). The second cycle was characterized by the use of IVEP in dairy breeds, particularly in Girolando (Gir x Holstein crossbreds). The availability of commercial X-sorted semen after 2005 was a turning point for the development of IVEP in dairy breeds. Previously, depending on the breed, sire, and culture conditions, the use of conventional semen for IVF resulted consistently in a higher proportion of male births (Alomar *et al.*, 2008; Camargo *et al.*, 2010; Rubessa *et al.*, 2011), which did not meet the expectations of dairy farmers. Therefore, the lack of X-sorted semen in the early 2000's may explain why the IVEP growth cycle occurred later in dairy, if compared to the early growth in beef breeds.

The emergence of IVEP in dairy breeds highlighted two major changes in the Brazilian embryo industry features. Firstly, IVEP became an alternative for large scale production of replacement heifers, particularly in crossbred herds (Pontes *et al.*, 2010), instead of a reproductive tool restricted to elite animal breeding programs and high-value donors. This represents an important shift in the perception of the potential (and therefore the impact) of IVEP outside Europe and North America - until recently, this technology was referred to as of little application in cattle breeding, particularly in developing countries (Rodriguez-Martinez, 2012). Secondly, the use of *Bos taurus* and crossbreds for IVEP increased dramatically and, in 2013, overcame the production of embryos in pure breed *Bos indicus* (Viana *et al.*, 2017).

More than a simple change in market demand, this shift towards *Bos taurus* suggests that the technical and operational improvements in IVEP made the technique economically interesting even for breeds with lower oocyte yields. Thus, the high number of COC recovered from breeds such as Nelore that might have supported the early emergence of IVEP in Brazil, was no longer a bottleneck for the adoption of *in vitro* technologies. This hypothesis is supported by the late development of IVEP in Argentina (Fig. 1), in which *Bos taurus* breeds are predominant. The increase in embryo production in dairy and *Bos taurus* breeds also turned the South American embryo industry more similar to the North American, with a relative balance between dairy and beef (Table 1).

A parallel development convergent with the new demands of the embryo industry was related to protocols for synchronization of ovulation. These protocols were initially developed for timed artificial insemination (TAI) and currently account for most of AI breedings in Brazil (Baruselli *et al.*, 2012; Sartori *et al.*, 2016). Protocols for timed embryo transfer (TET) were soon adapted for the preparation of embryo recipients (Baruselli *et al*., 2011; Bó *et al*., 2011), and are currently being used in commercial ET programs, with results similar to those obtained using TAI (Pellegrino *et al*., 2016). The main advantage for the use of TET protocols is to increase the synchrony between the embryo developmental stage and the recipient's uterus, required for achievement of higher pregnancy rates after ET, besides eliminating the well-known problems of estrous detection efficiency (Senger, 1994). Protocols for TET were particularly useful due to the preference for the transfer of fresh IVP embryos (77.9% in South America in 2016; Perry, 2017) and the need to optimize the use of recipients in dairy herds, usually smaller than beef operations. This also have certainly contributed to the long-lasting cycle of growth of IVEP in dairy breeds.

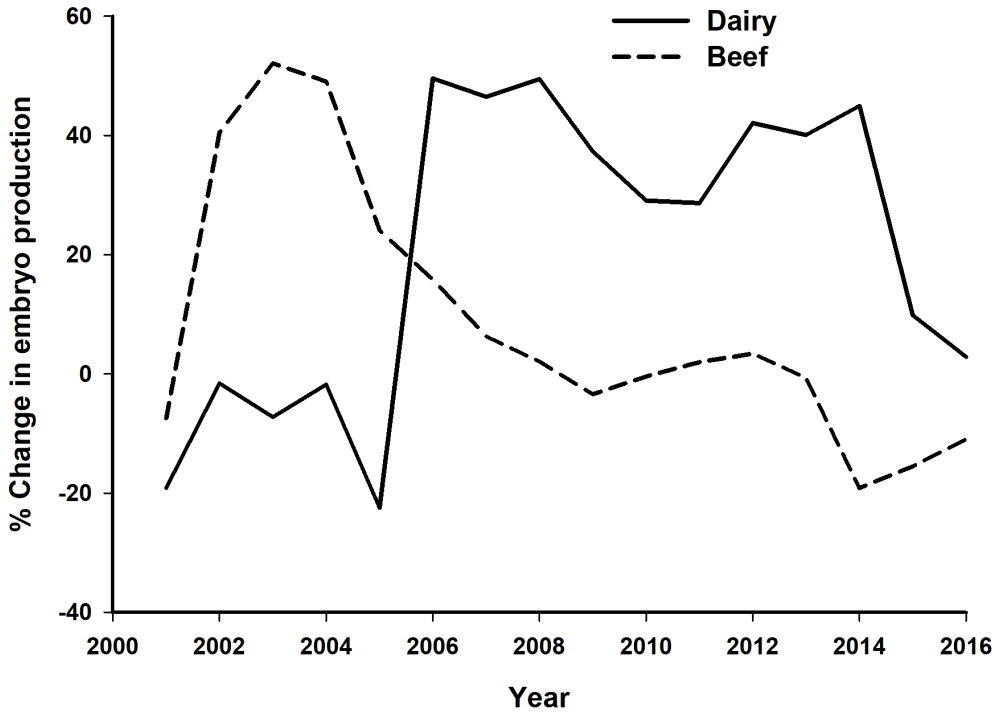

Figure 4. Proportion of changes in the Brazilian embryo production, according to sector (dairy: solid line; beef: dashed line), in the period 2001-2016. Values were calculated based on the variation from the previous year and data was corrected using moving means (3-year average) to reduce the effect of occasional fluctuations.

Table 1. World embryo production in 2016, according to region, sector (dairy or beef) and technology (IVD, *in vivo* derived; or IVP, *in vitro* produced).

	Dairy			Beef		
Region	IVD	IVP	Total (%)	IVD	IVP	Total (%)
Africa	240	0	240 (3.8)	3,863	2,167	6,030 (96.2)
Asia	13,226	0	13,226 (11.7)	99,372	0	99,372 (88.3)
Europe	99,693	16,678	116,371 (78.2)	29,184	3,296	32,480 (21.8)
NA	111,575	136,204	247,779 (41.8)	220,677	124,370	345,047 (58.2)
Oceania	160	1,956	2,116 (15.3)	7,332	4,364	11,696 (84.7)
South America	17,552	194,357	211,909 (49.6)	29,764	185,445	215,209 (50.4)
Total	242,446	349,195	591,641 (45.5)	390,192	319,642	709,834 (54.5)

Other embryo-related technologies

The growth of embryo production in Brazil and, particularly, the emergence of IVEP have had direct and indirect effects upon other embryo-related technologies, bringing new challenges for research, but also new market opportunities. One of the indirect consequences was the increased availability of laboratory infrastructure, as well as of qualified technicians. The first Brazilian *in vitro* fertilization company was established in 1998 and, by 2016, the Brazilian Ministry of Agriculture and Livestock have already registered >50 IVEP laboratories (MAPA, unpublished data). This network of laboratories is a platform for the development of other technologies, particularly those that require substantial investments in laboratory equipment (e.g. micromanipulation, intracytoplasmic sperm injection [ICSI], somatic cell nuclear transfer [SCNT], etc.) but have limited commercial use *per se*. In research, IVEP has shifted the focus of some lines of investigation. For instance, due to the replacement of embryo production *in vivo* by *in vitro*, studies on ovarian superstimulation now aim to improve the number and quality of COC per OPU per donor (Vieira *et al.*, 2014; Silva *et al.*, 2017).

A number of research groups are currently developing embryo biotechnologies in Universities and research Institutes throughout South American countries and some of these technologies, such as micromanipulation for embryo sexing or genotyping are beginning to be used commercially. Only recently, however, this kind of data started to be collected by IETS (Perry, 2017) and there are still no comprehensive data for analysis. Bovine clones have been produced in Brazil and in Argentina (Meirelles *et al.*, 2010; Cánepa *et al.*, 2014). Table 2 shows the number of birth records from zebu breeds in Brazil that were derived from SCNT. Despite the low efficiency of embryo reconstruction and very high rates of embryonic loss, abortion, and stillbirths (Chavatte-Palmer *et al.*, 2012), SCNT numbers have increased in the past few years, demonstrating that the demand for the use of the technology has overcome the technical difficulties and, consequently, the high production cost. As expected, most clones were from Nelore (62.5%) and Gir (26.9%), which are the most important beef and dairy zebu breeds in Brazil, respectively. Nonetheless, it is remarkable that the majority of calves (142 of 160, 89.0%) were females and this was true for all breeds. This is probably evidence of a greater interest in cloning high-value oocyte donors, though clones of important sires have also been produced.

Table 2. Number of birth records (RGN) from the Brazilian Zebu Cattle Breeders Association that were derived from somatic cell nuclear transfer embryos in the period 2010-2015, stratified by breed, sex, and year of birth.

Breed	2010		2011		2012		2013		2014		2015		Total (%)
Sex	M	F	M	F	M	F	M	F	M	F	M	F	
Gir	0	2	0	0	0	5	2	14	0	6	0	14	43 (26.9)
Guzera	0	0	0	0	0	1	0	2	0	3	0	0	6 (3.8)
Nelore	0	3	1	22	3	11	3	11	3	5	6	32	100 (62.5)
Tabapua	0	0	0	0	0	0	0	6	0	0	0	0	6 (3.8)
Brahman	0	0	0	0	0	2	0	3	0	0	0	0	5 (3.1)
Total	5		23		22		41		17		52		160 (100)

From Associação Brasileira dos Criadores de Zebu - ABCZ, 2017.

Conclusions

Over the past few decades, embryo industry in South America has been very active, particularly in cattle, with some particular differences among countries. The adoption of IVEP in this region directed significant changes in total embryo production, as well as in the main features of the cattle embryo industry. This phenomenon was first observed in Brazil, which allowed this country to become a reference in the use of *in vitro* embryo technologies and supported the development of other embryo-related technologies. Finally, as IVEP is currently in open expansion in other countries, similar effects are likely to be observed in those regions as well.

Acknowledgments

The authors thank the SBTE statistics committee and the Data Retrieval Committee of the IETS for the Brazilian and world embryo production data. They also thank the following breeders associations for the information on embryo transfers: ABCZ, ABCBRH, ABCGPS, ABCG, ABCM, ABC - Herd Book Collares, ABCSS.

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DOI: 10.21451/1984-3143-AR2018-0043

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Assisted reproductive technologies (ART) in water buffaloes

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Abstract

Our expanding knowledge of ovarian function during the buffalo estrous cycle has given new approaches for the precise synchronization of follicular development and ovulation to apply consistently assisted reproductive technologies (ART). Recent synchronization protocols are designed to control both luteal and follicular function and permit fixed-time AI with high pregnancy rates during the breeding (autumn-winter) and nonbreeding (spring-summer) seasons. Additionally, allow the initiation of superstimulatory treatments at a self-appointed time and provide opportunities to do fixed-time AI in donors and fixed-time embryo transfer in recipients. However, due the scarce results of *in vivo* embryo recovery in superovulated buffaloes, the association of ovum pick-up (OPU) with *in vitro* embryo production (IVEP) represents an alternative method of exploiting the genetics of high yeld buffaloes. Nevertheless, several factors appear to be critical to OPU/IVEP efficiency, including antral follicle population, follicular diameter, environment, farm and category of donor. This review discusses a number of key points related to the manipulation of ovarian follicular growth to improve assisted reproductive technologies in buffalo.

Keywords: artificial insemination, embryo transfer, synchronization.

Introduction

The combined use of assisted reproductive technologies (ART), such as, timed-artificial insemination (TAI), superstimulation (SOV), ovum pick-up (OPU), *in vitro* embryo production (IVEP) and timed-embryo transfer (TET) has a great potential to improve reproductive outcomes and disseminate selected genetics, improving milk and beef production in buffalo herds.

However, the success of ART is closely related to the control of ovarian follicular development and ovulation. Buffalo is a seasonal reproductive species and becomes sexually active in response to a decreasing day length (short-days) in late summer to early autumn (Zicarelli, 1997). During the nonbreeding season, buffalo often exhibit anestrus, which extends the anovulatory period and consequently, reduces reproductive performance (Zicarelli, 2007).

In recent decades, several therapies have been proposed for manipulating ovarian follicle growth and ovulation in buffalo, regardless of reproductive seasonality (Baruselli *et al*., 2007; Campanile *et al*., 2010; Carvalho *et al*., 2016). These hormonal manipulations have been successfully used to optimize the reproductive outcomes following the application of various biotechnologies.

This review aims to elucidate some factors that affect the efficiency of assisted reproductive technologies (ART) in buffalo.

Ovarian physiology in buffalo

The understanding of follicular dynamics in buffalo is necessary for developing new techniques and improving the currently used regimens for the manipulation of the estrous cycle. Ovarian follicular dynamics in buffalo are similar to those in cattle. The 2-wave cycle is the most common in buffalo (63.3%; Fig. 1; Baruselli *et al*., 1997) and the follicle deviation occurs 2.6 days after ovulation, when the diameters of the dominant and subordinate follicle are 7.2 and 6.4 mm, respectively (Gimenes *et al*., 2011). As in cattle, the number of waves in a cycle is also associated with the luteal phase and with the estrous cycle length.

However, the number of follicles recruited per follicular wave is lower in buffalo than in cattle (Baruselli *et al*., 1997; Gimenes *et al*., 2009; Campanile *et al*., 2010). The number of primordial cells in buffalo ovaries varies from 10,000 to 12,000 (Danell, 1987), which is about 10-fold lower than in cattle (Manik *et al*., 2002). Furthermore, it was verified that 92 to 95% of follicles are estrogen inactive/atretic at random stages of the reproductive cycle. Van Ty *et al*. (1989) also observed the existence of a lesser number of antral follicles in buffalo, when compared to cattle. These authors found that buffalo ovaries have about 20% of the number of antral follicles found in cattle (47.5 ± 23.8 *vs*. 233.0 ± 95.8; $P < 0.002$).

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Received: February 28, 2018
Accepted: May 24, 2018

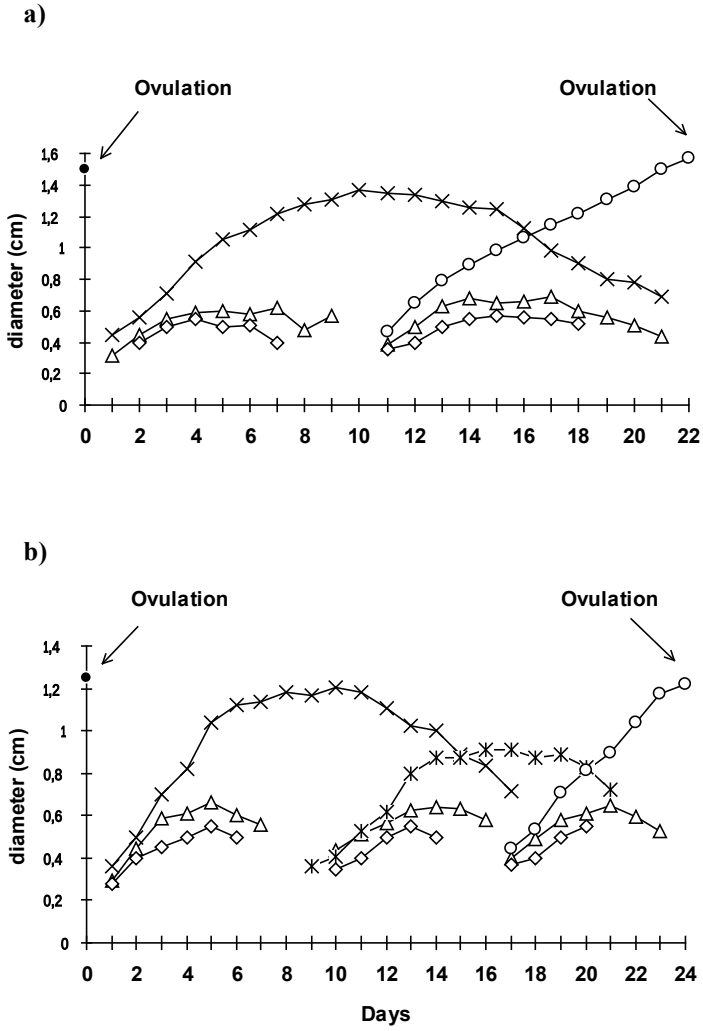

Figure 1. Standardized diameters of ovarian follicles (dominant follicle, largest and second largest subordinate follicle) in buffalo cows with a) two wave (n = 19) and b) three wave (n = 10) estrous cycles. Adapted from Baruselli *et al*. (1997).

Pharmacological control of follicular development and ovulation

Prostaglandin F2α (PGF)

Estrus synchronization with prostaglandin F2α (PGF2α) is an effective and economical tool for induction of luteal regression, improving the estrous detection efficiency and the use of ART in buffaloes. Studies have shown that PGF2α treatment caused 100% luteolysis in buffalo (plasma progesterone <1 ng/ml within 48 h of administration), regardless of the luteal phase (early or late luteal phase; 6-9 or 11-14 days after estrus, respectively, (Porto Filho *et al*., 2014). Ovulation can occur up to 6 days after PGF2α administration, depending on the responsiveness of the corpus luteum (CL) and the stage of ovarian follicle development at the time of PGF2α treatment (Porto Filho *et al*., 2014). However, the major limitation of PGF2α in buffalo to apply efficient ART is the poor estrous behavior, and the lack of efficiency in females without a responsive CL (e.g., females within 5-6 days of a previous estrus) or in pre-pubertal heifers and postpartum anestrous cows. These particularities compromise the efficient use of only PGF2α treatment in reproductive programs in buffaloes.

GnRH

The GnRH administration induces the emergence of a new follicular wave after induction of ovulation in cattle (Macmillan and Thatcher, 1991; Twagiramungu *et al*., 1992a, b,1995; Wolfenson *et al*., 1994; Schmitt *et al*., 1996). This information became the basis for subsequent development of programs to control timed ovulation.

In buffalo, 60.6% (20/33) of the cows ovulated after GnRH treatment at random stages of estrous cycle (Baruselli *et al*., 2013). The responses of GnRH depend on the diameter of the largest follicle at the moment of the treatment (Neglia *et al*., 2016). Buffalo that ovulated after GnRH treatment presented a larger follicle than animals that did not ovulate (9.5 ± 1.7 *vs*. 6.7 ± 2.4 mm; $P < 0.01$). However, no effect of the progesterone (P4) concentrations at the time of GnRH treatment and the GnRH dose (10 *vs*. 20 μg of buserelin) on the ovulation rate and the time of ovulation were observed. Furthermore, the interval between GnRH treatment and

ovulation was between 28 to 33.0 h (Berber *et al*., 2002; Baruselli *et al*., 2003b; Campanile *et al*., 2008; Jacomini *et al*., 2014), similar to the interval observed in cattle (Wiltbank and Pursley, 2014).

Estradiol plus progesterone to synchronize wave emergence

The combination of progesterone (P4) and estradiol (E2) treatment induces follicular atresia by suppressing FSH and LH release after the treatment and then synchronous emergence of a new follicular wave in response to the subsequent FSH release in cattle (reviewed by Bó *et al*., 2003) and buffaloes (reviewed in Baruselli *et al*., 2007) was observed.

The administration of 1 mg (Bartolomeu, 2003) or 1, 2.5 or 5.0 mg of estradiol benzoate (Moura, 2003) in progestin-treated buffalo results in emergence of a new follicular wave between 3 to 6 days after treatment in more than 90% of buffalo cows. However, a delay in the onset of follicular wave (8.7 ± 0.27 days) was observed when estradiol valerate was administrated (Bartolomeu, 2003). Treatment with P4 + E2 can be used efficiently to synchronize the emergence of a new follicular wave in buffaloes.

Equine chorionic gonadotropin (eCG)

The treatment with equine chorionic gonadotropin (eCG) has been demonstrated as an alternative to increase final follicular development (follicular growth from luteolysis to ovulation) and pregnancy per TAI, mostly in anestrous buffalo during the non-breeding season (Carvalho *et al*., 2013). In buffalo with insufficient pulsatile release of LH to support the final stages of ovarian follicular development, treatment with eCG can improve the ovulatory response to the synchronization protocol and pregnancy outcome. The use of eCG in the synchronization protocol increases the diameter of the dominant follicle at TAI (13.7 ± 0.4 *vs*. 12.6 ± 0.6 mm, P = 0.09) and the ovulation rate (66.7 *vs*. 44.8%; P = 0.05). Moreover, eCG treatment results in increased CL diameter (15.8 ± 0.92 *vs*. 12.7 ± 0.77 mm, P = 0.03), increased P4 concentrations (0.59 ± 0.08 *vs*. 0.27 ± 0.05 ng/ml, P = 0.01) at the subsequent diestrus and increased pregnancy rate (52.7 *vs*. 39.4%, P = 0.03; Carvalho *et al*., 2013).

LH, hCG, GnRH and estradiol benzoate to synchronize ovulation

After luteolysis, synchronization protocols require the use of inducers of ovulation to achieve a synchronized ovulation. Timed artificial insemination (TAI) protocols generally incorporate gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), human chorionic gonadotropin (hCG) and estradiol esters to synchronize ovulation. The endocrine and follicular responses in buffalo to these different treatments are presented in Table 1.

All treatments for ovulation induction have satisfactory results in buffalo, with only particularities in the endocrine and follicular responses. Plasma P4 concentration at the subsequent diestrus was lower in GnRH (2.94 ± 1.51 ng/ml) than in hCG (4.02 ± 2.34 ng/ml; P < 0.05) treated buffalo for ovulation induction (Carvalho *et al*., 2007b). Furthermore, there is evidence that EB induces a greater release of LH compared with GnRH (Berber *et al*., 2007). and pre-exposure to P4 before EB administration anticipated the preovulatory-like LH surge in buffalo cows (Jacomini *et al*., 2014).

Table 1. Interval between treatment to induce ovulation and peak of LH, time to ovulation and ovulation rate in buffalo.

Treatment	Interval treatment to LH surge (h)	Interval treatment to ovulation (h)	Ovulation rate (%)	Reference
LH	-	24	93	Berber *et al*., 2002
hCG	-	24	81	Baruselli *et al*., 2003a; Carvalho *et al*., 2007a, b
GnRH	1-3	26-28	75-85	Berber *et al*., 2002, 2007; Baruselli *et al*., 2003b; Carvalho *et al*., 2013, 2017; Jacomini *et al*., 2014
Estradiol Benzoate	23-27	44	78-82	Berber *et al*., 2007; Jacomini *et al*., 2014; Carvalho *et al*., 2017

ART for artificial insemination

Artificial insemination (AI) has proven to be a reliable technology for buffalo producers to improve genetic progress and control venereal diseases in their herds. However, the traditional AI program is impaired by the low estrous detection efficiency due to the poor manifestation of the symptoms of estrus in buffalo and to operational difficulties to detect estrus (Baruselli *et al*., 2007). Currently, timed artificial insemination (TAI) can be applied routinely in the reproductive programs on farms. TAI protocols are designed to control of both luteal and follicular function, permitting the TAI without estrus detection with satisfactory pregnancy per AI (P/AI), during the breeding and non-breeding season.

Among the hormonal therapies developed for cattle, GnRH plus PGF2a-based TAI protocols (Ovsynch; Pursley *et al*., 1995) resulted in follicular response with effective synchronization of ovulation in cycling buffaloes during the breeding season (Baruselli *et al*., 2003b). However, when the Ovsynch protocol was used in anestrous buffaloes (without CL), results

were inferior to those obtained with cycling buffaloes. Souza *et al*. (2015b) verified that buffaloes without a CL at the beginning of the Ovsynch protocol responded poorly to the first (42.0 *vs*. 89.8% ovulation rate) and second (52.0 *vs*. 87.8% ovulation rate) GnRH treatments, and this resulted in a lower pregnancy rate after TAI (20.0 *vs*. 65.3%, respectively) compared to the animals with a CL. Results of several other studies revealed a high incidence of anestrus during the nonbreeding season (spring and summer), and lower pregnancy rates after TAI were reported when the Ovsynch protocol was used (7.0-30.0%; Baruselli *et al*., 1999, 2002, 2003b, 2007; De Rensis *et al*., 2005; Ali and Fahmy, 2007; Vecchio *et al*., 2013). Therefore, during the nonbreeding season, when a high incidence of anestrus is expected, lower pregnancy rates are encoutered in buffaloes synchronized with the Ovsynch protocol for TAI. On the contrary, studies have demonstrated similar pregnancy per TAI in both breeding and nonbreeding seasons after the use P4, E2, and eCG-based protocols (Baruselli *et al*., 2013; Carvalho *et al*., 2013; Monteiro, 2018; Table 2).

These data demonstrate that it is possible to establish an effective AI program in buffaloes throughout the year, however it is relevant to understand the interactions between ovulation synchronization treatments and the season of the year.

Table 2. Pregnancy per AI in lactating buffalo cows subjected to GnRH plus PGF2α (Ovsynch) or P4/E2 and eCG based protocol during the breeding and nonbreeding season.

	Breeding season	Nonbreeding season	P value
GnRH plus PGF2 (Ovsynch) [1]			
Pregnancy rate per TAI	48.8% (472/967)	6.9% (6/86)	0.001
P4/E2 and eCG[2]			
Pregnancy rate per TAI	66.7% (112/168)	62.7% (111/177)	0.31

[1]Baruselli *et al*. (2003b); Monteiro (2018).

ART for embryo production

Reproductive technologies, such as superstimulation for *in vivo* embryo production and ovum-pick-up (OPU) for *in vitro* embryo production (IVEP) can rapidly enhance genetics in buffaloes through both the female and male superior lineage. The *in vivo*-derived (IVD) embryo production has been shown to be feasible in buffalo, however low efficiency and limited commercial application has been documented (Baruselli *et al*., 2000; Campanile *et al*., 2010). Currently, a series of recent studies have demonstrated the potential of *in vitro* embryo production (IVP) in buffalo. Studies on the particularities of these biotechnologies in buffalo will be discussed.

Production of in vivo*-derived (IVD) embryos*

The multiple ovulation followed by TAI for *in vivo* embryo production is a technique that generates greater numbers of embryos per donor in cattle (Mapletoft *et al*., 2002). These techniques, which are associated with ET to recipients, are powerful tools to accelerate the gain in genetic programs (Bó *et al*., 2002; Baruselli *et al*., 2011). However, buffalo donors generally have lower embryo recovery rates than bovines. While buffaloes have shown follicular responses after superovulation treatment (mean of 15 follicles >8 mm), only a moderate ovulation rate (approximately 60%) and CL yield at the time of flushing (approximately 9 CL) and low embryo recovery rates (34.8%) have been obtained (Baruselli *et al*., 2000). The embryo recovery rate in superovulated buffaloes (approximately 20 to 40%) is lower than in cattle (63 to 80%; Boland *et al*., 1991; Adams, 1994; Vos *et al*., 1994; Shaw *et al*., 1995). This divergence in embryo recovery rates was hypothesized to be related to a failure of oocyte capture and/or of oocyte transport along the oviduct (Baruselli *et al*., 2000). In rabbits, the administration of sequential doses of $PGF_{2\alpha}$ during the periovulatory period stimulated the contraction of oviduct smooth muscles, allowing the activation of the oviduct fimbriae to capture the oocytes (Osada *et al*., 1999). Based on this observation, our research group (Soares, 2015) performed an experiment that evaluated the use of PGF2α (injectable or using a mini osmotic pump; OP) during the periovulatory period in superovulated buffaloes. However, no differences were found on the total number of recovered structures (G-CONT = 2.1 ± 0.8 *vs*. GPGF-IM = 2.1 ± 0.6 *vs*. G-PGF-OP = 1.4 ± 0.4; P = 0.58). The low embryo production per donor impairs the use of this biotechnology by buffalo producers.

In vitro *embryo production (IVEP)*

Due the scarce results of *in vivo* embryo recovery in superovulated buffaloes, the association of OPU with IVEP represents an alternative method of exploiting and multiplying genetic for superior merit (Boni *et al*., 1996; Neglia *et al*., 2003; Sá Filho *et al*., 2009). Historically, OPU-IVEP in buffaloes produced lower outcomes (Gasparrini, 2002; Sá Filho *et al*., 2009; Gimenes *et al*., 2010) than in bovines (Lonergan and Fair, 2008; Pontes *et al*., 2011). However, a series of recent studies have demonstrated the commercial potential of these techniques in the buffalo specie (Baruselli *et al*., 2013).

Two main biological problems seem to be related to the low efficiency of the OPU-IVEP technique in buffaloes: 1) low number of follicles on the ovary that results in low oocyte recovery per OPU and; 2) poor oocyte quality retrieved (only 27.3 to 31.3 % of

oocytes are classified as viable (Campanile *et al*., 2003).

The first limitation can be related to the lower number of follicles recruited per follicular wave (Baruselli *et al*., 1997), as observed in studies comparing buffaloes with *Bos indicus* cattle (Ohashi *et al*., 1998; Gimenes *et al*., 2015). Additionally, a higher level of follicular atresia was reported (Danell, 1987; Van Ty *et al*., 1989) and, consequently, a lower number of total recoverable and viable oocytes. Buffaloes and cattle raised with contemporary nutrition and management were compared *post mortem* by Ohashi *et al*. (1998), and *in vivo* by Gimenes *et al*. (2015). In both studies, lower number of follicles and viable oocytes were observed in buffaloes than in *Bos indicus* cattle.

The second limitation can be attributed to a more fragile zona pellucida (Mondadori *et al*., 2010) and a more fragile bonding between cumulus cells and the oocyte (Ohashi *et al*., 1998; Gasparrini, 2002) in buffaloes than in cattle.

Thus, to improve oocyte quality and recovery, studies were conducted by our research group to upgrade this biotechnology in buffaloes. Initially, we tested the hypothesis that bST could elevate circulating IGF-1 levels, promoting recruitment of a greater number of follicles and enhancing oocyte quality (Sá Filho *et al*., 2009). Although bST treatment resulted in greater numbers of aspirated follicles and retrieved oocytes per donor per session, reduced blastocyst production rate was observed (Ferraz *et al*., 2007, 2015; Sá Filho *et al*., 2009).

The phase of the estrous cycle is an important factor that directly influences the quantity and quality of oocytes obtained by OPU and, consequently IVP efficiency (Vassena *et al*., 2003). Thus, in another study buffalo (*Bubalus bubalis*), Nelore (*Bos indicus*) and Holstein (*Bos taurus*) heifers were synchronized to be submitted to OPU 1, 3 or 5 days after wave emergence. No effects were observed on the OPU-IVEP efficiency according to the different phases of the synchronized ovarian follicular wave in all genetic groups. However, the OPU-IVEP procedure was less efficient in buffalo and Holstein than in Nelore heifers (Gimenes *et al*., 2015).

The influence of season (winter; breeding season or summer; nonbreeding season) on oocyte viability (number of viable oocytes and mitochondrial DNA amount) was investigated in nulliparous and multiparous buffaloes (Macabelli *et al*., 2012). During summer, the amount of mtDNA was lower in oocytes from nulliparous than those from multiparous, but during winter mtDNA amount was greater in oocytes from nulliparous than those from multiparous. The mtDNA analyses do not suggest a negative effect of summer on oocyte viability in buffalo. Therefore, in tropical climates, the season would not appear to adversely affect oocyte quality and fertility. However, other studies carried out in buffalo showed an effect of season on either the number of follicles/viable oocytes or oocyte developmental competence, at different latitudes (Manjunatha *et al*., 2009; Di Francesco *et al*., 2011, 2012).

Number of oocytes retrieved per buffalo and its relationship with in vitro *embryo production and pregnancy*

The number of antral follicles in the early follicular phase is directly correlated with the ovarian reserve (Frattarelli *et al*., 2000). Indeed, the antral follicular population (AFP) directly represents the follicle cohort in the ovaries, which is associated with the number of oocytes retrieved per OPU for IVEP.

A large variability of AFP is reported among different females, however AFP count is highly repeatable within animal (Burns *et al*., 2005; Ireland *et al*., 2007), and anti-Müllerian hormone (AMH) can be considered a reliable endocrine marker of ovarian reserve (Ireland *et al*., 2007, 2008; Monniaux *et al*., 2012). In cattle, circulating AMH concentration can help veterinarians to predict AFP in ovaries (Ireland *et al*., 2008; Rico *et al*., 2009; Batista *et al*., 2014), response to SOV treatments (Rico *et al*., 2009; Monniaux *et al*., 2010a, b; Souza *et al*., 2015a), and more recently as a marker to predict IVEP performance of *Bos taurus* (Guerreiro *et al*., 2014 Gamarra *et al*., 2015; Vernunft *et al*., 2015) and *Bos indicus* breeds (Guerreiro *et al*., 2014).

Aiming to determine the relation between AMH and AFP we recently conducted a study in buffalo and cattle (Baldrighi *et al*., 2014; Liang *et al*., 2016). Despite the high variability in AFP among individuals within each genetic group, the AFP count was greater in Gir (*Bos indicus*) than in Holstein (*Bos taurus*) and Murrah (*Bubalus bubalis*) heifers ($P = 0.01$). Similarly, AMH concentration was lower ($P < 0.01$) for Holstein and Murrah heifers than for Gir heifers. In spite of the differences between genetic groups, a positive relationship among AFP and AMH concentration was detected within buffalo. These studies suggest AMH as endocrine marker to predict AFP and IVEP performance in buffalo.

Recently we have studied the relationship between AFP and *in vitro* embryo production and pregnancy rate in buffalo. The number of oocytes recovered per OPU (analyzed by tertile) had no effect on viable oocyte and blastocyst rates (Table 3). However, the number of blastocysts per OPU was greater when higher number of oocytes were recovered per OPU. Pregnancy rate following ET in buffalo was lower in donors with greater amounts of oocytes retrieved per OPU.

The results demonstrate that the number of oocytes recovered per OPU had a minor effect after ET both on blastocyst rate and pregnancy rates. However as more oocytes are collected, the number of produced blastocysts improves (Fig. 2). These results highlight the relevance to identify donors with greater potential to oocyte recovery per OPU to assure greater IVEP success, especially in buffalo that yield fewer oocytes per OPU than bovine. There was great variation in the number of oocytes retrieved per OPU (from 0 to 30), with a mean of 8.9 ± 5.0 per donor (Fig. 3). Therefore, a holistic approach selecting donors with greater genetic value (through genomics) and oocyte population (through AMH assays or ultrasound for quantify AFP) is highly advisable.

Table 3. Effect of retrieved numbers of oocytes per OPU from Murrah buffalo (*Bubalus bubalis*) donors on IVEP.

Items	TERTILE			P value
	Low	Medium	High	
Tertile, n	60	59	60	-
Retrieved oocytes, n	4.1 ± 0.14[c]	8.2 ± 0.19[b]	14.5 ± 0.5[a]	<0.0001
Viable oocytes, n	2.1 ± 0.17[c]	3.9 ± 0.24[b]	7.7 ± 0.37[a]	0.0002
Viable oocyte rate, %	51.8	47.8	53.2	0.31
Blastocyst per OPU, n	0.83 ± 0.11[c]	1.19 ± 0.13[b]	2.17 ± 0.24[a]	<0.0001
Blastocyst rate, %[1]	20.3	14.5	14.9	0.15
Pregnancy rate, %	44.2 (22/50)[a]	29.6 (21/70)[ab]	25.3 (33/130)[b]	0.05

[1]No. blastocysts/no. retrieved oocytes; Adapted from Soares *et al*. (2018); Centro de Pesquisa em Urologia, Escola Paulista de Medicina, São Paulo, SP, Brazil; unpublished data.

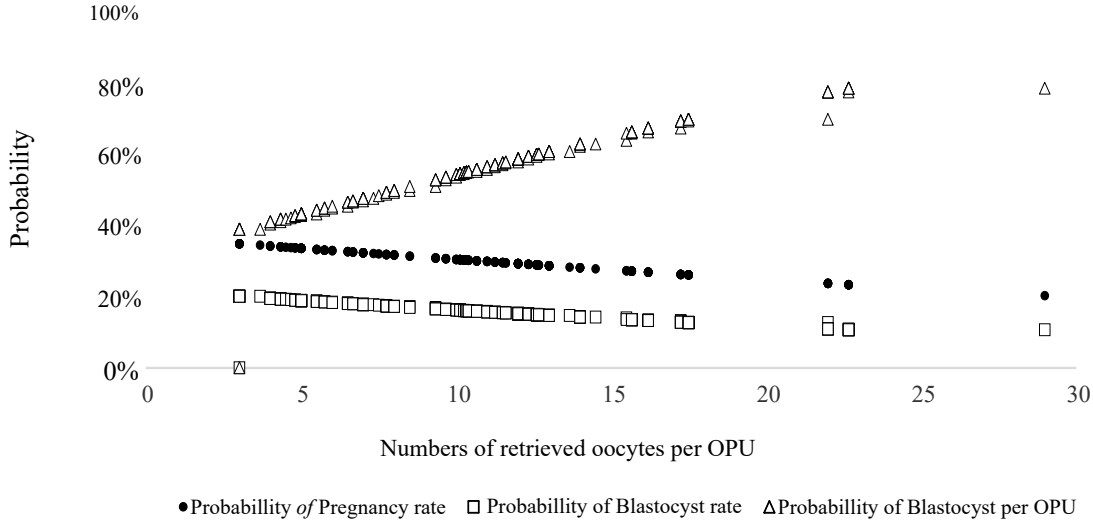

Figure 2. Probability of blastocyst rate (□), pregnancy rate (●) and blastocisty per OPU (Δ) as a function of numbers of retrieved oocytes per OPU in Murrah buffalo (*Bubalus bubalis*) donors (n = 179). Probability_blastocyst_rate = EXP (-0.0375* Oocytes_retrived -1.2673) / [1+ EXP (-0.0375 * Oocytes_retrived - 1.2673)]; P = 0.07; r2 = 0,02 Probability_pregnancy_rate = EXP (-0.0287 * Oocytes_retrived -0.5366) / [1+ EXP (-0.0287 * Oocytes_retrived - 0.5366)]; P = 0.41; r2 = 0.0025. Probability_blastocyst per OPU = EXP (+0.0891 * Oocytes_retrived -0.7164) / [1+ EXP (+0.0891 * Oocytes_retrived - 0.7164)]; P < 0.001; r2 = 0.35.

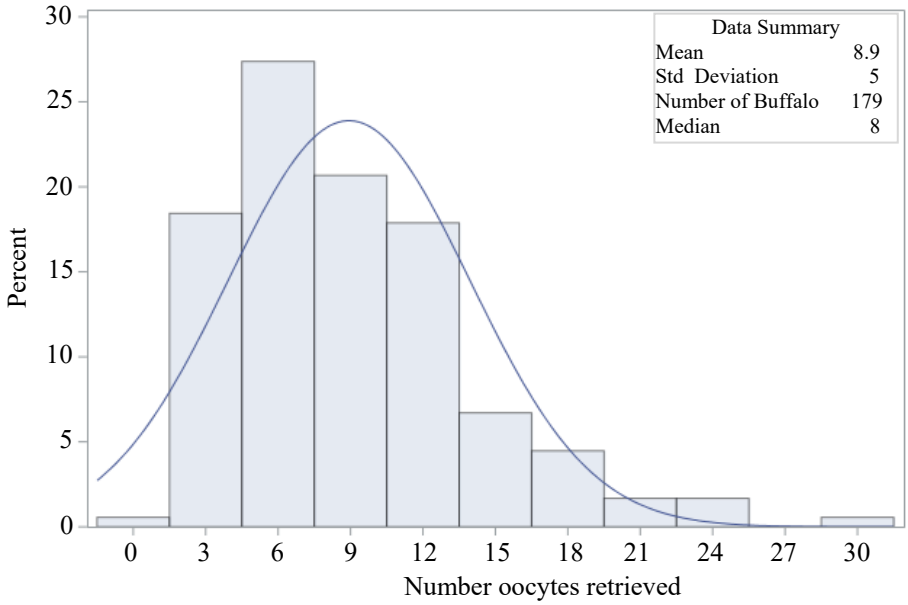

Figure 3. Distribution of oocytes retrieved per OPU in Murrah buffalo donor (n = 179).

Factors affecting OPU/IVF efficiency in buffaloes

Numerous factors may interfere with the efficiency of OPU/IVEP in buffaloes. Table 4 shows the effect of farm, category, postpartum period, reproductive status (pregnant or non-pregnant at the OPU) and BCS on IVEP production in buffalo donors (Carvalho *et al*., 2018; Unidade de Pesquisa e Desenvolvimento de Registro, Instituto de Zootecnia, Registro, SP, Brazil; unpublished data). The HPMIXED procedure of SAS through the best linear unbiased prediction (BLUP) analysis was utilized to rank sires, farms, category, postpartum period and BCS in terms of oocytes per OPU, number of blastocysts and blastocyst rate. Effects of farm (P = 0.05), category (P = 0.07) and reproductive status (P = 0.02) on the number of retrieved oocytes per OPU were found. Nulliparous and primiparous produced higher number of retrieved oocytes per OPU than multiparous. Furthermore, pregnant buffaloes (30 to 120 days of gestation) produced lower number of retrieved oocytes per OPU than non-pregnant. However, no effects were observed in the number of embryo produced per OPU and embryo rate (Table 4).

There is also a strong effect of the bull on the efficiency of IVF in buffaloes (Fig. 4). It is clear that semen used during *in vitro* procedures potentially influence IVEP and field fertility results (Watanabe *et al*., 2017). Top ranking sires yielded outstanding blastocyst rates, while poor sires produced low blastocyst rates.

Table 4. Effect of different variables in the IVEP production in buffalo donors.

Variable	Number of retrieved oocytes	P value	Embryo produced per OPU	P value	Embryo rate (%)	P value
Farm		0.05		0.75		0.54
A (n = 114)	9.6 ± 0.5[a]		1.7 ± 0.2		18.5%	
B (n = 269)	8.9 ± 0.3[ab]		1.7 ± 0.1		20.0%	
C (n = 38)	6.9 ± 0.9[b]		1.5 ± 0.3		26.4%	
Category		0.07		0.48		0.62
Nuliparous (n = 57)	10.2 ± 0.7		1.7 ± 0.2		17.9%	
Primiparous (n = 39)	11.1 ± 0.9		2.0 ± 0.3		21.2%	
Multiparous (n = 245)	8.34 ± 0.4		1.6 ± 0.1		18.4%	
Post partum period		0.92		0.45		0.26
≤117d (n = 68)	9.5 ± 0.8		2.1 ± 0.2		24.4%	
117d to 217d (n = 68)	9.1 ± 0.6		2.2 ± 0.3		17.7%	
>217d (n = 69)	8.5 ± 0.5		1.6 ± 0.2		26.0%	
Reproductive status		0.02		0.80		0.13
Pregnant (n = 52)	7.9 ± 0.6[b]		1.7 ± 0.2		23.3%	
Non pregnant (n = 139)	10.0 ± 0.5[a]		1.8 ± 0.1		17.5%	
BCS		0.98		0.88		0.44
≤3.0 (n = 25)	9.4 ± 1.3		2.0 ± 0.5		20.3%	
3.0 to 4.0 (n = 42)	9.6 ± 0.9		1.3 ± 0.3		16.8%	
>4.0 (n = 47)	9.8 ± 0.8		2.0 ± 0.3		19.1%	

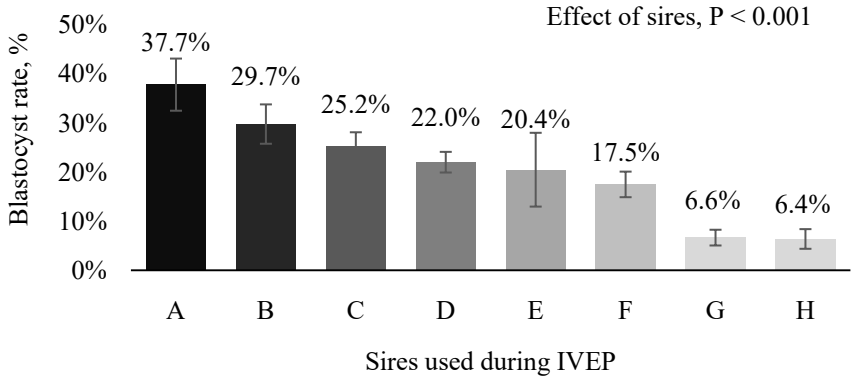

Figure 4. Blastocyst rate (%) according to sires used (n = 8) during IVEP from buffalo (*Bubalus bubalis*) donors (n = 379).

Superstimulation with FSH prior to ovum pick-up

Superstimulation with FSH previous to OPU has been used successfully for IVP programs in cattle, resulting in increased total embryo yields per OPU session (Goodhand *et al*., 1999; Sendag *et al*., 2008; Vieira *et al*., 2014), possibly because of the greater follicular diameters of the aspirated follicles. The FSH treatment for superstimulation can promote the growth of a homogeneous follicle population and to recover competent oocytes suitable for IVEP procedures.

In buffalo, superstimulation with FSH prior to OPU increased the proportion of large and medium-sized follicles available for the OPU procedure (Fig. 5). Consequently, the treatment enhanced the proportion of oocytes suitable for culture and resulted in greater blastocyst rates and embryo yield per OPU-IVEP session (Table 5).

These results provide evidence that superstimulation with FSH increased the proportion of medium-sized follicles available for the OPU procedure. Consequently, the treatment also enhanced the proportion of viable oocytes for culture and resulted in greater blastocyst rates and embryo yield per OPU-IVP session in buffalo.

Table 5. Summary of oocyte and embryo production (mean ± SEM) after OPU-IVEP in control and p-FSH-treated buffalo donors (heifers, primiparous and multiparous).

	Heifers		Primiparous		Multiparous		P value		
Item	Control	FSH	Control	FSH	Control	FSH	Treat	Cat	Treat*Cat
No.	18	18	15	15	21	21			
Total follicles aspirated, n	20.3 ± 2.4	18.3 ± 1.6	21.3 ± 4.4	17.7 ± 2.9	18.1 ± 2.2	17.6 ± 1.7	0.53	0.73	0.85
Total oocytes retrieved, n	11.7 ± 1.6	12.3 ± 1.0	11.5 ± 2.0	9.0 ± 1.2	8.7 ± 1.0	9.3 ± 1.2	0.85	0.05	0.46
Recovery rate, %	68%	73%	66%	55%	53%	53%	0.92	0.71	0.92
Viable oocyte, n	6.6 ± 1.3	7.8 ± 0.9	5.9 ± 1.5	5.67 ± 1.1	4.3 ± 0.7	5.6 ± 0.9	0.26	0.08	0.72
Viable rate, %	50%	58%	47%	56%	50%	57%	0.03	0.46	0.95
Embryo per OPU	1.8 ± 0.5	3.7 ± 0.7	2.4 ± 0.6	2.7 ± 0.8	2.0 ± 0.5	2.6 ± 0.7	0.07	0.25	0.22
Blastocyst rate, %	17%	34%	27%	28%	24%	32%	0.03	0.89	0.25

Adapted from Soares *et al.* (2018); Centro de Pesquisa em Urologia, Escola Paulista de Medicina, São Paulo, SP, Brazil; unpublished data.

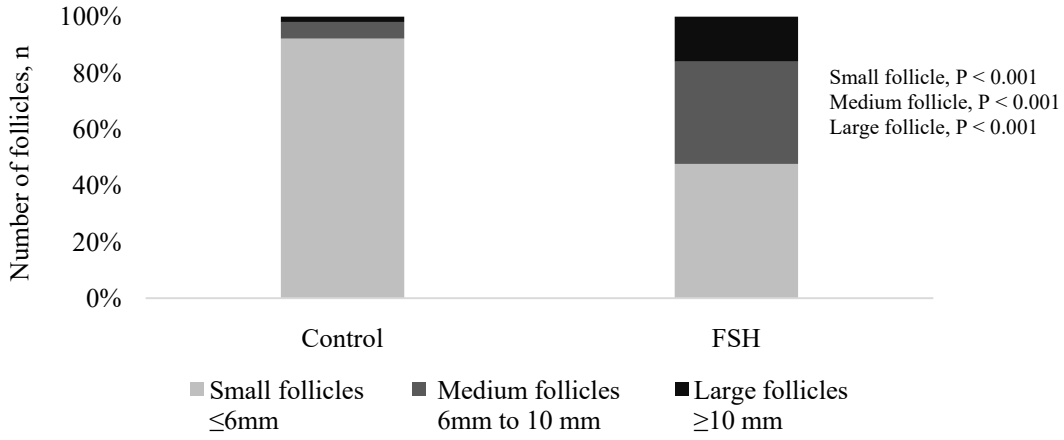

Figure 5. Proportion of small (<6 mm), medium (6-10 mm), and large follicles (>10 mm) in buffalo donor submitted to OPU with and without FSH superstimulation prior to OPU.

Buffalo calves as oocyte donors

With the advent of genomic technology in association with the traditional genetic evaluation, the use of calves as oocyte donors is an important strategy to accelerate genetic gain by decreasing generation intervals (Armstrong *et al*., 1992; Lohuis, 1995; Camargo *et al*., 2005). Several research groups have successfully produced viable embryos from prepubertal heifers (Armstrong *et al*., 1992; Revel *et al*., 1995; Fry *et al*., 1998; et al., 1998; Taneja *et al*., 2000; Baruselli *et al*., 2016) in cattle. However, there are some concerns that oocytes from young females have a lower developmental capacity than those from adult donors (Khatir *et al*., 1996; Presicce *et al*., 1997; Majerus *et al*., 1999; Palma *et al*., 2001). In buffalo, our group compared the embryo production of calves (from 2 to 4 months of age) in relation to prepubertal heifers (from 13 to 15 months of age) and lactating buffalo cows (Silva *et al*., 2017). The calves received sheep intravaginal P4 device (day 0) and were treated with 140 mg of FSH in 4 decreasing doses at 12h

intervals on day 5 and day 6. Calves were aspirated on day 7 by laparoscopy (LOPU - Laparoscopy Ovum Pick Up) and prepubertal heifers and adult lactating cows by intravaginal follicular aspiration (OPU). Both LOPU and OPU were performed on the same day and the same sire was used for IVF. Data are shown in the Table 6.

The calves embryos produced (n = 8) were transferred to synchronized recipients at the São Paulo University Campus and three pregnancies were diagnosed (pregnant/transferred = 38%; 3/8) at the 30 and 60 days of gestation and three healthy calves were born, demonstrating the viability of this biotechnology for buffalo.

Table 6. Number oocytes retrieved and blastocysts produced (mean ± SEM) after LOPU-IVEP in buffalo donor calves and after OPU - IVEP in prepubertal heifers and cows.

	Category			P value
	Calves	Pre-pubertal heifers	Lactating cows	
No.	8	10	10	
Total oocytes retrieved, n	10.9 ± 3.3[ab]	15.5 ± 2.1[a]	5.8 ± 1.3[b]	0.007
Viable oocytes, n	7.6 ± 2.7	6.2 ± 1.6	3.2 ± 0.9	0.11
Viable oocytes rate, %[a]	63.9[a]	39.3[b]	54.1[a]	0.01
Total oocytes cleaved, n	2.7 ± 0,9	3.1 ± 0.7	2.1 ± 0.4	0.52
Cleavage rate, %[b]	30.3[ab]	20.8[b]	37.6[a]	0.04
Viable embryos, n	1.0 ± 0.6[b]	1.5 ± 0.3[a]	1.1 ± 0.4[ab]	0.02
Embryos rate, %[c]	5.1[b]	9.3[a]	15.4[a]	0.05

Adapted from Silva *et al*. (2017).

Embryo recipient synchronization

The inefficiency in estrus detection, especially in buffalo, has limited its widespread application and greatly increased the cost of embryo transfer commercial operations. The incorporation of techniques designed to control follicular wave dynamics and ovulation reduces the problem of estrus detection and provides possibilities for the application of efficient FTET programs in buffalo. At unknown days of the estrous cycle (day 0), buffalo recipients were treated with intravaginal progesterone device plus 2 mg of EB (im). Nine days later (day 9), the P4 device was removed and the recipients received PGF and eCG (400 IU). On day 11, recipients were treated with GnRH and on day 17 recipients received a FTET (Saliba *et al*., 2013; Soares *et al*., 2015). The results showed similar efficiency for FTET when different categories of recipients (nulliparous, primiparous and multiparous) were used (Soares *et al*., 2015).

Summary and conclusions

Currently there is technology overall to establish efficient programs for the use of ART in buffalo. The control of follicular wave emergence and ovulation at predetermined times, without estrus detection, has facilitated the AI programs and the donor and recipient management. Synchronization protocols are designed to control both luteal and follicular function and permit fixed-time AI with high pregnancy rates during the breeding (autumn–winter) and nonbreeding (spring and summer) seasons. The OPU/IVEP is showing promising results, and has become an alternative to superovulation for *in vivo* embryo production. The use of this biotechnology makes possible to promote a rapid enhancement in genetics through both the female and male lineage. Therefore, the ART are being established and can collaborate for genetic improvement and reproductive efficiency, increasing the meat and milk production of the buffalo herds.

Acknowledgements

The authors would like to acknowledge the support from FAPESP, CNPq and CAPES of Brazil. We also would like to thank Otavio Bernardes (Fazenda Paineiras da Ingai) for the great collaboration in the development of these research projects.

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DOI: 10.21451/1984-3143-AR2018-0022

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

From reproductive technologies to genome editing in small ruminants: an embryo's journey

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Abstract

The beginning of this century has witnessed great advances in the understanding of ovarian physiology and embryo development, in the improvement of assisted reproductive technologies (ARTs), and in the arrival of the revolutionary genome editing technology through zygote manipulation. Particularly in sheep and goats, the current knowledge on follicular dynamics enables the design of novel strategies for ovarian control, enhancing artificial insemination and embryo production programs applied to genetic improvement. *In vitro* embryo production (IVEP) has evolved due to a better understanding of the processes that occur during oocyte maturation, fertilization and early embryo development. Moreover, interesting advances have been achieved in embryo and oocyte cryopreservation, thereby reducing the gap between the bench and on-farm application of IVEP technology. Nevertheless, the major breakthrough of this century has been the arrival of the CRISPR/Cas system for genome editing. By joining diverse disciplines such as molecular biology, genetic engineering and reproductive technologies, CRISPR allows the generation of knock-out and knock-in animals in a novel way never achieved before. The innumerable applications of this disruptive biotechnology are challenging the imagination of those who intend to build the animals of the future.

Keywords: cryopreservation, genome modification, IVF, MOET, ovine, transgenesis.

Introduction

Sheep and goats have been used in science not only because both species have great relevance as suppliers of food and wool/hair, but also due to their plasticity as experimental models for different purposes. Like Dolly - the world's most famous sheep – these animals have been studied for basic reproductive physiology as well as for developing novel biotechnologies. In this review, we briefly describe the main advances of the last 20 years in both species related to ovarian physiology, the progress of reproductive technologies, and the contribution of embryo manipulation to genome editing (Fig. 1). Because extensive information has been discussed in previous reviews, we just highlight the latest advances and focus on the main results recently obtained in our laboratory.

Follicular dynamics in sheep and goats

Since a deep knowledge of ovarian physiology is required for the control of reproduction and the application of assisted reproductive technologies (ARTs; Fig. 1), a brief update of ovarian follicular dynamics is presented. The follicular wave pattern in sheep and goats was clearly described in the 1990s with the advent of transrectal ultrasonography for the study of ovarian physiology (reviewed in sheep by Evans, 2003, and in goats by Rubianes and Menchaca, 2003). Follicular waves in these species have been reported during the estrous cycle, prepubertal period, seasonal anestrus and early gestation. This phenomenon is determined by the precise action of the endocrine system pathways through the combined action of gonadotropic hormones and steroids, as well as through the differential ability to express hormonal receptivity of the dominant or subordinate, growing or regressing, large or small follicles. The interrelationship between these endogenous factors has direct implications on the exogenous control of ovarian function for estrus synchronization and superovulation. During follicular wave emergence, the recruitment of small follicles is promoted by an FSH surge that precedes each wave, while after selection, the growth of medium and large follicles is supported by the LH hormone. Endogenous (and exogenous) progesterone influences follicular waves; high hormonal levels promote follicular turnover mainly by inhibition of LH support, while low progesterone levels promote the growth of the largest follicle, inducing a persistent follicle that negatively affects fertility. The emergence of each wave is unpredictable with the exception of wave 1, which emerges on day 0 in the interovulatory interval, soon after ovulation, and has practical implications for exogenous ovarian control. These mechanisms related to follicular waves pattern were extensively studied in several reports and are further described in previous reviews on sheep (Evans, 2003; Bartlewski *et al*., 2011) and goats (Rubianes and Menchaca, 2003).

Since follicular waves – especially follicular recruitment and dominance – have a substantial effect on the response to gonadotrophin and steroid administration, new hormonal protocols have been designed to improve pregnancy rates with a single insemination without the need for estrus detection (i.e., fixed-time artificial insemination or FTAI) or to enhance multiple ovulation and embryo transfer (MOET) programs.

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Received: February 4, 2018
Accepted: May 3, 2018

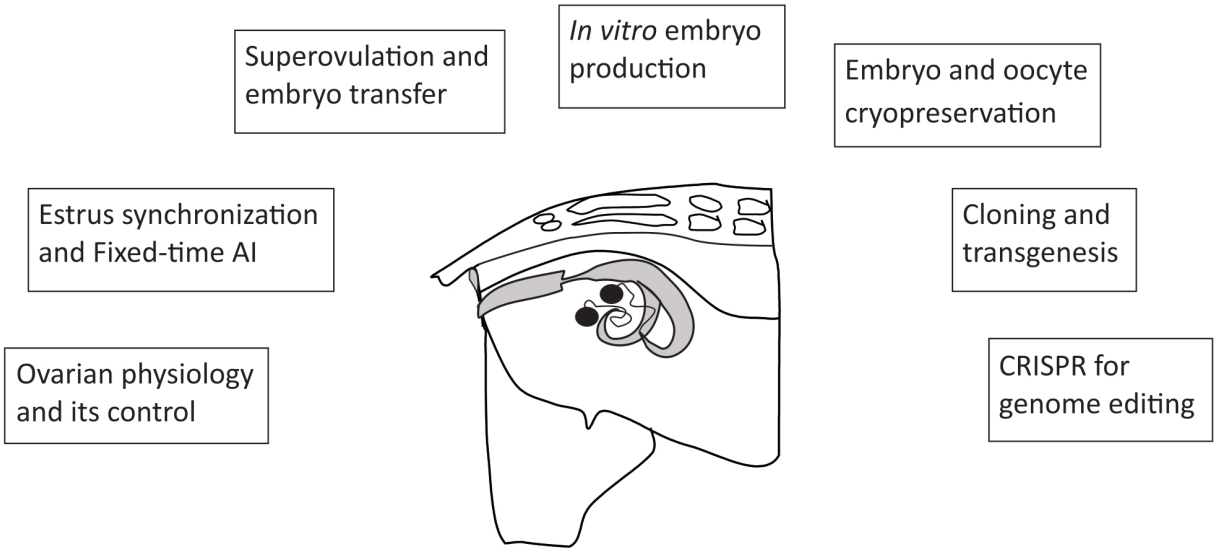

Figure 1. Contribution of the ewe/goat to the development of reproductive technologies. The understanding of ovarian physiology, as well as oocyte maturation, fertilization and embryo development, has allowed new advances in fixed-time artificial insemination (AI), in superovulation and in embryo transfer technologies. This has led to greater efficiency of *in vitro* embryo production and cryopreservation, application of modern technologies such as cloning and transgenesis, and more recently, embryo-related technologies have contributed with the arrival of the CRISPR/Cas system for genome editing.

Synchronization of ovulation for FTAI

The information reported on follicular dynamics during the 1990s and 2000s was not considered in the traditional treatments for estrus synchronization, as they were designed in the 1970s-1980s. The implementation of artificial insemination, particularly without estrous detection, requires a precise control of a) luteal function, b) follicular development, and c) ovulation. Traditional protocols were designed with the aim of controlling the luteal function by exogenous progesterone/progestogens administration for 10-14 days. The new protocols for FTAI achieve a better control of follicular development and ovulation that enhances fertility, mainly by reducing progesterone exposure from 10-14 days to 5-7 days (short-term protocols). This simple strategy avoids the detrimental effect of low progesterone concentrations during long periods when the intravaginal devices are placed for many days. These short-term protocols for FTAI (Menchaca and Rubianes, 2004) consist of exposure to exogenous progesterone (usually in a CIDR-type intravaginal device) for 5-7 days, associated with a dose of equine chorionic gonadotropin (eCG) and prostaglandin (PG) F2α at the time of device removal. This protocol induces high progesterone concentrations that promote follicular turnover soon after device insertion (low LH support), and leads to the growth of a new follicle that reaches a preovulatory diameter 5-7 days after the intravaginal device insertion. Estrus, LH peak and ovulation occur approximately 30, 40 and 60 h after device removal, respectively (goats: Menchaca *et al*., 2007a; Vilariño *et al*., 2011; sheep: Vilariño *et al*., 2010, 2013). The pregnancy rate obtained with the short-term protocol associated with FTAI, and associated with natural mating or conventional artificial insemination, has been previously published in several reports in sheep and goats (Ungerfeld and Rubianes, 1999; Menchaca and Rubianes 2004, 2007; Fonseca *et al*., 2005, 2017). In addition, we have recently generated new information on large-scale FTAI programs on more than 13,000 ewes (Menchaca, 2018; IRAUy, Montevideo, Uruguay; unpublished results). In these programs, progesterone priming was administered by using intravaginal devices containing 0.3 g of progesterone (DICO, Syntex, Argentina) as described previously (Vilariño *et al*., 2010; Santos-Neto *et al*., 2015a). In one experiment, the short-term (6 days) *vs.* long-term (14 days) protocol was compared in 1,750 multiparous sheep that received intrauterine insemination by laparoscopy. The pregnancy rate was significantly higher with the short-term rather than the long-term treatment (43.5 *vs.* 37.8%, respectively; $P < 0.05$). In a following experiment, to further compare high *vs.* low progesterone concentrations, 922 females were treated for 6 days with a new intravaginal device (high progesterone for a short time) or for 14 days with a second-use device previously used for 6 days (low progesterone for an extended length). The pregnancy rate was also higher for the shorter treatment (41.2 *vs.* 29.1%, respectively, $P < 0.05$). These results confirm previous studies reported in sheep and goats (Menchaca and Rubianes, 2004), adding more evidence to the concept that fertility falls as the progesterone levels decreases when using intravaginal devices for long periods.

In another experiment on 3,893 multiparous ewes, we evaluated the best moment for FTAI with this 6-day protocol followed by cervical or intrauterine insemination from 46 to 56 h after device removal (the progesterone device was removed in the morning on day 6; Menchaca, 2018; IRAUy, Montevideo, Uruguay;

unpublished results). When new devices were used, the greatest pregnancy rate with cervical insemination was obtained when FTAI was performed on the morning of day 8 (i.e., 46 to 50 h from device removal) rather than in the afternoon (i.e., 52 to 56 h), while with intrauterine insemination greater pregnancy rate was obtained with FTAI in the afternoon. Interestingly, the pregnancy rate with second-use devices was similar between those ewes with FTAI in the morning and in the afternoon, both by cervical and intrauterine insemination. This difference between new and used devices is probably related to a wide period of ovulation in the females treated with used devices (Fig. 2). The Short-term protocol for FTAI in sheep has also been evaluated by transcervical insemination route through cervical retraction, achieving an intermediate pregnancy rate between conventional cervical and laparoscopic intrauterine insemination (Casali *et al*., 2017).

In summary, different studies reported during the last few years show that short-term protocols using intravaginal progesterone devices result in a series of benefits compared with the long protocols used previously, namely, better control of follicular response and ovulation, acceptable pregnancy rates, shorter periods for implementation, and eventually, the possibility of reuse of silicone devices, thus reducing the cost of the treatment. The current protocol for FTAI applied in our practice is depicted in Fig. 2.

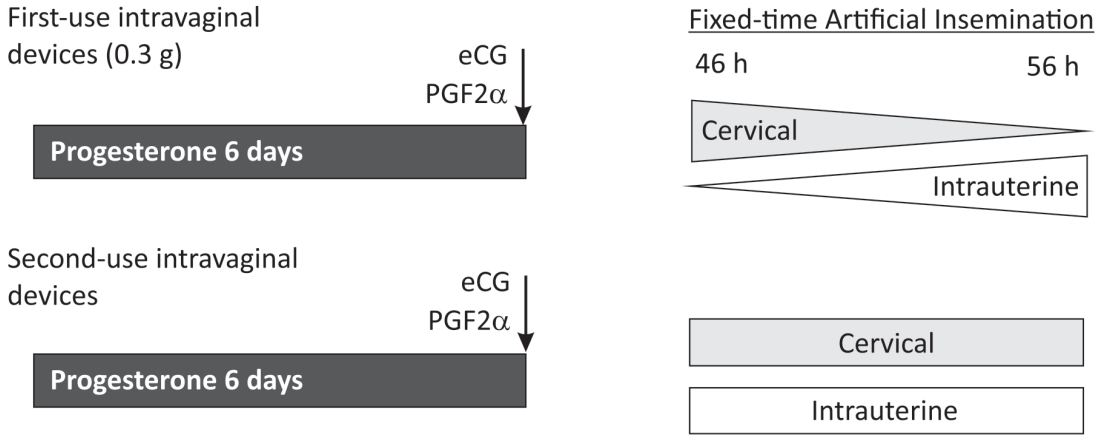

Figure 2. The short-term protocol for Fixed-time Artificial Insemination (FTAI) in sheep and goats. The protocol consists of progesterone treatment, administered by intravaginal devices (e.g., CIDR or DICO) for 5-7 days associated with one dose of equine chorionic gonadotrophin (eCG) and prostaglandin (PG) F2α at the time of device removal. First-use (new devices) or second-use (used previously by 5-7 days) intravaginal devices can be used. In sheep, for first-use devices, FTAI should be performed on the morning of day 8 (46-50 h after device removal) by the cervical route, or in the afternoon (52-56 h) by the intrauterine route. For second-use devices, FTAI could be performed by both insemination routes in the morning or in the afternoon without affecting fertility.

Superovulation and *in vivo* embryo production

The current knowledge about follicular dynamics has also enabled the development of new superstimulatory treatments for embryo production. It has been shown that the presence of a dominant follicle at the beginning of a superstimulatory treatment has a detrimental effect on the response to superovulation and embryo production (reviewed by Menchaca *et al*., 2010). Because 70 to 85% of donors have a dominant follicle at the moment of the first FSH administration in conventional treatments (Veiga-Lopez *et al*., 2005; Menchaca *et al*., 2007b), at least three new alternatives to synchronize the emergence of a new follicular wave before FSH administration have been proposed by different authors (Menchaca *et al*., 2002, 2007b; Cognié *et al*., 2003; Bartlewski *et al*., 2008). In general, these three strategies results in a better control of follicular dynamics and a greater superovulatory response, taking advantage of the spontaneous recruitment that normally occurs within the emergence of a follicular wave. One of these treatments is known as the Day 0 protocol, which consists of the superstimulation of wave 1 (Menchaca *et al*., 2002, 2007b, 2009, 2010). This protocol initiates FSH treatment when the first follicular wave emerges at the time of ovulation (i.e., on day 0 of the cycle), thus requires the synchronization of the ovulation of the dominant follicle to promote follicular turnover and the emergence of wave 1. The Day 0 protocol is depicted in Fig. 3. This treatment has improved ovarian response and embryo production compared to traditional treatments, both in sheep and goats (Menchaca *et al*., 2010).

We have recently demonstrated in sheep the convenience of exposing the oocyte to high progesterone concentrations prior to maturation, i.e., during preovulatory follicular development (Cuadro *et al*., 2018; IRAUy, Montevideo, Uruguay; submitted article). Interestingly, the induction of high progesterone levels for three days before luteolysis (i.e., during the FSH treatment) improves fertilization rate and embryo yield. In a subsequent study in which the oocytes were aspirated and subjected to *in vitro* fertilization, it was demonstrated that this enhancement was due to a greater oocyte developmental competence (Menchaca *et al*., 2018). For this reason, we have added into the Day 0 protocol

the administration of an intravaginal device with progesterone during the FSH treatment (Fig. 3). Because the use of progestogens instead of progesterone does not always induce the same response (Santos Neto *et al*., 2015a), the use of progestogens should be evaluated before its application in this superstimulatory treatment. These and other refinements have been incorporated in the Day 0 protocol by different authors (Tasdemir *et al*., 2011; Balaro *et al*., 2016; Lima *et al*., 2016; Mogase *et al*., 2016; Souza-Fabjan *et al*., 2017).

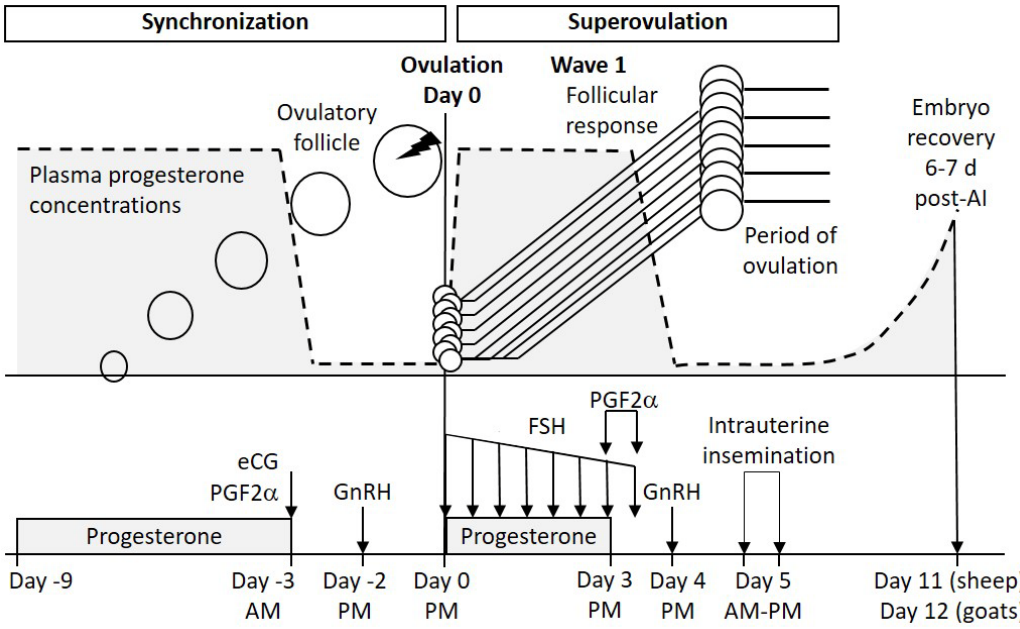

Figure 3. Ovarian superstimulatory treatment (Day 0 protocol) for embryo production in sheep and goats (adapted from Menchaca et al., 2010; Cuadro et al., 2018). During the synchronization period (left), the ovulation is induced to synchronize the emergence of wave 1. During the superovulation period (right), the FSH is administered to promote follicular recruitment of wave 1 in absence of a large dominant follicle. Additionally, progesterone treatment using an intravaginal device (i.e., CIDR-type device) is given during FSH administration to induce greater progesterone levels (gray color under dotted line) during superstimulated follicular growth. Prostaglandin (PG) F2α is administered in two half doses 12 h apart (the first one is given at device removal), GnRH is given 24 h later, and intrauterine insemination is performed 16-24 h after GnRH. Uterine flushing is performed 6 and 7 days after insemination in sheep and goats.

Before these new approaches for superovulation were developed through the control of follicular waves, some improvements to the traditional protocols had been proposed. In a large-scale program with 4,262 produced sheep embryos (Menchaca *et al.*, 2009), we attempted to enhance traditional protocols consisting of 12-14 days of exposure to progesterone before FSH administration. In one of these experiments on 239 donor sheep, the length of progesterone priming was evaluated to determine whether 12-14 days of exposure was necessary. Ewes were exposed to progesterone using CIDR-G (0.3 g of progesterone, Zoetis) for 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 days (23 to 25 donors in each experimental group). The results showed no significant differences in any of the evaluated variables with a similar embryo production, concluding that the length of the treatment could be more flexible, which has practical advantages for application in large-scale programs (Menchaca *et al*., 2009). In another experiment, the use of eCG associated with the administration of FSH was evaluated on 264 donor ewes. This has interesting implications because, even though no clear evidence was available, many practitioners use one dose of eCG at device removal during the FSH treatment, assuming that its LH action could promote final follicular development, enhancing embryo production. However, in this study, the treatment with eCG reduced the quantity and quality of produced embryos, suggesting the elimination of eCG at the end of FSH administration (Menchaca *et al*., 2009). In another experiment, in order to improve the synchronization of the ovulation and improve the embryo yield, the effect of GnRH administration 24 hours after CIDR removal was evaluated on 161 donor ewes. The GnRH treatment increased the fertilization rate and enhanced embryo production, and thus, we recommend the systematic use of GnRH after FSH administration (Menchaca *et al*., 2009). In summary, although traditional protocols have shown certain weaknesses that can affect the quantity and quality of the produced embryos (compared to the protocol for wave 1), for those practitioners that still use these treatments, the adjustments described above are recommended.

Superovulation and embryo production is a well-known technology, and for this reason, many other intrinsic and extrinsic factors affecting its success have been clearly identified. Since not all of the published

information can be included, for further information we recommend previous reviews encompassing more global ideas of this technology (Gonzalez Bulnes *et al*., 2004; Menchaca *et al*., 2010; Bartlewski *et al*., 2016).

In vitro embryo production

Significant fine-tuning of *in vitro* embryo production (IVEP) technology has been achieved from a better understanding of different molecular and biochemical events that occur during oocyte maturation, fertilization and early embryo development. In addition to the advantages of *in vivo* embryo production related to selective breeding, the *in vitro* system in livestock also allows the production of offspring from females that would not be able to reproduce using artificial insemination or MOET, such as prepubertal animals. The IVEP system is also useful for species conservation programs, and represents a valuable research tool in developmental biology and in the study of human infertility treatments. Even more interestingly, this technology provides the platform for the implementation of other technologies such as cloning, transgenesis and genome editing.

The success of an IVEP program depends largely on the availability of a continuous number of good quality oocytes. Although slaughterhouses represent a low-cost and abundant source of oocytes useful for research projects, oocytes from live animals are required for commercial application of IVEP. For this purpose, follicular aspiration by laparoscopy (LOPU) is mandatory in sheep and goats, providing approximately 10-14 oocytes per female in each session (Baldassarre *et al*., 2002, 2003b; Teixeira *et al*., 2011). Follicular aspiration of live animals needs to be associated with ovarian stimulation, usually achieved by using a single dose of FSH and eCG 36 h before LOPU (Baldassarre *et al*., 1996; Gibbons *et al*., 2007). The control of follicular dynamics previous to aspiration to improve *in vitro* oocyte developmental competence has been recently proposed (Menchaca *et al*., 2018) and further investigations are required.

Once cumulus oocyte complexes (COCs) are obtained, the success of the following steps depends, in addition to oocyte quality, also on the *in vitro* culture environment. Thus, culture media composition and protocols are determining factors for *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC), having a direct impact on pregnancy rate and some long-term consequences on offspring traits (Thompson *et al*., 2007). There are different *in vitro* media systems proposed and adopted, some of which are made in the laboratory and some of which are commercially available. The most commonly used medium for IVM in sheep and goats is tissue culture medium (TCM199) supplemented with estrus sheep/goat serum, gonadotrophins, cysteamine and antibiotics. For IVF, usually synthetic oviduct fluid (SOF) supplemented with heparin, hypotaurine and estrus sheep serum is used. After fertilization, the recommended IVC system in general consists of serum-free media under defined or semi-defined conditions, sequential or not, and always designed to suit embryo requirements. The embryo culture media and procedures most likely differ between laboratories, which also represents a source of variation. The main features of the procedures used in our laboratory are available in detail in previous reports (see Menchaca *et al*., 2016b). Under these conditions, the expected cleavage rate is approximately 80-90%, and the blastocyst rate is approximately 30 to 40% (number of blastocysts on day 6 from COCs in IVF). For further information about other factors affecting the success of IVEP, see recent reviews by Souza-Fabjan *et al*. (2014), Paramio and Izquierdo (2016) and Menchaca *et al*. (2016b).

Embryo cryopreservation

Embryo cryopreservation in sheep and goats was first reported in the 1970s by the slow freezing method for *in vivo* derived embryos, which has received moderate improvements during the recent years. On the other hand, novel information has been published with vitrification by minimum volume methods, mainly focusing on the cryotolerance of *in vitro* produced embryos.

Slow freezing is the default method for *in vivo* derived embryos used by many practitioners worldwide, resulting in good embryo cryotolerance and acceptable pregnancy rates. However, when slow freezing is applied to *in vitro* produced embryos, low outcomes are achieved (Massip, 2001, Santos-Neto *et al*, 2017). Substantial efforts and some interesting strategies have been proposed to improve the survival rate of *in vitro* produced embryos subjected to slow freezing, mainly in bovine embryos (Sudano *et al*., 2013; Sanches *et al*., 2016). However, the application of slow freezing to IVEP programs remains controversial. Multiple factors are associated with the lower cryotolerance of embryos produced *in vitro* compared with embryos produced *in vivo* (Seidel, 2006), such as excessive cytoplasmic lipid content, changes in the structural, physic and chemical characteristics of the embryo, the stage of embryo development, media composition, and protocols. Usually, to avoid the low embryo survival after cryopreservation, IVEP programs are conducted with fresh embryos. For this type of programs (IVEP with fresh embryos) in large-scale operations with many embryos being produced every week during long periods, requires a large number of ready-to-use recipients. In addition, all the well-known advantages of cryopreservation related to international trade and genetics dissemination remain limited for IVEP technology. In this context, new approaches for embryo cryopreservation deserve to be considered.

Since the 1990s, several methods of vitrification have been proposed in small ruminants as an alternative to slow freezing, both for *in vivo* derived and *in vitro* produced embryos. Vitrification has been reported in these species with different success rates, in reports comparing different types of cryoprotectants and times of exposure, cryo-devices and protocols (Traldi *et al*., 1999; Dattena *et al*., 2000; Papadopoulos *et al*., 2002; Cognié *et al*., 2003; Martínez *et al*., 2006;

Gibbons *et al*., 2011; Ferreira-Silva *et al*., 2017). More recently, the novel concept of minimum volume vitrification, with ultra-high cooling rates and high media viscosity, has appeared as a renewed hope for progress in embryo cryopreservation in various species (Arav, 2014). This idea has also been evaluated in caprine (Morató *et al*., 2011) and ovine embryos (Santos-Neto *et al*., 2015b; 2017) with promising results. We have been conducting a series of experiments with ovine embryos using the new minimum volume vitrification methods Cryotop and Spatula MVD. Both vitrification methods were reported for the first time in humans (Kuwayama, 2007) and mice embryos (Tsang and Chow, 2009), respectively and are routinely used in our laboratory for ovine (Santos-Neto *et al*., 2015b; 2017) and murine embryos (Meikle *et al*., 2018; Institut Pasteur, Montevideo, Uruguay, submitted article). In brief, the ovine IVP embryos vitrified with both minimum volume methods at different stages (at 2 and 6 days after IVF) showed acceptable *in vitro* survival, development and hatching rates (Santos-Neto *et al*., 2015b). In a subsequent study (Santos-Neto *et al*., 2017), we compared the pregnancy outcomes of 437 *in vivo* derived and *in vitro* produced embryos submitted to vitrification by the Cryotop or the Spatula MVD methods, or submitted to conventional freezing. Interestingly, the pregnancy rate after fixed-time embryo transfer was significantly greater for the Cryotop method, both for *in vivo* and *in vitro* embryos. For *in vivo*-derived embryos, vitrification by Cryotop reached a remarkable pregnancy rate of 67.1% (pregnant/transferred embryos), while for slow freezing it was 45.6% ($P < 0.05$) that is considered normal for frozen embryos. For *in vitro*-produced embryos, the pregnancy rate was 55.1% and 7.3% for Cryotop and conventional freezing, respectively ($P < 0.05$), which confirms the extremely low outcomes with slow freezing and demonstrates the acceptable performance with the Cryotop method. The results of the Spatula MVD method were intermediate (Santos-Neto *et al*., 2017). Therefore, vitrification by minimum volume methods appears to be an interesting cryopreservation tool for future implementation of IVEP programs, at least in sheep, and may be an alternative for replacement of slow freezing technology in conventional MOET programs. Vitrification by minimum volume methods is depicted in Fig. 4.

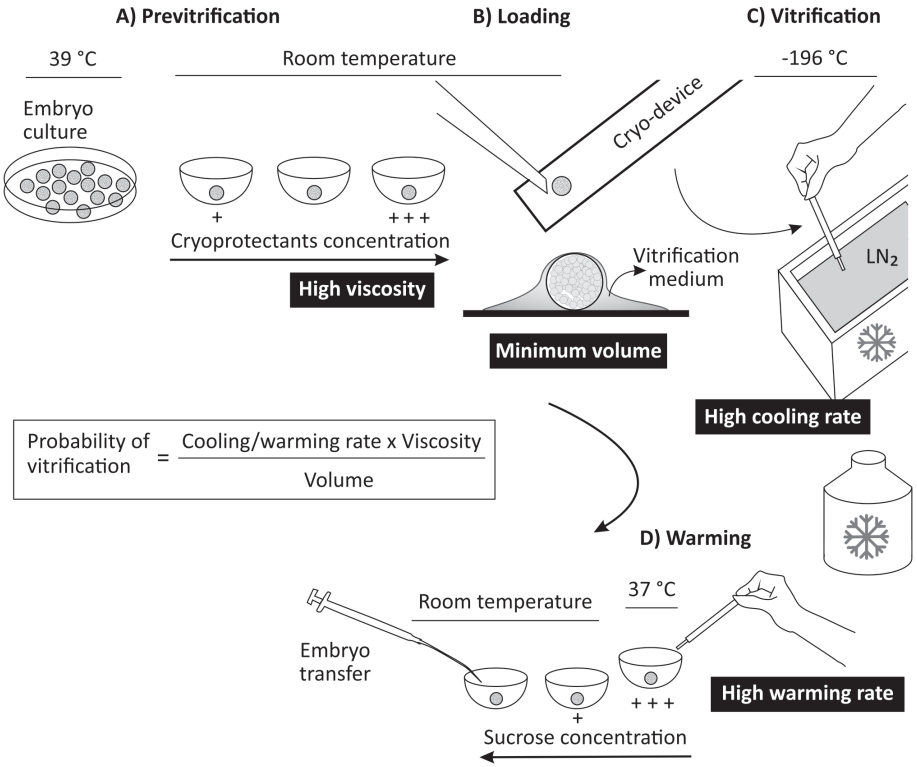

Figure 4. Embryo cryopreservation by minimum volume vitrification (e.g., Cryotop or Spatula) used in our Laboratory (dos Santos et al., 2015; 2017; Meikle et al., 2018; Barrera et al., 2018). Before vitrification (A), the embryos are equilibrated at room temperature with increasing concentrations of cryoprotectants to reach high viscosity. The embryos are loaded on a cryo-device (Cryotop or Spatula) (B), and the vitrification media is removed from the surface until reaching to reach minimum volume (i.e., ~ 1 µl). Then, the loaded device is rapidly plugged into liquid nitrogen (LN_2) to reach an ultra-fast cooling rate (C). Warming is performed by plugging the device directly from LN_2 into a sucrose containing media at 37°C (D). Embryos are washed successively in decreasing sucrose concentrations and handling medium, and finally are transferred into recipient females. This protocol sticks to the concept proposed by Arav (2014) to enhance the probability of vitrification (i.e., high cooling/warming rate, high viscosity, minimum volume). The methods have been modified from Kuwayama (2007) and Tsang and Chow (2009).

Recently, we have included the minimum volume method Cryotop in the routine production of microinjected embryos for CRISPR gene editing technology. The possibility of temporary dissociation among embryo production and transfer, simplifies the complex operation of carrying out IVEP, micromanipulation protocols for CRISPR injection, recipient management, estrous synchronization, and embryo transfer simultaneously. This is particularly important in this kind of large-scale projects. Additionally, regarding the relevance of oocyte cryopreservation for different species (Vajta, 2000; Ledda *et al*., 2007), vitrification by minimum volume methods has been evaluated also in sheep oocytes with promising results (Succu *et al*., 2008; Barrera *et al*., 2018). However, even though important advances have been achieved on cryopreservation of *in vitro* produced embryos and oocytes, further investigations and some refinements are still necessary in order to obtain an easy, fast, low-cost and effective method for a wider application of this ART.

Embryo micromanipulation for genetic modification

Cell plasticity in terms of totipotency and pluripotency in zygotes and early embryos, respectively, enables novel strategies for genetic manipulation in experimental and farm animals. Traditionally, the ovine and caprine zygote has been microinjected into the pronucleus in order to add exogenous DNA to obtain transgenic founders for a specific gene (reviewed by Menchaca *et al*., 2016a). In these species, the pronucleus is difficult to visualize mainly because they contain a huge quantity of lipid droplets inside the cytoplasm, and centrifugation is usually required. The injection pipette loaded with the DNA fragment is inserted into the pronucleus and the DNA is released inside until swelling occurs. Although pronuclear microinjection was the unique technique for genome modification for many years, it has some disadvantages in livestock: i.e., <10% transgenic offspring efficiency, unpredictable gene integration and expression, high cost and time consuming projects, eventually with low feasibility and sometimes with frustrating results (Menchaca *et al*., 2016a). Some technical difficulties were overcome by other available techniques, such as SCNT or lentiviral vectors. We have reported interesting results with transgenesis mediated by lentivirus in sheep (100% gene integration and 88.9% of expression in 9 lambs produced with GFP reporter gene), showing some additional advantages such as high efficiency through perivitelline microinjection of zygotes or 2-cell embryos instead of pronuclear microinjection or nuclear transfer (Crispo *et al*., 2015b). Transposons system (Bevacqua *et al*., 2017) and sperm-mediated gene transfer (Pereyra-Bonnet *et al*., 2011) have been reported in sheep or goats although scarce information is available in these species.

Since Dolly (Wilmut *et al*., 1997), and particularly since Polly and Molly (Schnieke *et al*., 1997), SCNT has been the default method for generating transgenic farm animals. The procedure has been extensively described in small ruminants (Keefer *et al*., 2001; Campbell, 2002; Menchaca *et al*., 2016a), and although some laboratories use this tool as routine, the technique is laborious and time consuming, with low final efficiency and fetal/placenta problems or newborn alterations (Martins *et al*., 2016). In sheep, some reports describe the use of this technique to generate transgenic animals expressing or overexpressing exogenous or endogenous genes, respectively (Schnieke *et al*., 1997; Deng *et al*., 2013; Zhang *et al*., 2013), or more recently, knock-out models combining the use of SCNT with cells edited through the use of endonucleases (Li *et al*., 2016). More information can be found in goats, with several reports describing a wide diversity of interesting models (e.g., Baguisi *et al*., 1999; Baldassarre *et al*., 2003a; Yu *et al*., 2012).

Recently described, the CRISPR/Cas (clustered regulatory interspaced short palindromic repeats/CRISPR associated protein) system allows the microinjection of single guides of RNA (sgRNA) directly into the cytoplasm, with no need to centrifuge the zygote as in pronuclear injection, or avoiding the nuclear reprogramming required for SCNT. In addition, it shows a high embryo survival and pregnancy rate with uncommon fetal and offspring losses, as well as milder ethical concerns. Most impressively, this tool allows not only add new DNA as the previous aforementioned tools, but also to silence or correct endogenous genes, or to introduce mutations in the genome in a way never achieved before. This biotechnology was selected as Science's 2015 Breakthrough of the Year (Travis, 2015) and represent one of the great advances, if not the greatest, of this century in biology and related fields.

CRISPR/Cas for genome editing

The first knock-out (KO) animal model produced with the CRISPR/Cas system was reported in mice in 2013 (Wang *et al*., 2013). Subsequently, new births were achieved in other species including sheep (Crispo *et al*., 2015*a*), goats (Wang *et al*., 2015), and more recently, cattle (Gao *et al*., 2017). CRISPR is the third generation of restriction endonucleases (enzymes with the ability to cut specific regions of DNA), and it has been proven to be much more efficient and easy to apply than its predecessors, namely, zinc finger nucleases (ZFN) and transcription activator–like effector nucleases (TALEN). The uniqueness of CRISPR/Cas lies in the use of RNA instead of proteins to confer target specificity. The different components of the system, including CRISPR RNA (crRNA), trans-activating crRNA (tracrRNA) and the Cas9 enzyme, work together to make the process effective and efficient. Basically, these molecular scissors recognize and bind a specific DNA sequence, producing double strand breaks that can be repaired by the host DNA repair mechanisms. This repairing can be done by non-homologous end joining (NHEJ) or by homology-directed repair (HDR) in order to produce insertions or deletions, which can cause a frameshift mutation and thus a null allele, or the exchange of a few nucleotides or even transgene insertion if the repair is through HDR. Due to its high efficiency, its easy and fast laboratory setup, and its

unlimited number of applications, the CRISPR/Cas system is a real revolution in several disciplines.

CRISPR technology applied in ruminants could be designed to enhance meat and wool production, increase the yield and quality of milk, generate disease resistant animals, provide animal resilience to hostile environments and enhance animal welfare, or to reproduce human diseases for biomedicine application. There is worldwide scientific consensus that this technology is far better than the previous tools. The relatively simply molecular biology setup, the ease of zygote microinjection into the cytoplasm, and the high mutation rate efficiency makes this tool available to many more laboratories working in different species. The sgRNA and Cas (RNA or protein) are mixed and loaded into the microinjection pipette, and few picoliters are injected into the cytoplasm or pronucleus of zygotes soon after fertilization. Surviving zygotes are left in culture until embryo analysis, cryopreservation or transfer (Fig. 5). In these conditions, acceptable outcomes have been obtained in sheep and goats with CRISPR injection into zygotes (Menchaca *et al*., 2016a). At the end of 2014, our first lambs edited by CRISPR/Cas9 were born (Crispo *et al*., 2015a) in a KO model to disrupt the myostatin gene (a gene encoding for a protein that inhibits muscle growth). We obtained 45.4% born lambs showing mutations at the myostatin locus, resulting in a body weight increase of 25% when compared to wild type counterparts. In a more recent sheep model of disease resistant animals (Menchaca, 2018; IRAUy, Montevideo, Uruguay; unpublished results), we obtained a 53.8% mutation rate by NHEJ in preimplantation embryos; additionally, in a following project to produce a human disease sheep model, we obtained 50.0% mutant embryos confirming the efficiency of this technique. Currently, about ten models for each species (sheep and goats) have reported successful births and several projects are ongoing in different laboratories worldwide. This technology is more recent in cattle, with the first birth of CRISPR-edited calves reported last year in China (Gao *et al*., 2017). These extraordinary outcomes and acceptable efficiencies encourage the widespread use of CRISPR/Cas to generate large animal models, including knock-outs and knock-ins with different purposes.

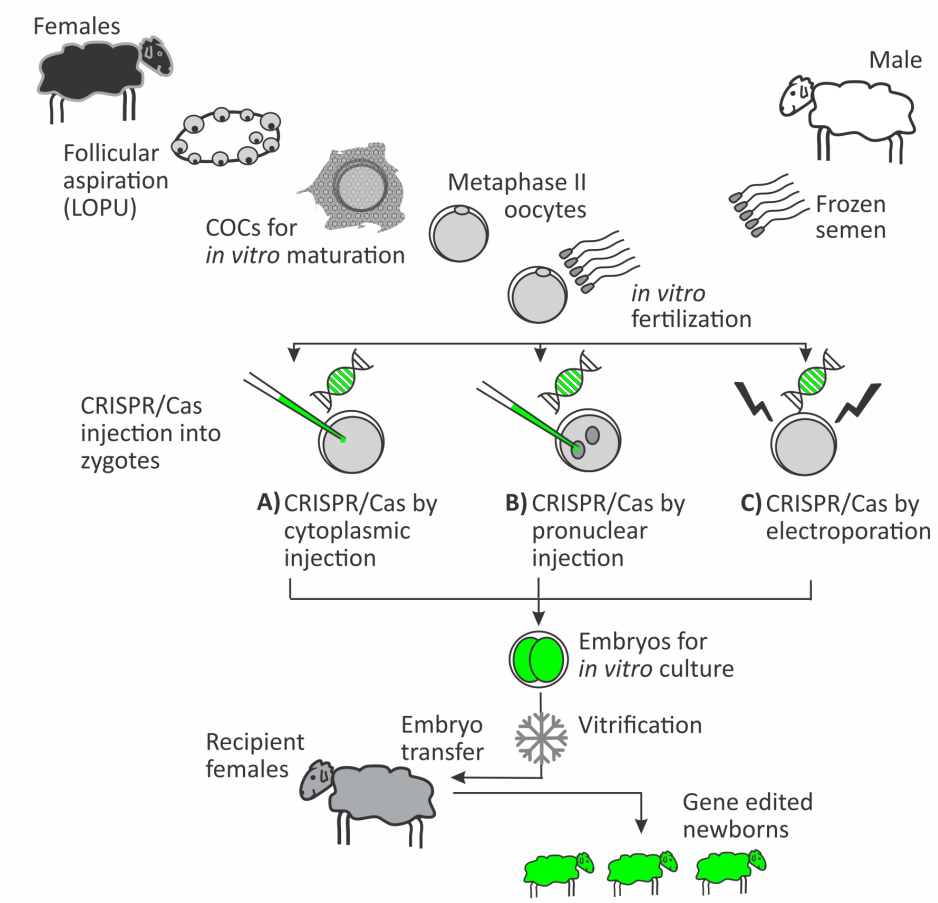

Figure 5. Schematic representation of embryo production and CRISPR microinjection for the generation of genome edited sheep and goats (adapted from Menchaca et al., 2016b). Immature oocytes can be obtained from live animals by Laparoscopic Ovum Pick-Up (LOPU) or from slaughterhouse ovaries. After *in vitro* maturation and fertilization, zygotes are prepared for CRISPR/Cas injection. The CRISPR delivery is performed by cytoplasmic microinjection (the default method in farm animals) (A), pronuclear microinjection (used in mice and rats) (B), or electroporation without the need of embryo micromanipulation (reported only in rats and mice) (C). Microinjected embryos are maintained in *in vitro* culture until fresh transfer or vitrification.

In order to refine and the CRISPR technology, different improvements are continuously being published. Reducing off-target sites by the use of nickases (Frock *et al*., 2015), improving HDR using SCR7 as an inhibitor of NHEJ (Vartak and Raghavan, 2015) or using Cas9 variants, Cas9 homologs and novel Cas proteins other than Cas9 (Nakade *et al*., 2017), will allow improvement of the efficiency and specificity of our models, including the targeting of multiple gene loci, generating knock-down or knock-in models, or include fluorescence imaging. Another improvement in overall efficiency is the introduction of CRISPR/Cas9 to zygotes through electroporation (Hashimoto *et al*., 2016; Remy *et al*., 2017), avoiding the use of expensive equipment and high skill human resources, with the possibility to edit hundreds of zygotes in few minutes. These and other improvements are envisioned for the following years, enhancing even more the power of this novel biotechnology.

Final remarks

Better understanding of ovarian physiology and embryo development has allowed the progress of artificial insemination and embryo transfer technology, mainly applied to genetic improvement and breeding programs. The *in vitro* technology for oocyte maturation, fertilization and embryo development open new opportunities for genetic improvement and, more importantly, for the development of innovative biotechnologies through embryo micromanipulation. Cryopreservation of *in vitro* produced embryos in small ruminants has been advanced recently, but further refinements are required. These advances have been useful but slightly modest, and they have mainly focused on the improvement and not on the disruption of existing technologies. On the other hand, genome editing appears to be a novel and powerful approach, and in contrast to previous breakthrough technologies that can take years of experience to master, the CRISPR system enables the rapid and widespread application of genome editing by new users in almost every species. In the coming years, this technology will be applied in livestock through the support of genetics industry, in public health through biomedical businesses, and in basic and applied research conducted by different scientific organizations. The animals of the future will be different, the CRISPR revolution has only just begun.

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DOI: 10.21451/1984-3143-AR2018-0033

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Natural and controlled ovulation in South American camelids

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Abstract

The four species of New World camelids and 2 species of Old World camelids derived from a common ancestor in North America. The reproductive characteristics, particularly those involving ovarian function and ovulation, are remarkably similar among the 6 living species of camelids, so much so that interspecies hybrids of nearly all possible combinations have been documented. Camelids are induced-ovulators, triggered by an ovulation-inducing factor in seminal plasma. The timing and mechanism of endocrine events leading to ovulation are discussed, as well as the discovery, identification and mode of action of the seminal factor responsible. The applied aspects of our present understanding are discussed with specific reference to controlled induction of ovulation, ovarian synchronization, and superovulation. Emphasis has been given to the literature on llamas and alpacas, with some reference to studies done in wild species of South American camelids and Old World camels.

Keywords: Camelid, ovary, ovulation, superovulation, synchronization.

Introduction

No large group of recently extinct placental mammals remains as evolutionarily cryptic as the approximately 280 genera grouped as 'South American native ungulates' (Welker *et al*., 2015). This is particularly true of the phylogeny and taxonomy of the family Camelidae. The crown family Camelidae originated in North America 40 to 45 million years ago (Ma) and evolved into two tribes, the Camelini (Old World camels) and Aucheniini (or Lamini, New World camelids) 11 to 17 Ma (Stanley *et al*. 1994; Heintzman *et al*. 2015). Recent paleogenomic data suggest that 2 major groups of the Camelini tribe, *Camelops* and *Paracamelus* gave rise to present day Camelus species (dromedary and Bactrian camels) after migrating to Asia across the Bering landbridge from 7 to 5 Ma. It is unclear if these 2 groups co-habited North America since *Paracamelus* died out in North America ~1 Ma, and *Camelops* (the largest of the ancestral camelids) died out in North America ~13 thousand years ago along with other of the Pleistocene megafauna (Heintzman *et al*., 2015).

Contrary to the previous notion of having descended from *Paleolama*, New World (or South American) camelids likely descended from another branch of the Aucheniini (Lamini) tribe called *Hemiauchenia* between 9-11 Ma (Wheeler, 1995; Scherer, 2013). After migration from North to South America across the Panamanian isthmus beginning ~2.7 Ma, only 2 genera (*Lama* and *Vicugna*) survived the end of the Pleistocene period ~10 thousand years ago, and only those in South America (Wheeler *et al*., 1995; Heintzmen *et al*., 2015). The Inca and Aymara empires of pre-colonial South America began domesticating South American camelids ~6 to 7 thousand years ago. However, as a result of severe population bottle-necks in both genera beginning at the time of the Spanish Conquest in 1532, and subsequent hybridization of domestic lines, the lineage of today's domestic species (alpaca and llama) is equivocal (Stanley *et al*., 1994). Currently, the domestic llama (*Lama glama*) is thought to have descended from the wild guanaco (*Lama guanicoe*) and the domestic alpaca (*Vicugna pacos*) from the wild vicuna (*Vicugna vicugna;* Wheeler *et al*., 1995).

All four species of New World camelids are capable of interbreeding and producing fertile offspring without apparent reduced fecundity, as are the two species of Old World camels (reviewed in Skidmore *at al*., 2001). Despite geographic separation for at least the last 11 million years, hybridization between Old and New World camelids has also been documented through the use of artificial insemination and transfer of hybrid embryos (Skidmore *et al*.,1999, 2001). Fecundity of Old x New World crosses, however, is very low. Of 102 artificial inseminations, pregnancy was detected in only 9 (9%) and only 1 live offspring was born (1%). While all camelid species have the same number of chromosomes (2n = 74), the genetic distance between Old and New world camelids is apparently sufficient to make the pairing of homologous chromosomes no longer possible.

The purpose herein is to provide an overview on what is known about ovulation in camelids. We've included a discussion of historical and contemporary studies on the nature and mechanism of ovulation, the role of ovulation-inducing factor (OIF) in semen, and implications for controlled induction of ovulation, ovarian synchronization, and superovulation. Emphasis has been given to the literature on llamas and alpacas, with some reference to studies done in wild species of South American camelids and Old World camels.

Camelids are induced ovulators

Gonadotropin releasing hormone (GnRH) is the central hypothalamic regulator of LH pulses in both

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Received: February 17, 2018
Accepted: May 5, 2018

spontaneous and induced ovulators. GnRH is produced by the hypothalamic neurons from a precursor polypeptide after enzymatic processing and then it is packaged in storage granules that are transported down neural axons to the median eminence (Fink, 1988). Immunocytochemical studies have indicated that GnRH neurons are scattered throughout the medio-basal hypothalamus (MBH), rostrally and dorsally to the preoptic area and ventromedial hypothalamic nuclei (Karsch, 1987). Although there are clear species differences in the localization of GnRH neurons, differences have not been related to the type of ovulation mechanism observed in domestic animals.

Spontaneous ovulators (cattle, sheep, horses, pig) have ovarian cycles where the periodic preovulatory LH surge and ovulation occur at regular intervals, controlled by the feedback effects of ovarian steroids on the pituitary gland and hypothalamus (Karsch, 1987; Turzillo and Net, 1999). In contrast, induced ovulators such as South American camelids do not have an ovarian cycle that is punctuated by regular periodic ovulation. Instead, copulation has been considered the required stimulus to induce ovulation (San Martin *et al*., 1968; England *et al*., 1969). In an early study designed to determine factors associated with eliciting ovulation in alpacas (Fernandez-Baca *et al*., 1970a), ovulation rate was compared among females that 1) were unmated, 2) were mounted only followed with or without artificial insemination, 3) had interrupted mating, 4) had sterile mating (vasectomized male) followed with or without artificial insemination, 5) had single or multiple uninterrupted mating (intact male), or 6) were given hCG. A high ovulation rate (80 to 100%) was observed in females mated by intact or vasectomized males or when hCG was given.

Timing of endocrine and ovarian events

In the first endocrine study to correlate circulating concentrations of LH with mating and ovulation in camelids (Bravo *et al*., 1990), LH increased at 15 min, peaked at 2 h, and declined to basal levels by 7 h after natural mating. The rapid increase in plasma LH after mating in llamas resembled that observed in rabbits after a single mating (Jones *et al*., 1976). Apparently, the number of matings did not increase either the ovulation rate in alpacas (Fernandez-Baca *et al*, 1970a) or the amplitude of the LH surge in llamas and alpacas (Bravo *et al*., 1992), in contrast to other induced ovulators such as the cat in which multiple mating increased both plasma LH amplitude and ovulation rate (Concannon *et al*., 1980).

The interval from stimulus to ovulation was not reported in the study by Fernandez-Baca *et al*. (1970a) because ovaries were collected from a slaughterhouse 3 days after treatment. In a study using one-time examination of the ovaries during necropsy at 2 to 6 h intervals after mating (1-5 alpacas/time interval; San Martin *et al*., 1968), ovulation had occurred as early as 26 h after mating (3/5 alpacas) but the mean interval to and distribution of ovulations was not reported. In a study using one-time laparoscopic examination of the ovaries of alpacas at 12 h intervals after mating (Sumar *et al*., 1993), the interval to ovulation was 30 to 72 h in 50% (38/76) and approximately 30 h in 24% (18/76) of females.

Incorporation of B-mode ultrasonography into studies of ovarian function enabled rapid advancement in our understanding of follicular and luteal dynamics and factors associated with ovulation in camelids. A wave-like pattern of ovarian follicular development has been documented in llamas (Adams *et al*., 1990), alpacas (Vaughan *et al*., 2004), vicunas (Aba *et al*., 2005) and guanacos (Riveros *et al*., 2010). The wave pattern was characterized by periodic increases (every 15 to 20 days) in the number of antral follicles and the selection of a single dominant follicle of ≥7 mm in both llamas and alpacas (Adams *et al*., 1990; Vaughan *et al*., 2004). It has been proposed that the ability of llamas and alpacas to ovulate in response to a mating stimulus is influenced by the developmental status of the dominant follicle at the time of mating (Adams *et al*., 1990). Based on daily ultrasonography of the ovaries in llamas, spontaneous ovulation occurred in 2 of 25 (8%) unmated llamas and failure to ovulate occurred in 5 of 49 (10%) mated llamas (Adams *et al*., 1990). In separate studies, ovulation occurred on the second day after mating in 75% (6/8, Adams *et al*., 1989) and 96% (26/27, Adams *et al*., 1990) of llamas. Collectively, the interval from the first mating to ovulation was 2.0 ± 0.1 days, and was not affected by lactational status or the type of mating (vasectomized *vs*. intact male). By ultrasonographic examination every 4 h (Ratto *et al*., 2006), the interval to ovulation was 30.0 ± 0.5, 29.3 ± 0.6, and 29.3 ± 0.7 h in llamas given natural mating or treated with either 12.5 mg LH or 50 ug GnRH analogue (gonadorelin acetate), respectively.

Changes in plasma progesterone concentration have been characterized after treatment with hCG to induce ovulation (Fernandez-Baca *et al*., 1970b; Adam *et al*., 1989), after mating with a vasectomized male (Sumar *et al*., 1988) or an intact male (Bravo *et al*., 1990, 1991), and throughout pregnancy (Leon *et al*., 1990). In a study involving daily examination and blood sampling (Adams *et al*., 1991), measurement of the diameter of the corpus luteum by transrectal ultrasonography was an accurate method of assessing luteal function (plasma progesterone concentration) in llamas ($r = 0.83$, $P < 0.0001$). Corpora lutea were not detected and plasma progesterone concentration did not exceed 0.4 ng/ml in anovulatory (nonmated) llamas. In ovulatory nonpregnant (vasectomy-mated) llamas, the corpus luteum reached maximal diameter (12.8 mm) on mean day 7 (day 0 = ovulation), and regressed between days 10 and 12. In pregnant llamas, luteal diameter continued to increase until mean day 21 (16.3 mm); maximal diameter was maintained for the remainder of the observational period (day 60). Similarly, the corpus luteum in alpacas reached a maximum diameter of 14 mm 8 to 9 days after mating, and regressed 8 to 12 days after mating. Maximum plasma progesterone concentrations in nonpregnanct alpacas and vicunas occurred at 7 to 8 days post mating (Sumar *et al*., 1988; Aba *et al*., 1995).

Ovulation-inducing factor in semen

Discovery

Studies in China on Bactrian camels were the first to report an ovulation-inducing effect of seminal plasma (reviewed in Adams *et al*., 2016). At the time, this finding was largely dismissed in favor of the established notion that the trigger for induced ovulation is coital stimulation. During the same time period, results of studies in pigs (spontaneous ovulators) revealed that infusion of semen in the uterus of sows increased litter size (Murray *et al*., 1983). Thus, the concept that seminal plasma may have direct effect on hypothalamic-hypophyseal-gondal axis of the female began to take root. Confirmation of the existence of an ovulation-inducing factor (OIF) in semen came 20 years later in a series of studies done in llamas and alpacas where the intramuscular administration of seminal plasma induced ovulation in a high proportion of females (Adams, 2005).

Paradoxically, intrauterine infusion of seminal plasma induced ovulation in llamas at a lower rate than intramuscular administration. However, the addition of endometrial curettage (mimicking copulatory mucosal erosion) with intrauterine administration of seminal plasma resulted in a marked increase in ovulatory response, and supported the hypothesis that the effect of OIF is mediated by absorption into systemic circulation of the female (i.e., increased by endometrial hyperemia; Ratto *et al*., 2005). Intrauterine infusion of a larger dose of OIF (i.e., commensurate with the amount present in a normal ejaculate) resulted in a 100% of ovulation rate in llamas (Silva *et al*., 2015). Irrespective of the route of administration, seminal plasma (or purified OIF) elicited a surge in circulating concentrations of LH, demonstrating that the effect is mediated centrally at the hypothalamic-pituitary unit. Despite having branched from other artiodactyls more than 45 Ma, camelids have interesting features in common with spontaneous ovulators. One is that while rising concentrations of estradiol do not trigger the preovulatory LH surge in camelids, it appears that estradiol modulates the LH secretory response to OIF (Silva *et al*., 2012a). More surprisingly, OIF has been detected in the seminal plasma of every spontaneous ovulator tested to date (reviewed in Adams *et al*., 2016), suggesting the existence of as yet unknown pathways influencing reproduction.

Identification

Chemical identification of OIF began by treating seminal plasma in ways intended to neutralize specific constituents and thereby ablate the ovulatory effect (reviewed in Adams *et al*., 2016). Treatment by heating (65ºC), charcoal-dextran, or proteinase K (38ºC for an hour) did not abolish the ovulation inducing effect of llama seminal plasma. However, treatment with pronase E, a complex mixture of proteases, ablated the ovulatory effect, suggesting that the molecule responsible of ovulation induction was in fact a protein. The use of a two-step chromatographic procedure allowed the isolation of a protein fraction that retained the ovulatory effect. The final identification of OIF was discovered during crystallography studies in which the amino acid sequence and protein structure of OIF was identical to the known neurotrophin beta-nerve growth factor (bNGF; Ratto *et al*., 2012). Similar chromatographic procedures have led to the identification and purification of OIF/bNGF in camel seminal plasma (Kumar *et al*., 2013) and its role in camel ovulation (Fatnassi *et al*., 2017).

Luteotrophic effect

Additionally, OIF has been shown to have a powerful luteotrophic effect. Plasma progesterone concentration on day 7 after ovulation induced by OIF were 2.5 times higher than in GnRH treated animals (Adams *et al*., 2005), and luteal function was enhanced independent of follicle size at the time of treatment with OIF (Silva *et al*., 2014). A mechanistic association between NGF and angiogenic factors has been reported in the rat ovary and human granulosa cells (Julio-Pipper *et al*., 2006, 2009). An angiogenic mechanism was implicated in more recent studies of the luteotrophic effect of OIF/NGF in which the preovulatory follicle of OIF-treated llamas displayed a transient increase in vascularity 4 h after treatment, and corpus luteum vascularity was greater at day 6 after treatment compared to GnRH-treated llamas (Ulloa-Leal *et al*., 2014). Further, OIF treatment in llamas was associated with a 3-fold increase in the mRNA of steroidogenic enzymes in the corpus luteum at day 4 after ovulation compared to llamas induced to ovulate with GnRH (Silva *et al*., 2017).

The luteotrophic effect has been attributed largely to the increased amounts of LH secreted under OIF stimulation (Adams *et al*., 2005), and dose-response effect on ovulation rate, LH secretion, and corpus luteum function in llamas (Tanco *et al*., 2011). Interestingly, a similar dose-response effect on LH secretion was observed with increasing doses of GnRH in llamas, but luteal function (e.g. progesterone concentration) did not differ at different GnRH dosages (Silva *et al*., 2012b). Perhaps then, the requirements for luteal development are fully met by minimal GnRH doses, but the administration of OIF provides an extra signal that promotes luteogenesis. This view is consistent with several reports of high- and low-affinity receptors for NGF in the ovaries of different species (Dissen *et al*., 1996, 2000; Levanti *et al*., 2005; Carrasco *et al*., 2016).

Proposed mechanism

It is well established that OIF stimulates LH secretion prior to ovulation in camelids, interacting directly or indirectly with the pituitary gland (reviewed in Adams *et al*., 2016). Consistent with an early report using rat pituitary cells and alpaca seminal plasma (Paolichi *et al*., 1999), treatment of primary cultures of llama and bovine pituitary cells with OIF induced LH secretion into the culture media (Bogle *et al*., 2012).

However, OIF treatment in vivo was not associated with a detectable rise in plasma LH or ovulation in prepubertal heifers (Tanco *et al*., 2012), nor in llamas pre-treated with a GnRH receptor blocker (Cetrorelix; Silva *et al*., 2011). These findings support the hypothesis that OIF acts upstream from the pituitary, most likely at the level of the hypothalamus, perhaps on GnRH neurons themselves. For this hypothesis to be plausible, OIF must cross the blood-brain-barrier and neurons in the hypothalamus must express NGF receptors. In mice, NGF has been shown to cross the blood-brain-barrier (Pan *et al*., 1998); however, the site and the mechanism by which it crosses remains unknown. In an immunofluorescent study to determine if llama GnRH neurons possess receptors for NGF (trkA and p75; high- and low-affinity receptors, respectively), we found no co-localization with GnRH neurons (Carrasco RA et al., 2018; Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, SK, Canada; submitted paper), suggesting that the neuronal target for OIF in the hypothalamus is a group of interneurons that synapses with GnRH neurons, stimulating its secretion into the portal system.

Ovulation synchronization

Experiments involving the empirical use of progesterone have been reported in llamas and alpacas, based on studies done in cattle and sheep. The rationale for using progesterone alone to synchronize follicular development in camelids, however, is unclear since regular luteal phases are not a characteristic of the ovarian pattern in camelids (i.e. induced ovulators) and follicular waves continue to emerge at regular intervals during progestational states (i.e. after sterile mating or during pregnancy; Adams *et al*., 1990). Induction of a luteal phase was associated with a decrease in the diameter profile of the dominant follicle and a shorter interval between follicular waves (Adams *et al*., 1990). Similarly, the use of an intravaginal progesterone-releasing device in llamas (Chaves *et al*., 2002) and vicunas (Aba *et al*., 2005) resulted in a decrease in the maximum diameter of the dominant follicle, but no data were reported regarding the emergence of a new follicular wave. In a controlled synchronization study (Ratto *et al*., 2003), llamas were treated with saline (control), a combination of estradiol plus progesterone, LH, or by transvaginal ultrasound-guided follicle ablation (n = 20 per group). Compared to controls, treatment with LH or follicle ablation were equally effective at synchronizing and shortening the interval to follicular wave emergence to 2 days after treatment, and to the day on which the new dominant follicle reached ≥7 mm (ovulatory capability; 5 days after treatment), while the steroid-treated group was intermediate in effect. Compared to controls, synchronization treatment resulted in a higher pregnancy rate to a single, timed mating (54 *vs*. 76%; Ratto *et al*., 2003).

Superovulation

Important limitations to implementing embryo transfer technology in South American camelids include an inconsistent ovarian superstimulatory response, the challenge of collecting and processing semen, low embryo collection efficiency, and the recovery of advanced-stage embryos (hatched blastocyst) that are difficult to handle and cryopreserve. Studies have involved superstimulatory treatments with FSH or eCG during the follicular or sexual receptivity phase or during natural or artificially induced luteal phases (review in Ratto *et al*., 2013). The superovulatory response in camelids, estimated by the total number of corpora lutea on the day of embryo collection, ranges from 0 to 17 per female, with an embryo recovery rate ranging from 0 to 45% (Del Campo *et al*., 1995; Ratto *et al*., 2013). In a retrospective analysis of 5547 single- or multiple-ovulation embryo transfers performed on commercial alpaca farms in Australia (Vaughan *et al*., 2013), factors found to have a significant impact on the success of embryo transfer were the day of flushing after mating (8 and 9 days after mating were best), embryo diameter (larger were better), embryo quality, day of transfer to recipients (7 and 8 days after GnRH were best), and the age of the recipient (≤15 years). Compared to single-ovulation donors, superovulation of donors resulted in an average of 6.4 ovulations and 3.6 times as many transferrable embryos (0.67 *vs*. 2.44) and offspring (0.29 *vs*. 1.02) per donor flushed. These results are in agreement with an earlier controlled study (Huanca *et al*., 2009) in which superstimulatory treatment with eCG (with or without progestin) induced an average of 9.3 ovulations and 4.3 embryos per donor flushed; 10.1 times as many ovulations and 5.9 times as many embryos as in unstimulated controls.

Although not critically examined in camelids, studies in cattle have documented the positive effect of initiating ovarian superstimulatory treatment at the time of follicular wave emergence (reviewed in Adams, 1994 and Adams *et al*., 2012). With successful development of protocols to control follicle development and ovulation, superstimulation may now be initiated at a pre-scheduled time to optimize the ovulatory response. Ovarian superstimulation with either FSH or eCG given during follicular wave emergence induced by follicle ablation were equally efficacious in inducing multiple follicle growth in llamas without progesterone/progestin treatment (Ratto *et al*., 2004). Similarly, eCG (with or without progestin) given to llamas at the time of follicular emergence induced by LH administration resulted in an average 8.6 and 10.1 corpora lutea (eCG with and without progestin, respectively) and an average of 3.7 and 4.9 embryos, respectively (Huanca *et al*., 2009).

Other camelid species

Most of studies on the reproductive physiology of South American Camelids have been conducted in the domestic species, llamas and alpacas, and results may not necessarily be extrapolated to the wild species. A wave-like pattern of ovarian follicular development similar to that described for llamas and alpacas has been reported in vicuñas (Aba *et al*., 2005) and guanacos

(Riveros *et al*., 2010). The maximum diameter of the dominant follicle was 8.9 ± 0.9 mm (range: 6.2-11.2) and 10.2 ± 2.1 mm (range: 7.2-16.1 mm) for vicuñas and guanacos, respectively. In another vicuña study (Miragaya *et al*., 2004), intramuscular administration of 750 IU of eCG with or without an intravaginal progesterone device induced the growth of 8 to 13 follicles ≥6 mm per female. No studies have been conducted in the wild species on the timing of ovulation after natural mating or administration of GnRH, hCG, or OIF.

The Old World camelids, dromedary and Bactrian camels, inhabit the dry desert of Africa, Arabia and the cold regions of China and Mongolia, respectively. They are seasonal breeders, induced ovulators, and also display a wave-like pattern of ovarian follicular development (Skidmore, 2011). The maximum diameter of the dominant follicle was 2.0 ± 0.1 cm (range: 1.5-2.5) in both dromedary and Bactrian camels; however, in about 50% of follicular waves in dromedaries the dominant follicle reached a maximum diameter of 4.0-6.4 cm. The diameter of the follicle on the day before ovulation was 1.3 ± 0.2 cm in diameter and ovulation was detected between 28 and 36 h after mating (Skidmore, 2011). A GnRH agonist, Buserelin, or 3000 IU of hCG have been used to induce ovulation or synchronize follicular wave emergence in camels. Treatment with GnRH or hCG induced ovulation in 85% of dromedary camels when given in the presence of a preovulatory follicle between 1.0 and 1.9 cm, but was ~12% when given in the presence of a follicles between 2.0 and 2.9 cm, and none ovulated when treatment was given when the largest follicle was ≤0.9 or ≥3.0 cm in diameter (Skidmore *et al*., 1996). In a more recent study in dromedaries, two GnRH injections 14 days apart, with or without PGF2a 7 days after the first GnRH treatment, were effective methods of synchronizing wave emergence and subsequent ovulation (Skidmore et al., 2009), but the efficacy of synchronization for fixed-time insemination has not been reported.

Conclusions

Much has been learned in the last 20 years about ovarian function in camelids. As the largest domestic species of induced ovulator, llamas, alpacas and camels have provided an opportunity to re-examine our understanding of factors and mechanisms involved in ovulation. Studies involving serial examination of ovarian and endocrine events permitted testing new hypotheses about the role of semen in these and other species of induced ovulators, as well as in spontaneous ovulators. The discovery of a factor in semen that has a direct effect on the hypothalamo-pituitary axis of the inseminated female is new and exciting, and may have broad implications. Basic and applied studies are on-going in the hope of elucidating the precise site of action, and neuro-endocrine cells involved in initiating the preovulatory surge in LH. A better understanding of ovarian follicular dynamics and treatments designed to control follicle growth and ovulation have enabled the use of reproductive techniques such as synchronization and timed-insemination, and have made viable the business of embryo transfer in both Old and New World camelids.

Acknowledgments

Research by the authors, referred to in this review, was supported by grants-in-aide from the Natural Sciences and Engineering Research Council of Canada, and the Chilean National Science and Technology Research Council (FONDECYT).

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DOI: 10.21451/1984-3143-AR2018-0007

Proceedings of the 10th International Ruminant Reproduction Symposium (IRRS 2018); Foz do Iguaçu, PR, Brazil, September 16th to 20th, 2018.

Evolution of knowledge on ovarian physiology and its contribution to the widespread application of reproductive biotechnologies in South American cattle

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Abstract

As our understanding of ovarian function in cattle has improved, our ability to control it has also increased. Luteal function in cattle has been studied in detail, and prostaglandin F2α has been used for several years for the elective induction of luteal regression. More recently, follicle wave dynamics has been studied and protocols designed to induce follicular wave emergence and ovulation have reduced, and even eliminated, the need for estrus detection. The addition of progestin-releasing devices, estradiol, GnRH and equine chorionic gonadotropin (eCG) have provided opportunities for fixed-time AI (FTAI) and possibilities for increased pregnancy rates. In embryo transfer programs, these same treatments have eliminated the need for estrus detection, permitting fixed-time embryo transfer and the initiation of superstimulatory treatments without regard to day of the estrous cycle. Collectively, new treatment protocols have facilitated the application of assisted reproductive technologies, and this is especially true in South America. Over the last 20 years, the use of AI in South America has increased, due largely to the use of FTAI. There has been more than a 10-fold increase in the use of FTAI in Brazil with more than 11 million treatments in 2016, representing 85% of all AI. Similar trends are occurring in Argentina and Uruguay. Production of *in vivo*-derived (IVD) embryos has remained relatively stable over the years, but *in vitro* embryo production (IVP) has increased dramatically over the past 10 to 15 years, especially in Brazil where more than 300,000 IVP embryos were produced in 2010. World-wide, more than 666,000 bovine IVP embryos were produced in 2016, of which more than 57% were produced in South America. The use of assisted reproductive technologies has facilitated the dissemination of improved genetics and increased reproductive performance; other South American countries are now following suit.

Keywords: AI, embryo transfer**,** estrus synchronization, follicular wave, superovulation.

Introduction

Increasing knowledge of ovarian physiology in cattle over the past 50 years has provided approaches for the manipulation of ovarian function. Protocols designed to control both luteal and follicular function have improved estrus synchronization and permitted fixed-time AI (FTAI) and fixed-time embryo transfer (FTET), and the initiation of superstimulatory treatments at a self-appointed time. Bovine practitioners around the world are now using these reproductive technologies, and this is especially the case in South America. This review will briefly describe improvements in our understanding of ovarian physiology in cattle and discuss how this has affected the application of assisted reproductive biotechnologies in South America, with special emphasis on Brazil, Argentina and Uruguay where much of the research and practical application is taking place.

A brief review of recent knowledge on ovarian physiology in cattle

Ovarian follicles in cattle grow in a wave-like fashion. A follicular wave consists of the synchronous emergence of a group of antral follicles 4 to 5 mm in diameter followed in 2 or 3 days by the selection of one follicle to become dominant, while subordinates become atretic (Ginther *et al*., 1989a). Estrous cycles in cattle are composed of either 2 or 3 follicular waves (Ginther *et al*., 1989b), although 4-wave cycles have been reported in *B. indicus* breeds (Bó *et al*., 2003). In both 2- and 3-wave cycles, emergence of the first follicular wave occurs on the day of ovulation (Day 0). Because of the presence of the mid-cycle CL, the dominant follicle of the first wave regresses, and a second wave emerges on Days 9 or 10 in 2-wave cycles, and Days 8 or 9 in 3-wave cycles, with the third wave emerging on Days 15 or 16. Follicular waves occur in heifers before puberty and in postpartum cows, before the first ovulation (reviewed in Adams, 1999).

Recruitment of follicular waves and selection of the dominant follicle are based on differential responsiveness of antral follicles to FSH and LH (reviewed in Adams, 1999). Surges in circulating FSH are followed in 1 to 2 days by the appearance of a group of follicles that are 4 to 5 mm in diameter. FSH is then suppressed by estradiol and inhibin produced by follicles in the wave (especially the future dominant follicle), leading to the selection of a dominant follicle approximately 3 days after wave emergence. The dominant follicle acquires more LH receptors on its granulosa cells than its subordinates and is able to shift its gonadotropin dependence to LH during the period of low FSH; it continues to grow while subordinates

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Received: May 24, 2018
Accepted: January 25, 2018

requiring FSH regress (Ginther *et al*., 2001). Suppression of LH by progesterone from the CL causes the dominant follicle of the first wave (and of the second wave in 3-wave cycles) to eventually cease its metabolic functions and regress, which leads to an FSH surge and emergence of a new follicular wave (Adams, 1999). Luteal regression results in increased LH pulse frequency, increased growth of the dominant follicle, elevated estradiol concentrations, an LH surge and ovulation.

The number of follicular waves in cattle depends on the duration of follicular dominance in the first wave; it is 3 days longer and the onset of regression occurs later during 2-wave cycles than 3-wave cycles. However, the onset of luteolysis occurs earlier in 2-wave cycles resulting in an interovulatory interval of 20 days, as compared to 23 days in 3-wave cycles (Adams, 1999; Sartori *et al*., 2004). The reason for difference in the duration of follicular dominance in the first wave between wave types is unknown. The dominant follicle present at the time of luteolysis becomes the ovulatory follicle, and emergence of the next wave is delayed until the ensuing ovulation (Kastelic and Ginther, 1991).

Follicular wave dynamics as described has been limited to follicles $\geq$ 4 mm because of the resolution of early ultrasound equipment. However, newer equipment has permitted the identification of the future dominant follicle at a diameter of 1 mm, suggesting that 1 to 3 mm follicles also develop in a wave-like manner (Fig. 1). Adams *et al*. (2008) pointed out that at a microscopic level, there is no morphologic distinction between follicles that are 1 to 3 mm and those that are more than 3 mm, and that at a cellular level, both size categories not only express FSH receptors but have a similar level of expression on a per granulosa cell basis (reviewed in Bao and Garvarick, 1998). They also provide evidence that these small antral follicles respond to transient elevations in circulating FSH and that their growth progresses over a period encompassing the entire FSH surge. Thus, 1 to 3 mm follicles may be an important component of the wave. This has implications for the optimization of superstimulation for both *in vivo* and *in vitro* embryo production in cattle (Garcia Guerra *et al*., 2015).

There are several differences in reproductive function between *B. indicus* and *B. taurus* breeds of cattle and these must be considered when designing assisted reproductive programs. Nutrition, post-partum anestrus and age of onset of puberty are especially important in *B. indicus* cattle. They also have a shorter estrus period, often expressed during the night, and although follicular dominance is similar, maximum diameters of the dominant follicle and CL are smaller (Bó *et al*., 2003), as are dominant follicle diameters at the time of selection and ovulation (Sartori *et al*., 2001; Sartorelli *et al*., 2005; Gimenes *et al*., 2008). In addition, *B. indicus* breeds tend to be more sensitive to steroid hormones which must be considered in designing estrus synchronization programs (Bó *et al*., 2003). However, *B. indicus* breeds also have greater antral follicle counts (Batista *et al*., 2014; Sartori *et al*., 2016a) which has important implications for superstimulation and ovum pick-up and *in vitro* fertilization (OPU/IVF).

Synchronization of estrus for artificial insemination or embryo transfer

Prostaglandin F2α

Prostaglandin F2α (PGF) has become the most common treatment for elective induction of luteal regression and synchronization of estrus in cattle (reviewed in Odde, 1990). However, cattle must be cycling and PGF will not induce luteolysis during the first 5 days of the cycle (Seguin, 1987). In addition, the onset of estrus may occur over several days; treatment when the dominant follicle is near mature will result in ovulation in 2 or 3 days, whereas treatment when it is no longer viable will result in ovulation of the dominant follicle of the next wave, 4 to 5 days later (Kastelic *et al*., 1990). In a two-dose PGF synchronization scheme, an interval of 10 or 11 days has been recommended to ensure that all cattle have a PGF-responsive CL at the time of the second treatment. Although an interval of 11 days between PGF treatments has been found to be acceptable for heifers (Selk *et al*., 1988), higher conception rates have been reported in lactating dairy cows with a 14-day interval (Folman *et al*., 1990). Most other methods of estrus synchronization utilize PGF to regress an existing or new CL.

Acceptable pregnancy rates in embryo transfer are partially dependent upon the onset of estrus in the recipient being within 24-hour synchrony of the embryo donor or stage of development of the embryo (Bó *et al*., 2012). Pregnancy rates do not differ whether recipients are selected following detection of estrus in untreated animals or estrus synchronization. As estrus in donors will occur 36 to 48 hours after treatment with PGF, recipients must be treated 12 to 24 hours earlier than donors (Bó and Mapletoft, 2014).

Progestins

Progesterone alters ovarian function in cattle by suppressing estrus and preventing ovulation, primarily by suppressing LH release. As progesterone does not suppress FSH secretion, follicle waves continue to emerge in the presence of a functional CL (Adams, 1999). Progestins given for intervals exceeding the lifespan of a CL result in synchronous estrus upon withdrawal, but fertility is usually low because exogenous progestins are generally less suppressive of LH than endogenous progesterone. The resulting high LH pulse frequency leads to a "persistent" follicle (Savio *et al*., 1993) with an oocyte that may be infertile (Revah and Butler, 1996).

Progesterone-releasing devices are now used to synchronize estrus in cattle (Mapletoft *et al*., 2003), and there are several such products available in Brazil and Argentina with different payloads of progesterone for different classes of cattle. Progestin devices are normally removed after 7 or 8 days and PGF is administered at that time (or 24 hours earlier); estrus occurs 48 to 72 hours later. Because of the short treatment period, persistent follicles do not occur and fertility following AI is usually normal. Progestin devices are well suited for the various

approaches used to synchronize follicular development and ovulation (Mapletoft *et al*., 2003).

As protocols designed for estrus synchronization have depended on estrus detection, results are often disappointing because estrus detection is time consuming and inaccurate (Washburn *et al*., 2002). While acceptable conception rates may occur following estrus detection, pregnancy rates are low because of low submission rates. Estrus detection efficiency also affects the use of assisted reproductive technologies e.g., when PGF is used to synchronize cycling recipients in a 2-injection protocol, 80% are expected to show signs of estrus within 5 days of the second treatment. However, due to inefficient estrus detection, less than 50% receive an embryo (Bó *et al*., 2012). The problem may be greater if the recipients are *B. indicus* or *B. indicus* crosses under pasture conditions (Sartori and Barros, 2011). An alternative is to eliminate the need for estrus detection by applying fixed-time protocols (Baruselli *et al*., 2010; Bó *et al*., 2013).

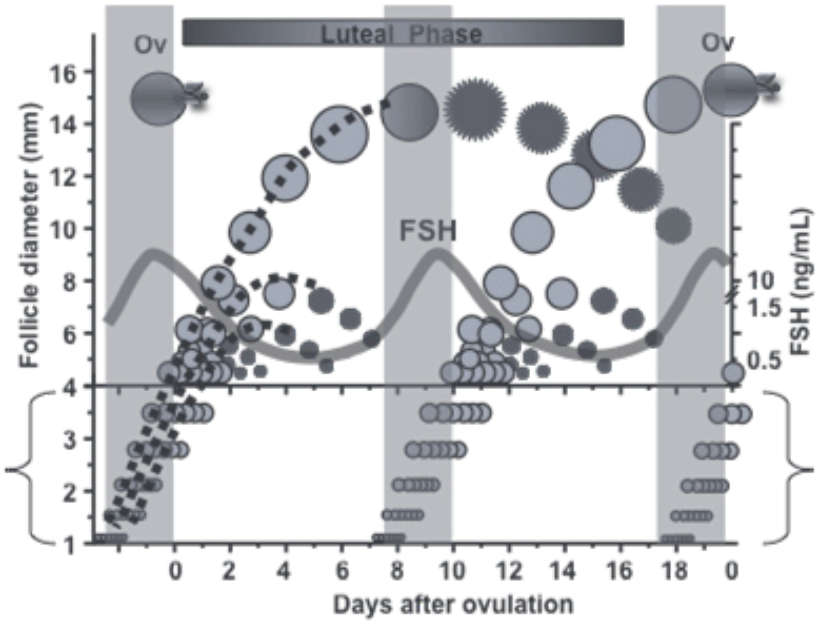

Figure 1. A two-wave ovarian follicular wave pattern detected in follicles as small as 1 mm in diameter. Small follicles (1 to 3 mm) in parentheses illustrate wave emergence 2.5 days earlier than previously detected (i.e., at 4 to 5 mm). Note that the growth rate of the follicle destined to become dominant (dotted line) is similar to others in the wave until about 5 days after wave emergence at 1 mm, and has a size advantage over those destined to become subordinate as small as 1 mm in size (Adams et al., 2008). Compliments of GP Adams.

Manipulation of ovarian function for fixed-time AI or fixed-time embryo transfer

Ultrasound-guided follicle ablation

Transvaginal ultrasound-guided ablation of antral follicles results in emergence of a new follicular wave in approximately 1.5 days by removing the suppressive effects of follicle products (e.g., estradiol and inhibin) on FSH release (Bergfelt *et al*., 1994; Fig. 2). Although follicle ablation in combination with PGF is very efficacious in synchronizing follicle wave emergence and ovulation, it is not practical in the field.

Estradiol and progesterone

Estradiol valerate was originally used at the start of a 9-day progestin protocol to cause uterine-induced luteolysis (Wiltbank *et al*., 1965), but it has also been shown to suppress antral follicles (Bó *et al*., 1995). The mechanism of estrogen-induced follicle atresia appears to be systemic, and involves suppression of FSH (Fig. 3). Once exogenously administered estradiol is metabolized, FSH surges and a new follicular wave emerges. The administration of 2.5 to 5 mg estradiol-17β (E-17β; reviewed in Bó *et al*., 2002) or 2 mg of estradiol benzoate (EB; Martinez *et al*., 2005) or estradiol valerate (EV; Colazo *et al*., 2005) in progestin-treated cattle results in emergence of a new follicular wave in 3 to 5 days.

In early estrus synchronization protocols, 2.5 mg EB is administered at the time of insertion of a progestin device which is removed 7 days later, at the time of administration of PGF (Mapletoft *et al*., 2003). A dose of 1 mg EB was given 24 hours later to induce an LH surge in 16 to 18 hours (Martinez *et al*., 2007) and ovulation approximately 24 hours later. This permitted FTAI with acceptable pregnancy rates. As an alternative, administration of 0.5 to 1.0 mg of estradiol cypionate (ECP; Colazo *et al*., 2003) at the time of progestin removal with FTAI 48 to 52 hours later is often used in South America (Sales *et al*., 2012: Martins *et al*., 2017). Pregnancy rates following FTAI have been shown to be improved in suckled beef cows and heifers, especially *B. indicus* crosses, when 300 to 400 IU of eCG is administered at the time of progestin removal (Baruselli *et al*., 2004; Bó *et al*., 2012; 2016). The administration of eCG stimulates dominant follicle growth and

maturation, and increases progesterone production by the subsequent CL (*B. taurus*, Nunez-Olivera *et al*., 2014; *B. indicus*, Baruselli *et al*., 2004). Estradiol, progesterone and eCG treatments are also very useful for fixed-time embryo transfer (FTET) with a greater proportion of recipients selected for embryo transfer and higher pregnancy rates following transfer (Baruselli *et al*., 2010; Bó *et al*., 2012; Rodrigues *et al*., 2010).

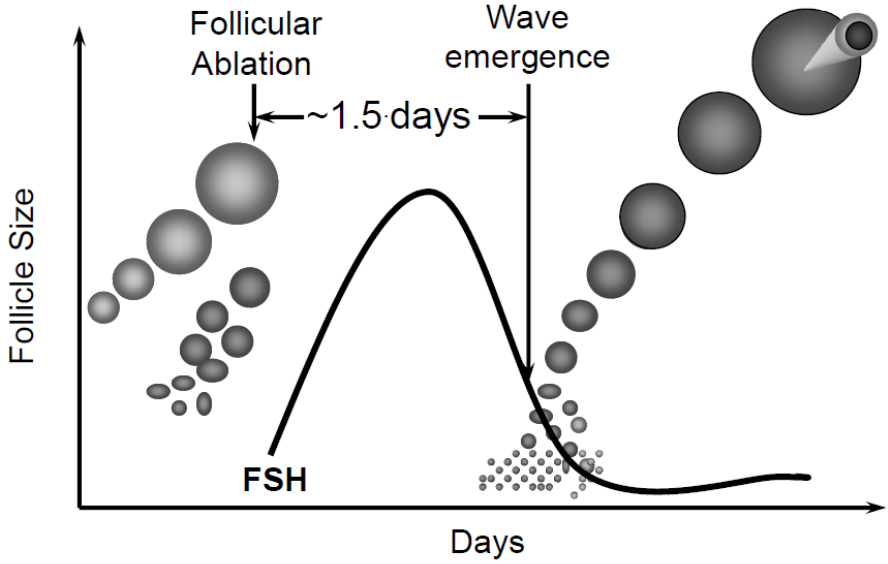

Figure 2. Synchronization of follicular wave emergence by ultrasound-guided follicle ablation. Aspiration of antral follicles causes the emergence of the next follicular wave by removing the suppressive effects of follicle products on FSH release. FSH surges and follicular wave emergence occurs 1 to 2 days later.

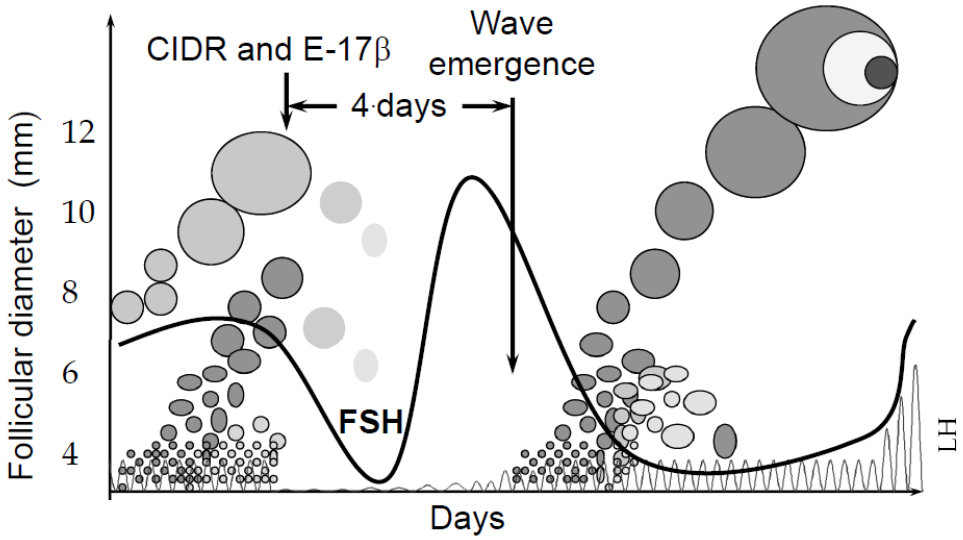

Figure 3. Synchronization of follicular wave emergence by estradiol/progestin treatment. Estradiol causes suppression of FSH and atresia of FSH-dependent follicles. Once the injected estradiol is metabolized, FSH surges and a new follicular wave emerges 3 to 5 days after treatment.

Gonadotropin releasing hormone (GnRH)

GnRH treatment of cattle with a growing dominant follicle will induce ovulation and emergence of a new follicular wave 1 to 2 days later (Macmillan and Thatcher, 1991) by inducing LH release, but only when ovulation occurs (Martinez *et al*., 1999; Fig. 4). An ovulation synchronization protocol for FTAI in lactating dairy cattle utilizing GnRH has been developed by Pursley *et al*. (1995; Ovsynch). Administration of GnRH is followed in 7 days by PGF and a second GnRH in 48 hours, with FTAI 16 to 18 hours later. More recently, it has been shown that pregnancy per AI (P/AI) is improved in lactating dairy cattle if the interval from PGF to the second GnRH is increased to 56 hours (Ovsynch-56; Brusveen *et al*., 2008). The Ovsynch protocol has been more efficacious in lactating dairy cows than in heifers (Seguin, 1997). Although the cause is not clear, ovulation following the first GnRH has been reported to be higher in cows than heifers and a higher percentage of heifers show estrus early, resulting in reduced fertility following FTAI

(Wiltbank, 1997).

Stage of development of the dominant follicle (Martinez *et al*., 1999), and stage of the estrous cycle (Vasconcelos *et al*., 1999; Moreira *et al*., 2000) affect response to the first GnRH. If the dominant follicle is immature or post-mature, ovulation may not occur and a new follicular wave will not emerge. Cattle respond most consistently between Days 5 and 12 of the estrous cycle, so a 2-injection PGF pre-synchronization treatment is often used before administration of the first GnRH (Moreira *et al*., 2001). Various other presynchronization protocols have been developed to improve P/AI following the use of the Ovsynch protocol (Stevenson, 2011). Alternatively, a progestin insert at the time of the first GnRH has also improved P/AI in beef heifers (Martinez *et al*., 2002), beef cows (Lamb *et al*., 2001) and anovulatory dairy cows (Stevenson *et al*., 2006; Bisinotto *et al*., 2013). Treatment with eCG at the time of progestin removal has also improved pregnancy rates in non-cycling beef cows (Bó *et al*., 2016).

More recently, a 5-day GnRH-based, Co-Synch protocol has been developed for beef cattle in North America, with higher P/AI than obtained with the more traditional 7-day Co-Synch protocol (Bridges *et al*., 2008; reviewed in Day, 2015). The physiological basis of this protocol is to reduce the progestin device insertion period to 5 days, avoiding the development of persistent follicles in cattle not ovulating to the first GnRH, and then lengthening the proestrus period to 72 hours to allow for greater dominant follicle development and higher circulating estrogen levels prior to ovulation. However, two injections of PGF are required with this protocol to induce luteal regression in animals that ovulate to the first GnRH. Several subsequent studies have been designed to eliminate the need for extra animal handling associated with two injections of PGF, but results have been inconsistent, possibly because of the inconsistent response to the first GnRH in heifers. Rabaglino *et al*. (2010) found no difference in P/AI, whether one or two injections of PGF were administered, while Peterson *et al*. (2011) reported that heifers given two injections of PGF tended to have higher P/AI. Colazo and Ambrose (2011) eliminated the initial administration of GnRH in the 5-day protocol without adversely affecting fertility. However, in a large study, Lima *et al*. (2013) found that P/AI was greater in heifers that received GnRH at progestin insertion and two injections of PGF after progestin removal (on Days 5 and 6) than in heifers that did not receive the initial GnRH, whether they received one or two injections of PGF. More recently, Kasimanickam *et al*. (2014) reported that the initial GnRH increased P/AI in beef heifers, but not in dairy heifers, and that P/AI in dairy heifers did not differ whether they received one or two injections of PGF. Perhaps, it is simply a matter of statistical power. In Argentina, an estradiol-based protocol with shortened progestin exposure and a lengthened proestrus, named J-Synch, has been developed (reviewed in Bó *et al*., 2016). This protocol has the advantage that an initial GnRH treatment (and subsequently, two injections of PGF) is not required. Use of a 6-day J-Synch protocol has resulted in higher P/AI in heifers than the conventional 8-day estradiol-based protocol (Bó *et al*., 2016).

GnRH-based protocols are also efficacious in the synchronization of ovulation in recipients (Bó *et al*., 2012). In *B. indicus* x *B. taurus* crossbred heifers, the overall pregnancy rate was higher in recipients treated with a 7-day GnRH-based protocol than with PGF alone, because more recipients had a CL on the day of embryo transfer (Baruselli *et al*., 2010). The inclusion of a progestin device in a 7-day GnRH-based protocol in embryo recipients has also resulted in higher pregnancy rates (Beal, 1999). In a field trial involving 1637 recipients treated with GnRH plus a CIDR device and without estrus detection, overall pregnancy rate following embryo transfer to recipients with a CL was 59.9%. The beneficial effects of the J-Synch protocol on fertility has been confirmed recently in a recipient synchronization program (Bó *et al*., 2016).

Manipulation of ovarian function for superstimulation

The objective of ovarian superstimulatory treatments in cattle is to obtain the maximum number of viable embryos by stimulating growth of antral follicles and ovulation of competent oocytes (Bó and Mapletoft, 2014). Two very important factors influencing variability in superstimulatory response are the intrinsic number of antral follicles in donors, and the stage of follicular development at the time of initiating FSH treatments. Response can be predicted by antral follicle counts done with ultrasonography (Singh *et al*., 2004; Ireland *et al*., 2008), or measurement of circulating concentrations of anti-Müllerian hormone (AMH; *B. taurus*, Rico *et al*., 2009; Ireland *et al*., 2011; *B. indicus*, Batista *et al*., 2014). High antral follicle counts resulted in more ovulations and a greater number of transferable embryos than low antral follicle counts (Ireland *et al*., 2007). Similarly, the top quartile of circulating AMH values was associated with a greater superovulatory response than the lowest quartile (Souza *et al*., 2014).

The conventional protocol of initiating ovarian superstimulation during mid-cycle has been based on anecdotal and experimental evidence suggesting a greater superovulatory response when gonadotropin treatments were initiated between Days 8 and 12 of the cycle (Bó and Mapletoft, 2014). It is now known that mid-cycle is the approximate time of emergence of the second follicular wave (Ginther *et al*., 1989b). However, day of second wave emergence varies between wave types (1 or 2 days later in 2-wave cycles than in 3-wave cycles). In this regard, Nasser *et al*. (1993) showed that superovulatory response was greater when gonadotropin treatments were initiated at the time of follicle wave emergence; 1-day asynchrony reduced the response. The necessity of waiting until mid-cycle to initiate FSH treatment implies monitoring estrus, the obligatory delay and an inability to group donors. An alternative is to superstimulate donors following synchronization of follicular wave emergence (Bó *et al*., 1995; 2002; 2014).

Follicle ablation

Transvaginal ultrasound-guided follicle ablation followed by FSH treatments 1 or 2 days later is very efficacious (Bergfelt *et al*., 1997), but requires specialized skill and equipment and is difficult to apply in the field. However, follicle aspiration for OPU/IVF will synchronize wave emergence and thus, embryos could be produced both *in vitro* and *in vivo,* from the same donor (Surjus *et al*., 2014).

Estradiol and progesterone

The preferred approach for synchronization of follicular wave emergence prior to superstimulation is administration of 5 mg E-17β plus 100 mg progesterone and insertion of a progestin device 4 days before initiating FSH treatments. Experimental (Bó *et al*., 1996) and commercial (Bó *et al*., 2002) results have shown that embryo production following this treatment at unknown stages of the estrous cycle is comparable to that initiated 8 to 12 days after observed estrus. By synchronizing follicle wave emergence, the full extent of the estrous cycle is available and the need to detect estrus and wait 8 to 12 days to initiate FSH treatments was eliminated. Although E-17β and its esters are available in most, if not all, countries in South America, this is not the case in many other countries around the world necessitating the use of alternatives to synchronize follicle wave emergence prior to superstimulation.

Gonadotropin releasing hormone

Attempts to synchronize follicular wave emergence for superstimulation with GnRH were initially unsuccessful; however, subsequent field data were more promising. In these cases, GnRH was administered 1.5 to 3.0 days after the insertion of a progestin device which may have increased the probability of an LH-responsive follicle. Indeed, Bó *et al*. (2008) reported on the strategic use of PGF, a progestin device and GnRH to induce ovulation prior to initiating FSH treatments. Basically, a persistent follicle was induced by treatment with PGF at the time of progestin device insertion; following administration of GnRH 7 days later; ovulation occurred in more than 95% of animals. Superstimulation initiated 36 hours after GnRH (with the progestin device remaining in place) resulted in a superovulatory response that did not differ from controls. More recently, Hinshaw *et al*. (2015) reported no difference in superovulatory response whether GnRH was administered 2 or 7 days after insertion of a progestin device.

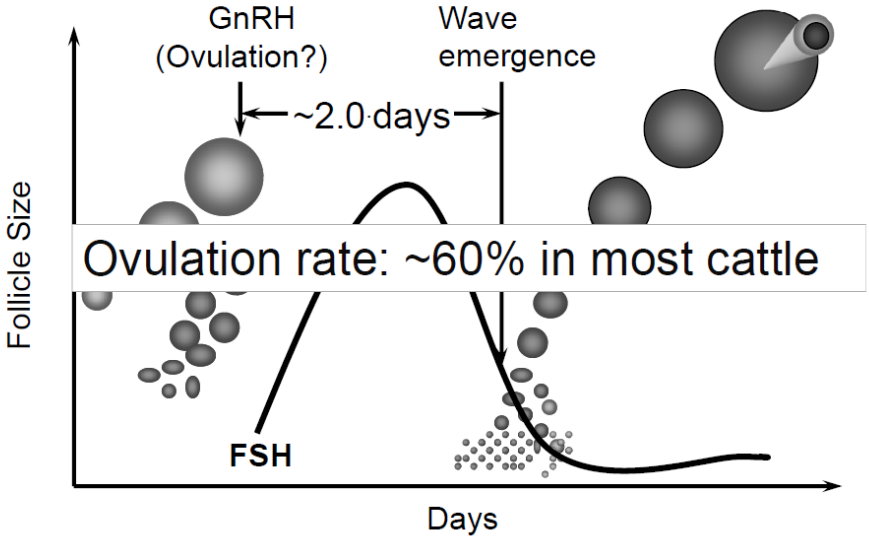

Figure 4. Synchronization of follicular wave emergence by GnRH treatment which causes ovulation of a growing dominant follicle. This removes the suppression of follicle products on FSH release; FSH surges and a new follicle wave emerges approximately 2 days after treatment. However, treatment at random stages of the estrous cycle results in less than 60% of animals ovulating and if ovulation does not occur, follicle wave emergence will not be synchronized.

The ruminant reproduction revolution in South America

South American countries are world leaders in beef production, while only Uruguay and possibly Argentina, have dairy industries large enough to exceed local demands. In 2016, Latin American countries had approximately 359 million cattle (accounting for 24% of the world's cattle population) and produced 15.1 million tonnes of beef (Food and Agriculture Organization of the United Nations-FAO, January, 2018). Brazil had 218.2 million cattle and produced 9.3 million tonnes of beef in 2016. Of the main beef-producing countries in South America, Brazil (60.8% of the total), Argentina (14.7%) and Uruguay (3.3%) are also prominent in development and application of assisted reproductive biotechnologies. Through training and continuing education programs, many of which one or more of the authors have organized or participated in, these technologies are being used increasingly in other South

American countries. It is through these efforts, in partnership with the pharmaceutical industry, that the ruminant reproductive revolution in South America has become widespread.

It is not possible in this manuscript to discuss all the contributions of South American scientists to the increased and improved understanding of bovine reproduction, especially in *B. indicus* breeds, nor describe in detail the application of reproductive biotechnologies in their breeding herds. However, the reference list in this manuscript (and many other similar publications) provides names of many South American nationals who have made important contributions. Brazil and Argentina, in particular, are very active in research, continuing education and post-graduate student training in reproductive biotechnology, especially in cattle.

Application of assisted reproductive technologies (Sartori *et al*., 2016b), and the state of the embryo transfer (Viana *et al*., 2017) and AI (Baruselli *et al*., 2017) industries in Brazil have been reviewed very recently. Therefore, this paper will focus on Brazil, recognizing that similar advances are taking place in other South American countries, especially Argentina and Uruguay.

The commercial embryo transfer industry began in North America in the early 1970s, and the technology soon spread to South America (Mapletoft, 2013). Although IVD bovine embryo numbers remained modest for several years, Brazil and Argentina consistently ranked in the top five countries outside North America and Europe. Perry (2017) has reported that in 2016 more than 632,000 IVD and 666,000 IVP bovine embryos were produced world-wide. North America accounted for more than 52% of the IVD embryos, but South America accounted for more than 57% of the IVP embryos. The use of IVP in Brazil has increased rapidly since 2000, driven primarily by *B. indicus* breeds which have large numbers of antral follicles from which large numbers of high quality oocytes can be recovered, without superstimulation. Viana *et al*. (2017) reported that embryo transfer accounted for 19.7% of all "Zebu" calves registered in Brazil between 2005 and 2015. *In vitro* embryo production in Brazil increased by 184.0% between 2005 and 2016, while numbers of IVD embryos decreased by 73.7% (Fig. 5; Viana, personal communication, 2017).

Commercial IVP in Brazil has been reported to have gone through three phases (Sartori *et al*., 2016b). The initial phase involved the use of proven donors of high genetic merit in both beef and dairy cattle, and the numbers of IVD and IVP embryos increased similarly. The second phase of growth occurred between 2003 and 2010, driven largely by the need to produce replacement bulls. In 2005, at the peak of this phase, 90.0% of IVP was in beef breeds with Nelore accounting for 82.7% of all embryos. The third phase has involved the use of sex-selected sperm and is associated with a shift in IVP from beef breeds to *B. taurus* dairy breeds. In 2014, IVP in dairy breeds increased by 46.5% (69.0% of the total), exceeding that of beef breeds for the first time.

A similar example of the application of assisted reproductive technologies involves the utilization of FTAI in cattle breeding. Most beef herds in Brazil are composed of *B. indicus* or *B. indicus* crosses, while Argentina and Uruguay tend to have more *B. taurus* breeds and their crosses. It is noteworthy that *B. indicus* breeds tend to have long periods of postpartum anestrus and low body condition scores on pasture, with an increased interval from calving to conception and low fertility (Bó *et al*., 2003). In a pasture-based cow-calf production system, synchronization protocols are necessary to produce pregnancies by AI during a short breeding season and because of problems with estrus detection, FTAI has been incorporated (Bó *et al*., 2013; 2016). Breeding objectives have been to inseminate early in the breeding season followed by early ultrasonographic pregnancy diagnosis and reinsemination of open cattle as soon as possible (Baruselli *et al*., 2017).

Because estradiol preparations have been available in South America, most FTAI protocols include estradiol as a means of synchronizing follicle wave emergence and ovulation (Bó *et al*., 2013). As herd cyclicity and body condition scores are usually low, progestin devices and eCG are usually included in the synchronization protocols (Baruselli *et al*., 2012; Bó *et al*., 2016). To minimize animal handling and the suppressive effect of progesterone on follicle growth, PGF is often administered at the beginning of the protocol, and ECP is administered at the time of progestin device removal (rather than EB 1 day later) with FTAI 48 hours later. Protocols of 6, 7, 8 or 9 days progestin treatment have resulted in similar P/AI, and shortened progestin protocols with a lengthened proestrus involving either estradiol or GnRH have been used with success (Bó *et al*., 2016). The administration of PGF on Day 7 (1 day before progestin removal) in dairy cows has increased P/AI following either FTAI or FTET (Pereira *et al*., 2013). The benefits of eCG in FTET treatment protocols have also been shown in crossbred recipients (Baruselli *et al*., 2010; Bó *et al*., 2012) and high-producing dairy cows (Rodrigues *et al*., 2010).

Resynchronization protocols have also been developed to reduce the interval between FTAI and reinsemination of nonpregnant animals (Bó *et al*., 2016). Traditionally, resynchronization has been done at pregnancy diagnosis 28 to 32 days after FTAI, with a breeding interval of approximately 40 days. Two recent protocols, developed in Brazil, beginning before pregnancy diagnosis (14 or 22 days after the FTAI) have reduced the interval between FTAI and reinsemination to 24 and 32 days, respectively (reviewed in Baruselli *et al*., 2017). The novelty of the 14-day protocol is the use of Doppler ultrasonography 22 days after FTAI for pregnancy diagnosis; a CL area of ≥2 cm² and/or CL blood flow of ≥25% are diagnostic of pregnancy. Resynchronization protocols have led to the adoption of management schemes exclusively for FTAI, eliminating the need for clean-up bulls. In one study, a cumulative pregnancy rate of 87.4% was achieved after three FTAI in a 64-day breeding season, which was greater than achieved with bull exposure after one FTAI (reviewed in Baruselli *et al*., 2017).

Data also indicate that FTAI is increasing the

use of AI, especially with *B. indicus* sires on *B. indicus* cows in Brazil. There has been more than a 10-fold increase in the use of FTAI in Brazil, from ~1 million protocols in 2005 (11% of all AI) to 10.5 million protocols in 2015 (77% of all AI; Sartori *et al*., 2016b), and a further increase to more than 11 million FTAI in 2016 (Baruselli *et al*., 2017; Fig. 6). Currently, FTAI procedures account for 85% of AI performed in Brazil. In addition, the proportion of dairy cows not inseminated by 70 DIM has decreased resulting in more cows pregnant by 103 DIM and a decrease of approximately 35 days open (Sartori *et al*., 2016b). Industry reports from Argentina and Uruguay for the 2016/17 breeding season indicate a similar trend, especially in beef cattle (3 million and 300,000 FTAI, respectively, representing approximately 10% of their breeding herds). In total, more than 15 million cattle were inseminated by FTAI in these three countries over the past year. Baruselli *et al*. (2017) suggested that the adoption of this technology is an excellent example of a technological change in the production sector emerging from scientific developments in the academic sector.

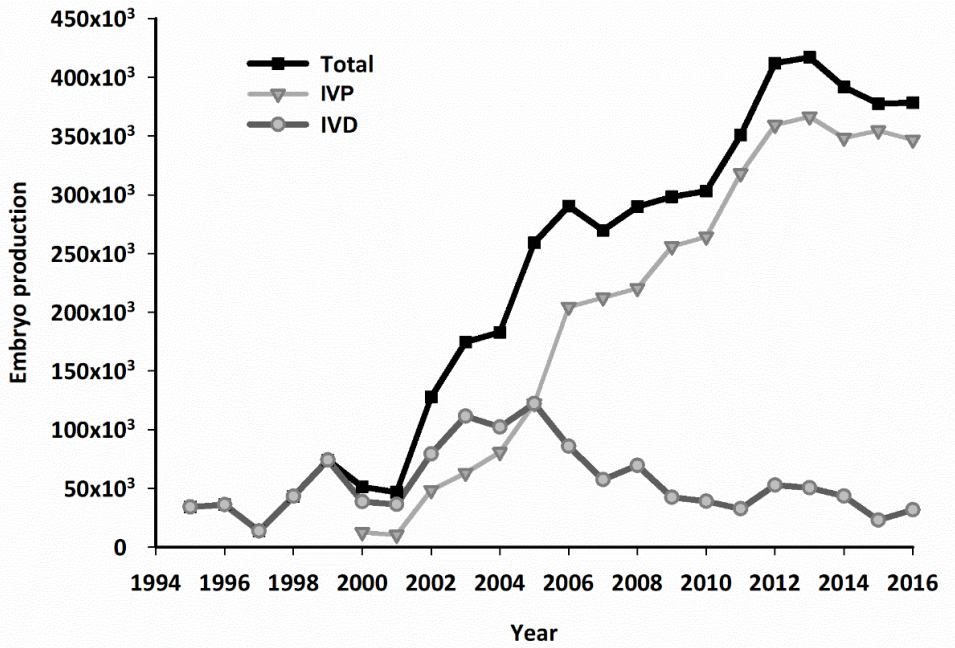

Figure5. Production of bovine embryos in Brazil from 1995 to 2016. Numbers of embryos produced are shown as Total (*in vivo* and *in vitro* embryos combined); IVP (embryos produced *in vitro*; OPU/IVF); IVD (embryos produced *in vivo;* superovulation and collection). Note that beginning in 2005 numbers of IVP embryos exceeded numbers of IVD embryos and the numbers of IVD embryos began to decline. Complements of Dr. JHM Viana.

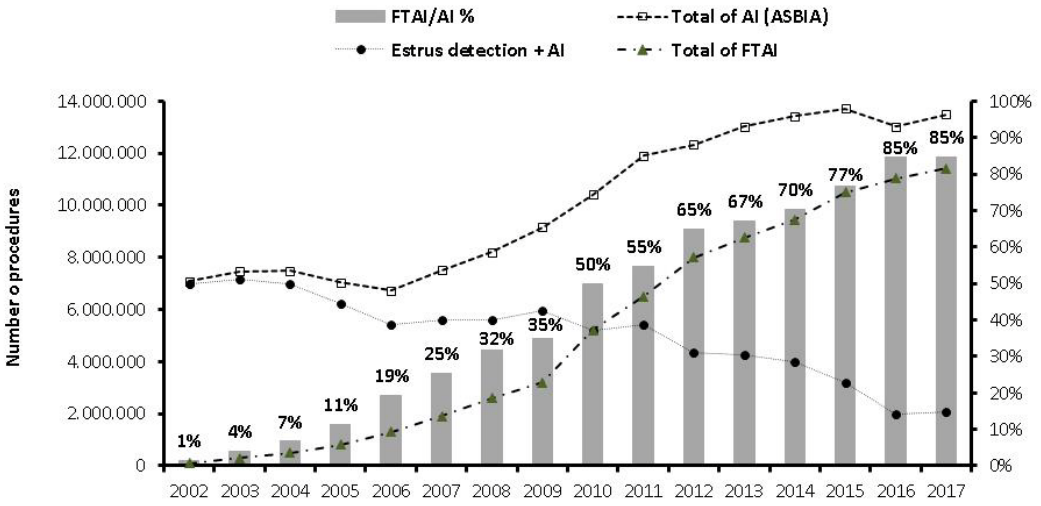

Figure 6. Illustration of the increased use of artificial insemination (AI) and fixed-time AI (FTAI) in Brazil. The numbers of AI were done after estrus detection, while the numbers of FTAI are an estimate based on the number of protocols sold (information provided by pharmaceutical companies in the sector) and the total number of AI is based on the numbers of semen straws sold (*ASBIA, 2017). Data were organized by Departamento de Reprodução Animal-FMVZ-USP, São Paulo, Brazil, 2017. Adapted from Baruselli *et al*., 2017. *ASBIA (Brazilian Artificial Insemination Association).

Financial benefits of the ruminant reproductive revolution in Brazil

It is estimated that the impact of FTAI on the dairy and beef industries in Brazil is ~U$800 million of extra income per year (Baruselli *et al*., 2017). The use of FTAI has resulted in an estimated 3,500 veterinarians being directly involved with ~U$175 million of economic activity, and there has been an increase of 8% in calf production, representing an additional income of ~U$253 million. Thus, FTAI in Brazil has increased income in the beef production chain by more than a half billion U$ per year. In the dairy industry, FTAI has added U$278 million per year by reducing calving intervals and increasing the use of genetically improved sires. It is estimated that FTAI has reduced the calving interval by 30 days and increased annual milk production by 10%, resulting in ~U$234 million of additional income per year for dairy producers.

Future considerations for continued development of the embryo transfer industry

Despite the considerable progress made in the application of bovine embryo transfer technologies in South America over the past two decades, especially with IVP in Brazil, donor selection has leaned heavily on phenotypic traits. For real genetic gain, generation intervals must be shortened, selection intensity must be increased and the accuracy of selection must be improved (Smith 1988). Genomic techniques have now become essential for the selection of donors (male and female) used in embryo transfer (Ponsart *et al*. 2014). In the dairy industry, genetic progress has been accelerated by the use of embryo transfer combined with genomic selection. This could have an even greater impact in the beef industry, as genomic traits for beef production are further developed.

Although the use IVP embryos in South America has increased greatly over the past 15 years, a major limitation has been that most IVP embryos had to be transferred fresh. Cryopreservation of IVP embryos, especially within *B. indicus* breeds, has been very difficult. Although improvements in culture conditions and embryo grading systems will likely increase success with cryopreservation, a recent publication by Sanches *et al*. (2016) provides convincing evidence that the Direct Transfer of frozen/thawed IVP embryos can result in satisfactory pregnancy rates. With continued improvements in this technology, the use of cryopreserved IVP embryos will no doubt increase.

Sex-selected sperm in the production of IVP embryos has obvious advantages, especially with the recent increase in the use of IVP in the generation of dairy embryos. However, Siqueira *et al*. (2017) have recently reported that the use of reverse X-sorted spermatozoa in the production of IVP embryos resulted in an alteration of embryonic programming that persisted postnatally and caused an effect on milk production in adulthood. Thus, benefits of the use of sex-selected semen in the production of IVP embryos could be offset by adverse programming events. It is not known whether regular sexing technologies have similar effects, and whether this effect can be overcome. Obviously, this finding raises concerns that must be resolved.

Summary and conclusions

The ability to control follicular wave emergence and ovulation in cattle has eliminated the need for estrus detection allowing for FTAI and FTET, and the initiation of superstimulation of embryo donors on predetermined schedules. In addition, the utilization of eCG in these protocols has increased pregnancy rates, especially in cattle experiencing postpartum anestrus. Practitioners in most South American countries are utilizing these protocols which has facilitated the application of assisted reproductive technologies. The utilization of FTAI has resulted in great increases in the use of AI, and the adaption of these protocols for FTET has increased the use of bovine embryo transfer, especially with IVP embryos. Currently, FTAI procedures account for 85% of all AI done in Brazil, and a similar trend is occurring in Argentina and Uruguay. Although the numbers of IVD embryos has tended to decrease, especially in Brazil, IVP has increased greatly with more than 57% of all IVP embryos in the world produced in South America. There has been a remarkable shift in the use of IVP from beef breeds to dairy breeds in Brazil, with only a modest decrease in embryo production. Collectively, the successful application of assisted reproductive technologies in South America is resulting in the dissemination of new and improved genetics and increased reproductive performance in all classes of cattle, with a corresponding increase in economic activity.

Acknowledgements

The authors thank Drs. GP Adams for Figure 1 and JHM Viana for Figure 4. Thanks also to Vetoquinol Inc. for support of Mapletoft and Bó.

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001 Reproductive Endocrinology

Effects of follicular ablation on follicular growth and codominance in beef cattle

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The process of follicular selection results in one or two dominant follicles that reach a size of > 9 mm of diameter. Selection of two dominant follicles with ovulatory capacity from a follicular wave is called co-dominance. The mechanisms by which two follicles reach dominance and acquire ovulatory capacity remains unclear. To increase our understanding about ovarian physiology, we evaluated the patterns of follicular growth in beef cows during the periovulatory period. Twenty multiparous cows weighing > 350 kg were selected, estrus was synchronized with intravaginal implant of progesterone (0.5 g), estradiol benzoate (2 mg i.m.) and prostaglandin F2α (Cloprostenol 500 µg i.m.). The cows were randomly divided into two groups: Group 1 (G1) ablated group: All follicles larger than 5 mm were ablated using a 20-gauge needle and transvaginal guided ultrasonography 52 h after removal of intravaginal progesterone and injection of prostaglandin F2α in ten cows; Group 2 (G2) control group: Ten cows without follicular ablation were scanned daily to examine follicular growth, appearance of co-dominance and corpus luteum development by transrectal ultrasonography. The largest follicle reached a diameter of > 9 mm between the second and third day of the follicular ablation. The cows of the control group (G2) ovulated between 3 and 4 days after prostaglandin F2α treatment. Ovulation in the ablated group occurred between 5 and 8 days after ablation. Five cows from the ablated group developed co-dominance and double ovulation, as confirmed by the presence of two corpora lutea. However, co-dominance and double ovulation was not observed in the control group. In conclusion, follicular aspiration resulted in an increase in co-dominance and double ovulation in beef cattle.
Acknowledgements: This research is supported by CONACYT (PINV 15-0023), Paraguay.

002 Reproductive Endocrinology

Supplementation of 17ß-estradiol and progesterone in the co-culture medium of bovine oviductal epithelial cells and ovine spermatozoa reduces sperm kinematics and capacitation

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In mammals, spermatozoa attach to oviductal epithelial cell and this interaction is able to maintain fertilizing ability until ovulation occurs. During the estrous cycle, sperm-oviduct interactions and oviduct-secreted proteins are influenced by the dynamics of the hormonal environment. Thus, this study assessed the effect of hormonally supplemented bovine oviductal epithelial cell (BOEC) co-culture on ram sperm function throughout 24 h of incubation. Ram cooled-stored spermatozoa were selected by swim-up and then co-cultured separately for 24 h at 38.5ºC under 5% CO_2 with either: (1) Fert-TALP medium (positive control – POSControl); (2) Fert-TALP medium without any capacitating substance (modified Fert-TALP) supplemented with 17beta-estradiol (E2) and progesterone (P4) at concentrations similar to follicular phase (Follicular NEGControl); (3) modified Fert-TALP medium supplemented with E2 and P4 concentrations similar to luteal phase (Luteal NEGControl); (4) BOEC cultured in the same medium of Follicular NEGControl group (Follicular BOEC group); (5) BOEC cultured in the same medium of Luteal NEGControl group (Luteal BOEC group). Sperm kinematics (analyzed by computer-assisted semen analysis - CASA), capacitation status and sperm plasma membrane integrity were evaluated in different intervals (0, 2, 4, 6, 18 and 24 h). The variables were subjected to a repeated measure two- way ANOVA (mixed model) and Bonferroni *post hoc* test ($P < 0.05$). Sperm plasma membrane integrity was not affected ($P > 0.05$) by BOEC co-culture, regardless the phase of the estrous cycle. At 2 and 4 h, the Luteal BOEC group presented lower ($P < 0.05$) progressive motility (PM) and total motility than the Luteal NEGControl group. At 4 h, the Follicular BOEC group showed lower ($P < 0.05$) velocimetric parameters (straight-line velocity, average path velocity, curvilinear velocity and beat/cross frequency) and PM than the Follicular NEGControl group. Throughout incubation, both BOEC co-culture groups showed a decrease ($P < 0.05$) in their capacitation rate compared with the POSControl group. On the other hand, the Luteal BOEC group had a greater ($P < 0.05$) noncapacitated rate than the POSControl group and Luteal NEGControl group. In conclusion, co-culture between ram cooled-stored spermatozoa and BOEC submitted to a hormonal environment similar to the follicular and luteal phases is able to suppress motility and also has a role on delaying sperm *in vitro* capacitation and consequently, prolonging the life span of spermatozoa. This study was supported by FINEP and FAPERJ.

003 Reproductive Endocrinology

Effect of zinc supplementation on the area of corpus luteum and progesterone serum concentration

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Zinc (Zn) is a relevant trace element in the body. The function of Zn involves a wide range of biological processes including cell proliferation, immune function, and defense against free radicals. Zinc is an essential component of Cu/Zn-SOD that plays a key role in the maintenance of a functional corpus luteum (CL), in its morphology and progesterone (P_4) production (Kawaguchi *et al*., 2013). The aim of this study was to evaluate the effect of parenteral Zn supplementation at the beginning of fixed-time artificial insemination (FTAI) on corpus luteum size, P_4 and Zn serum concentration. Multiparous Aberdeen Angus cows (n =27) were randomly assigned to two groups: Control (n=16) and Zinc (n=11) group supplemented with 400 mg $ZnSO_4$ injected at the beginning of FTAI protocol (day 0). Follicular waves were synchronized by intravaginal insertion of a CIDR for seven days and an intramuscular (i.m.) injection of estradiol (E_2) benzoate (day 0). At day 7, CIDR was removed and a i.m. injection of PGF2α and E_2 cypionate was applied. All cows were inseminated on day 9. Blood samples were collected on day 0, 7, 9 and 16. The variables assessed were Zn serum concentration (day 0, 7, 9 and 16), area of preovulatory follicle (**APF**), and E_2 serum concentration (**E_2SC**) at insemination time (day 9), area of corpus luteum (**ACL**) and P_4 serum concentration (**P_4SC**) at day 16 and pregnancy rate (day 40). The statistical analysis was carried out with SAS. Continuous response variables were analyzed with linear models and pregnancy rate (percentage) was analyzed by logistic regression. Serum zinc concentrations (Mean ± SEM) were not affected by Zn supplementation for Control= 92,8 ± 8,3; 130,4 ± 8,3; 99 ± 8,3; 89,3± 8,3 µg/dL; and Zinc= 89,6 ± 10; 121,6 ± 10; 100,8 ± 10; 90,6 ± 10 µg/dL at days 0, 7, 9 and 16 respectively ($P > 0.05$). These results showed that 66.6 % cows (18/27) had serum Zn deficiency (< 90 µg/dL) at the beginning of FTAI protocol (day 0). Zinc supplementation did not modify APF (Control= 10.1 ± 1.0; Zn= 12.9 ± 1.2 mm^2), E_2SC (Control= 17.8 ± 1.0; Zn= 16.3 ± 1.2 pg/mL) and ACL (Control= 34.8 ± 2.7; Zn= 38.6 ± 3.7 mm^2) when all cows were considered. However, Zn supplementation increased ACL (Control= 32.6 ± 2.9; Zn= 43.5 ± 3.9 mm^2; $P < 0.05$) in Zn deficient cow and tended to increase APF (Control= 9.7 ± 1.2; Zn= 13.6 ± 1.5 mm^2; $P = 0.097$). The P_4SC were increased by Zn supplementation when all cows were considered (Control= 4.2 ± 0.4; Zn= 5.7 ± 0.5 ng/mL; $P < 0.05$). The P_4SC of deficient cow were similar between treatments (Control= 4.1 ± 0.6; Zn= 5.4 ± 0.8 ng/mL; $P > 0.05$). Pregnancy rates at day 40 was higher but not significantly different for cows injected with Zn respect to Control group (Control= 46.1%, 50%; Zn= 80%, 100%, considering all and deficient cows respectively $P > 0.05$). In conclusion, Zn supplementation at the beginning of the FTAI protocol in deficient cows increased corpus luteum area, and increase serum progesterone concentrations when all cows were considered in the analyses. This study provide evidence that parenteral Zn supplementation may enhance pregnancy rates, even in those cows that present adequate Zn serum concentrations.

004 Reproductive Endocrinology

Delayed time of luteolysis using ovulatory doses of GnRH on days 8 and 15 after insemination in dairy cows

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Avoidance of corpus luteum (CL) regression during the critical period after fertilization is a main goal for reproductive success. Synthesis of uterine prostaglandin F2-alpha is stimulated by the increased responsiveness of the endometrial epithelium to oxytocin pulses following a coordinated action of progesterone (P4) and estradiol (E2) on their receptors (Silvia *et al*., 1991. Biol Reprod, 45:655-663). Experiments using follicular ablation between days 9-15 after ovulation managed to decrease E2 concentrations and delay the time of luteolysis (Araujo *et al*., 2009. Biol Reprod, 81:426-437). This study aimed to test the following hypotheses: 1) Cows receiving Gonadotropin Releasing Hormone (GnRH) on day 8 after artificial insemination (AI) will generate accessory CL and start a new follicular wave. 2) Cows receiving GnRH 15 days after AI will ovulate the dominant follicle and have delayed luteolysis. 3) The combination of treatments would improve the number of pregnant cows (P/AI). Four treatments were randomly assigned to 200 lactating Holstein cows submitted to fixed time AI 72 days after calving using double-ovsynch (Souza *et al*., 2008. Theriogenology, 70:208-215) synchronization protocol: 1) Control: No further treatment received (n=51); 2) G8: Cows received an intramuscular injection of 200µl of GnRH (Gonadorelin Acetate, Gonabreed, Parnell Pharmaceuticals, Overland Park, KS, USA) on day 8 after AI (n=46); 3) G15: Cows received 200µl of GnRH on day 15 after AI (n=51); 4) G8-15: Cows received 200µl of GnRH on day 8 and 200µl of GnRH on day 15 after AI (n=52). Blood samples were taken on days -3, -1, 8, 15, 18, 20, 22, 25, and 27 relative to AI to determine P4 concentrations, and ultrasound of ovarian structures was performed in order to determine CL regression in the non-pregnant cows. Undetermined CL regressions or ovulations were excluded from the analysis. Ovulatory response to GnRH on day 8 (80.6%, 79/98) was higher (P< 0.0001) than on day 15 (48.5%, 50/103), with no differences between G8 and G8-15 (P=0.44) nor G15 and G8-15 (P=1.0). Timing of luteolysis in non-pregnant cows was delayed in the G15 treatment (22.07±0.82d, n=14) compared to control (19.71±0.43d, n=17; P= 0.01) and G8 (19.62±0.67d, n=21; P= 0.03). For cows receiving GnRH on day 15, timing of luteolysis was longer for ovulating (24.71±0.80d, n=7) compared to not-ovulating cows (21.18±0.75, n=11; P= 0.007). However, not-ovulating cows to day 15 GnRH tended (P=0.09) to have longer time of luteolysis than cows not receiving GnRH (19.66±0.42, n=38). Overall conception rate (P/AI) was 45% (90/200) for pregnancy diagnose at day 32, with no difference detected between groups (control 41.2% (21/51); G8 37.0% (17/46); G15 52.9% (27/51); and G8-15 48.1% (25/52); P=0.40). Injection of GnRH on day 15 after AI causes ovulation of dominant follicle present in about half of the cows treated and extends the life of the CL increasing the risk of pregnancy success. Experiments with larger number of animals are required to determine whether injecting GnRH at day 15 after AI will improve P/AI, and also to determine whether steroidogenesis of E2 is affected after treatment regardless ovulation occurs or not.

005 Reproductive Endocrinology

The anti-equine Chorionic Gonadotrophin (eCG) antibody response after an eCG treatment during the non-breeding season does not affect the semen quality during the following breeding season in bucks

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Equine Chorionic Gonadotrophin (eCG or PMSG) is a glycoprotein with FSH/LH effects that has been widely used to induce follicular growth and ovulation in females. However, it may also be used to improve male reproduction. In a previous study we administered 5 doses of eCG (an initial dose of 800 IU, followed by 4 doses of 500 IU administered every 5 days), to bucks during the non-breeding season, observing an increase of testosterone secretion and an enhancement of fresh and frozen-thawed semen quality during the period in which eCG was administered. However, we also observed an increase in anti-eCG antibodies titer (435.6 ± 31.3 nmol/L vs 29.6 ± 4.0 nmol/L in treated vs untreated bucks). In female goats, repeated treatment with eCG is associated with a decrease in fertility caused by a possible cross-reaction, as endogenous LH or FSH can be blocked by circulating anti-eCG antibodies. Therefore, the aim of the present study was to determine if eCG treatment of bucks during the non- breeding season had negative consequences on testosterone concentration or seminal parameters during the first breeding season following the treatment. The study was performed during the breeding season (February – March, summer in the southern hemisphere) for 30 days, starting 91 days after the last eCG administration (treated group: n=10, untreated group: n=9). Testosterone was measured in serum from samples collected every 3 days by radioimmunoassay, using a solid phase kit. Anti-eCG antibodies were quantified with an ELISA kit in serum from samples collected on Day 91 and 98. Semen was collected weekly by electroejaculation. Statistical analysis was performed using a mixed model of SAS, considering the treatment (treated vs untreated bucks), the time (day of collection), and the interaction between treatment and time. The anti-eCG titer was still greater in treated than in untreated bucks (181.7 ± 44.0 vs 28.1± 7.6 nmol/L; P=0.009). However, there were no treatment differences, nor an interaction between treatment and time in testosterone concentration, sperm concentration, motility, percentages of motile spermatozoa, progressive motility of spermatozoa, or of spermatozoa with a functional membrane. Overall, it was concluded that although the bucks that were previously treated with eCG had greater amount of anti-eCG antibodies, there were no negative consequences on testosterone concentration or seminal parameters during the first breeding season following the treatment. We acknowledge Milton Pintos, María Noel Viera, Gerardo Less and Matias Fiorelli for their help during the collection of the samples. Also to Syntex Uruguay for providing the hormone used in this study. This study was supported by CSIC (Universidad de la República, Uruguay). FB received a scholarship from Agencia Nacional de Investigación e Innovación (ANII, Uruguay).

006 Reproductive Endocrinology

Timing of regression of contralateral accessory corpora lutea in pregnant lactating dairy cows

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Preliminary data have suggested that in cows with accessory corpus luteum (ACL) pregnancy loss is greater when this ACL is contralateral to the pregnancy. The aim of the study was to investigate an experimental model related to regression of ACL that were ipsilateral or contralateral to the pregnancy (until day 70 of gestation) in dairy cows. Sixteen lactating Holstein cows bred by fixed-time artificial insemination (FTAI; D0) were treated with GnRH 5 d after AI (D5) to form an ACL. Ovarian and uterine ultrasound evaluations were performed and blood samples were collected daily until D70 of pregnancy to evaluate ovulation, pregnancy, CL size and maintenance, and to analyze circulating progesterone (P4) concentrations. On D28, 13 cows were confirmed pregnant and all had ACL. On D70, cows were submitted to an oxytocin challenge with 100 IU oxytocin i.v. and concentrations of PGFM (pg/mL) were analyzed 0, 30, 60, 90, and 120 min after the oxytocin infusion. Statistical analyzes were performed using PROC GLIMMIX and MIXED of SAS 9.4 (LSM ± SEM; $P \leq 0.05$). In 9 pregnant cows, the ACL was contralateral to the original CL and in 5 pregnant cows, the CL was ipsilateral (1 cow had ACL in both ovaries). There were 2 periods in which luteolysis of the contralateral ACL occurred: between D22-24 (early luteolysis) or D48-53 (late luteolysis) after AI. Luteolysis occurred more frequently in cows with contralateral ACL [88.9% (8/9)] than in cows with ipsilateral ACL [0% (0/5)]. Interestingly, most [75% (6/8)] of contralateral ACL regressed late (average of day 50.3 after AI). The P4 concentration (ng/mL) was high and did not differ in cows that did not undergo early (11.1 ± 0.6) or late (10.4 ± 0.8) CL regression. Circulating P4 of cows that had either early or late luteolysis was similar on days -2, -1 and on the day of the onset of luteolysis (8.5 ± 3.3) determined by CL size using ultrasound. However, 1, 2 and 3 d after the onset of luteolysis (early or late luteolysis) P4 was lower for cows with CL regression than for cows without luteolysis (5.6 ± 2.9 vs 10.5 ± 0.8). The oxytocin challenge on D70 of pregnancy increased PGFM concentrations at 30, 60 and 90 min after oxytocin compared to time 0 or 120 min after oxytocin (19.5 ± 4.3^{a}, 40.3 ± 8.8^{b}; 35.0 ± 6.2^{b}, 33.5 ± 4.6^{b}, and 17.5 ± 4.2^{a}; for 0, 30, 60, 90, and 120 min, respectively). Despite PGF release after oxytocin, no cow underwent luteolysis of the original CL or had pregnancy loss after the oxytocin challenge. In summary, ipsilateral ACL did not regress during the first 70 days of pregnancy, whereas most contralateral CL regressed during this period, especially with late luteolysis, providing evidence for the involvement of local mechanisms in regression of ACL and in protection of the ipsilateral CL during the first and second month of pregnancy. In addition, oxytocin-induced release of PGF without regression of the original CL or loss of pregnancy at 70 d of pregnancy provides evidence that the pregnancy maintains the ipsilateral CL via mechanisms that prevent either transport or action of PGF on the CL. Wisconsin Experiment Station (WIS01240), BARD (IS-4799-15), FAPESP, CNPq, and CAPES.

007 Reproductive Endocrinology

Fractal analysis is a useful tool to evaluate bovine luteal development

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The *corpus luteum* (CL) is a temporary endocrine gland that is formed after ovulation and is the structure responsible for the initial maintenance of gestation. The tissue remodeling by which CL is formed is poorly described, particularly with respect to extracellular matrix components. To assist in evaluating and quantifying tissue changes, fractal dimension (FD) becomes a useful method, being used as a diagnostic tool for retinopathies, histopathological studies of neoplasms, hepatocyte morphometry, liver fibrosis and cardiac studies. Furthermore, it is a useful technique for quantifying the organization in an image from fractals that describe the amount of space and the self-similarity of the structure, once FD detects subtle morphological changes and performs functional quantitative measures. Based on this, we hypothesized that fractal analysis will be different between functional and regressing bovine CLs. For this, c*orpora lutea* was morphologically classified into two developmental stages as previously described (Ireland *et al*.,1980): functional CL (mid-cycle CL) showed well developed vasculature often visible at the apex and were completely orange or yellow, measuring from 1.6 to 2.0 cm; regressed CL showed no visible vasculature at the surface and were pale yellow to incolor measuring less than 1 cm diameter. Ten CLs were collected from a local abattoir (SP): five of functional CLs and five of regressed CL. The CLs were dissected, fixed in methacarn and processed for light microscope. The slides stained with H&E were designed to fractal analysis. One section of each CL was used to acquire five images/ section (totalizing 25 images per CL stage). After, images were binarized for reading and were the estimated by the box-counting method, through the software Image J. The result can be quantitatively expressed as FD = (Log Nr / log r-1) and, for this reason, the dimension will always quantify between 0 and 2. The means were compared with t-test using JMP software (SAS InstituteCary, NC). Differences were considered significant when $P \leq 0.05$. The results demonstrated a higher fractal dimension in functional CL (1.79±0.009) compared with regressed CL (1.66±0.02; P=0.0016). In conclusion, the higher FD observed during luteal regression showed that this analysis is an effective method to evaluate the tissue changes observed during luteal development in cattle. The authors are grateful to grant #2013/11480-3, São Paulo Research Foundation (FAPESP).

008 Reproductive Endocrinology

Hormonal treatment post insemination for induction of accessory corpora lutea and production of progesterone in sheep

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Different therapeutic strategies have been used with the objective of increasing the concentration of progesterone (P_4) and improving luteal function to reduce embryonic losses. Hormonal treatments in sheep were carried out in different breeds, animal categories and seasons of the year, using gonadotrophin releasing hormone (GnRH) or human chorionic gonadotrophin (hCG) in the early or late luteal phase. The aim of the study was to evaluate the effect of the administration of GnRH or hCG at day 4 post fixed-time artificial insemination (FTAI) on the induction and maintenance of accessory corpora lutea (acc-CL) and on the production of serum P_4 concentration. Multiparous Merino ewes (n= 36) were treated for estrus synchronization using intravaginal progestogen sponges (60 mg of MAP; Progespon®, Syntex, Argentina), during 14 days and a single dose of equine Chorionic Gonadotropin (200 IU of eCG, i.m.; Novormon®, Syntex, Argentina) was administered at the end of progestagen treatment. At 53-56 h after sponge removal, FTAI was performed vaginally with a dose of 100 million spermatozoa of fresh semen. The ewes were assigned randomly to three groups on day 4 post FTAI: 1. GnRH group (n= 12, 4 μg of GnRH, i.m., Buserelin, Receptal®, Intervet, Argentina), 2. hCG group (n= 12, 300 IU of hCG, i.m., Gonacor®, Ferring, Argentina) and 3. Control group (n= 12, 1 mL of saline solution, i.m.). Laparoscopic observation of the ovaries at day 4, 10 and 21 post FTAI was performed to determine the presence of ovulatory CL (days 4, 10 and 21) and acc-CL (days 10 and 21). Serum P_4 concentration was assessed by chemiluminescence on days 4, 7, 10, 14, 17, 21, 28 and 35 post FTAI. The number of acc-CL was compared by one-way ANOVA. The serum P_4 concentration in pregnant ewes was analyzed by a generalized linear model with time-repeated measurements. Statistical significance was accepted from $P<0.05$. The hCG group showed higher mean concentrations of P_4 on days 7, 10, 14, 21, 28 and 35 post FTAI compared with the GnRH group and the Control group ($P<0.05$), while no differences were observed between these two latter groups ($P>0.05$). In all groups, an increase of serum P_4 concentration was observed on day 35 post FTAI ($P<0.05$). The presence of an acc-CL was observed in 85 and 50 % of ewes treated with hCG or GnRH, respectively, whereas no ewes with an acc-CL were observed in the Control group ($P<0.05$). The hCG group had higher concentration of P_4 in the animals that had an acc-CL compared to those that did not generate acc-CL ($P<0.05$), while no differences were observed in the GnRH Group ($P>0.05$). The acc-CL were maintained until 21 days post FTAI in pregnant sheep treated hormonally. In conclusion, administration of hCG or GnRH at 4 days post FTAI induced and maintained the formation of an acc-CL. However, serum concentration of P_4 increased only in the hCG group and maintained until day 35 post FTAI. Differences in the pharmacodynamics of these two hormones might induce acc-CL with different steroidogenic capacity. Further research should be done to assess the effect of these hormones on the histological and functional characteristic of acc-CL. Funded by Projects PNSA 1115053 (INTA) and PICT 2012-2238 (FONCyT).

009 Reproductive Endocrinology

Dietary restriction in sheep: uterine functionality in ewes with different body reserves during early gestation

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The aim of this work was to study the reproductive response to a food restriction in ewes with different body condition score (0, emaciated-5, obese) at the beginning of the experiment (BCSi). During the breeding season, 36 Rasa Aragonesa ewes were divided into 2 groups with different BCS: BCS> 2.75 (high, H, 2.9 ± 0.04) and BCS <2.25 (low, L, 2.1 ± 0.04). Both groups received a diet to cover energy and protein maintenance requirements for 20 days, after which they were randomly assigned to two nutritional treatments: 1.5 (control, C) or 0.5 (undernourishment, S) times the daily maintenance requirements establishing four groups: high BCSi fed the control diet (HC, n = 9), high BCSi undernourished (HS, n = 10), low BCSi control (LC, n = 9) and low BCSi undernourished (LS, n = 8). The first day of the experimental diet, ewes were estrous synchronized with intravaginal sponges for 12 days and mated by ten rams (estrus=Day 0). Embryos were recovered on Day 5, and embryo viability was estimated *in vitro* after incubation for 48 h. Only ewes that had embryos were included in this study. Gene expression of uterine receptors of progesterone (PR), estrogen (ER), growth hormone (GHR), insulin (INSR), leptin (LEPR), adiponectin (ADIPOR2) and IGF-I, IGF-II and IGF binding proteins (IGFBP2-6), were determined by RT-PCR. All the variables were analyzed by ANOVA using a mixed procedure that included in the model BCSi, the nutritional treatment and their interaction. Embryo data was evaluated using Proc Genmod. Undernutrition decreased liveweight and insulin concentration but had no effect on leptin and IGF-I levels. BCSi affected all hormones, but the effect was more pronounced on IGF-I (395 *vs* 261 ng/mL for H and L groups respectively, P<0.05). Embryos from ewes with greater BCS tended to have higher viability rates (83.3 *vs* 58.3%, P=0.09) and *in vitro* embryo development (75.0 *vs* 48.4%, P=0.1). Uterine gene expression of most of the studied genes was not affected by BCSi or nutritional treatment. Ewes with low BCSi tended to have more *INSR* mRNA (P=0.07) than ewes with high BCSi, and LC ewes tended to have more *GHR* mRNA than HC ewes (P=0.10). *GHR* mRNA expression was higher in undernourished ewes than in control ewes (P=0.02). The changes in uterine gene expression suggest a compensatory mechanism that increases hormone tissue sensitivity when the hormone is decreased. Besides, *BP2* mRNA tended to be higher in undernourished than in control group (P=0.08). Overall, BCSi – but not undernutrition- effect on embryo viability is consistent with the marked differences found in IGF-I concentrations, which is a main promoter of embryo development and uterine function. Surprisingly, even if aligned in a similar pattern, BCSi and undernutrition affected few uterine genes. It should be taken into account that gene expression does not always reflect actual protein content. In conclusion, in the present study, embryo viability depended mainly on metabolic history (body reserves) than on more recent feeding level.

010 Reproductive Endocrinology

Testosterone concentration profiles in bucks treated with a GnRH-agonist implant or immunized against GnRH

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The chronic use of GnRH-agonists or the immunization against GnRH are reversible contraceptive methods that inhibit temporarily the hypothalamic-pituitary-testicular axis activity. The aim of this study was to compare the changes in testosterone profile in bucks treated with a GnRH-agonist or immunized against GnRH. Seven bucks were treated with deslorelin (4.7 mg) subcutaneous implants (GnRH agonist that is released continuously; group AGO), 7 with an anti-GnRH vaccine s.c. (300 µg GnRH-protein conjugate, two doses, group IMM), and 9 bucks remained untreated as controls (group CON). The study lasted 18 months, beginning the treatments during late spring (Week 0). Blood samples for measuring testosterone concentration (Tc) were weekly collected from Week -4 to 3 (Month 1), and monthly from Month 2 to 17. Data were analyzed using a mixed model considering as main effects the treatment, time, and the interaction between treatment and time, and are presented as LSmeans ± SEM. Bucks from AGO group had greater Tc than CON and IMM bucks in Months 1 (19.6±2.2 nmol/L, 12.5±1.8 nmol/L and 7.6±2.1 nmol/L, respectively, $P<0.05$ for all comparisons) and 2 (24.0±3.4 nmol/L, 5.9±2.9 nmol/L and 1.0±3.4 nmol/L, respectively, $P<0.05$ for all comparisons). Bucks from CON group had greater Tc than AGO and IMM bucks in Months 3 (31.8±2.9 nmol/L, 11.0±3.4 nmol/L and 7.9±3.4 nmol/L, respectively, $P<0.05$ for all comparisons) and 4 (19.2±2.9 nmol/L, 7.3±3.4 nmol/L and 7.9±3.4 nmol/L, respectively, $P<0.05$ for all comparisons). From Months 5 to 14 there were not differences in Tc between groups. Bucks from CON and IMM groups had greater Tc than AGO bucks in Months 15 (25.1±3.6 nmol/L, 29.3±3.7 nmol/L and 10.4±3.7 nmol/L, respectively, $P<0.05$ for all comparisons) and 16 (19.4±2.9 nmol/L, 23.5±3.7 nmol/L and 6.7±3.7 nmol/L, respectively, $P<0.05$ for all comparisons). Bucks from IMM group had greater Tc (33.6±3.7 nmol/L) than CON (16.4±3.1 nmol/L, $P<0.05$) and AGO (8.4±3.7 nmol/L, $P<0.05$) bucks in Month 17. At the beginning of the study AGO bucks had greater Tc due to the administration of the GnRH agonist ("flare up" effect). However, it later decreased and remained low, probably due to the inhibition of the pituitary or the testes, effect that was not overcome in 17 months. On the other hand, Tc decreased in IMM bucks but recovered in coincidence with the increase in CON bucks, during the onset of the breeding season. However, at the end of the study IMM bucks had greater Tc than CON bucks. The anti-GnRH vaccine probably lost the effect due to a decrease of the immune response, followed by a rebound effect. In sum, the effects elicited by the chronic use of a GnRH agonist lasted longer and produced a more pronounced decrease in Tc than the immunization against GnRH.

011 Reproductive Endocrinology

Follicle and hormone profiles during selection of the dominant follicle under different physiologic conditions in Holstein heifers

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Diameter deviation during selection of the future dominant follicle (F1) from the future largest subordinate follicle (F2) is defined as continued growth rate of F1 and decreased growth rate of F2. Expected diameter deviation in Holstein heifers begins when F1 is 8.5 mm and has been classified as conventional (F2 ≥ 7.0 mm at deviation) or undersized (F2 < 7.0 mm). Our overall objective was to understand the circulating hormones and ovarian physiology associated with conventional and undersized deviation. This study compared circulating FSH, LH, and P4 with the follicle dynamics during three different physiological conditions (spontaneous wave 2 vs a wave 2 induced by aspiration of wave-1 follicles, wave 1 vs 2, and conventional vs undersized deviations). Holstein dairy heifers (N = 24) were evaluated during wave 1 and randomized 6 days after ovulation into an induced wave 2 and a spontaneous wave 2. Values were normalized to the day of expected diameter deviation (day 0) and compared for days −2 to 0 and 0 to 2 using the SAS PROC MIXED procedure. Hypothesis 1 was supported that an induced wave 2 and spontaneous wave 2 have similar follicle dynamics. However, the peak of the FSH surge was more prominent at the emergence of an induced wave 2 ($P < 0.003$). Hypothesis 2 was supported that waves 1 and 2 differ in follicle and hormone events. Circulating P4 was less and LH was greater ($P < 0.01$) encompassing deviation in wave 1 than wave 2. Diameter of F1 did not differ but diameter of F2 was greater ($P < 0.01$) on day 0 in wave 1 (7.3 ± 0.2 mm) than in wave 2 (6.6 ± 0.2 mm). Differences between waves were not found when deviation in each follicular wave was classified as conventional vs undersized deviation and analyzed separately. Hypothesis 3 was supported that hormone differences occur between conventional and undersized deviations. Growth rate of F2 differed ($P < 0.0005$) during days −2 to 0 (conventional, 2.6 ± 0.2 mm/2d; undersized, 1.4 ± 0.3 mm/2d). However, circulating FSH and P4 concentration on days −1 and 0 tended to be greater ($P < 0.06$) in undersized deviations. Thus, the effect of different hormonal conditions on follicle dynamics was observed for F2 and not for F1. Understanding the physiology that produces conventional vs undersized deviations accounted for most differences in follicle dynamics and circulating FSH in these different physiological conditions. In addition, study of wave 2 may be facilitated by inducing a wave 2 since it was similar in follicle dynamics to a spontaneous wave 2. The authors thank the funding support from Eutheria Foundation, University of Wisconsin Experiment Station, CNPq, CAPES, and COLFUTURO.

012 Reproductive Endocrinology

The Periovulatory Endocrine Milieu Affects the Metabolomic Profile of the Oviductal Fluid in Beef Cows

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Oviductal secretions regulate the environment in which sperm storage and capacitation, fertilization, and early embryo development occur; however, molecular control of oviduct receptivity to the embryo is poorly understood. A model for receptivity based on the manipulation of the size of the pre-ovulatory follicle (POF) was used to compare oviductal fluid (OvF) composition on day 4 of the estrous cycle. The central hypothesis was that the size of POF modulates the periovulatory endocrine milieu and affects the composition of the OvF. Cycling, non-lactating, multiparous Nelore cows were presynchronized prior to receiving cloprostenol (large follicle [LF] group) or not (small follicle [SF] group), along with a progesterone (P4) device on Day (D) -10. Devices were withdrawn and cloprostenol administered 42–60 h (LF) or 30-36 h (SF) before GnRH agonist treatment (D0). As a result, greater proestrus estrogen concentrations, corpora lutea and early diestrus progesterone concentrations were also observed in LF group in comparison to SF group. Four days after GnRH-induced ovulation, the oviduct was dissected and lumen was flushed using 2 mL of sterile PBS to obtain OvF. The OvF was centrifuged to remove cells and debris. Next, the supernatant was frozen in liquid Nitrogen, and stored at -80°C for further analysis. Quantitative mass spectrometry was used to determine the concentration of 21 amino acids (AA), 21 biogenic amines (BA), 40 acylcarnitines (AC), 76 phosphatidylcholines (PC), 14 lysophosphatidylcholines (LP), 15 sphingomyelins (SM), 29 hexoses (HX), and 17 prostaglandins and related compounds (PGC). Multivariate analyses using the software MetaboAnalyst 3.0 were performed to identify which metabolites better explained the separation of experimental groups and which could be potentially used as markers of receptivity. Analytes with 50% or more of missing data were excluded from analyses. Partial Least Squares Discriminant Analysis (PLS-DA) was used to create a scores plot between the two groups and to identify the most important explanatory variables. The PLS-DA showed that the overall metabolite profiles of the LF-LCL and SF-SCL groups were significantly different and that samples from each group were divided clearly into two non-overlapping clusters. The most influential variables to separate the two groups included AAs, PCs, LPs and arachidonic acid. These results were further confirmed by univariate analyses. There were statistical differences in the concentration of 31 metabolites ($P \leq 0.05$) between groups. We concluded that the composition of the OvF is different between cows with contrasting receptivity and fertility status. Although further studies and analyses are needed, it could be assumed that molecules in OvF presenting different concentrations between groups can be used as biomarkers of receptivity. Additionally, it will be critical to identify the function of each of these compounds during early embryo development and to evaluate their potential use as supplements for in vitro production of embryos. The authors thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq): AMGD grant number 150844/2017-4 and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP): MB grant number 2011/03226-4.

013 Reproductive Endocrinology

Local effect of the corpus luteum (CL) on reproductive tract functionality in the ewe

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This study was designed to evaluate the effect of the laterality of CL on embryonic development and oviductal-uterine functionality in the ewe. For this purpose, *in vitro* produced embryos (Day 0: IVF) were transferred on Day 1 into oviducts ipsilateral (ILAT) and contralateral (CLAT) to the CL (18 to 20 embryos per oviduct, 38 to 40 embryos per ewe) in 12 estrous synchronized ewes one day after ovulation. On Day 6, the reproductive tract was obtained at slaughter, and the uterine horns were flushed to collect the embryos. A greater recovery rate, better embryo development, and less degenerated embryos were obtained when transferring zygotes to the ILAT than the CLAT oviduct (de Brun *et al*., 2016. Proc 30th Annu Mtg of Brazilian Embryo Technol Soc (SBTE 2016), p.599). Progesterone, estradiol, insulin, IGF1, leptin and adiponectin concentrations were determined in plasma, uterine fluid, and uterine and oviduct tissue, ILAT and CLAT to the CL. Gene expression of *ERa*, *PR, IGF1, IGF1R, INSR, IGFBP2-6, LEPR*, *AdipoR1y 2* were determined in ILAT and CLAT uterine horns. All statistical analysis were performed using the Statistical Analysis System (SAS), PROC MIXED, where the model was the side relative to ovulation-CL. The ILAT oviductal tissue presented 4 times higher P4 concentrations compared to the CLAT oviduct (57.3 ± 15.1 vs. 11.6 ± 4.8 ng/g of tissue, $P<0.05$); nevertheless, no other differences according to the side of CL were found in hormones concentrations in uterine or oviductal tissues. On the other hand, ILAT uterine fluid presented lower insulin concentrations compared to the CLAT (5.9 ± 0.3 vs. 4.6 ± 0.2 µIU/mL, $P=0.05$), perhaps associated with higher uptake of insulin by the embryo in the ILAT horn. The ILAT uterine horn also had greater expression of *ERa* (1.8 ± 0.2 vs. 0.9 ± 0.3, $P<0.05$), which may be related to a greater previous exposure to preovulatory estradiol. In addition, *LEPR* (1.3 ± 0.2 vs. 0.7 ± 0.2, $P<0.05$) and *IGFBP3* (1.1 ± 0.1 vs. 0.7 ± 0.1, $P=0.08$) mRNA expression was greater or tended to be greater in the ILAT vs. the CLAT uterine horn, and both factors have been positively associated with embryo growth in ruminants (Cerro and Pintar, 1997. Dev Biol, 184:278-95). In conclusion, we suggest a local effect of CL, which is evidenced by the concentrations of progesterone in oviductal tissue, insulin in uterine fluid and the differential gene expression of some target genes according to the side of the reproductive tract with respect location of the CL, which is associated with greater embryo recovery and development in the ipsilateral horn.

014 Reproductive Endocrinology

Functional transitions in the corpus luteum are associated with changes in NR5A2 abundance, which regulates luteal progesterone production

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Nuclear receptor subfamily 5 group A member 2 (NR5A2) has roles in ovulation and early luteal development. However, little is known about its function in the bovine corpus luteum (CL) during acquisition of luteolytic capacity (ALC), luteolysis, or luteal rescue. To identify key regulators of luteal regression or survival, differentially abundant mRNA from an RNAseq study of CL on day 17 of the estrous cycle and pregnancy were compared to a microarray dataset accessed via the NCBI Gene Expression Omnibus that compared luteal response to $PGF_{2\alpha}$ on day 4 and day 11 of the estrous cycle. NR5A2 was among mRNA that were both greater in early pregnancy and lesser after $PGF_{2\alpha}$ on day 11, but not on day 4. The abundance of NR5A2 during functional transitions in the CL was investigated using qPCR and western blot. Luteal abundance of NR5A2 mRNA increased on day 6 compared to day 4 of the estrous cycle ($P < 0.05$), while abundance of its protein was unchanged. Both mRNA and protein for NR5A2 decreased with time during induced luteolysis ($P < 0.05$), but protein decreased earlier than mRNA. NR5A2 mRNA declined by 8 hr, whereas NR5A2 protein tended to decrease by 0.5 hr and decreased by 2 hr after $PGF_{2\alpha}$ injection. During early pregnancy, NR5A2 mRNA changed ($P < 0.05$), with both day 20 and day 23 having lesser mRNA than day 14, but no days differing from day 17; protein abundance did not change. Discrepancies between NR5A2 mRNA and protein during ALC, luteal regression, and early pregnancy indicate that protein abundance may be regulated by miRNA. Thus, miRNA expression during early pregnancy and ALC was measured by Nanostring technology. Twelve miRNA that differed between day 4 and 6 CL and five miRNA that changed (padj < 0.1) during early pregnancy (day 14-23) were predicted to target NR5A2. Most notably, miR-432-5p fit the expression pattern expected for inhibition of NR5A2 during ALC and early pregnancy. Because of the decrease in abundance of NR5A2 during luteolysis and stabilization during early pregnancy, a role in luteal progesterone production was hypothesized. Cultured luteal cells were treated with the NR5A2 antagonist Calbiochem # 505601 (10 μM). There was no effect of treatment on cell viability. However, the NR5A2 antagonist decreased progesterone production ($P < 0.05$). Overall, these data demonstrate a role for NR5A2 as a regulator of progesterone production and implicate miRNA as regulators of NR5A2. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2012-67015-30212 from the USDA National Institute of Food and Agriculture to JLP, Multistate Project NE 1227 (Hatch WV 476), USDA NIFA predoctoral fellowship no. 2017-67011-26062 to CKH, and the Erickson Discovery Grant to AR.

015 Reproductive Endocrinology

The involvement of resistin in the regulation of gonadotropin secretion in sheep

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Relationships between energy metabolism and fertility have been observed in many species. However, there are several metabolic signaling pathways, including those involving the adipokine resistin, that have not been fully explored. Work in cattle and rodents has shown that resistin, in addition to its roles in insulin resistance and inflammation, is involved in the regulation of gonadal and testicular steroidogenesis and gametogenesis. However, its role in the regulation of reproductive processes in other species such as the seasonal breeding sheep is unknown. Herein, we tested the hypothesis that resistin can influence secretion of anterior pituitary hormones and that its effect is dependent on day-length in ewes. Thirty ewes of the Polish Longwool breed, a breed that exhibits strong seasonal reproduction, were ovariectomized with estrogen replacement using subcutaneously inserted estradiol implants. Ewes were fed at libitum and housed in natural photoperiod (longitude: 19°57' E, latitude: 50° 04' N). Intravenous treatments consisted of control or recombinant bovine resistin (rbresistin) in saline: 1) Control (saline; n = 10), 2) Low resistin (1.0 µg/kg BW; n = 10), and 3) High resistin (10.0 µg /kg BW; n= 10). Experiments were conducted during both short (SD) and long days (LD). Blood samples were collected every 10 minutes during 4 h. Blood plasma concentrations of FSH and LH, were assayed using RIA. Pulse parameters of LH and FSH secretion were calculated using the Pulsar Computer Program. A season x dose interaction was observed for all hormonal variables measured. Greater concentrations ($P < 0.001$) of LH and FSH were observed during SD compared for LD in all groups. During SD, the High dose (10 µg/kg BW) decreased ($P<0.001$) basal LH and amplitude ($P < 0.05$) of LH pulses and increased ($P < 0.001$) circulating concentrations of FSH. However, the lower dose of resistin decreased ($P < 0.001$) FSH concentrations compared to Controls. During LD, both the Low and High resistin doses increased mean concentrations of LH ($P < 0.001$ and $P < 0.05$, respectively) and FSH ($P < 0.001$). These results demonstrate for the first time that resistin is involved in the regulation of pituitary gonadotropin secretion and this effect is differentially mediated during LD and SD. Further studies are underway to clarify the potential roles of resistin in modulating GnRH/gonadotropin secretion in seasonally-breeding sheep. Research supported by NCN 2015/19/B/NZ9/01314 to DAZ.

016 Reproductive Endocrinology

Expression of inhibin α subunit in bovine theca cells: does inhibin α contribute to the regulation of ovarian androgen production?

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Ovarian follicular fluid contains considerable amounts of 'free' inhibin α subunit but its functional significance remains obscure. Here, we report the unexpected finding of comparable expression levels of INHA mRNA in theca interna and granulosa layers of small/medium size bovine antral follicles (1-8 mm in diameter). Immunoreactive inhibin α subunit protein was also evident in the follicular theca layer and in cultured theca cells. Different molecular weight forms of inhibin α were secreted by cultured theca cells, including pro-αC isolated previously from bovine FF. These observations suggest that the theca interna layer may contribute significantly to the inhibin α subunit content of peripheral blood and antral follicular fluid, hitherto considered to be solely of granulosal origin. In vitro experiments revealed that RNAi-mediated knockdown of thecal INHA inhibited INSL3 and CYP17A1 expression and androgen production ($P < 0.01$) while INSL3 knockdown reduced INHA and inhibin α secretion ($P < 0.01$). These findings suggest a local inhibitory role of thecal inhibin α on androgen production. Despite this, treatment of cultured theca cells with purified pro-αC had no effect on basal or LH-induced androgen production. BMP treatment reduced thecal INHA expression, inhibin α protein secretion and androgen production in an inhibin-reversible manner. However, an in vitro experiment to test the hypothesis that free inhibin α subunit acts locally to modulate the effects of BMP and/or inhibin on androgen production yielded inconclusive results with no significant effect observed. Furthermore, neither circulating nor intrafollicular androgen concentrations were found to differ between control heifers and heifers actively immunized against inhibin α subunit, casting further doubt on thecal inhibin α subunit as having a significant physiological role in modulating androgen production. Further research is required to establish what intra-ovarian or peripheral endocrine role(s), if any, are played by theca-derived inhibin α subunit. supported by BBSRC.

017 Reproductive Endocrinology

Development of a physiological model of proestrus in cows using exogenous hormones

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The aim of this study was to develop a model of proestrus similar to the physiological status using only exogenous hormones. Two total doses of estradiol benzoate (EB) were used to determine the protocol that would more closely mimic the peak circulating E2 (6-12 pg/mL) and morphological and behavioral changes observed during natural proestrus (evaluated in a parallel experiment). Nonlactating multiparous Holstein cows (n = 18) were pre-synchronized with EB (2 mg) and an intravaginal P4 device (1 g) on D-7. On D0 the device was removed, PGF was administrated (0.526 mg) and 2 new P4 devices (2 g each) were introduced. On D1 another PGF was administered. On D4 and D5 all follicles =5 mm were aspirated (OPU). After OPU on D5, one of the P4 devices was removed and cows were randomized into 2 groups (n = 9/group): Low (L) E2 (total EB = 0.4 mg) or High (H) E2 (total EB = 0.8 mg). The EB total doses were divided into 8 treatments given 6 h apart (from 0 to 42 h) using increasing EB amounts (first 2 doses = 10%, 3rd and 4th = 20%, 5th and 6th = 30%, and the last 2 doses = 40% of the total). The second P4 device was removed 18 h after withdrawal of the first one. Blood samples were collected for E2 analysis every 6 h, from D5 to 7, just before each EB treatment. The uterus was examined using transrectal ultrasound to evaluate changes in endometrial thickness (ET) at 0, 12, 24 and 48 h after the beginning of EB treatments. Expression of estrus was observed using tail chalk. Continuous variables were analyzed using PROC MIXED and binomial data using Chi-Square analysis in SAS (P = 0.05; tendency = 0.05 < P < 0.1). Circulating E2 (pg/mL) was very low in all cows from both groups at time 0 (L = 0.1 ± 0.04 vs H = 0.03 ± 0.01). When data were normalized to the time of E2 peak, there was a progressive increase in circulating E2 over time with approximately double the E2 in H than L resulting in greater peak circulating E2 in H than L cows (7.2 ± 0.5 vs 3.5 ± 0.4). Similarly, when data were normalized to the time of EB treatment, circulating E2 increased progressively with an effect of time in both L (greater E2 at 36, 42 and 48 h than time 0) and H (greater E2 at 24, 30, 36, 42 and 48 h than time 0). Further, there was time*treatment interaction, with group H having higher E2 at time 42 (5.6 ± 0.8 vs 3.0 ± 0.3) and 48 (6.8 ± 0.6 vs 2.9 ± 0.5) than L. There was no effect of treatment on ET (mm) but there was an effect of time in both groups with greater ET at time 24 and 48 [L (0 h = 6.6 ± 0.3^{a}; 12 h = 7.4 ± 0.4^{a}; 24 h = 9.3 ± 0.2^{b}; 48 h = 10.6 ± 0.3^{b}); H (0 h = 6.5 ± 0.3^{a}; 12 h = 7.0 ± 0.4^{a}; 24 h = 9.2 ± 0.4^{b}; 48 h = 10.2 ± 0.4^{b})]. Group H tended to have greater expression of estrus (100% vs 66.7%). In conclusion, the model of proestrus using exogenous hormonal treatments had similar hormonal dynamics, changes in ET, and expression of estrus as reported during natural proestrus. Both groups had similar ET changes, however, the group treated with higher EB doses had greater circulating E2 with peak concentrations that more closely mimicked physiological concentrations with all cows expressing normal behavioral estrus. Therefore, a protocol using a total dose of 0.8 mg of EB seems to represent the natural circulating E2 dynamics during proestrus and may be useful for future studies on the mechanisms involved in the physiological changes that occur during proestrus. FAPESP, CAPES and CNPq.

018 Reproductive Endocrinology

Intensity of estrous expression detected by automated monitor and its relationship with concentration of pregnancy-associated glycoprotein

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The aim of the current project was to assess the relationship between intensity of estrous expression, detected by an automated activity monitor system, and the concentration of pregnancy-associated glycoprotein (PAGs) at 24 and 31 days post artificial insemination (AI) in serum and milk. A total of 442 events from 256 lactating Holstein cows were enrolled. Cows were continuously monitored for activity by an AAM (Afimilk). At the time of an alert of increase in activity, all animals had their body condition scored and activity data recorded to determine the intensity of estrous expression. Cows had a sample of milk and blood harvested at 24 and 31 days post AI, for the analysis of the concentration of PAGs. All animals were examined by ultrasonography on day 31 ± 3 and 60 ± 3 days post-AI for the detection of a viable embryo. Pregnancy losses were determined as the percent animals that lost their pregnancy between 31 and 60 days post-AI. Pregnancy data were analyzing using logistic regression with the GLIMMIX procedure and continuous variables were analyzed using ANOVA with the MIXED procedure of SAS. Activity was classified as high or low using the median of the peak activity. Animals that had high activity had higher fertility when compared with animals that had low activity (48.5% vs 35.2%, retrospectively; $P < 0.05$). Pregnancy losses tended to be higher in animals that had low activity at the moment of AI (18.2% vs 8.9%, $P = 0.07$). Animals that had higher activity had higher PAGs in serum at day 24 (2.05 ± 0.2ng/mL vs 2.84 ± 0.3ng/mL; $P = 0.05$) but not milk ($P = 0.18$). PAGs at day 31 post AI were higher in both serum and milk in animals that had a higher increase in activity at the moment of AI than those that did not (serum: 10.41 ± 0.6ng/mL vs. 8.89 ± 0.6ng/mL; $P = 0.04$; milk: 1.24 ± 0.06ng/mL vs 1.01 ± 0.05ng/mL; $P = 0.01$). When we considered only pregnant animals into the analyses animals that had high activity had higher concentration of PAGs in serum (3.43 ± 0.3ng/mL vs. 2.54 ± 0.3ng/mL; $P < 0.01$) but not in milk ($P = 0.29$). PAGs at day 31 post AI were higher in both serum and milk in animals that had a higher increase in activity at the moment of AI than those that did not (serum: 10.18 ± 0.6ng/mL vs. 8.77 ± 0.5ng/mL; $P < 0.05$; milk: 1.18 ± 0.06ng/mL vs 0.97 ± 0.05ng/mL; $P = 0.01$). No difference in PAG concentration at day 24 post-AI were found in serum or milk for those that later lost their pregnancy, however, those that went on to lose their pregnancy had lower PAG concentration at day 31 post-AI both serum and milk (serum: 8.45 ± 1.04ng/mL vs. 9.80 ± 0.4ng/mL; $P = 0.02$, and milk: 0.85 ± 0.12ng/mL vs. 1.10 ± 0.05ng/mL; $P = 0.02$). In conclusion, estrus expression detected by the automated activity monitors were associated with concentration of PAGs at day 24 in serum but not in milk and at day 31 in both serum and milk. Pregnancy losses were found to occur in animals with lower activity and lower concentrations of PAGs at day 31. Authors would like to thank Dairy Farmers of Canada, NSERC and BC Dairy Association.

019 Reproductive Endocrinology

Administration of endothelin-1 induces complete luteolysis in cyclic ewes

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Luteal regression is initiated in domestic ruminants by surges of prostaglandin F-2α (PGF-2α) from the uterus. Studies have demonstrated that ET-1 is required for the manifestation of the luteolytic effects of PGF-2α. A series of experiments was performed to determine if multiple injections of ET-1 would induce luteolysis and reduce the length of the estrous cycles in ewes. Ewes were treated (5/group) with saline, or varying amounts and frequencies of ET-1 as follows: 100 μg (single injection - 1x), 25 μg (4x), 50 μg (2x) and 50 μg (4x), on d 9 of the estrous cycle. Multiple injections of ET-1 were administered at 2 h intervals and jugular venous blood samples were collected before and at frequent intervals through 24 h after treatment and twice daily until ewes returned to estrus and analyzed for concentrations of progesterone. Treatments with ET-1 (100 μg-1x; 25 μg-4x; 50 μg-2x) resulted in a transient decrease ($P<0.05$) in progesterone; levels were similar to control values by 24 h and lengths of the estrous cycle were not affected. Complete luteolysis was induced in all ewes treated with 50 μg (4x) and the length of the estrous cycle was reduced (11.4 d; $P<0.01$). In a second experiment, ewes were treated with 50 μg ET-1 (4x) or saline (n=6/group) on d 9 and concentrations of progesterone and PGFM were determined for 24 h after treatment. ET-1 induced luteolysis in all 6 ewes treated and there was an increase ($P<0.05$) in PGFM from 2 through 18 h after treatment. Exogenous ET-1 induces ovine luteolysis and is associated with concommitant increases in PGFM.

020 Reproductive Endocrinology

The response of ovarian follicle, at the early static phase, to eCG-GnRH in Holstein heifers

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Equine chorionic gonadotropin (eCG) is a potent hormone to enhance follicle growth in cattle. More recently, we have found that 84 h after administrating eCG at the early static phase of follicle development, there is a dominant follicle at growing phase available on the ovary (Hosseini, A. *et al*. 2018 IJVR 19:15-21). The objective of this study was to investigate the effect of ovulating agent (GnRH) on ovarian follicles at the early static phase that is primed with eCG. Holstein heifers were synchronised using two injections of prostaglandin F_2a analogue, 14 days apart. On the second day in which ovarian follicle remained nearly similar size (approximately Day 8 of oestrous cycle, early static phase; Day 0 of experiment), heifers were randomly assigned into two experimental groups (n=5 in each group). On Day 0, Heifers in the treatment group received an i.m. injection of eCG (500 IU; Folligon®; Intervet, Holland). At the same time, heifers in the control group received saline. Heifers in both groups received GnRH analogue (100µg Gonadorelin acetate, GONAbreed®, Parnell, Australia) 84 hrs after receiving eCG/saline. On a daily basis, ultrasound examination was carried out to investigate follicle development and blood sampling was conducted to measure progesterone concentrations. Data were analyzed using GLM procedure. On Day 0, the diameter of the ovarian follicle was similar between control (13.5±0.29 mm) and treatment groups (13±0.32 mm; P>0.05). However, at the time of GnRH administration, the diameter of the ovarian follicle reached at 12.2±0.75 mm and 17.2±0.45 mm in control and treatment group, respectively (P<0.05). From Day 0 to GnRH administration, in the treatment group, follicles were in the growing phase, with the growth rate of 1.2±0.14 mm/day; however, at the same period, ovarian follicles in the control group were in the regressing phase with the growth rate of -0.4±0.18 mm/day. Following GnRH administration, all heifers in the treatment group ovulated, whereas no heifers in the control group ovulated (P<0.05). The average progesterone concentrations between Day 8 to 15 of oestrous cycle were 5.1±0.1 and 6.2±0.26 ng/mL, in control and treatment groups, respectively (P<0.05). In conclusion, administration of eCG in the early static phase of follicle development could change the fate of the ovarian follicle from a regressing to a growing follicle, responsive to an ovulating agent. The authors expressed their sincere appreciation for the financial and academic support of Faculty of Veterinary Medicine, University of Tehran. Special thanks to the Institute of Veterinary Research, Faculty of Veterinary Medicine, University of Tehran, for their kind collaboration for smooth running of this study.

021 Reproductive Endocrinology

Effect of IGF2 on bovine luteinising follicular angiogenesis and progesterone production *in-vitro*

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Insulin-like growth factor (IGF) 2, acting through the IGF type 1 receptor (IGF1R), is likely to play an important role in the follicle-luteal transition in the cow. Importantly, IGF2 promotes granulosa cell proliferation and differentiation and is present in greater concentrations than IGF1 in follicular fluid. *IGF2* mRNA is localised to both granulosa and theca cells with *IGF2* mRNA levels in theca cells greater in dominant follicles than other follicles. Therefore, this study tested the hypothesis that IGF2 will promote angiogenesis and steroid production in bovine luteinising follicular cells *in vitro*. Granulosa and theca cells were dispersed from abattoir-derived bovine antral follicles (10-16 mm, n=4 cultures) and co-cultured under serum-free conditions. In Expt 1, cells were treated with IGF2 (0, 10 or 100 ng/mL) in the absence and presence of FGF2/VEGFA (both 1 ng/mL). On culture day 5, endothelial cell (EC) networks were quantified by von Willebrand factor immunohistochemistry and image analysis. Spent media was assayed for progesterone by ELISA while cell growth/viability was determined by MTT assay. In Expt 2, cells were treated with picropodophyllin (PPP, specific IGF1R inhibitor) in the presence and absence of IGF2 (10 ng/mL). Organised intricate EC networks were formed in the absence and presence of angiogenic-stimulation. In Expt 1, IGF2 decreased total EC network area ($P<0.05$) and branch points ($P<0.05$) under both basal and angiogenic-stimulated conditions ($P>0.05$). Progesterone concentrations were 4-fold greater ($P<0.001$) on day 5 than 3 of culture and were unaffected by treatment with IGF2 ($P>0.05$). The number of viable cells was increased by 15% by IGF2 treatment ($P<0.001$) on day 5 of culture. In Expt 2, IGF2 had no effect on EC network area but PPP reduced total EC area and perimeter, degree of branching, number of EC islands and branch points by 60-70% ($P<0.001$) irrespective of IGF2 treatment. Additionally, PPP decreased progesterone production by 12% on day 3 ($P<0.05$) and by 65% on day 5 ($P<0.001$) in the presence and absence of IGF2 treatment. In conclusion, exogenous IGF2 had limited effect on luteinising follicular angiogenesis, but inhibition of IGF1 receptor reduced luteal endothelial cell network formation and progesterone production in luteinising follicular cells. This suggests that endogenous production of either IGF1 or IGF2 stimulates follicular-luteal angiogenesis *in vitro*. Funded by TETFund, Nigeria.

022 Reproductive Endocrinology

Luteal regression is compromised in high producing lactating Holstein cows independent of embryonic mortality

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The aim of this study was to compare the length of the luteal phase in lactating Holstein cows that were previously exposed to artificial insemination (AI) or not (Control). Non-pregnant cows (n=714) were submitted after the voluntary waiting period to a synchronization protocol: an intravaginal progesterone device (CIDR), 100 μg of GnRH and 2.0 mg of estradiol benzoate on d -11; 25 mg of PGF2α on d -4; and CIDR removal, 1.0 mg of estradiol cypionate and PGF2α on d -2. Cows were then randomly assigned at estrus (d 0) to the AI or the Control (sham) treatments. Only cows observed in estrus, captured by activity monitors prior to treatment, and with ovulation confirmed by ultrasonography 72 h after estrus were enrolled in the study. An ovarian map, using ultrasonography, of each individual cow was performed to evaluate the corpus luteum (CL) presence on d 17, 24 and 31, and to determine its regression status. For the analysis of length of luteal phase, only cows detected with a CL on d 17 and later not diagnosed pregnant were included in the analysis (n = 413; Control = 122; AI = 291). Cows with the original CL (d 17, 24, 31), not rebred during the period and diagnosed non-pregnant on d 31 were classified as having an abnormally long luteal phase. Pregnant cows that were retrospectively removed from the luteal phase calculations and rebred cows between d 17 and 31 were assumed to have a normal luteal regression. The body condition score (BCS) and a size and position score of the uterus (SPS) were measured by per rectum palpation at d 0 and included in the analyses of risk factors associated with pregnancy and CL dynamics. Binomial data were analyzed using PROC LOGISTIC of SAS with a backward stepwise elimination procedure. Statistical models included treatment, parity, BCS, SPS and interactions. Proportions were collected through frequency tables (PROC FREQ of SAS). Cows on both treatments were similarly distributed by parity, BCS and SPS (P>0.42). Pregnancy per AI at d 31 and 60 in the AI group was 21.0% and 17.2%, respectively, whereas pregnancy loss was 18.2%. Major risk factors for a successful pregnancy were parity (P<0.02) and SPS (P=0.04), as primiparous and cows with a SPS of 1 and 2 had greater pregnancy per AI. Presence of a CL was similar between Control and AI groups (P>0.24) prior to initiation of the synchronization protocol (57.3 vs 51%) and at d 24 (70.2 vs 69.7%) and 31 (50.8 vs 45.7%). Similarly, the frequency of spontaneous or induced re-breedings after d 17 of the treatments was not different between Control and AI groups (50.0 vs 46.4%; P=0.88). Treatment did not affect the proportion of cows with a persistent CL at d 31 (41.1 and 38.8% for Control and AI groups, respectively) and none of the explanatory variables were significant as risk factors to explain the presence of a long luteal phases. In conclusion, this study demonstrated, as shown by the lack of differences between the AI and control groups, that a large proportion (~ 40%) of lactating Holstein cows failed to properly regress the CL in a timely manner and consequently endured an abnormally long luteal phase that does not seem to be associated with the presence of an embryo. Authors would like to thank Colorado Dairy, Conapec Jr., CNPq and NSERC.

023 Reproductive Endocrinology

Proinflammatory cytokine gene expression in endometrium and fertility in timed AI postpartum beef cows

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The objective of this study was to evaluate the uterine health and fertility of postpartum beef cows subjected to TAI protocols at different moments in the postpartum. Multiparous lactating Nelore cows (*Bos indicus*; *n* = 244) from two comercial beef farms in Rondônia - Brazil, were used in this study. The TAI protocols were initiated between 20 and 60 days postpartum (DPP). Cows were given 2 mg of estradiol benzoate i.m. and received an intravaginal progesterone-releasing device (1.9 g progesterone, CIDR®) to synchronize follicular wave emergence on Day 0. The CIDR was removed and cows were given 150 µg of d-Cloprostenol i.m. (PGF2α-analogue; Croniben®), 1 mg of ECP im (E.C.P.®), and 300 IU of eCG (Novormon®) i.m. on Day 8. All cows were TAI 48 h after CIDR removal. The cows were divided into three groups according to the days postpartum (PP) that the hormonal treatment was initiated, as follows: 1) Early PP (n=64), cows between 20 to 30 DPP; 2) Middle PP (n=115), cows between 31 to 45 DPP; and 3) Late PP (n=65), cows between 46 to 60 DPP, on the Day 0 of the TAI protocol. At Day 0 of the protocol, endometrial cytobrush samples were collected from a subset of cows (n=148). Slides for citology were prepared before the same cytobrush was transfered to a microtube containing 1 mL of RNA later reagent. Total RNA was extracted from 40 cytobrush samples (14, 11 and 15 from Early, Middle and Late Group, respectively) and analysis of *il1*, *il6*, *il8*, *tnf*, *GAPDH* and *βactin* gene expression was performed using quantitative real-time PCR. Cows from the Early group had lower (P<0.05) pregnancy per AI (P/AI) than cows from Middle and Late groups; 29.7% (19/64), 45.2% (52/115), and 52.31% (34/65), respectively. Accordingly, the Early group had higher (P<0.05) proportion of polymorphonuclear cells in the uterus than Middle and Late groups; 9.0%, 3.8%, and 2.2%, respectively. Relative expression of *il1* and *il8* were higher (P<0.05) in the Early group than Middle and Late groups. In contrast, expression of *il6* and *tnf* did not change among groups. These results demonstrated that cows subjected to TAI protocols early (< 30 DPP) in the reproduction season are less likely to become pregnant. Moreover, proinflammatory cytokines *il1* and *il8* are less active in the uterus after 30 DPP. Jéssica Andrade, Paulo Marcos Neves, and Izabela Lemos have scholarship from CAPES. Elizangela Moreira has scholarship from FAPERO/CNPq. George Silva has scholarship from CNPq. This research project is supported by Universal CNPq/2016 from MCT and by Macroprograma MP1 project from Embrapa.

024 Reproductive Endocrinology

Health status and resumption of ovarian ciclicity in dairy cows

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The objectives were to assess if health status interacted with parity to affect the resumption of ovarian ciclicity (ROC) and haptoglobin (Hp), insulin-like growth factor-I (IGF-I) and insulin profiles during the transition period of primiparous (PP, n=116) and multiparous (MP, n=172) Holstein cows. Cows were fed a total mixed ration according to requirements, milked 3 times daily and bled once a week from -2 to +7 weeks relative to calving. A prospective observational cohort study was followed including only healthy cows at the beginning of the study. Peripartum diseases were diagnosed by a trained veterinarian and cows were classified in 3 categories: healthy, 1 event or 2 events. For statistical analysis PROC FREQ, PROC MIXED, and multivariable logistic regression (MLR) were performed. From 288 cows, 45.8% become sick and 1.9% (n=6) were discarded or died within 30 DIM. The proportion of cows and their health status by parity were: MP: 53.5%, 29.4% and 17% and PP: 55%, 31.3% and 13.5% for healthy, 1 event and 2 events cows respectively (Ruprechter *et al*., 2018). Primiparous produced less milk than MP cows (6,866 ± 272 *vs* 8,341 ± 213 L, P<0.01) and healthy produced more than sick cows (8,165 ± 199, 7,547 ± 276 and 7,098 ± 387 L) for healthy, 1 and 2 events cows respectively (P<0.05). Insulin, IGF-I and Hp profiles were affected by the interaction among parity, health status and week. Concentrations of Hp were greater in sick than healthy cows at weeks +1 and +3 (P<0.01), as expected for this acute phase protein, being also higher in 2 events MP than 1 event MP cows (P<0.01). Overall, IGF-I and insulin concentrations were greater in PP than MP cows, since PP are still growing animals. While healthy MP cows presented a sharp IGF-I decrease after calving, healthy PP cows maintained IGF-I concentrations, being this consistent with the lower milk production and the reported attenuated uncoupling somatotropic axis of PP cows. Independent of parity, all sick cows had a sharp IGF-I decrease around calving suggesting a lower dry matter intake during the transition period (greater net energy balance). From 288 cows, 59.7% (n=172) become cyclic during the first 7 weeks postpartum while 40.3% (n=116) did not. Health status and parity affected ROC (P<0.05). More MP cows reinitiated than PP cows (64%, 110/172 *vs* 52%, 60/116 respectively). A greater percentage of healthy cows reinitiated ovarian ciclicity (68%, 106/156) when compared to 1 event (53%, 46/87) and 2 events cows (44%, 20/45). After performing MLR, insulin and IGF-I resulted predictive for ROC (P<0.05); Insulin: (OR [IC]); 1.09 [1.01-1.17] at week -1 and IGF-I: 1.01 [1.00-1.02] at week +1. In conclusion, health status interacted with parity to model ROC. Besides IGF-I and insulin concentrations around calving were both predictive for ROC arguing for the importance of these anabolic hormones signaling the metabolic status to the reproductive axis.

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Protein supplementation control on luteal progesterone and expresion of its receptor in ovarian tissues of Boer goats

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Nutritional effects on reproductive performance such as ovulation rate and embryo survival in sheep are well documented, but there are still debates among researchers and farmers regarding this matter in goats. There are reports in the literature showing in goats that high energy-protein supplementation has no influence on ovulation rates but improves embryo survivability and maintenance of pregnancy. Therefore, we examined the effects of short-term protein supplementation (commercial pellet; double maintenance) on ovulation rate, mRNA expression of progesterone receptor gene in corpus luteum and the concentration of plasma progesterone. In this study, seventeen female Boer goats were divided into two groups; 1) Control group (n=9) received maintenance diet (commercial protein pellet and Napier grass) and 2) Treated group (n=8) received double maintenance diet (commercial protein pallet-2x M and Napier grass). The feeding treatment begun 5 days before CIDR removal (Day 0) for 25 days. The body weight and BCS were recorded every two weeks until all animals were slaughtered (Day 27). The ovaries of all animals were collected, and the number of CL was counted and kept in RNAlater for analysis of gene expression through qPCR. Results show that short-term supplementation of high protein does not affect ovulation rate (Control = 1.00 ± 0.24; Treated = 1.25 ± 0.24; $P>0.05$) or change body weight or body condition during the experimental period. However, the plasma progesterone concentration (Day 27; Control = 7.88 ± 0.26; Treated = 10.77 ± 0.28 ng/mL) and the expression of progesterone receptors in corpus luteum (Control = 1.00 ± 0.48; Treated = 3.79 ± 0.38) were higher in treated does ($P<0.05$). In conclusion, we suggest that protein supplementation appears to control the structural and functional integrity of CL tissue, thus increasing the production of progesterone. I would like to acknowledge the Fundamental Research Grant Scheme (01-01-15-1713FR) from Ministry of Education (MOE) Malaysia and Putra Grant (GPIPS/2016/9493100) from Universiti Putra Malaysia for financial support. Also, we appreciate the team members of Animal Sciences Research & Technology (AnSTECH) for their kind participation in these experiments.

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Resistin regulates prolactin concentrations from the ovine adenohypophysis depending on season

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Adipokines are hormones that are mainly produced by white adipose tissue, an endocrine organ involved in energy homeostasis. However, there are several metabolic signaling pathways, including those involving the adipokine resistin, that have not been fully explored. One prominent feature of season in animals is its effects on the neuroendocrine regulation of pituitary hormone release, including prolactin (PRL). This axis is highly sensitive to photoperiod in all seasonally breeding mammals, with marked activation of PRL secretion by long days. However, no studies in seasonally-breeding sheep have examined the role of resistin in regulating the secretion of PRL, particularly in the context of the effect of day length. Thus, in the present in vivo study, we investigated the effect of different doses of resistin on PRL secretion during contrasting photoperiods. Thirty Polish Longwool ewes, a breed that exhibits a strong seasonal reproductive pattern, were ovariectomized with estrogen replacement using subcutaneously inserted estradiol implants. Ewes were fed ad libitum and housed under natural photoperiod (longitude: 19°57' E, latitude: 50° 04' N). Within season and replicate ewes were assigned randomly to one of three treatments (five ewes/treatment/season) and infused intravenously once at time 0. Treatments consisted of control or recombinant bovine resistin (rbresistin) in saline: 1) Control (saline; n = 10), 2) Low resistin dose (R1; 1.0 µg/kg BW; n = 10), and 3) High resistin dose (R2; 10.0 µg/kg BW; n = 10). Jugular blood samples were collected at 10-min intervals beginning immediately before the start of infusions and continuing for 4 hr. Experiments were conducted during both short (SD) and long (LD) days. Plasma concentrations of PRL were assayed using RIA. All data are expressed as the mean ± standard error of the mean [SEM]. Hormone data were analyzed by a series of 2-way analyses of variance (two-way ANOVA) using SigmaPlot statistical software (version 11.0; Systat Software Inc., Richmond, CA, USA) for repeated measures. A season x dose interaction was observed for PRL concentrations. Within both control and treated groups (R1 and R2), mean PRL concentrations were greater ($P < 0.001$) during LD than during SD. However, only the high dose of rbresistin increased PRL concentrations during both LD – 120.4 ± 2.1 ng/mL ($P < 0.001$) and SD – 23.05 ± 0.6 ng/mL ($P < 0.05$) compared to controls (LD – 78.1 ± 0.9 ng/mL and SD – 12.04 ± 0.8 ng/mL) the R1 group ($P < 0.001$ and $P < 0.05$, respectively for LD – 59.3 ± 0.3 ng/mL and SD – 16.02 ± 0.5 ng/mL). Results suggest that there may be a link between adipose tissue production of resistin and the control of PRL release. Such an effect would likely involve a direct influence of resistin at the pituitary depending on the length of the day. In summary, the current data indicate that resistin may serve as an additional adipokine engaged in the regulation of PRL, an essential hormone for mammary gland development and milk production in ruminants. Research supported by NCN 2015/19/B/NZ9 01314 to DAZ.

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Association of antral follicle count and peripheral anti-Müllerian hormone concentrations with fertility in beef cows

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While it has been reported that reproductive performance in cows with high antral follicle count (AFC) was improved compared with those with low AFC, other research groups have demonstrated that there was no difference in conception rate between cows with high AFC and those with low AFC. On the other hand, anti-Müllerian hormone (AMH) may be a useful biomarker for evaluating fertility in cattle. Objectives of this study were to clarify effect of the stage of estrous cycle on AFC and to elucidate whether there is an association between AFC, peripheral AMH concentrations and fertility in beef cows. A total of 71 Japanese black cows (33.0 ± 2.7 days postpartum) were treated with Ovsynch protocol on day -11 and ovulation was confirmed on day 0. The cows were divided into three groups; cows treated with prostaglandin $F_{2\alpha}$ (PG) on day 7 (Day 7 group, n = 24), those treated on day 11 (Day 11 group, n = 23) and those treated on day 15 (Day 15 group, n = 24). All cows received GnRH 56 hours after PG administration and were inseminated at fixed time (16-20 hours after GnRH administration). AFC was defined as the total number of antral follicles (2 mm or larger in diameter, detectable by ultrasonography) in the both ovaries. AFC and size of the largest follicle were recorded by ultrasonography at PG, at GnRH and one week after timed AI, and blood samples were collected at PG and one week after timed AI for determination of plasma AMH and progesterone concentrations. Plasma AMH concentrations were measured by ELISA and plasma progesterone concentrations were measured by Enzyme-Linked Fluorescent Assay. All the cows were also divided into two groups based on the median AFC: cows with high AFC (equal to or higher than the median; High AFC) and cows with low AFC (lower than the median; Low AFC). Pregnancy diagnosis was made at 30 to 50 days after timed AI. There were no significant differences in AFC at PG, size of the largest follicle at GnRH, plasma progesterone concentrations and volume of corpus luteum one week after timed AI and conception rate among the three groups. The median AFC was 37. Low AFC group had higher ($P < 0.05$) conception rate (24/35, 68.6%) than High AFC group (13/36, 36.1%). Plasma AMH concentrations in Low AFC group (854 ± 460 pg/mL) were lower ($P < 0.05$) than those in high AFC group (1625 ± 905 pg/mL). In conclusion, although the stage of estrous cycle does not seem to affect AFC, both AFC and peripheral AMH concentrations had an association with subsequent fertility in beef cows. We are grateful to the staff of the farms for their care and management of the cows. We would also like to thank Aska Animal Health for providing the hormonal drugs and MTF for providing the ultrasound equipment used in this study.

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Use of FSH in FTAI protocol in lactating dairy cows

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The aim of the present study was to evaluate the use of follicle stimulating hormone (FSH) as an alternative to equine chorionic gonadotropin (eCG) to improve pregnancy rates in fixed-time artificial insemination (FTAI) protocols in high producing dairy cows. It was also evaluated the effect of the hormonal protocol over the dairy production. For that, 111 crossbred multiparous cows (*Bos taurus* vs *Bos indicus*), lactating, 45-100 days postpartum, with body condition score between 3 and 4 (1 to 5 scale), milk production from 20 to 40 liters per day and randomly distributed in three groups were used. All females were evaluated ultrasonographically for the presence of a corpus luteum (CL) at day zero and were randomly distributed in experimental groups. Group1 (n=34) were submitted to a FTAI protocol and the cows were treated with a progesterone device (PRIMER™, Agener União, Brazil) for 8 days, two estradiol benzoate doses (EB; RIC-BE™, Agener União, Brazil) applied intramuscularly (IM), the first one (2 mg) were administered at the time of implant placement – D0 and the second dose (1 mg), 24 hours after progesterone device removal – D9, a dose (0.500 mg) of PGF2α (Prolise™, ARSA, Argentina) that was administered at the time of progesterone device removal – D8 and a dose of GnRH (25 µg buserelin acetate) (Gestran Plus™, Agener União, Brazil) administered 24 hours after removal of the device – D9. The animals were inseminated 52 hours after progesterone device removal with freezing semen from the same bull. Group 2 (n=37) were submitted to the same FTAI protocol plus the application of 0.75 mL (15 mg) of FSH (Folltropin™-V, Vetoquinol, Canada) IM at D8. At the time of FTAI all cows had their ovary ultrasonographically scanned for the presence of dominant follicle (diameter ≥ 10mm). The third group (n=40) was not submitted to a hormonal protocol. Cows were milked thrice daily and were maintained in feed lane with natural and artificial shadow and with access to water and mineral mixture *ad libitum*. Pregnancy was diagnosed 40 days after IA by ultrasonography. For statistical analysis frequencies (presence of CL at D0, presence of dominant follicle at D10 and pregnancy rate) were compared by Chi-square test with 5% of significance. For milk production comparison among the three groups, data were evaluated by the Kruskal-Wallis test followed by Dunn test with 5% of significance. There was no difference in pregnancy rate between Group 1 (44.11%; 15/34) and Group 2 (45.94%; 17/37) ($P > 0.05$). Considering the presence of a dominant follicle at D10, it was present in 67.64% (23/34) of the cows in Group 1 and in 97.30% (36/37) of the cows in Group 2. It was also observed that milk production did not vary throughout the evaluated moments in Groups 1, 2 and 3 and that milk production did not vary among evaluated groups. Based on the results obtained it can be concluded that treatment with 15 mg of Folltropin® showed a larger number of animals with dominant follicles on the day of AI, but the use of this hormone was unable to increase pregnancy rate. Moreover, the hormonal protocol and more handlings did not affect the dairy production, and the presence of a CL at the beginning of the protocol did not improve pregnancy rate. The authors thank Cariocão Farm, CNPq-Brazil and Fapemig-Brazil.

029 Reproductive Endocrinology

Creep feeding has no effect on antral follicle count at weaning

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Creep feeding (CF) is a nutritional tool that improves growth and development of calves, is associated with an increase in IGF1 concentrations at weaning and reduces the age at puberty (Guggeri *et al*., 2014. Livestock Science 167:455-462). Primordial follicles develop during pre-natal life, but nutrition increases the number of gonadotrophin responsive follicles that can be assessed by ultrasonography (Scaramuzzi *et al*., 2011. Reprod Fertil Dev, 23:444-467). Since antral follicle count (AFC) is a phenotypic marker of fertility (Evans *et al*., 2010. Soc Reprod Fertil Suppl, 67:421-429), we hypothesized that high nutrition in early life would have a positive effect on AFC. Fifty-four Hereford cows and their calves of 73 ± 1.5 days of age were assigned to two groups, with two replicates: 1) Without CF (-CF; n=27); 3) With CF (+CF; n=27). The cows grazed on *Campos* natural grassland with initial adjustment on forage allowance higher than 8 kg DM/kg BW for all plots. Calves supplementation was performed during 98 days with distiller's dried grains with solubles supplied to 40% of the diet daily and adjusting the supplement every 2 weeks, according to the body weight evolution and the respective requirements. Body weight and daily weight gain were evaluated in all calves. At the end of the supplementation period, females calves (n=26 +CF and n=22 –CF) were submitted to transrectal ultrasonography for 4 consecutive days and follicles > 3 mm counted using an Aloka 500 with a transrectal 7.5 MHz probe. Body weight and daily gain were analysed by ANOVA, using the mixed procedure in SAS. An average of the 4 days of follicle count was obtained, and analysed using the GLM procedure of SAS. Values were considered significant if $P < 0.05$. Creep feeding had a positive impact on average daily gain (+CF: 0.982±0.02 kg/d vs –CF: 0.832±0.02 kg/d; $P<0.05$) and weaning weights (+CF: 205±1.6 kg vs –CF: 191±1.6 kg; $P<0.05$). However, it was not associated with differences in AFC, that were similar in +CF (14.3±1.6 follicles) and –CF calves (16.8±1.8 follicles; $P>0.05$). We concluded that CF does not modify ACF of calves at weaning. The authors would like to thank the team working at Glencoe Research Station, INIA, Tacuarembó.

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Mechanism of LH release after peripheral administration of kisspeptin in cattle

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Kisspeptin (Kp) elicits LH release in cattle but whether the effect is mediated by activation of GnRH neurons in the hypothalamus is unknown. Experiments were done to determine if administration of Kp will increase LH secretion through activation of GnRH neurons and to determine if pre-treatment with a GnRH receptor blocker will alter the pattern of Kp-induced LH release and ovulation. In Expt1, Holstein cows were assigned randomly to two groups (n = 3 per group) 24 h after administration of PGF2α and given either Kp (3 doses of 15 mg hKp10 iv at 60-min intervals), or control (saline). Blood samples were collected every 15 min from -30 min to 150 min (0 min = treatment). Cows were euthanized at 150 min. The head was perfused with 4% paraformaldehyde via the carotid artery to fix the brain *in situ*. The mid-brain (pre-optic area to mammillary body) was excised, cryoprotected in saturated sucrose solution, frozen at -80ºC and sectioned serially at a thickness of 50 mm using a cryostat microtome. Every 20th free-floating section was processed for double immuostaining for cFos (Nickel-DAB) and GnRH (DAB) using sequential immuno-peroxidase reactions. The number of neuron cell bodies was counted in the pre-optic area and the hypothalamus by bright-field microscopy. In Expt 2, pubertal heifers (n = 5 per group) were assigned randomly to 1) hKp10 group: 3 doses of 15 mg hKp10 iv at 60-min intervals, 2) Cetrorelix+hKp10 group: pretreatment with a GnRH antagonist (Cetrorelix, im) before hKp10 treatment or 3) control group: 3 dose saline iv at 60-min intervals. Treatment was initiated 6 days after emergence of a follicular wave induced by ultrasound-guided follicle ablation (performed 3 days after ovulation). A CIDR was placed in vagina at the time of ablation and heifers were given PGF2α at 4.5 and 5 days after follicle ablation. Blood samples were collected at 15 min intervals from -60 min to 240 min of treatment to measure plasma LH concentrations. Ovaries were examined daily by transrectal ultrasonography. Data were compared among groups by ANOVA for repeated measures. In Expt1, hKp10 induced higher plasma LH concentrations from 15 to 150 min after treatment than in controls (P=0.01), but the proportion of GnRH cells expressing cFos did not differ between hKp10 and control groups (5.8% and 3.5%, respectively; P=0.11). In Expt2, a rise in plasma LH concentration was detected from 15 to 240 min in the hKp10 group but not in the groups treated with Cetrorelix or saline (P<0.001). Similarly, ovulation was detected in the hKp10 group but not in the groups treated with Cetrorelix or saline (4/5, 0/5, and 0/5, respectively; P=0.02). In summary, treatment with hKp10 induced LH release and ovulation in cattle, but was not associated with GnRH neuron activation, and the effect was blocked by a GnRH antagonist. Results support the hypothesis that the effect of kisspeptin is mediated downstream of GnRH synthesis, perhaps by inducing release of pre-synthesized GnRH from the nerve terminals in the median eminence. Research supported by the Natural Sciences and Engineering Council of Canada.

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Estrous cycle stage-specific actions of exogenous prostaglandin F_{2a} on angiogenic and cell-death pathways in bovine Corpus Luteum may depend on its local or systemic administration

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Prostaglandin F_{2a} (PGF) and its analogues are extensively used to induce regression of the bovine corpus luteum (CL). This process consists of functional and structural luteolysis. Although apoptosis was considered as the most potent mechanism of cell death, a caspase-independent cell death pathway-necroptosis was found as alternative process of CL regression in cows. However, the newly formed bovine CL (Days 1-5 of the cycle) is resistant to the luteolytic actions of PGF. Moreover, local luteal PGF may play a role in angiogenesis and CL formation after ovulation. The mechanisms underlying these differential PGF effects are poorly understood. The goal of this study was to examine differences in expression of genes related to: angiogenesis (FGF2, VEGF and their receptors), necroptosis (RIPK1, RIPK3, CYLD, and MLKL) and apoptosis (CASP3, CASP8, Bax, and Bcl-2) in response to the local or the systemic administration of PGF in early (Day 4 of the cycle) versus mid (Day 10) CL. Cows at Day 4 (n=24, 6/treatment) or Day 10 (n=24, 6/treatment) were treated as follow: (1) intramuscular (i.m.) Saline injection (control), (2) i.m. PGF injection (25 mg; Dinoprost), (3) intra-luteal Saline injection (control) or (4) intra-luteal PGF injection (2.5 mg). CLs were collected by ovariectomy 4-h after treatment. Gene and protein expression was investigated by qRT-PCR and Western Blotting, respectively, and localization of select proteins was evaluated by immunohistochemistry. Intra-luteal and i.m. PGF injections up-regulated FGF2 expression, but decreased expression of VEGF and its receptors in the early and mid CL ($P<0.05$). In mid CL, whereas both local and systemic PGF injections increased the expression of necroptosis related genes RIPK1 and RIPK3 ($P<0.05$), induction of CYLD was responsive only to local PGF administration ($P<0.05$). In early CL, intra- luteal PGF induced upregulation of RIPK1 and MLKL ($P<0.05$) but downregulated RIPK3 ($P<0.05$). Intramuscular PGF administration resulted in a decrease in RIPK1 and RIPK3 expression, but in upregulation of CYLD ($P<0.05$). In mid CL, both routes of PGF administration resulted in an increase in pro-apoptotic Bax and a decrease in anti-apoptotic Bcl-2 expression ($P<0.05$). In early CL, whereas intra-luteal PGF resulted in increased Bax expression, systemic PGF resulted in decreased Bax expression but induced expression of Bcl- 2. In mid CL, whereas both routes of PGF treatment increased CASP3 expression, CASP8 induction was responsive only to systemic PGF ($P<0.05$). In the early CL, both routes of PGF administration resulted in a decreased expression of CASP3 but upregulation of CASP8. In conclusion, genes and proteins related to angiogenesis, necroptosis and apoptosis reveal stage- specific responses to PGF administration depending on whether it was administered locally or systemically. Although local PGF may play a luteoprotective role by inhibiting necroptosis and apoptosis pathways in the early CL, RIPK-depending necroptosis is a potent mechanism responsible for structural CL regression during luteolysis in cattle induced by PGF. Supported by NSC grant DEC-2017/X/NZ9/00363. DJS was supported by KNOW Consortium "Healthy Animal - Safe Food", MS&HE Decision No. 05-1/KNOW2/2015.

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Elevated free fatty acid concentrations alter gene expression, cell proliferation and steroid hormone production in cultured bovine granulosa cells

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Following the onset of lactation, high-yielding dairy cows enter a state of negative energy balance (NEB) when the energy demand for maintenance and lactation exceeds that of dietary energy intake (Bauman & Currie 1980). One of the major metabolic consequences of NEB is the increased concentration of non-esterified fatty acids (NEFA) in the serum due to fat mobilization. Elevated NEFA levels have been found detrimental for bovine (Vanholder *et al*., 2005) and human (Mu *et al*., 2001) granulosa cell growth and function *in vitro*. Elevated concentrations of saturated fatty acids result in deleterious effects on cell survival by induction of the apoptotic signaling cascade, while mono-unsaturated fatty acids like oleic acid have shown to rescue cells from such toxic effects either by channeling fatty acids towards lipid storage in lipid droplet or by increasing β-oxidation in mitochondria (Listenberger and Brown 2008). In the present study, we investigated the effects of physiological concentrations of three main NEFAs on granulosa cell viability, proliferation, steroid production and gene expression in a bovine E2 active granulosa cell culture model. Initially, granulosa cells from small to medium sized follicles (2-6 mm) were cultured for 48 h. Subsequently, the spent media were replaced with media containing different concentration of Palmitic acid (C16:0) (PA), Stearic acid (C18:0) (SA) and Oleic acid (C18:1) (OA) during the next 6 d of culture with regular media change every 48 h. Treatment with all three NEFAs increased the transcript levels of the fatty acid translocase *CD36* ($P < 0.05$) indicating the active uptake of free fatty acids by granulosa cells. In addition, both PA and SA treatment at 200 μM upregulated the of the gonadotrophin receptors *FSHR, LHCGR*, aromatase *CYP19A1* and the cell cycle regulator *CCND2* whereas OA treatment at 400 μM ($P < 0.05$) downregulated the transcript abundance significantly. Also, Oestradiol 17β production was stimulated with both PA (200 μM) and SA (200 μM) ($P < 0.05$), while reduced with OA treatment (400 μM) ($P < 0.05$). Thus, our results indicate that elevated free fatty acid concentrations specifically alter the granulosa cell functionality *in vitro*. Suggestively, this could be a possible mechanism through which free fatty acids influence folliculogenesis during the early postpartum period in high-yielding dairy cows.

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Prostaglandin profile in pregnant cows during initiation of active placentation

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In cattle, the incidence of late embryonic mortality (EM) varies from 3.2 to 42.7% of pregnancies. Relatively little is known about the causes or mechanisms associated with late EM, most of which occurs around the time of placentation. Usually associated with negative reproductive outcomes, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) may play a role in the initiation of placental attachment and proper placentome formation. The objective of the study was to evaluate $PGF_{2\alpha}$ secretion during active placentation from day 29 to 38 in pregnant cows that maintained pregnancy or experienced EM. Non-lactating cows were artificially inseminated to high fertility bulls (n=40) or sham inseminated (control; n=5) with heat treated semen at day 0. Control cows received a CIDR at day 17, which was replaced with a new CIDR at day 27, to maintain elevated circulating progesterone (P4) concentrations. Pregnancy diagnosis was performed at day 29 via ultrasound. Pregnant cows (n=23) and control cows (n=4) underwent coccygeal vein cannulation at day 29. A polyethylene catheter (BD Intramedic) was inserted 65 cm into the caudal vena cava via the coccygeal vein of all cows for sampling of utero/ovarian drainage. Blood samples were collected every 6 hours until day 38. Uteri of pregnant cows were examined by ultrasonography daily to monitor pregnancy until catheters were removed at day 38. Final pregnancy diagnosis occurred at day 70 of gestation. Cows pregnant at day 29 but lacking a fetus with a viable heartbeat at day 70 were considered to have undergone embryonic mortality (n=3). Serum concentrations of prostaglandin $F_{2\alpha}$ metabolite (PGFM) and $PGF_{2\alpha}$ were measured with a validated commercial ELISA and P4, a validated commercial RIA. Pregnancy associated glycoprotein (PAG) levels were measured daily using an in house ELISA. Data were analyzed using repeated measures in SAS 9.4 and pulses were identified using AutoDecon Pulse2. Concentrations of PGFM were significantly elevated in pregnant cows compared to control cows ($P<0.05$) across the sampling period as well as between sampling periods ($P<0.01$) during days 29 to 38 of gestation. Number of PGFM pulses did not differ between control cows (1.60±0.40 pulses) and cows that lost (2.25±0.63 pulses) or maintained pregnancy (1.67±0.18 pulses) but, in cows with 2 or more PGFM pulses, cows that maintained pregnancy had an increased pulse interval compared to cows that lost pregnancy (61.8±12.4 hours vs 26.4±9.5 hours). However, there was no difference in $PGF_{2\alpha}$ levels between control and pregnant cows. Control cows and pregnant cows had similar levels of P4 during the sampling period ($P>0.05$); however, the pregnant cows had increased PAG in circulation ($P<0.001$). There was no difference between circulating PGFM, $PGF_{2\alpha,}$ or P4 in cows that maintained a pregnancy until day 70 of gestation versus those that experienced EM ($P>0.05$); however, PAGs were decreased at day 29 of gestation ($P<0.05$). Based on these results pregnant cows have increased circulating concentrations of prostaglandin, but it remains unclear if prostaglandin is playing a role in cows undergoing embryonic mortality. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2017-67015-26457 from the USDA National Institute of Food and Agriculture.

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Components of *Biserrula pelecinus* that affect *in vitro* maturation of ovine oocytes, and subsequent fertilisation and embryo development

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New pasture legumes need to be tested for deleterious effects on reproduction in grazing livestock. Measurement of these effects in grazing animals is difficult, so *in vitro* techniques are preferred for initial screening. Using ovine oocytes and embryos, we have previously observed the effects of the isoflavones responsible for clover disease and the effects of a crude extract of *B. pelecinus*. Here, we go to the next stage by attempting to identify the plant secondary metabolites produced by *B. pelecinus* that could be responsible for the effect. Experiment 1: *B. pelecinus* was extracted with 1:1 (v/v) $MeOH/CHCl_3$ and the extract was fractionated using rapid silica filtration (RSF) with solvents of varying polarities; dried fractions were added, at final concentrations of 0, 100 or 200 μg mL^{-1}, to the medium used for *in vitro* maturation of cumulus-oocyte complexes (COCs) derived from abattoir-sourced adult ewe ovaries; matured oocytes were then taken through *in vitro* fertilisation and embryo culture so we could quantify embryo development (cleavage rate, blastocyst rate, hatching rate, blastocyst efficiency) and total blastocyst cell number (TCN). Data for embryonic development were analysed using CATMOD in SAS while TCN data were analysed using the GLM procedure in SAS. Of seven fractions tested, one fraction reducing cleavage rate by 9.8% and three fractions reduced blastocyst development by 14-22%. However, one of the active fractions (designated BP6) at 200 μg mL^{-1} increased hatching rate by 20% ($P<0.05$). Experiment 2: fraction BP6 was further fractionated by semi-preparative HPLC and loliolide was clearly the most abundant compound. Pure loliolide at 25 μg mL^{-1} increased hatching rate ($P<0.05$), consistent with the effects observed with fraction BP6. All oocytes supplemented with *B. pelecinus* fractions and loliolide reached the final stage of embryo development, blastocyst hatching, with no effect on TCN. We conclude that the ability of *B. pelecinus*, present during *in vitro* oocyte maturation, to improve fertilisation and embryo development, is at least partly due to the presence of loliolide. We now need to test the concept *in vivo*.

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Effect of supplementation with Cysteine, Glycine and Glutamate during *in vitro* fertilization of bovine oocytes

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The constitutive amino acids involved in glutathione synthesis are glycine (Gly), glutamate (Glu) and cysteine (Cys). In a previous study, we demonstrated that the supplementation with Cys, Gly and Glu during *in vitro* maturation of bovine oocytes improves its subsequent developmental capacity (Furnus *et al*., 2008. Metabolic requirements associated with GSH synthesis during in vitro maturation of cattle oocytes. Anim Reprod Sci. 109(1-4):88-99). The aim of this study was to evaluate the effect of Cys, Gly and Glu during *in vitro* fertilization (FIV). Cumulus–oocyte complexes (COC) obtained from cattle ovaries collected in an abattoir were matured *in vitro* in TCM 199 medium containing 10% FCS and hormones. The incubations were performed at 39 ºC in an atmosphere of 5% CO_2 in air with saturated humidity for 24 h. Then matured COC were washed twice in HEPES-TALP, and placed into 50 µl drops of IVF under mineral oil. COC were *in vitro* fertilized in a) TALP medium alone (Control) or supplemented with b) 0.6 mM Cys + 0.9 mM Glu + 0.6 mM Gly (TALP aa); c) 0.6 mM Gly + 0.9 mM Glu (TALP Gly+Glu); d) 0.6 mM Cys + 0.9 mM Glu (TALP Cys+Glu); and e) 0.6 mM Cys + 0.6 mM Gly (TALP Cys+Gly). To determine the effect of amino acids on subsequent embryo development, the presumptive zygotes were cultured for 9 days in SOFm medium. Cleavage rates were recorded 48 h after insemination. At the end of incubations, stages of embryo development were evaluated with an inverted microscope. For this purpose, 440 COC were matured in five replicates. A completely randomized block designs were used. Statistical model included the random effects of block and the fixed effect of treatment. Cleavage, blastocyst and hatching rates were analyzed by logistic regression using GENMOD procedure (SAS Institute). Percentages of cleavage (CL) and blastocyst (BL) for Control (CL: 75.88 - BL: 36.62%); TALP Gly+Glu (CL: 65.21- BL: 32.64%); TALP Cys+Glu (CL: 59.18- BL: 21.08%), TALP Cys+Gly (CL: 71.37 - BL: 35.22%) and TALPaa (CL: 57.05 - BL: 19.66%) were significantly lower in both, TALP Cys+Glu and TALPaa, compared with Control ($P<0.05$). On the other hand, no differences were found in hatching rates among the different treatments (62.21, 60.3, 40.84, 56.17 and 39.87% for Control, TALP Gly+Glu, TALP Cys+Glu, TALP Cys+Gly and TALPaa, respectively; $P>0.05$). In conclusion, IVF medium supplementation with Cys, Gly and/or Glu did not show an improvement effect on subsequent embryo development. On the contrary, the combination of Cys + Glu decreased cleavage and blastocyst rates.

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Heat shock compromises bovine oocyte endoplasmic reticulum reorganization during *in vitro* maturation

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Oocyte maturation is characterized by a dynamic reorganization of the endoplasmic reticulum (ER) increasing the ability of the oocyte to release Ca^{2+} during fertilization (Zhang *et al*., 2013. Maternal diabetes causes abnormal dynamic changes of endoplasmic reticulum during mouse oocyte maturation and early embryo development. Reproductive Biology and Endocrinology. 11:31). There is evidence that oocyte maturation is susceptible to disruption by elevated temperature compromising the capacity of the oocyte to undergo adequate fertilization and embryonic development. Heat shock (HS) during in vitro maturation (IVM) has been shown to damage the oocyte cytoskeleton (Rodrigues *et al*., 2016. Thermoprotective effect of insulin-like growth factor 1 on in vitro matured bovine oocyte exposed to heat shock. Theriogenology. 86:2028-2039) which may affect ER migration. Therefore, the objective of this study was to determine whether exposure of bovine oocytes to a moderate HS during IVM compromises ER reorganization. Grade I and II cumulus-oocyte complexes (COCs) obtained from slaughterhouse ovaries were examined immediately after collection (immature oocyte - 0 h) or subjected to IVM at control (38.5°C) and heat shock (40°C) for 24 h. COCs from all experimental groups were denuded by vortex in 0.1% hyaluronidase and washed twice in TCM 199 HEPES. Denuded oocytes were incubated in TCM 199 HEPES containing 100 nM ER-Tracker Red for 30 min on humidified chamber at 38.5°C and 5% CO_2. After incubation, oocytes were washed in TCM 199 HEPES and transferred to slides mounted with coverslips. Samples were analyzed in Zeiss Imager.A2 epifluorescence microscope. The most prevalent ER distribution categories were: uniform ER distribution through the oocyte cytoplasm for immature control oocytes and cortical ER localization for mature control oocytes. Data were analyzed by least-squares analysis of variance using the General Linear Models procedure of SAS. In vitro maturation at 38.5°C increased ($P < 0.005$) the percentage of oocytes with cortical ER distribution as compared to 0h (65.0 + 6.5% vs 10.0 + 6.5%, respectively). Exposure of oocytes to 40°C during IVM decreased ($P < 0.05$) the percentage of oocytes with cortical ER distribution as compared to 38.5°C (29.1 ± 8.0% vs 65.0 ± 6.5% respectively). Moreover, the percentage of oocytes with uniform ER distribution through the cytoplasm was 74.2 ± 1.0, 19.8 ± 1.0 and 43.4 ± 1.3% for immature oocytes and oocytes matured at 38.5ºC and 40ºC, respectively. In conclusion, moderate HS of 40°C during 24 h IVM reduced ER reorganization to the cortical region of the oocyte which could negatively impact fertilization. Previous experiments demonstrated that HS during IVM reduced cortical microfilament organization in bovine oocytes. Therefore, it is possible that heat-induced cytoskeletal changes mediate the negative effect of HS on ER distribution. This research was supported by Sao Paulo Research Foundation (FAPESP; 2017/13082-6) and Coordination for the Improvement of Higher Level Education – Personnel (CAPES) - Program attraction of young Talents (CSF-PAJT - 88887.068701/2014-00).

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Influence of sodium pump on ram sperm parameters

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The sodium pump (NaP) is a transmembrane protein affecting sperm motility parameters (mouse and human) and capacitation (bulls). However, there are few reports regarding the role of NaP in ram sperm. Preliminary data indicate that NaP is localized mainly in the middle piece of ram sperm flagellum (unpublished data). The goal of this study was to determine the effect of NaP on ram sperm parameters. Four semen collections (48-h intervals) were performed in five rams using artificial vagina. Fresh ejaculates from the same day were pooled, split in two tubes and centrifuged (600 × g, 37°C for 10 min) with TALPm (1:10, v:v) to remove seminal plasma. Thereafter, samples were re-suspended (50 × 10^6 sperm/mL) in TALPm + 2.5% estrous sheep serum with (Treated) or without (Control) addition of NaP inhibitor ouabain (10^{-4} M) and incubated for 3 h (38.5°C). Flow cytometric analyses were performed (Amnis ImageStreamx Mark II, Millipore Corp.) to evaluate acrosomal and plasma membrane integrity (FITC-PNA and IP), plasma membrane stability in viable cells (M540 and Yo-Pro-1), and mitochondrial membrane potential (JC-1). Kinematic parameters (total (TM) and progressive motility (PM), linearity (LIN), straightness (STR), curvilinear velocity (VCL), progressive velocity (VSL), path velocity (VAP), amplitude of lateral head displacement (ALH), and beat frequency of the tail (BCF) were assessed with a CASA system (SCATM; Microptics, S.L., Version 5.1). Differences between Control and Treated samples, at 0 and 3 h of incubation, were assessed using ANOVA, followed by Mann-Whitney pairwise with PAST software Version 3.18. For all analyses, untransformed data were used and $P < 0.05$ was considered significant. At 0 h of incubation, all endpoints assessed were similar between Treated and Control samples ($P > 0.05$). There was no influence of treatment or incubation time on percentage of live sperm with membrane stability ($P > 0.05$). The percentage of sperm with intact acrosomal and plasma membranes was higher in Treated than Control group ($P < 0.05$) after 3 h of incubation. Ouabain reduced the percentage of sperm with high mitochondrial membrane potential (HMMP) after 3 h compared to 0 h ($P < 0.05$), whereas in Control samples, HMMP was similar throughout incubation. There was no influence of treatment or incubation on LIN, STR, VAP and BCF. However, whereas in Control samples there was a significant reduction of MT, but not MP, treatment with ouabain led to the opposite response, with significant reduction in MP but not MT, and no difference between treatments at the same incubation time. In both Treated and Control samples, incubation reduced VCL ($P < 0.05$), but there was no difference between Control and Treated samples within time. Treated samples had reduced VSL after incubation ($P < 0.05$), with no change in Control samples. Conversely, ALH was similar in Treated samples at 0 and 3 h, but there was a significant reduction in Control. In conclusion, NaP affected kinematic parameters of ram sperm and, as an ionic channel. Effects were attributed to its influence on mitochondrial function. FACEPE-financial support.

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Ghrelin antagonist: possible solution for *in vitro* oocyte maturation from cows in negative energy balance

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Ghrelin is a gut hormone released when cows are in negative energy balance (NEB) with adverse effects on oocyte in vitro maturation (IVM) (Sirini *et al*., 2017. Zygote, 25:601-611). The aim of this study was to investigate the effect of ghrelin antagonist (Lys3-GHRP-6). For this purpose, cumulus oocyte-complexes (COC) were obtained from slaughterhouse ovaries; and then maturated for 24h in TCM199 with 0.4% BSA, FSH (1µg/mL) and estradiol 17-beta (1µg/mL). The COC were matured in IVM medium: a) alone (Control: C); or supplemented with b) 60pm/mL ghrelin (G); c) 60pm/mL ghrelin+20pM/mL Lys3-GHRP-6 (ghrelin antagonist 1: GA1); d) 60pm/mL ghrelin+60pM/mL Lys3-GHRP-6 (ghrelin antagonist 2: GA2); e) 60pm/mL of ghrelin+100pM/mL Lys3-GHRP-6 (ghrelin antagonist 3: GA3). After IVM oocyte nuclear maturation was analyzed by Hoechst 33342, cumulus expansion area (CEA) was calculated with microscopic photographs by ImageJ, cumulus cells (CC) viability was evaluated by FDA, CC apoptosis and necrosis was classified by Anexina V and propidium iodide (Pläsier *et al*., 1999. J Immunol Methods, 229:81-95) and CC DNA damage was estimated by comet assay. Results of oocyte nuclear maturation, CC viability and rates of apoptosis and necrosis were analyzed by logistic regression using GENMOD procedure, and CEA and genetic damage index were analyzed by mixed model (SAS Institute, Cary, NC, USA). There were not differences among groups in CEA and nuclear maturation rates at any IVM condition analyzed ($P>0.1$). On the other hand, CC viability was significantly higher in C than G or ghrelin plus high ghrelin antagonist supplementation (C 137.39±9.07 vs G 95.5±9.07, GA2 101.52±9.3 and GA3 96.68±9.04; $P<0.01$). However, no differences were observed between C and GA1 ($P>0.1$; 124.34±9.07 vs. 137.39±9.07, respectively). Apoptosis was not significant different between treatments. However, there was a significant difference in CC necrosis between C and G ($P<0.01$; 15.5% vs 38.8%), while C results were similar to GA1, GA2 and GA3 ($P>0.1$; 15.5%, 13.5%, 17%, and 14.3%, respectively). Comet assay data demonstrated that genetic damage index (GDI) was significantly higher in G ($P<0.01$), while C, GA1 and GA2 showed similar GDI. In addition, GA3 presented the lowest GDI ($P<0.01$). In conclusion, the presence of ghrelin antagonist during bovine IVM prevented negative ghrelin effects on oocyte maturation, and GA3 might be a protector to decrease DNA damage.

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Oxidative stress in newborn calves with intrauterine growth retardation is associated with a deficiency of selenium and copper

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Intrauterine growth retardation (IUGR) is defined as the discrepancy between the size of the embryos and fetuses and the term of their gestation (Wu *et al*., 2006). The immature antioxidant defense system of newborn animals with IUGR predisposes to the development of oxidative stress and postnatal metabolic disorders (Che *et al*., 2015). The aim of the research was to study the dependence of the activity of antioxidant enzymes in the blood of newborn calves with IUGR on their supply with selenium, copper, zinc and magnesium during the intrauterine period. Fifty-three calves of red-mottled breed were examined within 24 hours after birth: 28 with IUGR, and 25 with normal physiological course of pregnancy in the mothers (control group, CG). Criteria for underdevelopment of embryos and fetuses were based on the coccyx- parietal size: (a) on 38th-45th days after insemination and conception, less than 16 mm and the diameter of the body less than 9 mm; at the age of 60- 65 days, less than 45 mm and 16 mm, respectively. On 110th-115th days, IUGR was defined as the diameter of the uterine horn less than 15 cm and the placenta less than 17 mm (Nezhdanov *et al*., 2014). In the blood of calves, the concentration of malonic dialdehyde (MDA) and the activity of catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) were determined with a UV-1700 spectrophotometer (Shimadzu, Japan), using the relevant methods described in the literature. The concentration of selenium, copper, zinc, magnesium in the tail hair of calves was determined with an atomic-absorption spectrophotometer AA6300 (Shimadzu). Results were presented as means +/- standard deviation. The reliability of the differences between the groups was determined by comparing the medians of the samples using the non-parametric Wilcoxon test. Correlations between the indicators were detected using Spearman's nonparametric test. It was found that in the hair of IUGR calves the content of selenium was reduced by 26.4% ($P<0.001$), copper by 28.3% ($P<0.001$), zinc by 10.7% ($P<0.001$), magnesium by 9.4% ($P<0.001$), respectively, compared to CG. An increase in the concentration of MDA in the blood of IUGR-calves by 26.8% ($P<0.001$) compared to CG indicated the oxidative stress progression. It is known that during the last 10-15% of the gestation period, the activity of antioxidant enzymes in the fetal tissues increases by 150-200% (Frank and Sosenko, 1987). Since the concentrations of chemical elements in the tail brush hair of newborn calves reflect its accumulation during the last 3 months of intrauterine development, it is obvious that the maturation of the antioxidant system in fetus with IUGR occurs with the deficiency of selenium, copper, zinc and magnesium. Significant correlations were found between the content of copper in the hair and the activity of SOD in the blood ($r=+0.55$, $P<0.05$) and between the content of selenium in the hair and the activity of GPx in the blood ($r=+0.84$, $P<0.01$). Correlations between CAT activity in the blood and the content of trace elements in the calves' hair were not statistically significant. We assume that oxidative stress in newborn IUGR calves is associated with intrauterine deficiency of selenium and copper.

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Lipid profile during bovine initial development unraveled by sensitive MRM approach

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Spatial and temporal changes in lipidome play fundamental biological roles throughout mammalian initial development, since lipids are involved in key metabolic processes. Recently, MS based techniques have been used to assess the lipid content of gametes and embryos but the lipidome coverage is still limited to a few dozen lipids. Thus, we propose a novel analytical strategy referred as *tailored MRM lipid profiling* to allow the sensitive screening of entire lipid classes. Here, we reported the lipid changes observed throughout preimplantation bovine development using this method. The transitions connecting the precursor ion and a typical fragment for each lipid class (multiple reaction monitoring – MRM) were organized for 10 lipid subclasses listed in the LipidMaps database (Phosphatidylcholine [PC], sphingomyelin [SM], phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylglycerol, acyl-carnitine, cholesteryl ester, free fatty acids and triacylglycerol). Around 15,000 entries were combined as constitutional isomers, and the resulting 1,588 MRMs were used to screen pooled lipid extracts (representative samples) of the following experimental groups: Single bovine oocytes (immature [IO] and mature [MO]), embryos at 3 different stages (2 cells [2C], 8-16 cells [8C] and blastocysts [BX]). Screening was performed by flow-injection into a triple quadrupole mass spectrometer equipped with an electrospray ionization source. Only transitions (n=389) yielding ion intensity higher than a blank sample (pure solvent) were selected to screen individual oocytes and embryos obtained from at least 3 replicates of each group. Data was processed using an in house built script to obtain the absolute ion intensity and then normalized by the total ion count of each sample. Values of relative ion intensity were submitted to univariate (ANOVA) and multivariate analysis (PCA). Phospholipids such as PC and SM, and many cholesteryl ester ions presented higher relative intensity in IO group compared to MO, indicating the IO tendency to accumulate lipids during pre-maturation stage to form membranes and produce energy during the rapid cell division after oocyte fertilization. The MO group presented significantly higher intensity of free fatty acids such as palmitic (16:0), stearic (18:0) and oleic (18:1) that may be used through the β-oxidation to produce energy and boost maturation progression. When embryos were evaluated by PCA, the first principal component explained 81.8% of the variance in the dataset. Lipid relative amounts were similar between 2C and 8C groups, but distinct from BX. A total of 331 lipids were significant ($P<0.05$) by ANOVA, of which 306 showed increased higher relative intensity in blastocyst stage. The dynamic change from 2C/8C to BX can be related to embryonic genome activation that increases the production of macromolecules to sustain preimplantation development and initial cell fate differentiation which is clear at the blastocyst stage. We conclude that the tailored MRM-profile method allows comprehensive lipidomics assessment of stage-specific lipid profile of bovine oocytes and *in vitro* produced embryos, paving the way for monitoring metabolic impairments associated to lipid metabolism in cattle FAPESP #2015/03381-0 and Capes.

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Addition of pregnancy-associated plasma protein-A (PAPP-A) during *in vitro* maturation did not impact on lipid content of *in vitro*-matured bovine oocytes

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Insulin-like growth factor (IGF) participates in lipid metabolism and adiposity regulation by the increasing of lipids β-oxidation. The IGF bioavailability is regulated by pregnancy-associated plasma protein-A (PAPP-A), which is able to disrupt IGFs of IGF binding protein (IGFPBs) increasing availabiltiy of free IGF. Recent findings showed that addition of PAPP-A during *in vitro* maturation increases free IGF and impacts on the expression of genes related to lipid metabolism in bovine blastocysts. In this context, the aim of the present work was to investigate the effects of PAPP-A during IVM of cumulus-oocyte complexes (COCs) on lipid content of bovine oocytes. For this, COCs from a local abattoir were submitted to IVM for 24h with TCM199 in presence or absence of 100 ng/mL PAPP-A, as well as of bovine fetal serum (BFS). Therefore, COCs were matured in four different media: bovine serum albumin (BSA), BSA+PAPP-A, BFS or BFS+PAPP-A. Thereafter, matured and denuded oocytes (n=60 oocytes/experimental group) were submitted to semi-quantitative lipid assay with Sudan-Black B dye. Samples were fixed in a 4% paraformaldehyde solution in PBS free of calcium and magnesium, and transferred to 50% ethanol before staining in 1% Sudan-black B (w/v) in 70% ethanol for 2 min. Samples were mounted in glycerol and examined under a light microscope at 400x magnification. The relative lipid content was estimated in Image J 1.41 software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Images of oocytes were converted to a gray-scale and the gray intensity per area was calculated. The effect of PAPP-A was tested by ANOVA using JMP software (SAS Institute Cary, NC). Differences were considered significant when $P \leq 0.05$. The results showed that addition of PAPP-A during oocyte *in vitro* maturation did not alter lipid content (arbitrary units/μm^2) in the presence or absence of bovine fetal serum ($P > 0.05$; BSA: 9.48±1.56; BSA+PAPP-A: 8.96±0.6; BFS:8.66±1.11 and BFS+PAPP-A: 9.46±1.46). In conclusion, PAPP-A added during oocyte *in vitro* maturation is not able to reduce lipid content of matured oocytes. However, this finding does not exclude the possibility that PAPP-A treatment may impact on lipid metabolism at a later stage of bovine embryo development. The authors are grateful to São Paulo Research Foundation (FAPESP; grant #2013/11480-3, #2017/19679-4).

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An investigation into the factors affecting milk pregnancy associated glycoproteins in seasonal-calving pasture-based dairy cows

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Early pregnancy diagnosis through testing for pregnancy associated glycoprotein (PAG) in milk has the potential to be a useful management tool in seasonal calving systems of milk production. The objective was to examine the effects of week of pregnancy (weeks 5 to 21 post-insemination), fertility index (FI; low <85, medium 85-119 & high ≥120), parity (1, 2, 3+), and interactions between week post insemination and both parity and FI on milk PAG S-N values. Milk PAG S-N values were determined weekly from weeks 5 to 21 post-insemination using the IDEXX Milk Pregnancy Test (IDEXX, USA). Out of the 367 cows enrolled in the study, only cows that conceived to first artificial insemination and maintained pregnancy were retained, resulting in in 2351 observations from 169 cows (n= 53, 76 & 37 for parity 1, 2, and 3+, respectively) available for statistical analysis. PAG S-N values were not normally distributed, an appropriate Box-Cox transformation was identified, and the transformed PAG S-N values were analysed using generalized linear mixed-model (presented as back-transformed LS Means and 95% CI). Week post-insemination (P<0.0001), parity (P 0.065), parity x week post-insemination (P < 0.013) and FI x week post-insemination (P < 0.0028) were all significantly associated with milk PAG S-N values. Peak PAG S-N values were observed on week 5 post-insemination (1.38 [1.25, 1.51]), nadir values on week 9 (0.701 [0.63, 0.77]), followed by recovery thereafter. There was a trend (P = 0.065) for declining milk PAG S-N values with increasing parity number (1.32 [1.23, 1.41], 1.25 [1.17, 1.33] and 1.15 [1.03, 1.27] for parity 1, 2, and 3+, respectively). The interaction between FI and week post-insemination (P = 0.0028) occurred because cows with high FI had a greater PAG S-N values at weeks 5 (1.73 [1.52, 1.96]) and 6 (1.48 [1.33, 1.63]) post-insemination compared with cows with medium FI (1.14 [0.94, 1.36] and 1.32 [1.18, 1.48], respectively) and low FI (1.27 [1.09, 1.48] and 1.12 [0.98, 1.27], respectively), but differences were not detected thereafter. An interaction between parity and week post-insemination was also observed. Parity 1 cows tended to have greater mean nadir PAG S-N values (0.79, [0.67, 0.92]) at week 8 post-insemination compared with parity 2 (0.69 [0.59, 0.79]) and parity 3+ (0.64 [0.49, 0.79]). Most of the differences in milk PAG concentrations were observed before week 12 post-insemination. The temporal variation in milk PAG S-N values in seasonal calving pasture-based cows are consistent with previous reports from high yielding cows fed a total mixed ration. Dam genetic merit for fertility traits may also contribute to the variation in the PAG signal during weeks 5 to 6 post-insemination.

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Distinct expression profile of microRNAs in maternal serum indicates early pregnancy status of cows

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In mammals, pregnancy establishment is an evolutionarily conserved and a complex physiological process, which begins with the formation of a zygote in the oviduct. Following the continuous cleavages, the embryo enters the uterine cavity and undergoes rapid changes in size and shape. At this stage, the release of conceptus-derived IFN-tau acts on the maternal endometrium and prevents the regression of corpus luteum and maintains the pregnancy. In modern dairy farms, it is important to confirm the pregnancy status of breeding cows at the very earliest day post-insemination (DPI), so as to rebreed the non-pregnant cows and increase the reproductive efficiency. The currently available pregnancy detection tools have limitations to determine the pregnancy status of cows early and accurately, which resulted in a decline in farm profitability. Thus, development of alternative pregnancy detection tools, which can accurately predict the early pregnancy status of cows, is of relevance. Mature microRNAs (miRNAs) are known to be present in the maternal circulation and have been associated with various pregnancy-related disorders. Here, we determined the expression profile of circulatory miRNAs in serum samples of pregnant and non-pregnant dairy cows, with the aim to identify circulating miRNAs, which can be used as indicators of early pregnancy. For this, lactating Holstein-Friesian cows were estrous synchronized and inseminated with frozen semen. Blood samples were collected 19 and 24 DPI. Following this, ultrasonography was used to determine the pregnancy status of cows 35 days later, and accordingly serum samples were retrospectively categorized. Total RNA enriched with miRNAs was isolated from triplicate pools (4 animals/pool) of serum samples from each category and subjected to cDNA synthesis. The expression signature of circulatory miRNAs was analyzed using a PCR array containing primers of 748 mature miRNAs. Results showed that a total of 302 and 316 miRNAs were detected in the circulation of day 19 pregnant and non-pregnant cows, respectively. Similarly, 356 and 325 miRNAs were detected in day 24 pregnant and non-pregnant cows, respectively. Principal component analysis separated cows according to the pregnancy status both at 19 and 24 DPI. Moreover, 8 and 23 miRNAs were found to be differentially expressed in the serum of pregnant cows at day 19 and 24, respectively. One miRNA (miR-433) and four miRNAs (miR-487b, miR-495-3p, miR-376b-3p, and miR-323a-3p), which are homologous to the human pregnancy-associated C14MC miRNA cluster were among the differentially expressed miRNAs at day 19 and 24 of pregnant cows, respectively. MiRNAs differentially expressed in the serum of pregnant cows were predicted to be involved in adherens junction and ECM-interaction pathways. In conclusion, the expression profile of miRNAs in maternal circulation could be associated with the pregnancy status of cows and be indicators of early pregnancy.

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The kinetics of the first cleavages impacts on the regulation and levels of histone acetylation of bovine blastocysts

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In bovine embryos, distinct kinetics during the first cleavages is associated with blastocysts with different metabolic activity, gene expression and DNA methylation levels. However, there are no previous reports of alterations in histone regulation. The present work investigated the presence of acetylation on lysine 9 of histone H3 (H3K9ac), one of the most abundant histone alterations in mammal cells. Also, this work evaluated the mRNA levels of H3F3a, H3F3b, HDAC2 and DNMT1, the first two encoding the same histone variant protein associated with active transcription regions, and the last two encoding enzymes that integrate a transcriptional repressor complex. Embryos were produced in vitro with sexed semen using standard protocols and classified at 40 hours post insemination (hpi) as Fast (F) (4 or more cells) and Slow (S) (2 cells). Embryos from both groups were collected at 40hpi (Cleavage [CL] - FCL; SCL), 96hpi (Embryonic genome activation [EGA] - FEGA; SEGA) and 168hpi (Blastocyst [BL] - FBL; SBL) for evaluation of H3K9ac using immunofluorescence (minimum of 5 and maximum of 10 embryos/group, from 3 replicates) and transcript quantification (12 blastocysts/group from 4 replicates) by RT-PCR. The fluorescence intensity from each nucleus was quantified using ImageJ (Total number of nucleus analyzed per stage: 63 CL, 184 EGA and 1192 BL), normalized by the nucleus area and compared by non-parametric Mann-Whitney (kinetics) or Kruskal-Wallis test (stage). For RT-PCR, PPIA and GAPDH were used as endogenous controls for ΔCt calculation and data were submitted to Mann-Whitney test. H3K9ac fluorescence intensity was lower in slow compared to fast embryos at CL and EGA stages (FCL 164.6±10.1 A.U. vs SCL 96.5±11.7A.U.; FEGA 119.1±5.6A.U. vs SEGA 71.1±4.9A.U.; $P<0.0001$) but was higher at BL stage (FBL 69.7±2.3 A.U. vs SBL 274.5±16.3 A.U.; $P<0.0001$). It is important to highlight that acetylation of H3K9 decreased through development ($P<0.0001$) for fast embryos, but increased from EGA to BL stage in slow embryos ($P<0.0001$). Regarding gene expression analysis, no difference was found between mRNA levels of H3F3b, only of H3F3a ($P=0.06$), which was overexpressed in SBL than FBL. Also, there was no difference for HDAC2 mRNA levels, only DNMT1 ($P=0.05$) was underexpressed in SBL. Transcription analysis suggests that there was a replacement of histone 3 with one variant associated with more active transcription regions in SBL and that the HDAC2-DNMT1 complex might be less active in SBL, leading to an increase in transcriptional activity in this group. Based on the results for H3K9ac and gene expression of H3F3a and DNMT1, it is possible to expect that FBL show lower transcription activity than SBL. The present study corroborates previous reports from our group in which FBL presented increased levels of DNA methylation, suggesting that this group has lower transcription activity than SBL. The present data also indicate that the difference between FBL and SBL originates in earlier stages and is associated with distinct status of H3K9 acetylation during preimplantation development. However, it is worth investigating if other variations in epigenetic factors are associated with alterations in gene expression. FAPESP #15/03381-0 and Capes.

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Gene expression related to meiosis resumption after associated use of NPPC and rhFSH during *in vitro* prematuration of bovine *cumulus*-oocyte complexes

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Studies have shown that natriuretic peptide C (NPPC) and its type 2-receptor (NPR2) are essential for the maintenance of oocyte meiotic arrest. FSH is widely used to stimulate *cumulus* cell expansion and meiosis progression. Since the active NPPC-NPR2 system can delay spontaneous resumption of meiosis *in vitro* and FSH possesses an antagonistic action, the combined use of those drugs is avoided when designing systems for in vitro maturation (IVM) or pre-IVM. The objective of this study was to evaluate the effect of NPPC, associated or not to rhFSH in a 6 h pre-IVM system on the mRNA relative abundance of genes associated with meiosis resumption in bovine oocytes. *Cumulus*-oocyte complexes (COCs) were matured according to the following groups: I) Experimental control: 6 h pre-IVM with basic medium (M199, BSA, pyruvate and amikacin); II) Pre-IVM-NPPC: 6 h pre-MIV with basic medium plus NPPC (100 nM); III) Pre-IVM-FSH: 6 h pre-IVM with basic medium plus rhFSH (0.1 IU/mL); IV) Pre-IVM-NPPC/FSH: 6 h pre-IVM with basic medium plus NPPC and rhFSH. COCs were collected at 9 h and 15 h of culture (pre-IVM time plus IVM time; *i.e.*, COCs from groups I, II, III and IV were collected after 3 h and 9 h of IVM). Five replicates were performed with 20 COCs/group and the oocytes were separated from their *cumulus* cells by successive pipetting. Total RNA was extracted by RNeasy® kit (Qiagen) and reverse transcribed using High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). For the analysis of the gene expression we used the integrated high performance microfluidic system Biomark® HD, (Fluidigm, San Francisco, CA, USA) with Applied Biosystems™ TaqMan® Assays, specific for *Bos taurus*. The expression of target genes was normalized by geometric mean of three reference genes (*B2M, PPIA* and *SDHA*). The effect of treatments was tested by one way-ANOVA and the means were compared with Tukey Test. Differences were considered significant when $P<0.05$. From ten genes analyzed (*ADCY3, ADCY6, ADCY9, BDNF, IMPDH1, IMPDH2, MAPK1, NOS2, NOS3* and *PDE5A*), a difference in transcript relative abundance was found for two of them: *NOS3* and *ADCY9* (respectively at 9 h and 15 h). At 9 h IVM, groups II (NPPC) and IV (NPPC/FSH) had decreased mRNA content in relation to group I (Control). At 15 h IVM, groups III (FSH) and IV (NPPC/FSH) were up regulated when compared with group I (Control). These results suggest that after 9 h and 15 h of maturation, there was not a difference in mRNA relative abundance in the analyzed genes related to resumption of meiosis in bovine oocytes submitted to antagonistic action treatments (separately or in a combined way). Funding: grant #2012/50533-2, São Paulo Research Foundation (FAPESP). *In vitro* maturation; Oocyte meiotic arrest; NPPC; FSH; Gene expression; *Bos taurus*.

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Dynamic changes in miRNAs during early embryonic development in bovine: From oocytes to *in vitro* produced blastocysts

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Upon fertilization maternal transcripts are gradually replaced by embryonic mRNAs until the major embryonic genome activation (EGA), which occurs around the 8-16 cells stage. Short non-coding RNAs, microRNAs (miRNAs), can regulate gene expression through post transcriptional mechanisms, and can play a critical role regulating transcripts such as the maternal mRNA during the maternal-to-embryonic transition as well as after the major EGA. The aim of this study was to determine the dynamics of miRNAs during early bovine embryonic development *in vitro*. Slaughterhouse ovaries were used to obtain oocytes from 3-6 mm follicles while bovine embryos were produced by *in vitro* fertilization (IVF). Samples were collected as denuded mature oocytes as well as day 2 (4 cells), day 3 (8-16 cells), and morulae and blastocysts following IVF. Mature oocytes were fertilized with semen from one bull. *In vitro* culture (IVC) was performed at 38.5°C, 5% CO2 and 5% O2. Samples were grouped in three pools of 20 denuded oocytes or 10 embryos collected on day 2 (D2), containing 4 cells, 10 embryos collected on day 3 (D3), containing between 8 and 16 cells, and 10 morulae and 10 blastocysts. Oocytes and embryos were collected from 6 different embryo production routines and homogeneously distributed among pools. Total RNA quality was determined by Nanodrop. Reverse transcription was performed with 100 ng of total RNA utilizing the miScript PCR System kit (Qiagen). Real- time PCR analysis of the miRNAs was performed with a custom miRNA profiler plate. Data were normalized by the geometric mean of 2 endogenous small RNAs (RNU43 snoRNA and bta-miR-99b). The relative levels ΔCt were analyzed by ANOVA followed by Tukey-Kramer HSD test comparing pools as experimental unit. A total of 157 miRNAs were commonly identified from oocyte to blastocyst stage. The total number of miRNAs identified in each stage was 277 in oocytes, 255 in D2, 278 in D3, 265 in morulae and 285 in blastocyst. The total number of miRNAs identified as unique for each stage were seven for oocytes, four for D2, six for D3, none for morulae and 13 for blastocyst. A total of 25 miRNAs were differentially expressed comparing from oocyte throughout blastocyst stage ($P<0.045$ to $P<0.01$). Additionally, a total of 36 miRNAs were differently expressed comparing oocytes, D2 and D3 ($P<0.046$ to $P<0.01$). Bioinformatics analysis indicated similar pathways regulated by the different miRNAs such as PI3K-AKT, ErbB signaling and signaling pathways regulating pluripotency and stem cells. However, pathways such as RNA degradation, oocyte meiosis as well as biosynthesis of amino acids and cell cycle were enriched when we compared oocytes throughout blastocysts and oocytes throughout D3 embryos, respectively. Thus, our analysis demonstrated that miRNA levels are dynamic during early embryo development, suggesting they may play an important role in modulating cell differentiation and metabolism. The results of this project will allow a better understanding of pathways regulated by miRNAs during early embryonic development, with important implications for the assisted reproduction industries in cattle and humans. Financial support: FAPESP 2014/22887-0, 2014/03281-3, 2015/21674-5, 2013/08135-2 and 2012/50533-2.

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Interferon-tau is induced in bovine Day-4 embryos by oviduct epithelium, which generates an anti-inflammatory response in immune cells

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Early cleavage-stage bovine embryos express *MHC I* transcript and thus they could be detected as foreign by the oviduct immune system. The molecular mechanism by which bovine embryos avoid maternal immune rejection has not been well characterized. Here, we used an *in vitro* model to investigate the effect of embryos on immune responses in the oviduct. First, zygotes were cultured with or without bovine oviduct epithelial cells (BOECs) for 4 days, when embryos had reached the 16-cell stage. At that time, interferon-tau (IFNT) was immunohistochemically identified in embryos co-cultured with BOECs, but not in embryos cultured alone (n=19 each). Co-culture of embryos (25-30/well, n=6) with BOECs induced an anti-inflammatory response in BOECs *via* suppression of *NFkBIA* and *NFkB2* (inflammatory response mediators; $P<0.01$), and stimulation of *PTGES* (enzyme involved in PGE2 synthesis; $P<0.05$), as well as secretion of PGE2 (immunosuppressive prostaglandin; $P<0.05$). Next, peripheral blood mononuclear cells (PBMCs) were incubated for 24 h either in media from embryo cultures or from co-cultures of embryos with BOECs. The medium from embryo cultures did not modulate PBMCs gene expression; whereas the embryo-BOEC co-culture medium increased interferon (IFN)-stimulated genes (*ISGs*: *ISG15*, *OAS1*, *MX2*), *STAT1* (IFN signaling factor), *PTGES* and *TGFB1* (anti-inflammatory cytokine) but suppressed *IL17* (pro-inflammatory cytokine) expression in PBMCs ($P<0.05$). Both IFNT-treated BOEC culture medium and IFNT-supplemented fresh medium without BOEC, modulated PBMCs gene expression, similar to the effects of embryo-BOEC co-culture medium. Further, specific antibody to IFNT neutralized the effect of embryo-BOEC co-culture medium. Our findings indicate that BOECs stimulate embryos to produce IFNT, which then acts on immune cells to promote an anti-inflammatory response in the oviduct. This study provides new insight into the molecular mechanism by which a semi-allogenic embryo avoids immune rejection in the bovine oviduct. This study was supported by Grant-in Aid for Scientific Research (16H05013) of Japan Society for the Promotion of Science (JSPS).

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Seasonal influence on spermatic parameters and testosterone levels of Santa Inês rams

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The time of the year elected to perform the reproductive management of an ovine breeding farm must be based on a series of factors, including the weather and the nutrition and reproductive capacity of the male. Testosterone concentrations as well as seminal characteristics are influenced by the photoperiod. In Brazil, the ovine reproductive season is concentrated around October. The present study aimed to evaluate the seasonal effects on sperm parameters and on the serum concentration of testosterone of Santa Inês rams. We used 10 males older than 10 months, housed in individual pens in an intensive system, fed a balanced ration of hay and mineral supplementation. An andrological examination was performed before the animals were selected and at the end of each season (Spring, Summer, Autumn and Winter). Semen was obtained by electroejaculation. Evaluated parameters were volume (mL), mass movement (1 to 5), motility (%), vigor (1 a 5), sperm concentration, major defects (%), minor defects (%), scrotal circumference (cm) and testosterone serum concentration (ng/dL). The statistical analysis was performed using ANOVA with 5% of significance, followed by Tukey test when necessary. The mean and SEM of the obtained results were: Spring - volume = 0.83 ± 0.09^{b}, mass movement = 4.67 ± 0.21^{a}, motility = 86.67 ± 2.11^{ab}, vigor = 4.65 ± 0.21^{ab}, spermatic concentration = $1.54 \pm 0.61x10^{9a}$, major defects = 2.50 ± 0.62^{b}, minor defects = 7.67 ± 1.84^{a}, scrotal circumference = 27.75 ± 1.07^{a} and testosterone = 1.11 ± 0.26^{b}; Summer - volume = 0.97 ± 0.16^{ab}, mass movement = 4.25 ± 0.17^{a}, motility = 82.50 ± 1.71^{b}, vigor = 4.17 ± 0.17^{b}, spermatic concentration = $0.70 \pm 0.11x10^{9b}$, major defects = 7.50 ± 2.32^{a}, minor defects = 6.17 ± 1.78^{a}, scrotal circumference = 28.67 ± 1.66^{a} e testosterone = 1.15 ± 0.24^{b}; Autumn - volume = 1.36 ± 0.15^{a}, mass movement = 4.73 ± 0.14^{a}, motility = 88.18 ± 1.22^{a}, vigor = 4.73 ± 0.14^{ab}, spermatic concentration = $1.38 \pm 0.14x10^{9a}$, major defects = 6.86 ± 1.39^{ab}, minor defects = 5.21 ± 0.87^{a}, scrotal circumference = 31.36 ± 0.90^{a} e testosterone = 3.76 ± 0.64^{a}; and Winter - volume = 1.20 ± 0.13^{ab}, mass movement = 4.92 ± 0.08^{a}, motility = 89.17 ± 0.84^{a}, vigor = 4.92 ± 0.08^{a}, spermatic concentration = $1.66 \pm 0.19x10^{9a}$, major defects = 5.50 ± 0.76^{ab}, minor defects = 5.33 ± 0.67^{a}, scrotal circumference = 30.42 ± 1.09^{a} and testosterone = 1.79 ± 0.40^{ab}. We conclude that season of the year affected spermatic parameters and serum concentration of testosterone in Santa Inês rams under the experimental conditions. Financial support: FAPESP (no. 2011/51503-7).

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Hormonal metabolic aspects of embryonic mortality of cows and the effectiveness of its prevention

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Embryonic mortality and high frequency of its manifestation among lactating cows is one of the reasons for their fertility impairment and large economic losses in dairy cattle breeding. It is assumed that the determinants of this pathology are metabolic imbalance, hypoprogesteronemia and disorders of the immune- trophic interrelations in the mother-embryo system. The objective of this study was to assess the hormonal, antioxidant and endotoxic status of cows during the physiological formation of the embryo, during its development, retardation and death, as well as the effectiveness of a series of biologically active medications to reduce embryonic losses. The research was performed on 196 black-motley Holstein cows with an average annual milk productivity of 6.5-7.5 thousand kg. Diagnosis of pregnancy, embryo state and its death was carried out on 19-23, 28-32 and 38-45 days after insemination by detecting the progesterone concentration level in serum and by means of transrectal ultrasound scan of reproductive organs. The metabolic status was assessed according to the content of selenium, vitamin E, malonic dialdehyde (MDA), medium molecular peptides (MMP), and glutathione peroxidase (GPO) catalase activity in the blood serum. To prevent the death of embryos such additives as progesterone, gonadotropin PMS (pregnant mare serum), selenium and interferon-tau were used, which were assigned to the cows within the first days after insemination. The embryonic death among intact animals was registered in 29% of cases and was associated with a deficiency of progesterone, estradiol- 17ß, selenium, with a functional disorder of the antioxidant defense system and an increased level of endogenous intoxication. The concentration of progesterone in their blood was lower by 30.6-43.3% ($P<0.05$-0.01), estradiol by 31.0-46.1% ($P<0.05$-0.01), selenium by 26.3-33.2% ($P<0.05$), vitamin E by 31.2% ($P<0.01$), catalase by 27.3% -51.2% ($P<0.001$), the MDA content by 38.6-58.5% ($P<0.01$), the MMP - by 23.9-34.7% ($P<0.05$) and the endogenous intoxication index by 28.8-30.0% ($P<0.001$). Pharmacological control of the hormonal and metabolic status of inseminated cows by prescribing the above mentioned medications, reduced the intrauterine death of the embryo by 12.5-16.7% and increased the effectiveness of insemination by 1.5-1.8 times. The most efficient results were registered in the group of complex use of gonadotropin PMS and selenium. The size of developing embryos among the cows from the experimental groups exceeded the one among the control animals by 35-40% ($P<0.01$).

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Protective effects of cysteamine on bovine oocytes matured *in vitro* with eicosapentaenoic acid (EPA): impact on early embryo development

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Data on fatty acid composition and their uptake and utilization by oocytes and embryos are relevant for culture *in vitro*, cryopreservation practices, and embryo quality improvement. Despite several studies *in vitro* suggesting enhanced embryo development with polyunsaturated fatty acids supplementation, results are not consistent and it would be important to determine which fatty acid is the most beneficial for embryo quality and survival (Santos *et al*., 2008). *In vitro*, embryos are sensitive to lipids effects unless adequate antioxidant protection is provided (Reis *et al*., 2003). The aim of this study was to determine the effect of eicosanopentaenoic acid (EPA; n-3, 20:5) alone or with cysteamine (CYS) on bovine oocyte during *in vitro* maturation (IVM). For this purpose, bovine cumulus-oocyte complexes (COC) from ovaries obtained in a slaughterhouse were matured *in vitro in* TCM- 199 supplemented with 0.1% essentially fatty acid–free BSA, 1 µg/mL FSH, 1 µg/mL estradiol 17ß at 39°C with 5% CO_2 in humidified air. COCs were matured 24 h in IVM media: (1) alone (Control); (2) supplemented with 1 nM, 10 nM and 100 nM EPA and, (3) with EPA + Cysteamine 100 µM. Oocyte intracellular ROS level with H2DCFDA, cumulus expansion area with digitalizing images, nuclear maturation with Hoechst 33342, and embryo developmental capacity were evaluated. Data were analyzed using Statgraphics Plus 5.1 software. The results demonstrated that: a) ROS levels were significantly higher in oocytes matured with 1mM and 100 mM EPA compared with Control ($P<0.05$). The presence of CYS diminished ROS levels induced by EPA ($P<0.05$); b) Cumulus expansion area was similar in COC matured with EPA and EPA+CYS ($P>0.05$); c) EPA (100 nM) resulted in a decrease of 10.95% in nuclear maturation rate ($P<0.05$). However, addition of CYS to EPA supplemented media attenuated this effect; and d) embryo development was not altered with EPA, but EPA 1nM + CYS increased significantly cleavage and blastocyst rates in relation to Control values ($P<0.05$). In conclusion, EPA supplementation during bovine IVM increased subsequent embryo development only when CYS was also present in IVM medium providing protection against intracellular ROS generated by EPA. These results are relevant for including EPA in *in vitro* embryo production (IVP) media with the purpose to enhance embryo quality for cryopreservation practices.

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Influence of preovulatory estradiol on uterine luminal fluid proteomics around maternal recognition of pregnancy in beef cattle

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Proteins within the uterine luminal fluid (ULF) are involved in elongation, recognition of pregnancy, implantation, and placentation, and previous research has established that elevated preovulatory estradiol increased embryo survival and pregnancy rates. However, on d 16 of pregnancy no differences in conceptus recovery rates or IFNT concentrations were detected between animals with elevated or low concentrations of estradiol prebreeding. The present study evaluated the effects of preovulatory estradiol and conceptus presence on the d 16 ULF proteome. Beef cows/heifers (n=29) were synchronized and artificially inseminated (d 0), and grouped into high (highE2) and low (lowE2) preovulatory estradiol based on expression of estrus and circulating estradiol concentrations (d-2 and d0). On d 16, animals were slaughtered and uteri were flushed. Two separate ULF pools were created based on the following groupings: highE2/no conceptus, highE2/conceptus, lowE2/no conceptus, and lowE2/conceptus. Pools were then analyzed using a 2D LC-MS/MS based 8plex iTRAQ quantitative method. Scaffold Q+ was used to quantitate peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95% probability by Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99% probability and contained at least 2 identified peptides. The FDR was adjusted using Benjamini-Hochberg procedure to identify significance ($P < 0.05$) based on permutation tests. String database was used to analyze upregulated and downregulated proteins in each grouping. There was a total of 6989 peptides and 1269 proteins identified, and a total of 48 significant differentially expressed proteins between the highE2/conceptus group and the lowE2/conceptus group, 19 of which were upregulated (GOT2, ACAA1, TSTD1, GPLD1, ALDH2) and 29 were downregulated (UTMP, ORM1, ANXA8, ANXA1, FGG) in the highE2/conceptus group. Proteins associated with cell differentiation, single organism process, and regulation of metabolic process were upregulated in highE2/conceptus group. Proteins associated with angiogenesis, regulation of vesicle mediated transport, and positive regulation of transport were downregulated in highE2/conceptus group. A previous study identified 30 unique proteins produced by a short term culture of d 16 concepti, 24 were identified in the current study (20 were upregulated and 4 were downregulated in the highE2/conceptus group). These data provide insight on differences in specific protein abundances in the ULF that may simply reflect developmental processes, but also may contribute to improved conceptus survival among highE2 animals. USDA is an equal opportunity provider and employer.

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Differential expression of LH receptor, LHR mRNA binding protein (LRBP) and bta-miR-222 in the developing bovine ovary

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Gonadotropins play an essential role during antral follicular development, as well as in late stages of preantral follicle development. Although the luteinizing hormone receptor (LHR) has been detected in preantral follicles of rats, rabbits, and pigs, the expression and localization of LHR in bovine fetal ovaries have not been assessed. Thus, the present study aimed to quantify the expression of LHR in the developing bovine ovary. In addition, mRNA abundance of LHR binding protein (*LRBP*) and levels of bta-miR-222 were also quantified. Twenty bovine fetuses (predominantly *Bos taurus indicus*) were obtained in a local slaughterhouse and divided into four groups (n=5/group) according to gestational age estimated by the crown-rump length (gestational ages: days 60, 90, 120 and 150). Both ovaries were removed and one was destined to immunohistochemical analysis, while the other was submitted to total RNA extraction. The expression of *LHCGR* and *LRBP* was investigated by RT-qPCR using the Sybr Green system and LHR immunolocalization was performed by immunohistochemistry. miRNA extraction was performed using 50 µg of total RNA into *mir*Vana™ miRNA Isolation Kit (Life Technologies®, São Paulo, Brazil) and for reverse transcription and qPCR of target miRNAs, TaqMan® Reverse Transcription Reagents following manufacturer's protocols were utilized. For immunostaining of LHR, slides were incubated with primary antibody (anti-LH receptor, ab96603, Abcam®, Cambridge, UK) and then incubated with biotinylated secondary antibody followed by a VECTASTIN ABC Kit (Vector Laboratories Ltd.). Slides of mature *corpus luteum* were used as positive control. The effect of gestational age was tested by ANOVA and means were compared by Tukey-test. Differences were significant when $P \leq 0.05$. In summary, the *LHCGR* expression was present in the bovine fetal ovary and showed lower mRNA abundance on day 150 compared with day 60 (P=0.04). Nevertheless, the mRNA abundance of *LRBP* presented an opposite pattern, showing higher expression on day 150 (P=0.03). Similar as *LRBP* expression, the abundance of bta-miR-222 was higher on day 150 compared with days 60 or 90 (P=0.02). The LHR protein was immunolocalized in oogonias, primordial, primary and secondary preantral follicles. Moreover, both oocyte and granulosa cells showed LHR immunostaining. Another important finding was stronger immunoreactivity for LHR in the ovarian cortex compared with the medullary region and staining in the ovarian surface epithelium and blood vessels. In conclusion, these findings may suggest that the *LHCGR/LRBP* regulation could be involved in the mechanisms regulating preantral follicle development, especially during establishment of secondary follicles. Furthermore, the present data suggest that lower expression of *LHR* mRNA in bovine fetal ovaries at day 150 could be related to higher expression of *LRBP* and bta-miR-222, as described in granulosa cells during follicular deviation in cattle. The authors are grateful to São Paulo Research Foundation (FAPESP; grant #2013/11480-3).

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Transcripts abundance modulation by AREG and FSH treatments during the *in vitro* maturation of bovine *cumulus*-oocyte complexes

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The LH surge induces in the ovarian follicle the prompt production of cAMP that, in turn, subsequently stimulates the synthesis of EGF-like peptides: Amphirregulin (AREG) Epirregulin (EREG) and Betacelulin (BTC) in the granulosa cells. They are cleaved, released and bind to their receptor in granulosa and *cumulus* cells (CC), where they act stimulating the expression of critical genes for the CC expansion among other pathways. Moreover, they mimic the *in vivo* LH action when they are added *in vitro* (*i.e.*, they promote nuclear and cytoplasmic maturation). LH action on CC is mediated by EGF-like peptides and previous data suggest that they are superior to the FSH and EGF in *in vitro* maturation (IVM). The aim of this work was to evaluate transcript abundance of a gene set downstream to the LH surge pathway (*e.g.*, FSHR, EGFR, LHCGR *etc*.) in cumulus-oocyte complexes (COCs) after IVM with FSH or AREG. Ovaries were obtained from an abattoir and follicles between 2-8mm of diameter were aspirated. COCs (n=25 COCs/group; 5 replicates) were *in vitro* matured in a medium with BSA and AREG (100 ng/mL) or rhFSH (0.1 IU/mL; Gonal-*f*® 75 IU) or in a control medium [rhFSH (0.1 IU/mL; Gonal-*f*® 75 IU) plus FBS] at 38.5°C and 5% CO_2 humid atmosphere for 15 or 24h. Oocytes were separated from CC by pipetting and vortexing and stored at -80°C. Total RNA was extracted, DNAse treated, reverse transcribed using random primers and submitted to qPCR using the integrated high performance microfluidic system Biomark® HD (Fluidigm). The results were normalized using the geometric mean of *ACTB*, *B2M* and *RPL30* and analyzed by ANOVA or Kruskal-Wallis test using the JMP® software. Significant differences were considered when $P \leq 0.05$. Regarding the expression of the hormone receptors (EGFR, FSHR and LHCGR), there was no difference among groups except for EGFR (higher in FSH than in AREG after 15h of maturation in CC; $P = 0.0397$). From the 96 genes tested (related to thermal stress, apoptosis, cell cycle, cumulus expansion, blastocyst quality prediction and others) 23 were less abundant in CC samples from AREG group when compared to the control or FSH groups (*e.g.*, *BAX, BCL, VNN1, HSP1, HSPA1A* and *FOXO3*), whereas in oocytes two genes were less abundant in the AREG group compared to the control or FSH groups (*EGFR* and *HAS*). We observed a non-overlapping gene expression effect of the two ligands, consistent with the hypothesis of different pathways being activated with AREG or FSH during the IVM. Funding: grant #2012/50533-2, São Paulo Research Foundation (FAPESP).

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Lack of association of neospora caninum infection with late embryo losses in grazing dairy cows

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The fertility of high-producing, lactating dairy cows has decreased during the last decades. Pregnancy losses in dairy cows are an important aspect of infertility leading to poor economic performance. Late embryo losses (LEL) are assumed to be of multifactorial origin, with *N. caninum* infection being a potential cause. The objective of this study was to evaluate the association of *N. caninum* infection with LEL in grazing dairy cows. A case-control study was carried out in a commercial dairy herd (32°49´ S, 62°52´ W, Argentina) in which Holstein cows (n=92) were enrolled. Pregnancy was diagnosed by ultrasonography at 30-44 days post-insemination. Lack of heart beats, identification of membrane detachment, and disorganization or echoic floating structures including embryo remnants were used as indicators of LEL (CASE cows). Cows with positive pregnancy diagnosis on the same date of each case were considered as control (CON) and the ratio of case-control was 1. For each case animal, the conceptus was sampled (day 0) with an insemination pistol attached to a 10-mL syringe and transported at 4°C in a 1.5-ml tube. Cows were bled on days 0 and 30 for serological studies. Blood samples were refrigerated at 4°C and transported to the laboratory within 24 h, centrifuged and sera were stored at -20°C until analysis. Sera were tested by indirect fluorescent antibody test (IFAT) for the detection of antibodies against *N. caninum* using a cut-off titer of 1:200 and processed to final titer. The DNA from conceptuses from seropositive cows was extracted using a commercial kit according the manufacturer's recommendations. The DNA samples were analyzed by PCR with the specific primer pair Np6+/Np21+ for *N. caninum*. Chi squared test was used to detect differences between groups and $P<0.05$ was considered as significant. The proportion of seropositive cows to *N. caninum* in one or both sampling points was similar (P=0.599) between CASE (26%, 12/46) and CON (19.6%, 9/46) groups. The proportion of cows showing seroconversion was 6.5% (3/46) and 4.3% (2/46) for CON and CASE groups, respectively (P=0.212). Finally, PCR analysis was negative for *N. caninum* DNA in all the conceptus from CASE cows (n=12). We conclude that *N. caninum* is not associated with LEL in grazing dairy cows from Argentina.

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Serum concentration of sex steroids in down-calving cows as predictors of the respiratory diseases progression among their posterity

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DoHaD (developmental origins of health and disease) hypothesis proposes that early-life environment has long-term effects on the postnatal ontogenesis (Fukuoka, J Nutr Sci Vitaminol 61:S2–S4, 2015). Of great interest is the study of the role of sex steroids in perinatal and early postnatal development, in order to identify their potential capacity in imprinting/programming phenomena. The aim of the study was to determine the diagnostic value of serum concentration of progesterone, estradiol and dihydroepiandrosterone sulfate (DHEA-C) in down-calving cows to predict the respiratory diseases progression among their posterity. Forty-five cows of red-motley breed with one-fetal pregnancy at gestation period of 248-255 days and calves obtained from them were examined. The concentration of progesterone, estradiol and DHEA-S in the serum of cows was determined by the method of enzyme-linked immunosorbent assay with commercial sets of NVO Immunotech Company (Russia) using the Uniplan AIFR-01 analyzer (Pikon Company, Russia). The time of the first clinical signs appearance and the height of bronchitis for the calves, the severity of the course and complication in the form of bronchopneumonia were taken into account. To assess the relationship between serum concentrations of hormones studied in cows and the indicators of clinical status of calves, nonparametric correlation criteria of Spearman and tau-Kendall were used. The diagnostic cut-off point of the analyzed factors was determined using ROC analysis in the IBM SPSS Statistics 20.0 program (IBM Corp., USA). It has been established that the predictor of bronchopneumonia progression of newborn calves can be the serum concentration of estradiol in their mothers. The indicator was characterized by good diagnostic value (the area under the AUC curve is 0.707), a high sensitivity of 77.8%, and a specificity of 77.3%; a critical value that cuts the risk group for bronchopneumonia progression - less than 71.2 pmol/l. To predict the early (within the first week of life) development of bronchitis of calves, high diagnostic value for estradiol concentration (AUC = 0.729) and progesterone / estradiol (AUC = 0.750) in the serum of their mothers was found: sensitivity 70,8% and 54,2%, specificity 85,7% and 85,7% respectively; critical values that cut the risk group for early development of bronchitis are less than 116.8 pmol/l and more than 571.0: 1, respectively. Thus, the determination of serum concentration of progesterone, estradiol and DHEA-C in cows 25-32 days before calving allows to predict the respiratory diseases progression among their posterity with high accuracy.

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HSPA5 and *in vitro* embryo production: effect of addition of the protein during *in vitro* embryo culture in cattle

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After fertilization, the early embryo is exposed to oviductal epithelial cells and surrounded by oviductal fluid. The oviduct provides important structural, environmental and nutritional support for early embryonic development. Although oviductal fluid is not totally defined, it is known to contain HSPA5 protein. The HSPA5 is a member of the heat shock 70 class of proteins, also known as GRP78, and seems to play an important role in embryonic cell growth. Thus, the present study aimed to investigate the effect of HSPA5 during *in vitro* embryo culture on blastocyst (BL) yield. First, c*umulus*-oocytes complexes (COCs) were recovered from ovaries of slaughtered cows and *in vitro* matured in TCM199 medium plus pyruvate, amicacin, BSA (bovine serum albumin) or FBS (fetal bovine serum) in a humidified atmosphere at 5% CO_2 for 24h. After *in vitro* fertilization, presumptive zygotes derived from BSA or FBS *in vitro* maturation were denuded and cultured in a commercial culture medium in the absence (control group) or presence of HSPA5 (1 ng/mL: G1 group or 100 ng/mL: G100 group) in humidified controlled atmosphere at 5% CO_2 and O_2 until the BL stage. Therefore, experimental groups were: i) embryos derived from BSA maturation medium and cultured in control, G1 group, or G100 group (n=20 COCs/group; 7 replicates); ii) embryos derived from FBS maturation medium and cultured in control, G1 group, or G100 group (n=20 COCs/group; 7 replicates). The medium was partially replaced at day 3 and 5 with glucose addition (1µL of a 0,09mg/mL solution) on the last day feeding. The BL rates were assessed on day 7 and 8 after *in vitro* culture (IVC). Therefore, the experimental design was a 2x3 factorial, including two supplements during IVM (FBS or BSA) and three supplements during embryo culture [FBS; HSPA5 (1ng) or HSPA1 (100ng)], totalizing 6 experimental groups. Data were analyzed by ANOVA, PROC GLIMIX (SAS) and Tukey test was used to compare means. Significant differences were considered when $P < 0.05$. The BL yield from COCs matured in the BSA control group was increased compared with the FBS control group (40.05±3.35 and 25.5±4.6 respectively; $P = 0.04$), independently of HSPA addition. On day 7, embryo yield from COCs matured with BSA medium was higher in the control group (40.05±3.35) when compared with the G1 group (23±4.43; $P = 0.04$), but not different compared to the G100 group (32.62±3.8). The BL yield on day 7 plus day 8 was not affected by the presence of HSPA5 in the IVC medium: COCs matured in BSA medium (control: 43.07%; G1: 45.09% and G100: 46.6%) or COCs matured in FBS medium (control: 50.26%, G1: 54.2% and G100: 54.6%). However, the percentage of BL was higher in those derived from COCs matured in BSA and cultured with 100 ng/mL of HSPA5 protein ($P = 0.01$; 59±3), compared to those derived from COCs matured in BSF medium (46.67±2.46). Taken together these results suggest that HSPA5 may have a positive effect on blastocyst yield when COCs are matured only with BSA. Financial support: FAPESP (grant #2013/11480-3).

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Effect of laterality of ovulation and the presence of embryo on uterine horn irrigation in llamas

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Llamas have several unique reproductive characteristics. The establishment of pregnancies almost exclusively in the left uterine horn, regardless of laterality of ovulation, is one of them. Llama uterus presents differences in the vascular irrigation and drainage arrangements between both uterine horns (Del Campo MR, Del Campo CH, Ginther OJ. 1996. Vascular provisions for a local utero-ovarian cross-over pathway in new world camelids. *Theriogenology* 46:983-991). The presence of a prominent cross-over arterial branch extending from the right uterine artery to the left horn suggests that this is irrigated with a greater blood flow. Since an adequate endometrial blood supply is essential for a successful embryo implantation (Raine-Fenning N. 2008. Doppler assessment of uterine artery blood flow for the prediction of pregnancy after assisted reproduction treatment. Ultrasound Obstet Gynecol, 31:371-375), studies on the spatial relationship between the location of the early embryo and the degree of endometrial vascular perfusion in llamas are required. The aim of this study was to determine if intrauterine embryo location differentially induces changes in endometrial vascular perfusion between left and right uterine horns, during and after embryo migration, elongation and implantation in llamas. Adult, non-pregnant and non-lactating llamas (n = 24) were subjected to daily B-mode ultrasound scanning of their ovaries. Llamas with a growing follicle ≥8 mm in diameter in the left (n = 12) and right (n = 12) ovary were assigned to a single mating with a fertile adult male. Color-Power Doppler ultrasonography was used to determine the area of endometrial vascularization (AEV) in a cross section of the middle segment of both uterine horns. AEV was determined by off-line measurements of the average of three still images of each horn using the ImageJ software. AEV measurements were performed before mating and on days 5,10,15,20,25 and 30 post copulation in pregnant (n = 6 llamas with left or right ovulations) and non pregnant females (n = 6 llamas with left or right ovulations). Also, during the first 48 hours after mating, ovulation was confirmed by the disappearance of a follicle (≥8 mm) detected previously. Pregnancy was confirmed by the presence of the embryo proper and heart beat was used as a sign of embryo viability. AEV were analyzed by one-way ANOVA for repeated measures using the MIXED Procedure in SAS. If significant ($P \leq 0.05$) main effects or interactions were detected, Tukey´s post-hoc test for multiple comparisons was used. First observation of uterine content and embryo (exclusively in the left uterine horn) was on day 15.8 ± 3.8 and 22 ± 2.7, and 16.7± 2.6 and 27.5 ± 2.8 for pregnant llamas ovulating in the right and left ovary respectively. Although the AEV of both uterine horns was affected by time ($P < 0.05$) in pregnant llamas, it was not affected by the presence of embryo ($P = 0.35$) or laterality of ovulation ($P = 0.4$). Contrary to expected, regardless of the laterality of ovulation, in pregnant llamas the left horn did not display a greater AEV before or after embryo arrival. This trent was observed during the entire evaluation period. Proyecto Fondecyt Iniciación 11140396 (M. Silva) y Regular 1160934 (M. Ratto).

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Treatment of bovine embryos with dickkopf-related protein 1 from the morula to blastocyst stages of development alters trophoblast elongation at day 15 of pregnancy

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Dickkopf-related protein 1 (DKK1) is an endogenous inhibitor of WNT signaling produced by the endometrium which can improve competence of in-vitro produced embryos to establish and maintain pregnancy after transfer into recipients. Here, it was tested whether actions of DKK1 from days 5 to 7 of development (when the embryo is a morula and blastocyst) improved the ability of the embryo to undergo trophoblast elongation at day 15 of pregnancy. Embryos were produced in-vitro using oocytes from slaughterhouse ovaries and were exposed to 0 or 100 ng/ml recombinant human DKK1 from day 5 to 7.5 of culture. Blastocysts were transferred into synchronized recipient cows on Day 7.5 (n = 23 for control and 17 for DKK1). On Day 15, cows were slaughtered and conceptuses recovered by flushing the uterus with 50 to 200 mL Dulbecco's phosphate buffered saline (DPBS). Recovery was 48% (n =11) for control and 65% (n = 11) for DDK1 embryos. Except for two DKK1 embryos, all conceptuses were filamentous. Treatment with DKK1 increased (P = 0.007) the length of filamentous conceptuses from 63.7 mm (control) to 153.7 mm (DKK1). Concentration of interferon-τ (IFNT) in uterine flushings was measured using a sandwich ELISA with a limit of detection of 32.00 pg/mL and total IFNT was calculated using IFNT concentration and the infused volume of DPBS. DKK1 increased (P = 0.01) the amount of IFNT in uterine flushings of cows with filamentous embryos from 9.42 µg (control) to 21.84 µg (DKK1). It was concluded that DKK1 can act on the morula-to-blastocyst stage embryo to modify subsequent trophoblast elongation. Higher pregnancy rates associated with transfer of DKK1-treated embryos may be due in part to enhancements of trophoblast growth and antiluteolytic signaling through IFNT secretion. Support: USDA-NIFA AFRI Grant 2017-67015-26452 and CAPES, Brazil.

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Proteome profile of extracellular vesicles isolated from culture media of pre-elongation stage bovine embryos

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In bovine, elongation of the preimplantation embryo is a prerequisite for successful implantation, likewise it is critically dependent on the correct embryo-maternal communication. Pre-implantation bovine embryos as well as other cell types secrete extracellular (EVs) vesicles. This suggest that EVs play a role during early embryo-maternal for normal development and conceptus implantation. The aim of this study was to identify the proteome profile of EVs secreted by bovine pre-elongation stage embryos. Pools of 25 to 30 zygotes were cultured in vitro in 500 µL in 4-well plates using SOFaa medium until day 7 post-IVF. At day 7, the blastocysts in developmental stages 5, 6 and 7 according to morphological criteria of the IETS, were placed individually in SOFaa medium for extended culture until day 9 post IVF. At Day 9, only blastocysts in developmental stages 8 and 9 were further cultured individually in conditioned medium depleted of EVs (SOFdep) until day 12 post IVF. Embryo culture media from these embryos, was collected individually matching with the corresponding embryo and preserved individually at -80°C until further analysis. The EVs were isolated and characterized using nanoparticle tracking analysis (NTA). Then, all samples of embryo culture media were pooled in a single group and compared to SOFdep media (reference group). Proteomic profile of EVs was carried out by gel electrophoresis and protein identification performed by MALDI-ToF Mass Spectrometry. In all, culture media from 56 blastocysts with diameter in a range of 477.94±173.62 µm, at day 12 were pooled. The morphological characteristics of isolated EVs from culture medium were 127.38 ±36.63 nm of mean size and 2.97± 1.11 x 10^9 particles per mL. Twenty-eight proteins were identified, 13 were common, 3 were unique in reference group and 12 exclusive of the embryo culture media. The embryo-exclusive proteins present in EVS were: adiponectin, kininogen-1, fetuin-B, afamin, beta-2-glycoprotein 1, angiotensinogen, hemopexin, ceruloplasmin, vitamin D-binding protein, apolipoprotein A-II, olfactomedin 3 and alpha-2-antiplasmin. Using the Uniprot database the identified proteins were mapped to subcellular locations or to be secreted. Interestingly most of them, eight are involved in active process during embryo implantation. For instances, adiponectin is crucial for human embryo implantation (Duval *et al*., 2017), increase in hemopexin is related with a normal pregnancy (Bakker *et al*., 2007). In mice, fetuin-B protects against zona pellucida hardening, and is critical before fertilization as well as a later stages of embryonic development and might be related with embryo hatching (Dietzel, 2014). These results lead us to hypothesize that secreted EVs play a crucial role during embryo implantation in the bovine. We appreciate the input of Dr. Eduardo Callegari from University of South Dakota and Dr. Mauricio Hernandez from AUSTRAL-Omics. Work supported by Grant Fondecyt 1170310, Ministry of Education, Chile.

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Prepuberal Sertoli cell proliferation differs between the bull and boar

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Sertoli cells support spermatozoa during their development and the number of Sertoli cells is a major determinant of sperm production capacity in a male. The total number of Sertoli cells is normally determined by prepuberal proliferation. In recent decades, two waves of postnatal, prepuberal proliferation have been described in pigs and primates but not in rodents. The usual interpretation has been that two postnatal waves of proliferation are typical but waves occur concurrently or only one exists in early maturing rodents. Defining the timing of proliferative waves will assist in manipulations to increase Sertoli cell numbers and adult sperm production capacity. For example, reducing endogenous testicular estradiol stimulates the first postnatal wave of Sertoli cell proliferation in the boar but does not affect the second wave. In our first study, groups of Angus-Hereford crossbred bull calves were castrated at 1, 2, 3, 4, 5, and 6 mo of age (n = 8 per age). Testes were weighed and equatorial segments fixed. Sertoli cell density was determined following immunohistochemical labeling of Sertoli cells with GATA-4 antibody in 30- micron thick sections. The number of Sertoli cells per testis increased linearly from 1 mo to 5 mo of age ($r = 0.77$; $P < 0.001$). Sertoli cell numbers appeared to have plateaued at 5 mo of age and luminal development also was present. Hence, only one postnatal wave of Sertoli cell proliferation was detectable in the bull. In the second study, Jersey bull caves were treated twice weekly with an aromatase inhibitor, letrozole, beginning at 2 weeks of age, with the control animals receiving the canola oil vehicle. At 6 mo of age, testes were retrieved, weighed, tissue fixed and Sertoli cell density determined. Testicular tissue was analyzed for estradiol and testosterone and serum was analyzed for FSH and LH. Testicular estradiol was reduced ($P < 0.05$) in letrozole-treated bulls, as anticipated. Consistent with results in the boar, no effects on testosterone or circulating FSH or LH were observed. In contrast to the boar, reducing testicular estradiol did not alter Sertoli cell numbers. This discrepancy between responses in the boar and the bull might be due to the relatively low level of estradiol present in the testis in control animals (approximately 1% of that in boars) such that estradiol was exerting minimal inhibitory effects in the control bulls. The absence of a clearly distinguishable first postnatal wave of Sertoli cell proliferation in bulls is a second possibility for the discrepancy as only the first postnatal wave of Sertoli cell proliferation responds to reduced testicular estradiol in the boar. A larger proportion of the interval between sexual differentiation of the gonad and puberty is encompassed by gestation in the bull than in the boar; hence Sertoli cell proliferation corresponding to the first postnatal wave in the boar may be occurring prenatally in the bull, a possibility that awaits further research. (Supported by UC Davis FAPESP and AJCC).

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Comprehensive evaluation of metabolic behavior in preimplantation embryo

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The study of key metabolic processes of the early embryo has been driven by the desire to define requirements for embryo growth and viability. This study was designed to monitor the metabolic response of embryos with distinct kinetics when submitted to a combination of common environmental stressors in the culture system. Using a factorial experimental design (2x2x3), Fast (4 or more cells at 22 hours post insemination) and Slow (2-3 cells) embryos were produced *in vitro* by standard protocols and cultured in 20% or 5% O_2, and also in different glucose concentrations (0, 2 or 5mM), resulting in 12 study groups. Blastocysts were evaluated for 92 characters including 85 genes [control of gene expression (14 genes), lipid metabolism (16), energy metabolism (15), cell death (5), response to oxidative stress (17), other cellular functions (18)]; 7 biochemical measures: consumption of glucose, glutamate and pyruvate, production of lactate and ATP, generation of reactive oxygen species (ROS), mitochondrial activity, and lipid content by mass spectrometry. Data were normalized and gathered in a matrix that was analyzed under parsimony, with 500 replicates and Tree-Bissection Reconection as the swapping algorithm (*TNT software*). All characters were additive and equally weighted. This analysis resulted in a single optimal tree, fully resolved, which provided a panoramic view of the system. Interestingly, all groups arranged in only 2 main clusters according to high or lower oxygen tension, suggesting that this category stands out in terms of developmental kinetics and glucose, except for the groups cultured in a hyperglycemic environment (5mM) that formed a separate cluster. Further investigation of these 2 clusters (correlation attribute evaluation – *Weka software*) was able to highlight characters that most contributed to differentiate groups cultured in 20% vs. 5% O_2. A total of 33 genes [control of gene expression (8); lipid metabolism (10); cell death (2); response to oxidative stress (10)] were found to be related to such differences. In general, embryos cultured at 20% of O_2 were oriented to greater glycolytic activity, since the transcripts for glucose transporters and PFK were increased. Especially in the 2mM glucose groups, increased consumption of pyruvate, mitochondrial activity and production of ROS, indicated that apart from glycolysis, these embryos were also performing more oxidative phosphorylation than those at 5% O_2. However, this greater "commitment" to producing energy was not reflected in the amount of ATP. Moreover, at 20% O_2, the higher transcription of genes related to lipid synthesis, elongation and desaturation was accompanied by augmented lipid content, which is usually an indicator of lower quality. In contrast, at 5% O_2 the presence of palmitoyl carnitine indicated that these embryos were resorting to ß-oxidation, a highly efficient mode of energy production for the cell. Considering the plasticity of embryonic responses, our results help unravel the complexity of culture system and indicate that oxygen tension is an important factor for the maintenance of proper metabolic status. Sao Paulo Research Foundation #2015/03381-0.

062 Gametes Biology, Embryology and Fetal Development

Differential transcript profiles in cumulus-oocyte complexes originating from pre-ovulatory follicles of varied physiological maturity in beef cows

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Dominant follicle diameter at exogenous gonadotropin releasing hormone (GnRH) induced ovulation, but not at spontaneous ovulation, influenced pregnancy rate and late embryonic mortality in postpartum beef cows. Furthermore, induced ovulation of a larger dominant follicle increased fertilization rate and the probability of recovering a transferable embryo on day 7 post insemination. Therefore, we hypothesized that the physiological status of a pre-ovulatory follicle at GnRH-induced ovulation has a direct effect on oocyte competence and subsequent embryo development. The objective of this study was to determine if the transcriptome of oocytes and associated cumulus cells (CC) differed between GnRH-induced small follicles (<11.7mm), GnRH-induced large follicles (>12.5 mm), or endogenous gonadotropin surge, spontaneous follicles (11.6-13.9 mm). Ovulation was synchronized in postpartum beef cows (n=250), and dominant follicles were trans-vaginally aspirated ~20 hours after GnRH-induced ovulation (small; large) or the onset of estrus (spontaneous). Oocytes and CC were individually snap-frozen from each cumulus-oocyte complex (COC). For library preparation, RNA was extracted from pools of 4 oocytes or CC from 4 COCs (n= 6 pools for small and large follicles; n=5 pools for spontaneous follicles). An average of 21 million uniquely aligned, single end reads per sample were generated and differential expression analysis between sample groups was performed by fitting the expression data to a general linear model using edgeR robust (significance determined at FDR<0.10). Comparisons of the oocyte transcriptome revealed relatively few differentially expressed genes (DEG; 11, 15, and 9), whereas 884, 1609, 1491 DEG were revealed between CC of small vs. large, small vs. spontaneous, and large vs. spontaneous follicles, respectively. Most noteworthy, when CC from small and large follicles were compared, 430 transcripts were more abundant in CC from small follicles and 454 were more abundant in CC from large follicles. Interrogation of these transcripts within KEGG pathways revealed significant enrichment of 'Glycolysis/Gluconeogenesis' (FDR<$2.2x10^{-5}$) in CC from large compared to small follicles. Oocytes have a poor capacity for metabolizing glucose and rely on CC to supply pyruvate for energy production necessary for maturation. Therefore, we conclude that an inefficient or immature glycolytic pathway in cumulus cells from small follicles, alongside additional DEGs and associated pathways, contributes to the decreased competency of oocytes from small follicles in comparison to large or spontaneous follicles. AFRI Grant no. 2013-67015-21076 from the USDA National Institute of Food and Agriculture.

063 Environment and Reproduction

Effect of different feeding strategies on reproductive parameters in dairy cows

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The objective of this study was to investigate the effect of different feeding strategies on the reinitiation of ovarian cyclicity, pregnancy rates, and metabolic profiles in spring calving Holstein dairy cows. Feeding strategies involved total mixed rations (TMR) or a combination of TMR and pastures (PMR). Multiparous cows (n=90) were assigned to the following treatments: TMR+TMR (100% TMR *ad-libitum* in spring and summer), PMR25+TMR (grazing + 25% TMR in spring and 100% TMR in summer), PMR25+PMR35 (grazing + 25% TMR in spring + 35% TMR in summer). In treatments that included pasture, cows grazed the same pasture but in separate groups. Individual milk production was determinated weekly and milk for progesterone was collected twice a week to estimate first postpartum ovulation. The voluntary waiting period was 45 days after calving. Blood samples were obtained every 15 days during the experimental period to determine non-esterified fatty acids (NEFA) and betahydroxybutyrate (BHB). Body condition score (BCS) was determined fortnightly. Response variables were analyzed using the GLIMMIX procedure of SAS (SAS 9.2, 2008). Milk production was affected ($P<0.0001$) by the feeding strategy and also by the interaction ($P=0.02$) between treatment and season. In spring, milk production was 33 ± 0.9^{a}, 26 ± 0.9^{b}, and 26 ± 0.9^{b} for TMR+TMR, PMR25+TMR and PMR25+PM35 cows, respectively. In summer, milk production was 31 ± 0.7^{a}, 28 ± 0.7^{b} and 25 ± 0.7^{c} for TMR+TMR, PMR25+TMR and PMR25+PM35 cows respectively. The BCS was greater in TMR+TMR cows (2.9 ± 0.05^{a}) compared with PM25+TMR cows (2.7 ± 0.05^{b}), presenting no difference with PM25+PM35 cows. The concentration of NEFA was not affected by treatment the feeding strategy ($P>0.05$), but the concentration of BHB tended ($P=0.06$) to be affected by the treatments: PMR25+PMR35 had greater ($P<0.05$) BHB concentration than the TMR+TMR group (0.70 ± 0.05 vs. 0.54 ± 0.05 mmol/L), presenting no difference with PMR25+TMR, probably related to the increased pasture intake in PMR25+PM35 cows. For NEFA and BHB concentrations, there was an effect of the day postpartum ($P<0.05$) but there was no interaction between treatment and day postpartum. No effect ($P>0.05$) of feeding strategy on the probability of reinitiation of ovarian cyclicity was observed. The calving to first ovulation intervals were 46.7 ± 17, 46 ± 22 and 49 ± 28 days for TMR+TMR, PMR25+TMR and PMR25+PM35. Calving to first service intervals were 62.6 ± 17, 55.8 ± 20 and 57 ± 21 days for TMR+TMR, PMR25+TMR and PMR25+PMRD35. There was no effect of the treatments on the probability of pregnancy at 120 days postpartum (0.46 ± 0.1, 0.35 ± 0.1 and 0.46 ± 0.1 for TMR+TMR, PMR25+TMR and PMR25+PMRD35, respectively). Data show that even if the different feeding strategies affected milk production, they did not affect the reproductive parameters measured. The greater milk production and similar reproductive performance of TMR+TMR cows may be the result of increased density of dietary nutrients and increased intake. Agencia Nacional de Investigación e Innovación (ANII) of Uruguay for financial support. Project N° FSA_1_2013_1_12442.

064 Environment and Reproduction

Effect of maternal nutrition on embryo survival, uterine environment and embryo transfer in sheep

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Maternal nutrition is an important factor that influences the processes of implantation and embryonic development. The objective of this study was to evaluate the effect of nutritional status of donor and recipient ewes on embryo survival and uterine gene expression. The study was carried out in the Laboratory of Small Ruminants Reproduction of INTA Bariloche during the breeding season. Merino donor (n = 36) and recipient (n = 75) ewes were randomly assigned to two treatment diets, receiving 1.5 (Supplemented, [S]) or 0.5 (Restricted, [R]) times daily maintenance requirements during 21 days before recovery and transfer of embryos. Estrous synchronization was performed with intravaginal sponges containing progesterone for 14 days and an i.m administration of equine chorionic gonadotropin (eCG) at time of sponge removal. Donor ewes received a superovulatory treatment, which consisted of the administration of 100 mg of follicle stimulating hormone (FSH) in 6 decreasing doses every 12 hours. Intrauterine artificial insemination with frozen semen (100 million spermatozoa) was performed 12 hours after detection of estrus. On day 7 post-estrus, embryos were recovered and evaluated from S and R donors and were transferred by semi-laparoscopy procedure to S and R recipients, defining the following groups: SS, SR, RS and RR. Embryo survival rate was determined by ultrasonography on day 22 post embryo transfer. At embryo recovery, endometrial tissue was collected for biopsy from donors (n = 26) and recipients (n = 10) and stored in liquid N_2 until analysis. The embryo survival rate was analyzed using the CATMOD procedure of SAS. Two-way ANOVA, followed by post- hoc Tuckey tests, was performed to compare relative mRNA expressions. Data are presented as least square means ± pooled standard errors. Means were considered different when $P \leq 0.05$, and tendency to differ when $P < 0.10$. The uterine gene expression of the receptors for progesterone (PR), insulin-like growth factor 1 (IGF-1R) and leptin (LEPR) was determined by real-time PCR. At the time of embryo transfer, R donors (0.14 ± 0.06) and R recipients (0.08 ± 0.03) had less ($P < 0.05$) uterine relative mRNA levels for PR compared with S donors (0.39 ± 0.15) and S recipients (0.86 ± 0.0). Likewise, IGF-1R and LEPR relative mRNA levels on day 7 post-estrus were greater ($P < 0,05$) in S (IGF-1R: 0.15 ± 0.03; LEPR: 0.17 ± 0.04) than in R (IGF-1R: 0.05 ± 0.01; LEPR: 0.04 ± 0.01) females. The embryo survival rate after transfer tended ($P < 0.10$) to be less in R recipients that received embryos from S donors (SR, 27%) compared with the other treatments (64, 64 and 57% for SS, RS and RR, respectively). In conclusion, nutritional status of donors and recipients during 21 days before embryo transfer affected uterine gene expression, which might have modified the uterine environment of both donor and recipient females leading to changes in embryo survival. Funded by Projects PNSA 1115053 and PRET 1281102 (INTA), PICT 2012-2238 (FONCyT).

065 Environment and Reproduction

Impact of postpartum locomotion score on reproductive performance in grazing dairy cows

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The association between lameness in the first 50 days in milk (DIM) and calving-to-first service interval was evaluated in grazing Holstein cows to test the hypothesis that lame cows in early lactation have a longer calving to first service interval. A prospective observational study was carried out on a commercial dairy farm located in Carlos Casares (35°37' S, 61°22' W), Argentina. A total of 96 cows were included in the study. Locomotion score (LS) was evaluated four times 15-d apart in early lactation by using a 5-point scale: 1 = normal, 2 = presence of a slightly asymmetric gait, 3 = the cow clearly favored 1 or more limbs (moderately lame), 4 = severely lame, to 5 = extremely lame (nonweight-bearing lame). A cow was considered lame if she had a LS over 3 in the last scoring before the end of voluntary waiting period (50 DIM). After the voluntary waiting period, cows were detected twice daily for estrus and artificially inseminated (AI) using the AM-PM rule. Cows were considered in estrus when over 50% of tail paint was removed. Pregnancy was checked by transrectal ultrasonography approximately 30 d after AI. Calving to first service interval was registered for all cows until 200 DIM. The association between lameness and interval from calving to first service was assesed using survival analysis by fitting Kaplan-Meier survival curves to the data and using the Cox's proportional hazard regression model (PROC LIFETEST and PROC PHREG, SAS ver. 9.4). Lame cows had a median (95% confidence interval; CI) calving to first service interval 7 days longer ($P \leq 0.05$) than non-lame cows (75 [73-82] vs. 68 [59-74], respectively. In addition, non-lame cows had greater ($P \leq 0.05$) hazard of first service than lame cows (hazard ratio = 1.65, 95% CI =1.06-2.58). Therefore, lameness in early lactation grazing dairy cows extends the interval postpartum to first service likely because it delays ovarian cyclicity.

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Seasonal changes in melatonin concentrations in female guanaco *(Lama guanicoe)*

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The guanaco is a wild South American camelid characterized by a strategy of seasonal reproduction of long days. In many ungulates, photoperiod is known as a major factor for the onset of the breeding season, but in Camelids this effect is not well described. The translation of the environmental light signal to hormonal changes is mediated through the secretion of melatonin from the pineal gland. Our previous study showed that these animals have a circadian pattern of melatonin secretion with maximum concentrations during the night and minimums during the day. The aim of this work was to evaluate the seasonal changes in plasma melatonin concentrations in female guanaco comparing short-day versus long-day photoperiods. The study was performed in a Mediterranean climate (33°'28´S) under a short-day (10 h daylight/14 h darkness) and long-day (14 h daylight/10 h darkness) photoperiod. The experimental group was formed by 9 adult, captive-bred, non-pregnant non-lactating and healthy guanacos. Blood samples were collected once a week during the morning (10 am), for 3 weeks. Melatonin was measured by competitive ELISA assay. Concentrations of melatonin during the short- and long-day photoperiods differed ($P < 0.001$) and averaged 23±10 and 9±3 pg/mL, respectively. Furthermore, a negative correlation between duration of light hours and melatonin concentration was observed ($r = -0.714$; $P < 0.001$). This pattern resembled that observed in other ungulates in different latitudes. Therefore, it is possible that changes in melatonin concentrations modulated by exposure to natural illumination might play a role in the reproductive seasonality in adult female guanacos. We suggest that secretion of melatonin is an important endocrine signal influenced by daylight exposure between short- and long-days in guanacos. This work was funded by CONICYT, BECA DOCTORADO NACIONAL 2016.

067 Environment and Reproduction

Effect of controlled heat stress on follicular dynamics and steroid production of dairy cows

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Heat stress (HS) has an enormous economic impact on the dairy industry. Reproductive losses in dairy cows associated with HS include reduction in duration and intensity of expression of estrus and increased incidence of anestrus and silent ovulations. This study aimed to investigate the effects of continuous and severe HS on follicular dynamics and steroidogenesis in Holstein cows. Healthy non-lactating Holstein cows were synchronized with the Ovsynch protocol concurrent with an intravaginal progesterone device. On the day of ovulation, cows were contemporaneously and randomly assigned to thermoneutral (TN; n=12) or HS (n=12) treatments. The temperature and relative humidity in the climate chamber were, respectively, 25.9°C and 73.0% for TN, and 36.3°C and 60.9% for HS. Transrectal ultrasonography of ovarian structures was performed twice daily over two follicular waves. On day 9 of the first follicular wave, the largest follicle (F1~12.0 mm) was individually aspirated and all remaining visible follicles were ablated. Subsequently, at expected deviation time (F1~8.5 mm), both the F1 and the second largest follicle (F2) were individually aspirated. Follicular estradiol and progesterone concentrations were determined by ELISA. Blood samples were collected daily to determine concentrations of FSH, inhibin, and estradiol by radioimmunoassay. The diameters of follicles and hormone concentrations were analyzed using ANOVA with repeated measures followed by Tukey post-hoc test with significance declared at $P \leq 0.05$. Exposure to HS had deleterious effect on follicle dynamics. During the first follicular wave, the diameter of the F1 was smaller ($P<0.001$), whereas the F2 was larger ($P>0.01$) for HS than TN cows. An enhanced growth of follicles in this wave in HS cows was associated with increased ($P<0.05$) circulating FSH concentration and coincided with decreased ($P<0.02$) concentration of inhibin in serum. In the second follicular wave, the follicular diameters for F1 and F2 were smaller ($P<0.01$) in HS than TN cows. Although greater ($P<0.01$) serum concentrations of FSH were observed in HS than TN cows, no difference was detected in serum concentrations of inhibin between treatments. In both follicular waves, treatment did not affect serum concentrations of estradiol. The intrafollicular concentrations of estradiol and progesterone differed ($P<0.001$) across the stages of follicle development, but no effect of treatment was detected or interaction between treatment and stages of follicle development. These results indicate that HS may impair follicular development by altered concentrations of FSH and inhibin, contributing with the low fertility observed in dairy cattle during the summer. This study was supported by FAPESP (Grant #2012/18297-7, Grant #2013/20083-8, and Grant #2014/21257-2).

068 Environment and Reproduction

Effects of temperament on reproductive performance of *Bos taurus* heifers enrolled in a 7 d CO-synch + CIDR protocol

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It has been reported previously that cattle temperament significantly impacts production traits such as reproduction. The objective of the present study was to assess the association betwee temperament and pregnancy to fixed-timed artificial insemination (FTAI) in *Bos taurus* beef heifers. A total of 297 Angus influenced heifers from 3 locations were evaluated for temperament based on chute score and exit velocity on the first day of the estrous synchronization protocol (d-9) and at FTAI (d0). Individual exit velocity score was calculated by separating exit velocity results into quintiles and assigning heifers with scores from 1 to 5 (1 = slowest; 5 = fastest). Temperament scores were determined by the average of individual chute scores and exit velocity scores. Heifers were then classified by temperament type according to the temperament score (score less than or equal to 3: calm; score > 3: excitable). Pregnancy status was determined by transrectal ultrasonography approximately 40 days after FTAI. Hair from the tail switch was collected on d-9 and on the day of FTAI for cortisol evaluation. Overall, 71% of heifers were classified as calm whereas 29% were classified as excitable. Pregnancy to FTAI was less ($P = 0.042$) in excitable compared with calm heifers (36% vs. 55%, respectively). Mean concentration of cortisol in the hair was reduced ($P < 0.001$) from d-9 (3.5 ± 0.3 pg/mg of hair) to d0 (1.74 ± 0.3 pg/mg of hair) in all heifers, independently of temperament. In addition, no differences for mean hair cortisol concentrations were found between calm (2.91 ± 0.2 pg/mg of hair) and excitable heifers (2.67 ± 0.2 pg/mg of hair). We conclude that heifer temperament is associated with fertility and excitable heifers have reduced pregnancy to FTAI. Also, the reduction in concentrations of cortisol in hair from d-9 to d0 suggests that cattle handling during the estrous synchronization protocol might have acclimated cattle to handling and minimized potential stress.

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Dose-dependent effect of astaxanthin on bovine spermatozoa exposed to heat shock

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Heat stress affects spermatogenesis and compromises sperm quality; however, the direct effect of elevated temperature on spermatozoa is not fully understood. Insemination of cows under heat stress exposes the mature spermatozoa to elevated temperature in the female reproductive tract. This can compromise sperm motility and fertilizing ability. Previous studies in our laboratory demonstrated that heat shock increased reactive oxygen species (ROS) production in bovine spermatozoa. Therefore, the objective of this study was to determine the dose-response effect of the carotenoid antioxidant astaxanthin on motility of bovine spermatozoa subjected to moderate heat shock. For each replicate, semen straws from 3 different bulls were thawed and subjected to Percoll gradient. Sperm concentration and motility was evaluated immediately after Percoll purification (0 h control group). Sperm cells (1.5 x 10^6 sperm/mL) were incubated in SP-TALP and SP-TALP-Ethanol (astaxanthin vehicle control: 0.15% ethanol) at 38.5 and 40°C for 4 h, or incubated at different astaxanthin concentrations (150, 200, 250, 300 and 350 nM) in SP-TALP-Ethanol at 40°C for 4 h. Data were analyzed by least-squares analysis of variance using the General Linear Models procedure of SAS. This experiment was replicated five times. Sperm motility at 0 h control (69.0 + 2.3%) was greater ($P< 0.0001$) than all other treatments. Sperm incubation at 38.5 and 40°C reduced motility ($P< 0.0001$) compared with 0 h control. However, such drop in motility was greater for sperm exposed to 40°C than 38.5°C. Heat shock at 40°C for 4 h reduced ($P< 0.0001$) sperm motility in both SP-TALP (20.0 + 2.3%) and SP-TALP-Ethanol (19.0 + 2.3%) compared with 38.5°C (39.0% for SP-TALP and 41.0% for SP-TALP-Ethanol; SEM=2.3). Supplementation of heat-shocked spermatozoa with astaxanthin concentrations of 200 (36.0 + 2.3%) and 250 nM (33.0 + 2.3%) recovered motility to values similar to 38.5°C control. Therefore, astaxanthin recovered sperm motility and attenuated the negative effect of heat shock possibly by counteracting heat-induced ROS in bovine spermatozoa. This research was supported by Coordination for the Improvement of Higher Level Education – Personnel (CAPES # 168277/2017-4).

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Effect of seminal plasma on the interval to ovulation, dominant follicle and corpus luteum size in Alpacas (*Vicugna Pacos*) and Llamas (*Lama glama)*

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Alpacas and llamas as other camelids are induced ovulators and require an external stimulus, mounting by the male, for ovulation. Nerve growth factor beta (NGF-ß), a protein present in the seminal plasma (SP) is reported as being responsible for stimulating ovulation; however, limited information exists on the dynamics of the follicular wave in alpacas and llamas post induction of ovulation with SP. To determine the effect of two external stimulations on the interval to ovulation and dominant follicle and corpus luteum size, adult female alpacas and llamas, 5-6 years old were assigned to one of two treatments when a dominant follicle ≥ 7 mm was present: SP (n = 6): 1 mL of SP i.m; GnRH (n = 6): 0.05 mg acetate of busereline. Animals were examined by ultrasonography using a 5.0 MHz transducer at 1- to 2-h intervals between 22 to 40 h after treatment or until ovulation occurred. All animals were evaluated by ultrasonography every day from day 2 to day 7 post-treatment, and again on days 9, 12 and 15 post-treatment. Data were analyzed using ANOVA. Interval from treatment to ovulation did not differ between SP and GnRH in alpacas (26.7 ± 0.8 vs 28.0 ± 1.7 h) or llamas (30.5 ± 1.4 vs 31.0 ± 1.5 h), but a significant difference ($P < 0.05$) was observed between the two species. Interval to determination of follicle ≥ 7 mm did not differ between SP or GnRH treatment in alpacas (12.3 ± 2.5 vs 13.3 ± 1.4 h) or llamas (8.6 ± 2.5 vs 7.9 ± 2.0 h), but it differed ($P < 0.05$) between species. Corpus luteum diameter was smaller ($P < 0.05$) in SP than GnRH treatment in alpacas (8.2 ± 0.8 vs 10.3 ± 0.5 mm), but not in llamas (9.8 ± 1.0 vs 9.2 ± 0.8 mm). In conclusion, treatment did not influence interval to ovulation, but affected CL diameter only in alpacas. Interval from treatment to ovulation was longer in alpacas than llamas. Research funded by Project "Role of seminal plasma in reproductive physiology and aplication of biotechnologies in camelids" -149-2017- CIENCIACTIVA – CONCYTEC.

071 Environment and Reproduction

Ewe fertility in group mating: Does the number of rams the ewe mates with affect fertility?

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In commercial sheep meat production, ewes are often mated in large groups with multiple rams present in each group. We hypothesised that ewe fertility would vary according to the number of rams the ewe mated with in the first breeding cycle. To test this hypothesis, yearling ewes ≈ 8 months of age or adult ewes 1.5 years of age were joined with rams in a group mating situation during two breeding seasons for each age. For the yearling ewes, 4 groups of ewes (n=159-177/group) were joined with 3 rams/group for a total of 35 days. For the adult ewes, 3 groups of ewes (n=287-300/group) were joined with 3 rams/group for a total of 33-38 days. In each group, each ram wore a marking harness with a different colour crayon. For the yearling ewes, the number of rams (#R) the ewe mated to in her first mating was determined using the crayon marks. For the adult ewes, #R the ewe mated to in the first 17 days of the mating season were recorded. The effects of #R a ewe mated with on ovulation rate (OR; measured by laparoscope) and the number of lambs born (NLB) were analysed with restricted estimated maximum likelihood. The model included flock and weight at 4 weeks befor (adults only) and at the start of the mating season. In the yearlings, of the 577 ewes with mating marks, 25.4 ± 4.1%, 48.3 ± 4.0 % and 26.2 ± 5.0% mated to 1, 2 or 3 rams, respectively. The #R the ewe mated to was not associated ($P > 0.05$) with differences in OR (1.89, 1.85, and 1.76 for #R 1, 2 or 3, respectively; SED = 0.08) or NLB (1.58, 1.39, and 1.44 for #R 1, 2 or 3, respectively; SED = 0.11). For the adult ewes, 19.0 ± 2.2%, 19.2 ± 3.5%, 34.4 ± 1.3%, and 27.4 ± 3.3% were marked by 0, 1, 2 or 3 rams, respectively. In general, as #R the ewe mated with increased, NLB increased (1.24, 1.54, 1.79, and 1.80 for 0, 1, 2 or 3 #R, respectively; $P < 0.01$, SED = 0.09), but OR did not change (2.14, 2.10, 2.19, and 2.18 for 0, 1, 2 or 3 #R, respectively; SED = 0.07). Limiting the data for the outcomes from the first breeding, overall fertilization/embryo survival increased ($P < 0.05$, SED = 5%) as #R mated to increased (60%, 77%, and 80% of oocytes ovulated represented by a lamb at birth for 1, 2 and 3 #R, respectively). In summary, although no associations were found between #R a yearling ewe mated to and her fertility, in adult ewes, as #R increased, ewe fertility increased. For ewes that failed to mate to any rams in the first breeding cycle, this was likely driven by having one less chance to become pregnant and thus an increased number of ewes that were not pregnant at the end of the breeding season as well as reduced embryo survival. However, decreased fertility was still observed in ewes mating to a single ram versus multiple rams, and decreased embryo survival likely underlies this observation. Funding provided by AgResearch's Strategic Science Investment Fund from NZ Ministry of Business, Innovation & Employment.

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Ovarian characteristics and somatic development in 2 to 5 months old female Nelore (*Bos taurus indicus*) calves

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The herd's genetic gain could be faster and the generation interval shorter by including prepubertal bovine females in assisted reproductive programs. However, *in vitro* embryo production from calves is still challanging, and factors affecting recovered oocyte quality and developmental potential are not fully understood. In this regard, the relationship between somatic development and age at puberty is well known, but the relationship with reproductive development during the prepuberal period is unclear. Thus, the aim of the present study was to evaluate ovarian characteristics in 2 to 5-month old Nelore calves and their possible association with somatic development endpoints. Eight females were evaluated by transrectal ultrasonography (MyLab 30 VetGold, Esaote, 5 to 7.5 MHz probe, Genoa, Italy) once a week, during 12 weeks, to measure ovarian diameter, follicle population, diameter of the largest follicle present, and diameter of uterine horns. Calves were also evaluated for the following biometrics endpoints: height at the withers and hip, body length, depth, thoracic perimeter, rump width at ischium and ilium, rump length, head length, head width, and body weight. The possible associations were analyzed by Spearman correlation test, and a value of $P < 0.05$ was considered significant. There was a moderate correlation between ovarian diameter and follicle population ($r = 0.45$; $P < 0.0001$) and uterine horn diameter ($r = 0.31$; $P = 0.002$). There was a strong correlation among all biometric parameters ($0.85 < R < 0.98$; $P < 0.05$). Ovarian diameter was positively correlated with all biometric parameters, with R values ranging from 0.31 to 0.48 ($P < 0.05$). The diameter of the largest follicle present also was associated with thoracic perimeter ($P = 0.039$; $R = 0.32$). However, there was no significant correlation between follicle population and any biometric endpoint. The present results show an association between somatic development and reproductive tract development during the initial prepuberal period. Thus, we can hypothesize that biometric parameters can also be associated to oocyte development potential in calves, as well as to the potential as oocyte donors in adulthood. Financial support: EMBRAPA, CAPES, FAPEMIG e FAP-DF.

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Milk yield, periparturient diseases and body condition score as factors affecting the risk of fetal losses in high-yielding Holstein cows

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Non-infectious causes of abortion play an important role in premature pregnancy termination. The infectious causes of abortion have been a primary focus of attention, because they may be controlled to some extent by vaccination. The cause of non-infectious abortions is difficult to diagnose, but in many herds non-infecitous abortions are more prevalent than infectious ones. The objective of this study was to assess various risk factors affecting fetal losses in high-yielding Holstein cows in a hot environment. In a retrospective observational study, 14,384 records from Holstein cows from a large dairy herd in northern Mexico (25°N; 23.5°C mean annual temperature) and vaccinated against some agents of reproductive diseases were used. Fetal loss between 43 and 260 days affected 23.8% of the pregnancies. Multivariable logistic models indicated that dry period >60 d was associated ($P \leq 0.05$) with less incidence (odds ratio, OR = 0.8; 95% confidence interval, CI = 0.8-0.9) of fetal loss compared with cows with dry periods <60 d. Cows with BCS >3.0 at calving and 15 days postpartum were at half the odds of fetal loss compared w ith similarly aged cows with BCS <3.0. Cows with peak milk yield > 38 kg had 5.5 times greater ($P < 0.01$) odds of having fetal loss than cows with peak milk yield < 38 kg (36.9 vs. 9.6%). Fetal loss increased when cows had 305-d milk yield >9000 kg (OR = 2.1) compared with cows with milk yield < 9000 kg. Retained placenta was associated ($P \leq 0.05$) with increased fetal loss (OR = 1.2; 95% CI = 1.1-1.4). Cows suffering premature parturition were greater ($P \leq 0.05$) risk of suffering fetal loss than cows with normal parturition (OR = 1.2; 95% CI = 1.0-1.4). A bimodal distribution of detection of fetal loss was observed with peaks around 50 and 220 days of pregnancy, likely because when pregnancy diagnoses were performed with more frequency. It was concluded that, in this particular farm with cows under hot environment, increased milk yield, long dry period, reduced BCS at calving, and incidence of retained placenta were all associated with increased risk of fetal loss.

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The tail in estrous tropical hair ewes (*Ovis aries*) is used as a proceptive signal and favors copulation

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Docking tails is a painful procedure, that also can affect possible tail functions related to estrus and copulation. Two experiments were performed to determine if the tail has an active role in: 1) estrous ewes' proceptive behavior, and 2) facilitating copulation in hair sheep. In the first study, the movements and position of the tail of estrous (ES) and non-estrous (NE) ewes in response to male presence and courtship were compared in 20 mature ewes. In the experiment 2, the rams' courtship and mating behavior toward intact (INT) or docked (DOCK) ewes, being ES or NE, were compared using 5 ewes/treatment arranged as a 2 x 2 factorial, with main factors as ES vs. NE, and INT vs. DOCK ewes. In both experiments, 25 rams were individually tested with restrained ewes during 3 min. In Experiment 1, tail movements occurred in short episodes in response to the physical contact of the male, but the rest of the time the tail of NE ewes was drawn inward to the body; in ES ewes the tail simply hung naturally straight downward. Ewes in ES performed more tail moving episodes with more movements per episode, movements that had greater amplitude and higher elevation angle from the body than in NE ewes (16.0 ± 1.3 vs. 4.1 ± 0.6; 6.2 ± 0.3 vs. 2.1 ± 0.3; 90.5 ± 4.0° vs. 7.5 ± 0.9°; 49.0 ± 2.3° vs 17.3 ± 0.9°; $P < 0.001$ for all the comparisons; ES and NE ewes, respectively). In Experiment 2, the reaction time (RT) of DOCK ewes was delayed but only when they were in ES (19.9 ± 5.5 vs. 47.5 ± 11.2 seconds; $P < 0.05$, for INT and DOCK ewes, respectively), with no difference in NE ewes (137.1 ± 17.0 vs. 138.8 ± 12.8 seconds; $P > 0.05$, for INT and DOCK ewes, respectively). There were less failed intromissions to INT than to DOCK ewes when they were ES (4.2 ± 1.2 vs 9.5 ± 1.8; $P < 0.05$), but not if they were NE (18.9 ± 5.3 vs 17.7 ± 4.9; $P > 0.05$). There were also more failed intromissions before ejaculation to ES (7.6 ± 0.9 vs 3.4 ± 0.5; $P < 0.05$), but not to NE ewes (7.1 ± 0.8 vs 5.6 ± 0.5; $P > 0.05$). In addition, there were no differences according to the physiological status in those with docked tail (7.6 ± 0.9 vs 7.1 ± 0.8; $P > 0.05$, for ES and NE ewes, respectively). There were more anal intromissions to DOCK ewes (52% vs 0% in INT ewes) when they were in estrus, with intermediate values for NE ewes regardless of the presence of the tail (9 and 33%, for INT and DOCK ewes, respectively). In addition, there was an interaction between being or not in ES and having or not the tail ($P < 0.05$) in the RT, and the number of failed and anal intromissions. Movements and position of the tail in ES ewes is a main component of the proceptive behavior, and facilitates mating and avoids failed and anal intromissions in hair sheep. Authors acknowledge Reyes Vázquez for his help with animal management.

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Pasture allowance in pregnant ewe: Effect on the development of the reproductive tract of their male offspring

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The aim of the work was to compare the effect of natural pasture allowances (PA) offered to pregnant ewes on scrotal circumference (SC), anogenital distance (AG), penis size (PS), testes weight (TW), diameter of seminiferous tubules (DTS) and percentual volume of seminiferous tubules (VOL) of their male offspring. Single-bearing multiparous Corriedale ewes were randomly assigned to two PA from 30 to 143 d of gestation: i) High (HPA, n = 6): 14, 15 and 20 kg DM/100 kg body weight (BW)/d during 30-60, 61-110 and 111-143 d of gestation, respectively; ii) Low PA (LPA, n = 7): 6, 5 and 10 kg DM/100 kg BW/d on the same intervals as of HPA, respectively. Each treatment had three replicates in one of three independent paddocks (randomized block design with three replicates). From 100 d of gestation until weaning, ewes were supplemented daily with 300 g/animal of rice bran (88% DM, 14% crude protein, 9% acid detergent fiber, and 24% neutral detergent fiber) and from lambing to weaning all ewes were kept in the same paddocks with grass *ad libitum*. After weaning until slaughter at 200 d of age, lambs were located in individual pens where they were offered 6% of their individual BW adjusted every 15 d of a mixed diet: alfalfa hay and a complete commercial mixed ration (16% protein; 2% ether extract, 13% moisture, 19% crude fiber, 9% minerals). All lambs were slaughtered following standard procedures (electrical stunning, bleeding, skinning and evisceration) in a motor slaughterhouse. Mother's BW were registered fortnightly during gestation. The lamb's BW were registered at birth, 45 and 90 d of age, and fortnightly during fattering. The SC, AG, PS were measured at 180, 190 and 200 d of age. After slaughter, tests were weighed and and tissue fixed in Bouin's solution. The histological sections stained in hematoxylin-eosin were analyzed (light microscope, video camera, pc with software Infinity®). Body weight, SC, AG and PS were analyzed as repeated measures using the MIXED procedure of SAS with day as repeated effect. The model included treatment, day, and interaction between treatment and day as fixed effects, and the block as random effect. Other variables were analyzed using mixed models with treatment as fixed effect and block as random effect. Statistical differences were considered when $P \leq 0.05$, and tendencies when $0.05 < P \leq 0.10$. Data were expressed as least square means ± s.e.m. Ewes of HPA had greater (P = 0.0002) BW (44.7 ± 0.7 kg) than LPA ewes (42.7 ± 0.7 kg). An interaction (P = 0.028) between treatment and day was detected because treatment did not affect lamb's BW at birth and 45 d, but HPA lambs were heavier ($P < 0.05$) than LPA lambs at 90 d of age. Treatment tended (P = 0.07) to influence SC (HPA: 31.7 ± 2.0 vs. LPA: 26.6 ± 1.9 cm), influenced (P = 0.04) AG (HPA: 40.9 ± 1.2 vs. LPA: 37. ± 1.2 cm), but did not affect PS. Treatment influenced ($P \leq 0.05$) DTS (HPA: 369.7 ± 19.0 vs. LPA: 313.4 ±17.6 µm) and VOL (HPA: 51.6 ±1.7 vs. BOF 46.2±1.7 %), but did not affect TW (0.6). Pasture allowance of ewes during gestation affects the development of the male offspring at 200 d of age.

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The permanent presence of ovariectomized, steroid-treated goats does not prevent the seasonal decrease of LH and testosterone in male goats

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Male goats exposed to females in estrus during the non-breeding season exhibit increased plasma luteinizing hormone (LH) and testosterone concentrations. Objectives were to determine whether the permanent presence of ovariectomized (OVX), steroid-treated goats, prevents the seasonal decrease in plasma concentrations of LH and testosterone observed during the non-breeding season. One group of bucks (n = 5) was kept in contact with 3 OVX goats (OVX-only), whereas the other group (n = 5) was kept in contact with 3 OVX treated with 1 mg of estradiol preceded by 2 i.m. injections of progesterone 72 h apart (10 and 5 mg, respectively) labeled as OVX-steroid. In both groups, females were exchanged once a week throughout the study. Plasma LH concentrations were determined every 20 min during 6 h (08:00-14:00) in October, February, March and June, 72 h after exchanges of females in each group of males. Plasma testosterone concentrations were determined once a week, 48 h after exchanges of females in each group of males from October to July. Plasma LH and testosterone concentrations were analyzed by a two-way ANOVA with repeated measurements to assess the effects of treatment and time. Plasma LH and testosterone concentrations varied over the study (time effect: P = 0.0001), but these variations differed between bucks in contact with OVX-only or OVX-steroid (interaction between treatment and time: P = 0.0001). However, plasma concentrations of LH from both treatments were less than 1 ng/mL throughout the study. Plasma testosterone concentrations in bucks in contact with OVX-steroid and OVX-only decreased from October (12 ± 3 and 12 ± 4 ng/mL, respectively), and reached basal concentrations in January (4 ± 1 and 3 ± 1 ng/mL), which continued until June. Thereafter, testosterone concentrations increased in July in bucks in contact with OVX-steroid and OVX-only (11 ± 2 and 10 ± 3 ng/mL, respectively). We concluded that the permanent presence of OVX, steroid-treated goats does not prevent the seasonal decrease in LH and testosterone in bucks. This study was supported by CONACYT-México, Ciencia Básica (254176).

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Effect of somatic cell count and its temporal association with service date on conception rate in grazing dairy cows

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Reproductive efficiency is one of the most important factors associated with dairy farm profitability and is negatively affected by diseases such as clinical and subclinical mastitis. High somatic cells count (SCC) are used as indicators of subclinical mastitis and has been reported as risk factor for reproduction success (Fuenzalida et al., 2015). Although several studies have been done in dairy cows under confinement systems, to our knowledge only few have been done in grazing dairy cows. The objective of this study was to assess SCC and its temporal association with service date on pregnancy per insemination in grazing dairy cows. A retrospective longitudinal study was conducted using a dataset including records from lactations started between January 1, 2000 and December 31, 2014 (1,930,376 lactations) from 867 dairy herds located in the Province of Buenos Aires, Argentina. Four categories of SSC were define from SCC records measured 43 days before and 30 days after each service (only the first four services were used). The categories (SCC_C) were: 1) Healthy when both SCC records had <150K cells/mL; 2) New Case: when the first SCC record had <150K cells/mL and the second had >150K cells/mL; 3) Cured when the first SCC record had >150K cells/mL and the second had <150K cells/mL; and 4) Chronic when both SCC records had >150K cell/ml (Fuenzalida et al., 2015). Data were analyzed with logistic model using mixed models (PROC GLIMMIX, SAS ver. 9.4) and the probability of pregnancy was modeled. The model included the fixed effects of SSC_C, number of services (1 to 4), and their interaction, days in milk at every service (DIM), parity (1, 2, 3 and ≥4), calving season [Summer (Dec 21 to Mar 20), Autumn (Mar 21 to Jun 20), Winter (Jun 21 to Sep 20) and Spring (Sep 21 to Dec 20)], and calving year (2000 to 2014). Model also included the random effect of lactation to take into account the correlation between services within lactation. Pregnancy was associated with SSC_C, number of service, and their interaction. Healthy cows were reference for comparison. The odds ratios (OR) for pregnancy at first service in cows classified as Cured, New Case or Chronic were 0.92 (95% CI: 0.90-0.94), 0.87 (95% CI: 0.85-0.88), and 0.84 (95% CI: 0.83-0.86) respectively. The odds of pregnancy were less for Cured, New Case, and Chronic than for Healthy cows in all the successive services, but the effect size decreased with service number. For example, after the first service, the OR for pregnancy were 0.95 (95% CI: 0.88-1.03), 0.91 (95% CI: 0.84-0.98), and 0.91 (95% CI: 0.86-0.98) for Cured, New Case and Chronic cows, respectively. Somatic cell count and its temporal association with date of service are important risk factors for pregnancy in grazing dairy cows. This work was supported by UNLP Incentive Program V11/230 grant to RLS. Fuenzalida, M.J. et al, 2015. The association between occurrence and severity of subclinical and clinical mastitis on pregnancies per artificial insemination at first service of Holstein cows. J. Dairy Sci. 98:3791-3805.

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Aerobic metabolism is maintained in rams under testicular hyperthermia due to increased testicular blood flow

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Heat stress is an important factor affecting reproduction of male animals. Increasing global temperatures elicit concerns regarding male fertility, since in most mammals, testes must remain ~3-4ºC cooler than body temperature for production of morphologically normal and motile sperm. The classical dogma is that when testes are exposed to increased temperatures, there is no increase in blood flow and therefore the resulting hypoxia affects spermatogenesis. However, in our previous studies regarding impacts of oxygen (O_2) concentration on sperm quality, damage caused after warming the testis was not replicated by hypoxia nor prevented by hyperoxia. The objective of this study was to understand impacts of increased testicular temperature on testicular blood flow, conductance (blood flow/aortic pressure), O_2 delivery and extraction, metabolic rate, and evidence of hypoxia. Nine crossbred yearling rams were maintained under general anesthesia and their testicular temperatures were increased in a step-wise fashion, at 33, 37 and 40ºC (±0.5ºC). Effects of temperature were analyzed by one-way analysis of variance for repeated measures, followed by a Dunnet's *t*-test. Testicular blood flow increased ($P<0.05$) as testicular temperature increased from 33 to 40ºC (13.2 ± 2.7 vs 17.7 ± 3.2 ml/min/100 g of testes; mean ± SEM), with more profound ($P<0.01$) increases in conductance (1.08 ± 0.20 vs 1.60 ± 0.27 ml/min/g/mmHq 10^3). Furthermore, an increase ($P<0.0001$) in metabolic rate (0.35 ± 0.04 vs 0.64 ± 0.06 mL O_2/min/100 g of testes) was followed by an elevation ($P<0.0001$) in O_2 extraction (31.2 ± 5.0 vs 33 47.3 ± 3.1%). Lastly, there were no significant differences in hypoxia or impaired metabolism markers such as lactate, pH, HCO3- or base excess, indicating no evidence of anaerobic metabolism. This is apparently the first report that heating testes of rams with an intact scrotum increased testicular blood flow. Furthermore, these results were in accordance with our previous findings that hyperthermia and not hypoxia apparently caused impaired spermatogenesis after increased testicular temperature. In conclusion, successive increases in testicular temperature nearly doubled testicular metabolic rate. Remarkably, there were no indications of testicular hypoxia or anaerobic metabolism, due to increases in blood flow (25%), conductance (48%) and, in particular, O_2 extraction (52%). Therefore, these data, in combination with other reports, challenged the paradigm that testicular hyperthermia fails to increase testicular blood flow and the ensuing hypoxia disrupts spermatogenesis.

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Associations between ovarian cyclicity, uterine health, body condition score, metabolic status and parity during the postpartum period in seasonal calving grazing dairy cows

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The objective was to evaluate the associations between ovarian cyclicity (OC), uterine health status (UHS), body condition score (BCS), metabolic status (MS), and parity during early lactation in seasonally-calving, pasture-based dairy cows. First and second lactation spring calving dairy cows (n=2,602) from 35 dairy farms located in Ireland were enrolled in the study. All farms were visited every 2 weeks, and at each visit animals that were at week 3 (wk3; range 14 to 27 DIM) and week 7 (wk7; range 42 to 55 DIM) post-calving were examined. The BCS was measured using a 1 to 5 scale in 0.25 increments. Transrectal ultrasound examinations were conducted to determine OC and UHS. Blood samples were collected at each visit and the concentration of glucose, beta-hydroxybutyrate (BHB) and nonesterified fatty acids (NEFA) were analysed by enzymatic colorimetry. Cows were grouped into three BCS categories: low (less or equal to 2.50), target (between 2.75 and 3.25) and high (> 3.25); 3 OC categories: cycling (CyC, corpus luteum (CL) present), anestrous (AN, CL absent and dominant follicle (DF) present) deep anoestrus (DA; CL and DF absent); 4 UHS categories based on ultrasound findings: healthy (H), low infection (LI), mild infection (MI), severe infection (SI); and 3 MS categories based on glucose, BHB and NEFA: good, moderate and poor. Fisher's Exact Test was used to test associations between these different categorical variables and was supplemented by logistic regression to calculate odds ratios and predicted probabilities. At wk3 and wk7, 45.0% and 92.4% of cows had resumed cyclicity, respectively, and 96.4% and 73.6% of cows had a uterine infection, respectively. On wk3, there was association between likelihood of having resumed OC and UHS, BCS and parity; cows with low BCS (OR=0.73, P=0.05), uterine infection (both MI and SI; OR=0.17 and 0.04, respectively; both P <0.0001) and parity 1 (OR=0.63, P=0.001) had lower likelihood of having resumed OC compared with cows at target BCS, absence of uterine infection (H) and parity 2, respectively. Correspondingly, cows with low BCS (OR=1.16, P<0.01), anestrous (both AN and DA; OR=7.9 and 7.1, respectively, both P=0.0001), and parity=1 (OR=1.19, P=0.05) had greater likelihood of having uterine infection compared with cows with target BCS, CyC and parity=2, respectively. There was an association between BCS and MS (P=0.05); cows with good MS were more likely to be in the target BCS category than cows in the moderate or poor MS categories. Similar associations were observed at wk7. In conclusion, OC, UHS, BCS and parity were associated with each other throughout the postpartum period. Supported by Irish Department of Agriculture, Food and the Marine (Grant 13S528).

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Expression of COX, PPARGC1-A and NRF-1 genes in granulosa cells of goats after short term dietary supplementation with high lipid levels

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In ruminants, the level of lipid inclusion used in diets does not exceed 5% of dry matter (DM) to control nutritional and reproductive consequences. High concentrations of lipids can lead to a decrease in oocyte quality due to the formation of cytotoxic highly reactive lipid peroxides, which are detrimental to organelles. Short dietary supplementation with lipids alters granulosa cells function in goats. Thus, the objective of this work was to verify in goats the effect of high lipid dietary supplementation for a short period on cholesterol (CHOL) and triglycerides (TG) concentration in follicular fluid (FF) and gene expression of mitochondrial function in granulosa cells (GCs). Twenty adult crossbred goats, with homogenous body condition (3.0±0.2; mean±SD) and age (28.2±3.4 months), were fed chopped elephant grass and concentrates (corn 60.3%, wheat bran 32.6%, and soybean meal 3.1%). Seven days before ovarian recovery, goats were assigned to a control group (CG, n=10) which received the diet described previously, or the lipid group (LG, n=10), in which ground flaxseed replaced 30% of the ingredients in the concentrate DM. The dietary fat contents were 2.6% and 6.8% on DM basis for CG and LG, respectively. Estrus was synchronized in all goats by insertion of a vaginal progesterone device on day 0. On day 6, the device was removed, and 0.075 mg of prostaglandin $F_{2\alpha}$ and 150 IU of equine chorionic gonadotropin were injected i.m. At 36 h after prostaglandin treatment, goats received 0.125 mg of gonadotropin-releasing homone. Starting on day 9 until day 11, 200 mg of follicle-stimulating hormone was injected i.m. with doses divided in application with 12 h intervals. Goats were slaughter on day 12 and ovaries were collected and follicular contents aspirated. Granulosa cells were collected and stored until RNA extraction. Cholesterol and TG concentrations of FF were analyzed by spectrophotometry (Mindray® BS-120; Guangdong, China). Enzymatic activity of glutathione peroxidase from FF was measured using commercial kit (Ransel, Randox, Crumlin, UK). Analysis of *COX*, *PPARGC1-A* and *NRF-1* genes expression in CGs was performed using SYBR Select Master Mix (Life technologies, USA). Total RNA was extracted using the kit CellsDirectTM One-Step qRT-PCR and the RPS9 reference gene was selected as endogenous control. Data log-transformed (logx10), were subjected to ANOVA using the GLM. Diets type (CG and LG) was the main effect tested. Pairwise comparisons were performed by student t test. No significant effect of diet was found for concentrations of CHOL and TG in FF, which averaged 28.1±7.8 and 13.7±3.8 mg/dL, respectively. Glutathione peroxidase activity was greater (P=0.005) in LG than CG (432.0±18.7 vs. 355.0±15.9 U/L). All genes were expressed in GCs, but treatment did not affect gene expression. Increasing the dietary lipid conent fed for a short period increased glutathione peroxidase activity in follicular fluid, but it did not influenced expression of mitochondrial genes of granulosa cells.

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Evaluation of the leucocitary response with the use of Pegbovigrastim in Holstein cows in the peripartum

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Pegbovigrastim is a modified form of bovine granulocyte colony stimulating factor (bG-CSF), which is a group of cytokines necessary for the proliferation and differentiation of hematopoietic stem cells, conjugated to a polyethylene glycol (PEG). Treatment of cows with pegbovigrastim induces a transitent leukocytosis with neutrophilia that might influence innate immune response. The objective of this study was to evaluate the leukocyte response with the use of pegbovigrastim in postpartum Holstein cows. The experiment was carried out at the experimental station of the Agronomic Research Institute located in São Bento do Una, in the state of Pernambuco. Twelve pregnant cows assigned to two treatments, control untreated cows (CG), and treated cows (TG) in which pegbovigrastim was administered at 7 days before and within 24 hours of calving. The CG received injections of saline solution (0.9% NaCl, wt/vol). Total and differential leukocyte counts were evaluated every 7 days from the first treatment application to 21 days postpartum. Endometrial cytology was evaluated every 7 days in the first 21days postpartum. Blood samples were collected by puncture of the jugular vein using a 25 x 8 mm (21G) needle attached to evacuated tubes containing an aqueous solution of ethylene diamine tetracetate tripotassium (K3-EDTA) at 15%. The leukocyte count was performed by the manual method in Neubauer's chamber. Two blood smears were made for differential leukocyte counting, these smears after drying were stained using the rapid pantype kit according to the manufacturer's recommendation. In each blood smear 100 leukocytes were identified and classified according to their morphological and dyeing characteristics. The experiment followed a completely randomized design with two treatments and six replicates per treatment, and data were analyzed for prepartum period, day of calving and postpartum period. The data were tested for distribution of residuals using the Kolmogorov-Smirnov test, being expressed in central tendency measurements. Data were analyzed by ANOVA with repeated measures using the GLM procedure of SAS. In the case of significance of ANOVA, means were patitioned by the least significant difference of the Student- Newman-Keuls test. A P-value of 0.05 was considered significant. Cows in TG had increased ($P<0.0001$) count of leukocytes, mainly because of increased segmented neutrophils. The concentration of of segmented neutrophils in blood averaged 10,467 cells/μL in TG and 4,278 cells/μL in CG ($P<0.004$). The reference values for segmented neutrophils in cattle is between 600 and 4,000 cells/μL of blood. There was no change in the percentage of segmented neutrophils present in the endometrium. It is concluded that the treatment is effective in increasing the concentration of leukocytes in blood, mainly segmented neutrophils. Support for this poject was provided by Elanco (donation of Pegbovigrastim), Facepe (scholarship), and IPA (cows).

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Maternal nutrient restriction followed by realimentation in beef cows alters maternal and fetal circulating amino acid profiles

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Inadequate maternal nutrition impairs offspring growth, fertility and carcass quality. A practical strategy for ameliorating these developmental programming consequences is through realimentation of the undernourished dam. Our lab has previously demonstrated that realimentation of nutrient restricted gestating beef cows increases uterine blood flow to the conceptus. The objective of this study was to evaluate the effects of realimentation on amino acid profiles in maternal and fetal circulation. On d 30 of pregnancy, multiparous cows (initial body weight [BW] = 620.5 ± 11.3 kg and body condition [BCS] = 5.1 ± 0.1) were assigned to one of three dietary treatments: control (CON; 100% NRC; n = 18) and restricted (RES; 60% NRC; n = 30). On d 85, cows were slaughtered (CON, n = 6; R, n = 6), remained on control (CC; n = 12) and restricted (RR; n = 12), or were realimented to control (RC; n = 11). On d 140, cows were slaughtered (CC, n = 6; RR, n = 6; RC, n = 5), remained on control (CCC, n = 6; RCC, n = 5), or were realimented to control (RRC, n = 6). On d 254, all remaining cows were slaughtered. Maternal serum, fetal serum, and amniotic fluids were collected at slaughter. Amino acid concentrations were determined using ultra performance liquid chromatography. Data were analyzed with generalized least squares using the mixed procedure of SAS to examine the effects of dietary treatment at each day of slaughter. On day 85, maternal serum from RES cows contained less (P = 0.06) histidine, isoleucine, alanine, and valine, and more (P = 0.09) glycine compared to CON. Day 85 fetal serum from CON cows had more (P = 0.01) tryptophan compared to RES, while amniotic fluid from CON cows had more (P = 0.09) glutamate, but less (P = 0.07) phosphoserine, tyrosine, and phenylalanine than RES cows. Minimal changes were observed on day 140 of gestation, with maternal serum from RC cows having greater aspartate (P = 0.03) than CC and RC cows. By day 254, RRC cows had greater (P = 0.04) arginine concentrations in maternal serum compared to RCC and CCC cows, while fetal serum from RRC cows contained greater (P < 0.06) citrilline, phenylalanine, and tryptophan compared to RCC and CCC cows. Amniotic fluid from RRC cows had greater (P < 0.05) concentrations of histidine and lysine compared to RCC and CCC cows. In conclusion, it appears that amino acid circulation in pregnant beef cows and their fetuses may be sensitive to maternal diet, including the duration of nutrient restriction and realimentation. Realimentation at the right time during pregnancy may be a viable feeding strategy for producers to improve pregnancy outcomes of malnourished beef dams. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2009–65203–05812 from the USDA National Institute of Food and Agriculture to KAV and KCS.

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Introduction of androgenized steers during the late luteal phase triggers an advancement of luteolysis

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The sudden introduction of males (biostimulation or bull effect) induces an increase of estrogen concentration, which may trigger the mechanisms resulting in luteolysis. The objective of the present experiment was to determine if the introduction of androgenized steers to beef heifers during their late luteal phase advances the luteolysic process. The trial was performed in a private farm in Uruguay (35º S) during May (autumn) with 12 Angus based heifers. Heifers remained isolated from any contact with males more than 4 weeks before beginning the trial (minimum distance = 500 m). Heifers' ovulation was synchronized with two doses of 500 µg i.m. of chlorprostenol (Ciclase DL, Syntex, Argentina) separated 11 days, and a dose of 100 µg i.m. of gonadorelin acetate (Gonasyn, Syntex, Argentina) 2 days after the second dose of chlorprostenol. The day in which each animal ovulated (day 0 of the estrous cycle) was determined by transrectal ultrasonography. Transrectal Doppler ultrasound observations of the corpus luteum (CL) were performed daily from day 10 to day 12 of the estrous cycle of all animals. On day 12, the animals were separated into two groups of 6 animals each that remained in paddocks of the same size and with the same pasture availability. While 2 androgenized steers were joined with the biostimulated heifers (group BT), the other heifers remained continuously isolated from males (group CON). From day 13 to 19 of the estrous cycle, CL were observed with Doppler ultrasound every 12 h. There was no effect of biostimulation on CL volume, but the area of the CL perfused was lower in BT than CON heifers (0.09 ± 0.02 cm^2 vs 0.16 ± 0.02 cm^2 respectively (P = 0.015), indicating an earlier luteolysis. Furthermore, the percentage of the CL area that was perfused was also lower in BT than CON heifers (2.4 ± 0.4% vs. 4.2 ± 0.4% P = 0.011). Overall, it was concluded that the introduction of androgenized steers during heifers' late luteal phase advanced the luteolytic process. Support provided by Javier Meilán, from Zoetis, for the donation of hormones; Victor Mayorga and Augusto Ryonosuke, for their help in the field work; Comisión Sectorial de Investigación Científica (CSIC), Universidad de la República, for the financial support.

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Timing and duration of nutrient restriction and its impacts on placental development and umbilical blood flow in sheep

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Nutrient-restriction beginning on day 50 of gestation in nulliparous ewe lambs decreased umbilical blood flow by day 80 (Lemley et al., 2012; AJP.302:R454). We hypothesized that ewes would experience a decrease in umbilical blood flow upon nutrient restriction (day 50 to 90) and that blood flow would recover to control values upon realimentation during late gestation (day 90 to 130), or remain reduced in ewes that continued to be nutrient restricted. On day 50 of gestation, young nulliparous white face ewes (6 to 8 mo; n = 41) carrying singletons were randomly assigned to two dietary treatments where ewes received 100% of NRC recommendations (CON) or 60% of CON (RES). On day 90 of gestation, ewes either remained on CON or RES until day 130, or CON ewes were RES from day 90 to 130, or RES ewes were realimented to CON from day 90 to 130. This resulted in 4 treatment groups: CON-CON, CON-RES, RES-RES, RES-CON. On day 50, and every 10 days until day 110, umbilical blood flow and placentome measurements were obtained via ultrasonography. The study had an end point of day 130 (data not shown). The study was conducted as a completely randomized design arrangement with repeated measures. Data were analyzed using the MIXED procedure of SAS. Non-significant interactions and main effects were removed from the models when $P \geq 0.25$. There were no three way interactions or main effect of late gestational treatment on umbilical blood flow or placentome size ($P \geq 0.25$). However, there was a significant interaction of mid gestational nutrition and day ($P < 0.01$) on umbilical blood flow with CON ewes having greater blood flow compared to RES by day 90. Similarly, placentome size was affected by mid gestational nutrition and day ($P \leq 0.05$). Placentomes from CON ewes were larger than placentomes from RES ewes by day 80. However, placentome sizes were similar among treatments on day 90 ($P \geq 0.22$). We reject our hypothesis that realimentation would return umbilical blood flow to control levels, and we were surprised CON-RES ewes had similar blood flow compared to CON-CON ewes. The majority of placental development occurs during the first two thirds of pregnancy. Our findings suggest that an adequate placental development during mid gestation could potentially “protect” the fetus from a decreased umbilical blood flow later on gestation when nutrients were limited by 40%. We are uncertain why realimentation did not alter umbilical blood flow by day 110 of gestation, but further analyses at day 130 may provide some insight into our study. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2016-67016-24884 from the USDA National Institute of Food and Agriculture.

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Effect of post-insemination intrauterine treatment with cephapirin on the subsequent reproductive performance of dairy cows with mild endometritis

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Cows in estrus but with signs of clinical endometritis (CE) are often not inseminated or undergo an intrauterine treatment. Decades ago, the so-called Åström method was described as intrauterine infusion of an iodine-potassium solution a few days after artificial insemination (AI). Nowadays, the use of antibiotics instead of iodine solution is common and the treatment is performed a few hours after AI. The objective of this study was to evaluate the effect of this modified Åström method on pregnancy per AI (P/AI; proportion of cows pregnant after AI). The study was conducted on a large dairy farm in Slovakia. A total of 323 Holstein Friesian cows were included in the study at the day of AI. Before enrollment, vaginal discharge was evaluated with the Metricheck device. Animals with clear discharge were assigned to a healthy comparison group (HE=112) and animals with cloudy discharge or fleck of pus in the mucus were divided into a treatment and a control group. The treatment group (MET; n=108) received an intrauterine treatment with 500 mg cephapirin (Metricure®, Intervet Deutschland GmbH) 6±1 hours after AI. The control group (CO; n=103) remained untreated. From a subset of cows with CE (n=62), bacteriological samples were taken from the uterus with the cytobrush technique and subjected to Fourier-transform infrared spectroscopy. The statistical evaluation was carried out with the software SPSS (version 24.0, IBM SPSS Inc., Munich, Germany). For the comparison of the P/AI between the three groups HE, MET and CO, Chi-square tests were performed. The level of significance was set at $P<0.05$. Pregnancy at first AI was did not differ among groups (HE 37%; MET 32%; CO 31%). Non-pregnant cows were re-inseminated at the next estrus. For cows with a second AI, P/AI was greater in the MET (59%) compared with CO (44%). Interestingly, the lowest P/AI was observed in HE (32%; $P<0.05$). Cumulated results of the first and second AI showed a similar trend with the highest proportion of pregnant cows in MET (73%) followed by CO (63%) and HE (58%), although differences were not significant. Most of the examined cows (56/62) were bacteriologically positive. The uterine microflora was highly diverse and 234 isolates were found, mainly Gram-positive bacteria. Most frequently detected genera were *Staphylococcus* (15%), *Bacillus* (9%) and *Corynebacterium* (7%). Pathogenic species, such as *Trueperella pyogenes* and *Escherichia coli* were not or rarely isolated (0 to 2%). In summary, post-insemination treatment with cephapirin had a significant positive effect on P/AI at the second AI but not for the AI immediately before treatment. One reason for this delayed effect could be that the time for recovery of the inflamed endometrium after treatment was too short before the embryo entered the uterus. This finding and the fact that typical uterine pathogenic bacteria were rarely detected questions the indication of an intrauterine antibiotic treatment in cows with mild CE at breeding.

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Determinants of cytological endometritis in multiparous Holstein cows

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Cytological endometritis (CE) significantly reduces the reproductive performance of cows. Our objective was to establish the determinants that predispose dairy cows to CE. Multiparous Holstein cows (n = 119) from one 1,200-cow dairy herd were used. Body condition score (BCS) wwas evaluated at 2 weeks before expected calving and cows were classified as thin (≤ 2.75), moderate (3.00 and 3.25) and moderately fat (3.50 and 3.75). Blood was collected at wk -2 prepartum, and at wk +1 and +3 postpartum. Endometrial samples were taken at 40 d postpartum using an endometrial brush attached to a stainless steel device for use in cows (EP2029026B1). The first 45-d cumulative milk yields were categorized as low (< 1,314 kg), moderate (≥ 1,314 to < 1,780 kg), or high (≥ 1,780 kg). Diseases were recorded. Plasma insulin and insulin-like growth factor 1 (IGF1) and serum amyloid A (SAA) were analysed by ELISA; all other plasma variables were measured using an autoanalyser. Optimal criterion values were obtained from receiver operating characteristic curve analysis. Data were analysed with logistic regression and multiple correspondence analysis. The CE prevalence was 30.3% with a cut-off of > 8% polymorphonuclear neutrophils. Multiple correspondence analysis revealed two distinct clusters of risk factors for CE. The determinants of CE corresponding to the first cluster were associated with metabolic imbalance indicated by low BCS (≤ 2.75 vs. 3.50 and 3.75, odds ratio [OR] = 5.76, P = 0.027), high non-esterified fatty acids (> 108 µekv/L, OR = 3.65, P < 0.011), high haptoglobin (> 0.08 mg/mL, OR = 3.38, P < 0.005) and low IGF1 (< 74.6 ng/mL, OR = 5.96, P < 0.001) levels at wk –2 prepartum; low IGF1 (< 36.5 ng/mL, OR = 3.88, P = 0.012), low insulin (< 0.33 ng/mL, OR = 2.59, P = 0.025) and low Ca (< 2.43 mmol/L, OR = 3.08, P = 0.009) levels at wk +3 postpartum; a high cumulative milk yield over the first 45 d of lactation (≥ 1,780 kg vs. ≥ 1,314 to < 1,780 kg, OR = 10.54, P < 0.001) and an increased parity (≥ 3 lactations vs. 2 lactations, OR = 2.32, P = 0.044). The determinants of CE corresponding to the second cluster were related to a high degree of systemic inflammation indicated by high haptoglobin (> 0.81 mg/mL, OR = 6.39, P < 0.001), high SAA (> 128 ng/mL, OR = 9.13, P < 0.001), low albumin (< 36.5 g/L, OR = 2.96, P = 0.009), low cholesterol (< 2.15 mmol/L, OR = 2.33, P = 0.044) and low IGF1 (< 13.2 ng/mL, OR = 5.96, P < 0.001) levels at wk +1 postpartum. This cluster included a low albumin level (< 38.1 g/L, OR = 3.92, P = 0.002) at wk +3 postpartum, ill health (OR = 2.93, P = 0.008) and a low cumulative milk yield (< 1,314 kg vs. ≥ 1,314 to < 1,780 kg, OR = 7.81, P < 0.001) over the first 45 d of lactation. In conclusion, two distinct clusters of cows experienced high risk for the development of CE: 1) cows with metabolic imbalance and a high milk yield over the first 45 d of lactation; 2) cows with a high degree of systemic inflammation, ill health and a low milk yield over the first 45 d of lactation. Supported by IUT8-1.

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Resistin acts as a link between reproduction and energy metabolism in sheep

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Resistin exerts a regulatory influence on numerous reproductive and metabolic processes, including maintenance of energy homeostasis during different reproductive states. It is particularly important in seasonally breeding sheep which must adapt their metabolic state to the changing environment. Metabolic factors such as insulin, leptin, and glucose affect various aspects of reproduction and are related to activity of resistin-secreting adipocytes; however, information regarding the interactions among them in sheep is limited. Numerous metabolic abnormalities can lead to the development of pathophysiological conditions such as the polycystic ovarian syndrome (PCOS) and recent studies indicate that resistin may have a role in PCOS development. Moreover, obesity is significantly related to declines in fertility. Females with obesity have abnormal plasma resistin profiles, which may help explain some types of infertility. Herein we examined the interaction of season and recombinant bovine resistin (rbresistin) on plasma concentrations of leptin, insulin and glucose in experiments conducted during both short (SD) and long (LD) days. Thirty ewes of the Polish Longwool breed, which exhibits strong seasonal reproduction, were ovariectomized and given estrogen replacement using subcutaneously inserted estradiol implants. Ewes were housed in natural photoperiod (longitude: 19°57' E, latitude: 50° 04' N). Animals were 2 to 3 years of age and had a mean body weight of 60±2 kg. Intravenous treatments at the beginning of experiment (Time 0) consisted of control or rbresistin in saline: 1) Control (C; saline; n = 10), 2) low resistin (R1; 1.0 µg/kg BW; n = 10), and 3) high resistin (R2; 10.0 µg /kg BW; n= 10). Blood samples were collected every 10 minutes during 4 h. Blood plasma concentrations of resistin, leptin and insulin were assayed using RIA and ELISA kits. Glucose was measured using a Roche AccuChek Active device. Plasma leptin concentrations were greater ($P < 0.05$) in C during LD compared to SD. The R1 and R2 resistin treatments resulted in 2- and 3-fold increases, respectively, in circulating concentrations of resistin ($P < 0.05$) during LD. Resistin infusion decreased ($P < 0.001$) mean circulating concentrations of leptin in a dose- dependent manner during both seasons. However, only R2 increased ($P < 0.05$) plasma concentrations of insulin and did so during both seasons. Both R1 and R2 decreased ($P < 0.05$) glucose concentrations in both seasons. There was no time by treatment interaction in response to resistin injection. To the best of our knowledge, this is the first study to report a role for resistin in modulating circulating leptin, insulin and glucose, and indicate that the ability of resistin to create this effect is somewhat seasonally-dependent. Further studies investigating the interaction of resistin and other adipokines such as leptin are warranted. Research supported by NCN 2015/19/B/NZ9/01314 to DA.

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Estrous expression improves the success of timed artificial insemination and embryo transfer

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Detection of estrus is important for reproductive performance. Use of automated activity monitors as tools for detection of estrus has become popular on dairy farms. This study aimed to evaluate the associations between estrous responses measured with automated activity monitors at the end of a timed artificial insemination (TAI) or embryo transfer (ET) protocol and fertility outcomes. In study 1, lactating Holstein cows had their estrous cycles synchronized using a protocol based on estradiol and progesterone and bred by TAI using conventional semen (n=1411 events from 1040 cows). In study 2, lactating Holstein cows had their estrous cycles synchronized using a protocol based on estradiol and progesterone and 7 d post-estrus received an embryo (n=1147 events from 657 cows). Embryos were transferred as either *in vivo* (fresh or frozen) or produced *in vitro* (only frozen). Pregnancy was confirmed at 31 ± 3 d post-TAI or estrus; for study 1, pregnancy losses were determined by a second pregnancy diagnosis at 60 d post-TAI. In both studies, the expression of estrus was monitored through a leg-mounted activity monitor (AfiMilk Pedometer Plus, Afikim, Israel). Estrus was determined to have occurred when the relative increase (RI) in activity of the cow exceeded > 100% of their baseline activity. At estrus, physical activity was categorized as high or low intensity using the median. Data were analyzed with the GLIMMIX procedure of SAS and cow was used as a random effect. In study 1, 82.0% of cows expressed estrus on the day of TAI or the preceding evening. Cows expressing estrus around TAI had greater ($P < 0.01$) pregnancy per AI (P/AI) than those that did not express estrus (32.7 vs. 6.2%). Cows that expressed estruses of high intensity had greater ($P < 0.01$) P/AI than those with low intensity of estrous expression or that did not express estrus at all (35.1 vs. 27.3 vs. 6.2%). Cows with high intensity of estrous expression had less ($P < 0.05$) incidence of pregnancy loss compared with cows with low intensity of estrous expression (13.9 vs. 21.7%). In study 2, 89.1% of cows expressed estrus before ET. Cows expressing estrus before ET had greater ($P < 0.01$) pregnancy per ET (P/ET) than those that did not express estrus (35.8 vs. 5.9%). Of the cows that expressed estrus, those with high intensity of estrous expression had greater ($P < 0.01$) P/ET than those with low intensity of estrous expression (41.5 vs. 30.6%). No interaction was found between estrous expression and source of embryo (in vivo fresh, in vivo frozen or in vitro frozen), but cows receiving *in vitro* frozen embryos had less ($P = 0.02$) P/ET than those receiving embryos produced *in vivo* and ans transferred fresh or frozen (30.0 vs. 38.7 vs. 39.0%). Expression of estrus is associated with improved pregnancy in dairy cows bred by timed AI and timed ET. Furthermore, cows with more intense estrous expression have improved maintenance of pregnancy either following AI or ET. Authors would like to thank Colorado Dairy, Conapec Jr., Dairy Farmers of Canada and NSERC.

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Heat stress induces proteomics changes in the follicular fluid of dairy cows

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Reduced fertility associated with heat stress (HS) is a complex and multifactorial problem involving hormone secretion, follicular development, oocyte and embryo quality, and uterine function. The aim of this study was to investigate the effects of experimentally induced HS on follicular fluid (FF) proteome of cows at key stages of follicular development. Nonlactating Holstein cows were synchronized with the Ovsynch protocol that incorporated an intravaginal progesterone device. On the day of ovulation, cows were contemporaneously and randomly assigned to thermoneutral (TN; n = 12) or HS (n = 12) treatments. The temperature and humidity in the climate chamber were, respectively, 25.9°C and 73.0% for TN, and 36.3°C and 60.9% for HS. Transrectal ultrasonography was performed every 12 h to monitor follicular dynamics. On day 9 of the first follicular wave, the largest follicle (F1~12 mm) was individually aspirated and all remaining visible follicles were ablated. At expected deviation time (F1~8.5 mm), both the F1 and the second largest follicle (F2) were individually aspirated. After albumin depletion, triplicates of FF were reduced, alkylated, and digested with trypsin. The resulting peptides were labelled with TMTsixplex (Pierce, Rockford, USA) and quantified using LC-MS/MS (Orbitrap Elite, Thermo, San Jose, USA). Quantitative proteomic data were compared by PROC GLM (SAS Institute, Cary, USA). A total of 158 unique proteins were identified in the FF, mainly as plasma-matched proteins. Twenty-eight differentially ($P < 0.05$) expressed proteins were found in FF of cows exposed to HS versus TN, of which seven were affected by the interaction between the stage of follicle development and treatment. Data analysis using IPA software (Qiagen, Redwood City, USA) revealed that the most significant canonical pathways associated with the proteins identified in the FF were acute phase response, liver X receptor (LXR), retinoid X receptor (RXR) and farnesoid X receptor (FXR) activation, complement system, and coagulation system. Many components of the immune system (IgGs, inter-alpha inhibitor H4, alpha-2-macroglobulin, transthyretin, complement components C4, C6, C7, C8, C9, complement factor B, and factor I) were up-regulated by HS, while components of the coagulation cascade (plasminogen, hemopexin, prothrombin, and vitronectin) were down-regulated in response to HS. In conclusion, these findings demonstrate that HS alters the protein expression of FF, which might affect follicular function as well as oocyte quality and could explain in part the reproductive failure of heat stressed dairy cows. FAPESP (Grants #2012/18297-7, #2013/20083-8, and #2014/21257-2).

090 Assisted Reproductive Technologies

Prostaglandin $F_{2\alpha}$ as an inducer of ovulation in Fixed Timed Artificial Insemiantion in Zebu cows

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Prostaglandin (PG) is a potent biological substance with varied applications in bovine reproductive control, and $PGF_{2\alpha}$ main effect is to induce luteolysis. Prostaglandin $PGF_{2\alpha}$ has been reported as an ovulatory stimulus in prepubertal heifers. Thus, the objective of this study was to evaluate the use of $PGF_{2\alpha}$ in fixed timed artificial insemination (FTAI) protocol as an inducer of ovulation in cows and characterize the blood perfusion of the pre-ovulatory follicles. The experiment was carried out at Embrapa Genetic Resources and Biotechnology. Twenty-four zebu cows were assigned to a 3 x 3 Latin square desing with 30 days between treatment periods. Cows received at random stages of the estrous cycle an intravaginal device containing 1g of progesterone (P4) concurrent with an i.m. injection of 2.0 mg of estradiol benzoate (EB). The day of EB injection was considered study day 0 (D0). The intraginal device was removed on D8. Cows were then randomly assigned to three treatments as estrous synchronization protocols. In the PGF2α-D7 treatment, cows received 500 µg of PGF_{2a} i.m. (cloprostenol) in two applications on D7 and D8. In PGF2α-D9 treatment, injections of 500 µg of PGF_{2a} i.m. were on D8 and D9. Control (CT) cows received 500 µg of $PGF_{2\alpha}$ i.m. on D8 and in D9 and 1 mg of EB on D9. Cows were evaluated with a color Doppler ultrasound (MyLab™30Gold VET, Italy) every 12 hours from D8 (0 hrs) to D10 (60 hrs), after which cows were evaluated every 6 hours until ovulation occurred or a maximum of 120 hours. Seven days after ovulation, cows were examined by ultrasound with color Doppler mode to evaluate the presence of luteal blood flow to assess functionality of the corpus luteum (CL). For follicular and luteal vascularization, a subjective classification of grades 1 to 5 was adopted, which considered the percentage of follicle wall or CL with blood circulation. Statistical comparison was performed for time of ovulation, and size of preovulatory follicle using ANOVA with the Tukey adjustment for treatment comparisons. Degree of vascularization of follicle wall and CL was analyzed used Friedman's test. Time of ovulation differed ($P<0.05$) among treatments and CT cows ovulated earlier (72.4±3.7 h) compared with the other treatments (PGF2α-D7 96.0±12.8 and PGF2α-D9 90.0±18.2 h). The mean preovulatory follicle size did not differ among treatments (PGF2α-D7 13.1±1.6; PGF2α-D9 13.4±1.8; CT 11.9±1.5mm). Similarly, the degree of irrigation of follicles did not differ among treatments (PGF2α-D7 3.7±1.0; PGF2α-D9 3.5±1.0; CT 2.3±1.8). Ovulation rate in PGF2α-D7 and PGF2α-D9 averaged 62.5% (15/24) and it did not differ from CT (58.4%; 14/24). Diameter of the CL (PGF2α-D7 15.5±3.4; PGF2α-D9 14.4 ± 3.2; CT 13.4±2.7 mm) and its vascularization (PGF2α-D7 4.7±0.6; PGF2α-D9 4.7±0.6; CT 4.7±0.4) did not differ among treatments. Administration of EB, but not PGF2α, reduced interval to ovulation, although ovulation rate did not differ among treatments.
EMBRAPA, UNB.

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Cryopreservation of prepubertal bovine testicular tissue

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Spermatogenesis is the complex process responsible for sperm generation in the testis. Spermatogonial stem cells (SSCs) are at the basis of the seminiferous epithelium constantly generating cohorts of daughter cells committed to differentiate. In the bull, type A spermatogonia population includes both SSCs and their differentiating daughter cells (de Rooij and Griswold, 2012). Germ stem cells can be cryopreserved and used for germoplasm preservation (Onofre *et al*., 2016) and particularly posthumous preservation of elite bulls. During the early prepubertal stage, before the first wave of spermatogenesis, gonocytes, the precursors of A spermatogonia, are the only germ cells in the testis (Aponte *et al*., 2005). We aimed at cryopreserving whole bovine testicular tissue as a fast procedure to save particular bovine male germ-lines. For this purpose, we took testicular tissue from 2-3 mo old Hosltein calves (n = 4), isolated germ and somatic cells through enzymatic digestions, differential plating and recorded initial (day 0) cell composition and survival. Approximately 1 cm^3 testicular pieces from the same testes were slowly infiltrated with either MEM + DMSO + FCS (Sol. A) or MEM + DMSO + BSA (Sol. B) in 3 fractions, dropwise, over 2 h, 4°C. Tissue pieces were then placed at -80°C for 3 d, and stored in liquid N_2 for 2 wk. Then, they were thawed in water at 39°C. Immediately, the tissues were subject to cell isolation. Cell composition and survival were compared pre and post-freezing through One Way ANOVA (SPSS, Windows, V. 17.0.). Results are expressed in mean ± SEM. Testicular morphology post-thawing was similar to that of fresh pre-freezing tissue. In both cases testes showed seminiferous tubules with Sertoli cells and gonocytes located near the basal membrane. The percentage of viable gonocytes in the resulting cell suspension was significantly lower after thawing, independently of the cryopreservation solution used (pre-freezing: 40.6 ± 14.7%; post-freezing: Sol A, 6.3 ± 3.3%; Sol B, 3.4 ± 0.4%; $P < 0.05$). Viability within the gonocyte population was similar among the treatments (pre-freezing: 93.1 ± 4.7%; post-freezing: Sol A, 81.0 ± 10.9%; Sol B, 81.5 ± 1.4%; $P > 0.05$). These results suggest that gonocytes are able to survive cryopreservation but absolute numbers obtained are considerably lower after thawing. This is perhaps caused by higher amounts of free DNA shed from dying cells during cryopreservation, making the surviving cells "sticky" and lost during cell isolation steps. Gonocyte numbers can probably be brought up by adding higher concentrations of DNAse in the media and by using further purification steps. Post-freezing, apparently intact germ cells can be the basis for further reproductive biotechnologies aimed to bovine species such as spermatogenesis *in vitro* and transgenesis.

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Effect of heat shock on developmental competence in bovine oocytes during *in vitro* maturation

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The aim of this study was to determine the effect of heat shock (HS) and its time of exposure during *in vitro* maturation (IVM) of bovine oocytes on meiotic progression and blastocyst yield. Intact immature cumulus-oocyte complexes (COCs) obtained from a local abattoir were IVM at 38.5°C for 24 h (control group, CG), or incubated for 6 h (group G-6h), 12 h (group G-12h), 18 h (group G-18h), or 22 h (group G-22h) at 41°C, and then matured at 38.5°C to complete 24 h. After maturation, oocytes from each group were fertilized *in vitro*, and the presumptive zygotes were cultured until reaching the blastocyst stage. The rate of meiotic progression of all oocytes was recorded at 24 h (n=60-68 oocytes/group) and analyzed by Chi-square test. Data on the percentage of cleavage (n=127-200 presumptive zygotes/group) on day 3, blastocyst rate, and total blastomeres in expanded blastocysts on day 9, were analyzed by one-way ANOVA using SAS PROC GLM. Exposure of bovine oocytes to 41°C for 12 h reduced ($P<0.05$) the percentage that reached the metaphase II (MII) stage. The rates of blastocyst development in CG and G-6h groups were greater than in G-12h, G-18h, and G-22 h (29.8±4.8 and 25.5±5.0 vs. 10.7±2.8; 18.9±3.9 and 7.5±5.3%, respectively; $P>0.05$). Moreover, the total number of blastomeres in CG (127.9±3) was greater ($P<0.05$) than all other groups, being similar among G-12h, G-18h, and G-22h groups (88.1±4; 86±6 and 90±5.7, respectively). In conclusion, exposure of bovine oocytes for 12 h to HS during IVM did not block development but reduced blastocyst rates. Therefore, exposure to HS during *in vitro* maturation reduces developmental competence of bovine oocytes.

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Use of interspecific fertilization as a tool to evaluate fertilizing ability of buffalo *(Bubalus bubalis)* bulls in an *in vitro* fertilization program

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In vitro production (IVP) of embryos is a wide spread biotechnology in cattle but it is not well developed in buffaloes, in which the maternal model for reproduction is highly applied. Buffalo and cattle are bovidae species and share significant physiological similarities, suggesting that no highly effective species-specific fertilization barrier exists. The limited availability of oocytes to evaluate the performance of candidate bulls for IVP programs is part of the problem. The objective of this work was to develop a strategy designed to evaluate the fertilizing capacity of buffalo males to be used for in vitro fertilization. This work was performed in Embriones del Sinú Laboratory, located in Monteria North Coast Colombia, during 2015. Frozen semen from buffalo bulls was evaluated using matured cattle *(Bos indicus)* oocytes after OPU. Oocytes were obtained from 3 to 8 mm follicles, and matured for 24 h in SOF-supplemented medium. After thawing, sperm were prepared using a swim-up method and groups of 20 Grade I oocytes were inseminated with 2 million/mL motile sperm in 50 µl drops. Presumptive zygotes were transferred to culture medium for development until day 7, and cleavage and blastocyst rates (BR) were recorded. Of 272 oocytes used, 166 (61%) cleaved and 88 (32%) reached the blastocyst stage. Cleavage rate ranged from 50 to 74% and BR from 22 to 40%, in all the cases interspecific BR was higher than monospecific production (18%). These results are similar to those obtained by Kochhar et al. and Xiang Li et al., 86.3% and 71.3% cleavage and 25.9% and 33.7% BR, respectively. The feasibility of using cattle oocytes to evaluate the fertilizing capacity and the potential performance of buffalo bulls to be used for IVP has been shown, and as expected there was high variation in BR among the buffaloes evaluated. It is very surprising the low BR of embryos when cattle oocytes are fertilized by buffalo sperm. Others have reported fertilization of cattle oocytes with African buffalo epididymal sperm in vitro. Interspecies hybrids offer clues in the search for answers to several questions of genetics like interbreeding depression including over-dominance, hybrid vigor, and segregation distortion. Our next research will be conducted to evaluate the usefulness of the model to discriminate potentially fertile and infertile males. Embriones del Sinú - Asociación Colombiana de Criadores de Búfalos.

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Comparison between ovine refrigerated spermatozoa from ejaculate and epididymal cauda

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Recuperation and preservation of viable spermatozoa after the sudden death of an important breeding animal can be vital to manage breeding programs. The aim of this study was to compare sperm viability on refrigeration at 5°C when spermatozoa were collected in an artificial vagina (AV) or recovered from epididymal cauda (EPD). Semen was collected in an AV and 1 week after collection the same rams were slaughtered. Epididymal spermatozoa were recovered by slicing method immediately after slaughter of four randomly selected epididymis. After collection in AV or EPD the samples were diluted in medium containing glycine, 5% egg yolk and milk in final concentration of 400 x 10^6 spermatozoa/mL and refrigerated at 5°C. The parameters of total motility (TM), progressive motility (PM) and plasma membrane integrity (PMI) in hypoosmotic swelling test were evaluated 0 (R0), 24 (R24), 48 (R48) and 72 (R72) hours after the onset of cooling. The analyzes were performed in duplicate. ANOVA test was used to identify differences between groups (AV and EPD) and Repeated measures ANOVA test was used to analyze differences between moments (time after the onset of cooling). Tuckey test was applied on previous tests when $P<0.05$. There was no difference ($P>0.05$) in TM, PM and PMI between groups at any cooling moment. The initial TM was 86.4 ± 1.0% and 79.0 ± 4.3% in AV and EPD groups, respectively. TM decayed ($P<0.05$) from 24 hours of refrigeration in both groups (AV: 68.6 ± 6.3^{b}% and EPD: 60.0 ± 2.5^{b}%). However, PM differed ($P<0.05$) from the initial moment more rapidly in AV group (R0: 73.2 ± 1.4%^{a}; R48: 20.4 ± 3.3%^{bc}; R72: 6.3 ± 2.1%^{c}) when compared to EPD (R0: 62.0 ± 4.4%^{a}; R48: 24.0 ± 4.3%^{ab}; R72: 5.0 ± 1.7%^{b}). PMI decreased ($P<0.05$) from 12 hours of cooling in the AV group (R0: 90.4 ± 1.3%^{a} and R12: 65.4 ± 2.9%^{b}). In EPD group, PMI remained the same throughout the refrigeration period (R0: 82.0 ± 0.6%; R72: 66.5 ± 3.9%). No difference in TM, PM and PMI was identified between AV and EPD groups during the preservation of spermatozoa in liquid stage at 5°C. Similar results were found when the spermatozoa were frozen (Bergstein-Galan *et al*., 2017. Quality and fertility of frozen ovine spermatozoa from epididymides stored at room temperature (18-25°C) for up to 48 hours post mortem. Theriogenology, 96: 69-75). However, undesired changes, as the decrease in PM and PMI, occurred later in the EPD group when compared to AV. These findings are probably the result of greater resistance of epididymal spermatozoa to cold shock. In conclusion, there are no differences between ejaculated and epididymal spermatozoa preserved in liquid stage and cooled to 5°C.

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Impact of GnRH administration at the time of AI on pregnancy and ovulation rates and its interaction with estrous expression

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Cows with reduced estrous expression have been found to have compromised fertility. The aim of this study was to determine if the administration of GnRH at the moment of AI could increase ovulation rates and fertility of animals expressing low estrous behavior. Cows were enrolled at the time of estrus from 3 commercial farms (n = 1629 AI events; Farm A: 757, Farm B: 305, Farm C: 567) and randomly assigned to receive GnRH at AI or not. At the same time, cows had their BCS and gait scored. On all herds, cows had their estrous expression monitored through leg-mounted activity monitors. Estrous expression was quantified as the maximum activity that occurred during the event; using the farm median, estrous expression was categorized as high or low. On Farm A, cows were assessed at alert and for ovulation at 24h (n = 160), 48h (n = 707) and 7d (total ovulation failure; n = 707) post-alert using transrectal ultrasonography; ovulation was determined by the disappearance of the dominant follicle. Pregnancy was confirmed at 31 ± 3d post-AI. Differences between treatments were tested using the GLIMMIX procedure of SAS where cow within farm was used as a random effect. Occurrence of ovulation at 24h was impacted by estrous expression, where animals with high expression had lower ovulation rates at 24h (23.3 ± 4.8 vs. 37.7 ± 5.5%; P = 0.05); no impact of GnRH was found. An interaction between GnRH and estrous expression was found for both the occurrence of ovulation at 48h and total ovulation failure. At 48h, ovulation rates were highest for cows with high expression that received GnRH, all other groups were the same (GnRH: High – 94.8 ± 2.5, Low – 85.9 ± 2.6; No GnRH: High – 88.5 ± 2.9, Low – 87.3 ± 2.6%; P = 0.05). Similarly, total ovulation failure was lowest for cows with high estrous expression that received GnRH (GnRH: High – 2.2 ± 1.9, Low – 6.2 ± 2.1; No GnRH: High – 8.2 ± 1.9, Low – 8.9 ± 2.0%; P = 0.05). Ovulation was not impacted by parity, gait or BCS. Fertility on all farms was impacted by parity, where primiparious cows had higher P/AI than multiparous (46.0 ± 3.1 vs. 35.6 ± 2.3 %; P < 0.01), but was not impacted by gait or BCS. Fertility was not impacted by treatment, but there was an interaction of treatment and estrous expression, where animals with low estrous expression receiving GnRH at AI had higher P/AI than those that did not receive GnRH; GnRH did not impact P/AI of high expression cows. In fact, cows with low estrous expression receiving GnRH had the same P/AI as those with high estrous expression with or without GnRH (GnRH: High – 43.3 ± 3.3, Low – 40.8 ± 3.6; No GnRH: High – 46.2 ± 3.5, Low – 33.0 ± 3.5%; P < 0.01). In conclusion, administration of GnRH at the time of AI resulted in an increased conception risk of animals with low estrous expression, however, this effect does not seem to be closely related to ovulation rates. Zoetis, Dairy Farmers of Canada and NSERC.

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Factors affecting the ovum pick-up and *in vitro* embryo production in buffaloes

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The objective of the present study was to evaluate the influence of variables related to donors and sires in the efficiency of OPU/IVEP in buffaloes. For this, data from 421 OPU/IVEP procedures (farm [A, B, C], parity [nulliparous-N, primiparous-P and multiparous-M], postpartum period [≤117d, 117d to 217d and >217d) reproductive status [pregnant-P or non-pregnant-NP at the OPU], body condition score [BCS; <3.0, 3.0 to 4.0 and >4.0] and sire used for IVEP [A, B, C, D, E, F, G and H) were evaluated. All buffaloes underwent a regular transvaginal ultrasound-guided ovum pick-up (OPU) for oocyte recovery at random stage of the estrous cycle. The HPMIXED procedure of SAS through the Best Linear Unbiased Prediction (BLUP) analysis was utilized to rank farms, parity, postpartum period, reproductive status, BCS and sires, in terms of oocytes per OPU, embryo produced per OPU and embryo rate. It was evidenced effect of farm (A=9.6±0.5^{a}, B=8.9±0.3^{ab} and C=6.9±0.9^{b}; P=0.05) and parity (N=10.2±0.7^{a}, P=11.1±0.9^{a} and M=8.34±0.4^{b}; P=0.07) on the number of retrieved oocytes per OPU. Nulliparous and primiparous produced higher number of retrieved oocytes per OPU than multiparous. Regarding the postpartum period and BCS, no differences were found for the number of retrieved oocytes (P=0.92 and P=0.98, respectively). Furthermore, according to the reproductive status, pregnant buffalo (30 to 120 days of gestation) produced lower number of retrieved oocytes per OPU than non-pregnant (P=7.9±0.6^{b} and NP=10.0±0.5^{a}; P=0.02). However, only the sire variable had an effect on the number of embryo produced per OPU and embryo rate. There was a strong effect of the bull (P<0.001) on the efficiency of IVEP in buffaloes. The embryo rate (%) according to sires used (n=8) during IVEP was A=37.7; B=29.7; C=25.2; D=22.0; E=20.4; F=17.5; G=6.6 and H=6.4. It was concluded that semen used during IVEP procedures potentially influence IVEP results. Top ranking sires yielded outstanding embryo rates, while poor sires performers produced low embryo rates. Although we verified effect of farm, parity and reproductive status on the number of retrieved oocytes, no differences were observed in the number of embryos produced per OPU in the present study.

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Modifications of a 5-d GnRH-based timed-AI protocol to optimize fertility in Holstein heifers inseminated with sex-selected semen

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This study evaluated the usefulness of an estrus detection (ED) aid, timing of GnRH administration and AI on pregnancy per AI (P/AI) in Holstein heifers subjected to a 5-d GnRH-based protocol and inseminated with sex-selected semen. In Expt 1, Holstein heifers (n=591) received a progesterone releasing device (CIDR) on d 0, and on d 5 CIDR were removed, 500 μg of cloprostenol (PGF) was administered and ED patches (Estrotect^{TM}) were applied. Patches were scored from 0 to 2, based on color change between initial application and 36 and 48 h after CIDR removal; 0 = unchanged, 1 = ≤ 50% color change, 2 = > 50% color change (defined as estrus). Heifers in the Control group (n=195) received 100 μg of GnRH and were inseminated 72 h after CIDR removal, regardless of estrus expression (TAI). Heifers in the treatment groups that were in estrus (n=110) were AI 56 h after CIDR removal and those not in estrus received 100 μg of GnRH at either 56 (GnRH56; n=142) or 72 h (GnRH72; n=144) after CIDR removal and were TAI at 72 h. In Expt 2, Holstein heifers (n=330) received CIDR and PGF treatments and ED patches as in Expt 1. Heifers in estrus at 36 or 48 h after CIDR removal were AI at 56 h as in Expt 1, but those not in estrus were TAI at either 72 (TAI72) or 80 h (TAI80) after CIDR removal, and those with an ED patch scored 0 or 1 at TAI received 100 μg of GnRH. All heifers in both experiments were inseminated by the same technician with frozen-thawed, sex-selected semen from sires available commercially. Pregnancy was diagnosed by ultrasound 27 d after AI. In Expt 1, the percentage of heifers detected in estrus was 31, 28 and 27% for Control, GnRH56 and GnRH72 groups. Overall, P/AI was greater (P=0.04) for GnRH72 (63%) than Control (55%), and intermediate for GnRH56 (59%) group. There was an interaction between estrus expression and treatment group; in heifers that expressed estrus, P/AI was greater (P=0.02) in GnRH56 (64%) and GnRH72 (65%) groups compared to Control group (43%). In Expt 2, 36% of heifers were AI at 56 h and P/AI was 63%. More heifers (P<0.01) in the TAI72 were given GnRH at TAI compared to TAI80 (40 vs. 22%), but P/AI did not differ between groups (68 vs. 66%). In summary, breeding heifers based on detected estrus increased P/AI with sex-selected semen. Administration of GnRH before TAI or delaying TAI did not increase P/AI, but delaying TAI reduced the number of heifers treated with GnRH. Estrus detection patches were considered useful to identify animals exhibiting estrus before TAI, increasing P/AI with sex-selected semen and reducing hormone usage. Authors thank Vetoquinol N.-A Inc. (Lavaltrie, QC, Canada) and Rockway Inc. (Spring Valley, WI, USA) for their in-kind support and Breevliet Ltd (Wetaskiwin, Alberta, Canada) for cooperation during the study.

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Color Doppler ultrasonography for early pregnancy diagnosis in goats

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Subjective luteal blood flow analysis by color Doppler ultrasonography (US) was previously demonstrated in cows and sheep as an early and accurate method for pregnancy diagnosis. This study aimed to establish the best day for such diagnosis in dairy goats. 131 Saanen does 2.0±0.5 years old were used. In the first study, after a hormonal protocol for induction of synchronous estrus and AI, 60 goats were evaluated from Day 15 to Day 23 of the estrous cycle (Day 1 or D1 = ovulation day), by a subjective Color Doppler US assessment (score 1-4, where score 1 means no pregnancy and, score 2-4 means positive pregnancy) using portable equipment (Sonoscape S6, Shenzhen, China) with a 7.5 MHz linear rectal transducer. In the second study, 71 does received the same protocol and had the ultrasound exam performed at Day 21 (the best day detected in the first study) for luteal blood flow assessment. In both studies, B-Mode US at Day 30 confirmed pregnancy diagnosis (gold standard). The performance of the subjective luteal blood flow analysis and its agreement with the gold standard outcome in both studies was classified calculating Sensitivity (SEN), Specificity (SPEC), Positive Predictive Value (PPV), Negative Predictive Value (NPV), and Kappa index (K). In study 1, pregnancy diagnosis by subjective luteal assessment by Color Doppler US was not feasible at D15 and D16 (SEN 100%; SPEC 0%; PPV 49%; NPV not calculable, and K = 0) as all CL were considered viable (vascularization score was = 2) and consequently all animals were diagnosed as pregnant. From D17 to D21, the overall performance of the technique progressively increased (D17: SEN 96%; SPEC 4%; PPV 49%; NPV 50%, and K = 0.01; D18: SEN 100%; SPEC 12%; PPV 52%; NPV 100%, and K = 0.11; D19: SEN 100%; SPEC 42%; PPV 62%; NPV 100%, and K = 0.42; D20: SEN 100%; SPEC 73%; PPV 78%; NPV 100%, and K = 0.73). Results did not change from D21 to D23 (SEN 100%; SPEC 92%; PPV 93%; NPV 100%, and K = 0.92). Two animals diagnosed as non-pregnant on Day 30 had a well vascularized CL until Day 23. On D17, a doe diagnosed as pregnant on Day 30 had the CL scored as 1, even though it was evaluated as score = 2 on the following days. In study 2, the assessment presented a similar pattern of sensibility and specificity observed in study 1 (SEN 100%; SPEC 93%; PPV 91%; NPV 100%, and K = 0.92). The results showed that subjective luteal vascularization assessment by color Doppler US was a reliable tool for early pregnancy diagnosis in goats and can be efficiently used as early as 21 days post-breeding. Universidade Federal Fluminense, Infra-LabPesq/PROPPI, FAPERJ and the dairy goat farm Capril Vale das Amalthéias.

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Effect of a single bST administration on follicular dynamics and ovulation during an interovulatory cycle in sheep

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Insulin and IGF1 are major peripheral signals from the metabolic axis, and their plasma levels are seen as important for sound follicular function and fertility due to their ability to synergize with gonadotropins during terminal follicular development. Bovine somatotropin (bST) increases plasma IGF1 and insulin in sheep (Carrera-Chávez *et al*., 2014. Anim Reprod Sci. doi.org/10.1016/j.anireprosci.2014.10.009) and was used in this study to assess its influence on follicular dynamics and ovulation as markers of ovarian function. The study used Highlander ewes housed in collective pens linked to a paddock. In Exp 1, 15 ewes were estrous-synchronized (P_4-6 days + PGF2α d -6) and then divided into bST-treated (50 and 100 mg, Lactotropin®; n=5 ewes each) and untreated groups to assess the activity of bST through plasma IGF1 (RIA). In Exp 2, 12 ewes were synchronized and at d 6, they were grouped into a bST-treated (100 mg) and an untreated control (n=6 each) group. Starting at d 6 and up to 22 d after ovulation, each ewe was subjected to daily US (10mHz probe) to assess follicular and luteal (CL) dynamics and ovulation. The ultrasound included general ovarian features (interovulatory interval, ovulatory follicle and CL diameters and ovulation rate) and specific follicular wave features (number and duration of waves, growth rate, persistency and large follicle diameter). In Exp 3, the effect of bST was assessed under the restriction of anestrus, with estrus-synchronized ewes allocated to a bST group (100 mg at the start of the treatment), an eCG group (400 IU at the end of synchronization, d0) and a control group (n=15 each), the last two exposed to a male effect. At 36 h after d 0, all ewes were induced to ovulate with GnRH (4.2 µg buserelin). Plasma estradiol was measured at d 0 and at GnRH treatment from 6 ewes in each group (RIA). Results showed that bST increased plasma IGF1 compared to controls by day 3 ($P<0.01$) and kept levels for at least 7 days before recovering pre-treatment levels. The IGF1 increase after bST doses was similar in terms of a day-to-day and AUC comparisons ($P>0.10$). In Exp 2, results showed that bST-treated ewes preserve all general and specific markers considered to monitor ovarian function in the study ($P>0.10$). However, in Exp 3, results showed that bST preserved the number, diameter and ovulatory potential of large follicles after synchronization, but reduced the estradiol production in term of mean plasma profiles ($P=0.03$) and in terms of ewes producing >10 pg/mL at GnRH administration ($P=0.04$), and also reduced the CL diameter at day 10 compared to eCG-treated ewes ($P=0.003$). Collective results in the study suggest that ewes respond to bST administration, and at high dosage it has no influence on follicular dynamics and ovulation during the reproductive season, but due to its ability to reduce E_2 production, bST may compromise ovarian function during the anestrous season.

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Progesterone priming during follicular growth of Wave 1 improves pregnancy rate after FTAI in sheep

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The objective was to evaluate the effect of a short progesterone (P4) supplementation during preovulatory follicular growth of Wave 1 on pregnancy rate after fixed-time artificial insemination (FTAI) in ewes. The experiment was conducted during breeding season (33º S, Uruguay) on 804 multiparous cycling ewes that received three doses of prostaglandin (PG) F2α analogue (125 µg of cloprostenol, Ciclase DL, Zoetis, Argentina) im 7 d apart. This treatment (so-called Synchrovine protocol; Menchaca and Rubianes. 2004. New treatments associated with Timed Artificial Insemination in small ruminants. Reprod Fertil Dev, 16:403-414) is effective to synchronize the ovulation on average 60 h after the second PG treatment (ovulation occurs in a narrow window of 24 h). The Day 0 was defined at the time of the second PG dose, and 72 h later (i.e., after expected ovulation). The ewes were allocated into two experimental groups to receive (n=409) or not (n=395) a P4 treatment from Day 3 to Day 7 (i.e., in the early luteal phase during follicular growth of Wave 1). Progesterone was administered by using an intravaginal P4 device (0.3 g, DICO, Syntex, Argentina). This experimental model was recently validated in our laboratory to compare females with high vs. low serum P4 concentrations (~5.0 vs. 1.5 ng/mL, respectively; P<0.05; Cuadro et al., 2018. Serum progesterone concentrations during FSH superstimulation of the first follicular wave affect embryo development in sheep. Theriogenology; submitted). In both groups, all females received a third PG treatment on Day 7 to synchronize ovulation. Half of the flock with or without P4 treatment received cervical (n=387) or intrauterine (n=417) FTAI in a 2x2 factorial design, performed 48 h or 54 h after the last PG respectively, with 150 or 70 millions of spermatozoa, respectively. Pregnancy diagnosis was determined by transrectal ultrasonography 35 d after insemination. Statistical analysis was performed by GLMM. Pregnancy rate was improved by P4 supplementation (45.5%, 186/409 vs. 37.0%, 146/395; P4 vs. no P4 treated ewes, respectively; P<0.05) and by intrauterine insemination (45.1%, 188/417 vs. 37.2%, 144/387; intrauterine vs. cervical FTAI, respectively; P<0.05), without interaction between treatment and insemination method (P=NS). The number of fetuses/pregnant ewes was not affected by the P4 treatment and insemination method (P=NS). In conclusion, a short priming with P4 during the preovulatory follicular growth of Wave 1 increases pregnancy rate in sheep.

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Effect of pre-maturation culture using EGFR kinase inhibitor on embryo development, lipid metabolism and gene expression profile

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The epidermal growth factor receptor (EGFR) pathway is directly involved in oocyte meiotic resumption induced by a gonadotropic stimulus. Here, we used an EGFR kinase inhibitor (AG1478) to inhibit meiosis resumption in bovine oocytes and assessed the competence of such oocytes for embryonic development, lipid metabolism and gene expression. COCs (n=926) were pre-matured (PM) during 8h in TCM-199 with 1 μM AG1478 (AG group). Next, COCs were washed for meiotic inhibitor removal and cultured for 22h in IVM medium (TCM-199 with bicarbonate, 0.5 mg/mL FSH, 100 IU/mL hCG, and 10% FCS). The control group (C group) was only cultured for 22h in IVM medium. After that, the matured oocytes were fertilized and cultured until day 7 or 9 to evaluate blastocyst and hatching rate, respectively. On day 7 of culture, expanded blastocysts (n=32) were collected and the abundance of 86 transcripts was assessed by RT-qPCR using a microfluidic platform (BioMark HD System™, Fluidigm®). Relative mRNA abundance was calculated by ΔCt (target genes were normalized by two reference genes: *ACTB* and *HPRT1*). Total cell number and neutral lipid content was evaluated on day 7 expanded blastocysts (n=31) and on day 9 hatched blastocysts (n=63), by Nile red and DAPI stain, respectively. Data with normal distribution were analyzed by t test, and non-parametric data were analyzed by Mann Whitney's test ($P<0.05$). Blastocysts rate on day 7 (40.8%, averaged) and hatching rate on day 9 (77.4%, averaged) were unaffected by treatment ($P>0.05$). Similarly, treatment did not affect ($P>0.05$) the total cell number on day 7 (C 121.5±7.1 and AG 116.6±6.3) and on day 9 (C 198.9±9.04 and AG 180.1±6.9). Abundance of several transcripts was up-regulated ($P<0.05$) in AG group, including genes related to embryo development and quality (*NANOG* and *RPLP0*), epigenetic regulation (*H2AFZ*), metabolism (*HMGCS1*), lipid metabolism (*ACSL1*, *GPAT3*, *FADS2*, *FASN* and *FDX1*), apoptosis (*BID*) and stress response (*GPX4*and *HIF1A*). Arbitrary lipid content in pixels did not differ between treatments ($P>0.05$) on day 7- (C 1.1±0.1 and AG 0.9±0.1) and on day 9-embryos (C 0.3±0.0 and AG 0.4±0.0). Our results indicate that PM culture with an EGFR kinase inhibitor prior to IVM does not affect the subsequent embryonic development until blastocyst stage and hatching rate besides the increase of mRNAs related to embryo development and quality. The up-regulation of transcripts related to lipid metabolism did not reflect in an increase on embryonic lipid content. Also, the gene expression pattern suggests better embryonic stress resistance, but we still need to investigate the impact of these changes on conception rate and pregnancy development to term. In conclusion, the PM culture with AG1478 was not detrimental to embryo development and lipid metabolism. Financial support: FAPESP (#2015/06733-5 and #2012/50533-2) and CNPq (#307416/2015-1).

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Autologous nuclear transfer in cattle does not increase cloning efficiency

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Correct nuclear-cytoplasmic interactions are vital to generate viable offspring following somatic cell nuclear transfer (SCNT). In standard (allogenic) SCNT, donor nuclei are introduced into foreign oocyte cytoplasm harbouring mitochondrial (mt)DNA from genetically unrelated animals. The two sources of mtDNA can result in clones displaying heteroplasmy, which is contrary to the unimaternal inheritance of mtDNA during mammalian sexual reproduction. Oocytes from specific maternal lines (Brüggerhoff *et al.* 2002, *Biol. Reprod*. 66:367) or with the same mitochondrial haplotype as the donor cell (Yan *et al*. 2010, *BMC Dev. Biol*. 10:31) are reported to be beneficial for development of SCNT embryos. To avoid any mismatch between nuclear and mitochondrial genomes, we investigated whether fusing cytoplasts and somatic cells from the same cow (autologous SCNT) could improve development. Cumulus oocyte complexes (COCs) were aspirated from pairs of ovaries from 16 commercially slaughtered cows, over six replicates. COCs were matured *in vitro* for 18-20h, keeping each cow separate. After maturation, metaphase II oocytes from each cow were allocated equally to either autologous or allogenic SCNT treatments and reconstructed using a zona-free methodology. Bovine follicular cells, collected from each cow during oocyte aspiration, were cultured in medium with 0.5% fetal calf serum for 18h before autologous SCNT. For allogenic SCNT, a proven ovarian follicular donor cell line from an unrelated cow was used, also after 18h serum starvation. Reconstructed embryos were cultured in modified synthetic oviduct fluid media for seven days. Selected SCNT embryos, plus *in vitro* fertilised (IVF) controls, were transferred singularly to synchronized recipients and pregnancy monitored until fetal recovery on Day 117-118 of gestation. Values are presented as mean ± SEM. Statistical significance was determined using Fisher's exact test for both *in vitro* embryo development (expressed as a percentage of embryos cultured) and *in vivo* survival. On average, 40±4 COCs were recovered per cow. There was no difference in the rate of donor cell fusion between the two treatments (92±2%). Rates of cleavage were also similar (92±3% *v*. 94±2% for autologous and allogenic SCNT, respectively). However, the rate of *in vitro* development to transferable quality (grade 1-2) late morulae to expanded blastocyst-stage embryos on Day 7 following autologous SCNT was lower than for allogenic SCNT (53/223 = 24±4% *v*. 74/211 = 33±4%; P=0.05). *In vivo* survival on Day 30 of gestation was similar between autologous and allogenic SCNT (3/8 = 38% *v*. 2/9 = 22%) and tended to be less than IVF (5/9 = 56%). Losses by Day 117-118 were greater in the autologous and allogenic SCNT groups compared to IVF, with embryo survival being 13%, 11% and 44% respectively. Average fetal weight for SCNT fetuses was similar to IVF (698±6g *v*. 768±39g). In conclusion, our finding that autologous SCNT results in lower development compared to standard allogenic SCNT is contrary to previous reports (Yang *et al*. 2006, *Reprod*. 132:733). Nonetheless, specific nuclear-mtDNA haplotypes might be beneficial for embryo development and subsequent health and phenotypic performance of clones. Supported by MBIE, NZ.

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Flow cytometry analysis of the mitochondrial membrane potential using two incubation times of JC1 probe in fresh bovine semen

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Sperm mitochondria is located in the midpiece and shows different characteristics from somatic mitochondria. In addition, it plays an important role in sperm function including ATP production, maturation, capacitation, and apoptosis. Furthermore, mitochondria are key structures in sperm function suffering major alterations during the cryopreservation process. Mitochondrial membrane potential (MMP) is a fertility marker that can be asses by the use of the 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), a lipophilic cationic probe that exhibits the MMP depending on its accumulation, showing a green fluorescence in the inactive or depolarized mitochondria ($\Delta\Psi_m$ < 80-100 mV), and an orange fluorescence when mitochondria exhibit high membrane potential. Despite the fact that MMP has become a routine analysis, remarkable variability in the results have been reported when used different times of incubation. We hypothesized that MMP would be a better marker of fertility after a shorter incubation period. The aim of this study was to evaluate differences in MMP between a 15 or 30 min incubation protocol of JC1 using fresh bovine semen. The MMP was assessed by using JC-1 dye (T4069, Sigma-Aldrich, Saint Louis, MO, EUA; excitation, 488 nm; emission, monomers 525–530 nm and aggregates 590 nm). The semen was collected by electroejaculation from 3 bulls in 5 ejaculates each, the bulls were classified as satisfactory potential breeders according to the standards of the Brazilian College of Animal Reproduction (CBRA). After collection, 3 μL of JC-1 (153 μM) was added to 500 μL of sperm diluted in PBS 0.1 M (5 x 10^6 spermatozoa/mL) and incubated for 15 or 30 min at 37ºC. The samples were analyzed by flow cytometer (BD FACSVerse™ - Beckton-Dickinson®, SunnyVale, CA, USA). Data were analyzed using linear mixed models with repeated measures in time, and bulls were considered as random effect. Correlations among variables were evaluated by Pearson's simple correlation coefficient. The MMP percentage was higher (P < 0.05) in 15 than 30 min of incubation time (49.1 ± 2.1 vs 40.3 ± 2.1, respectively). The average sperm motility was 73.1 ± 2.9%; with minimum and maximum values of 55% and 90%, respectively. The MMP evaluated in both times were highly correlated between them (r = 0.90). However, there was no correlation between sperm motility and the MMP. Thus, the decrease of MMP in the 30 min protocol could be explained by the longer incubation time. Therefore, 15 min may be a more suitable incubation period for a MMP analysis in bovine fresh semen. This study was funded by CNPq, CAPES, and FAPEMIG.

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Follicular emergence in zebu cows actively immunized against GnRH

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Cystic ovarian disease (COD) is a frequent problem in cows intensively used as oocyte donors for in vitro embryo production (IVEP) (Faria *et al*. 2017 Anim Reprod 14:782). Many of these animals fail to respond to conventional treatments with LH or GnRH analogs, and oocyte yield is compromised. Previous studies demonstrated that active immunization against GnRH resulted in remise of COD, without affecting oocyte development potential in vitro (Faria *et al*. 2018 Reprod Fertil Develop 30:190). The aim of the present study was to characterize the follicular dynamics in cows immunized against GnRH. Nelore (*Bos indicus*) cows were assigned to control (n=5) or treatment (n=8) groups. Cows in the treatment group received two SC injections of 1.0 mL anti-GnRH vaccine (Bopriva, Zoetis, Brazil), 28 d apart (weeks 0 and 4). Effectiveness of immunization (E-IM) was confirmed when no follicles ≥ 5.0 mm were detected on the ovaries, only cows with E-IM were used (n=5). The control group underwent a conventional follicular wave synchronization protocol, based on the insertion of 1 g intravaginal progesterone device and injection of 2 mg estradiol benzoate. Both groups were submitted to transvaginal ultrasound-guided follicle aspiration (OPU), aiming to remove all follicles larger than 2 mm after E-IM (treatment group) or 5 d after follicular wave synchronization (control group). Transrectal ultrasonography was performed daily to evaluate the number and size of follicles emerging after OPU. SAS MIXED procedure with repeated measure statement was used to evaluate the effects of treatment, time, and interactions on ovarian endpoints; and SAS GLM procedure to analyze follicular dynamics data. Results are shown as mean±SEM. There was no difference in the follicle population 96 h after OPU between treatment and control groups (10.8±2.8 vs 13.8±1.5, respectively; P>0.05). However, only in the control group the number of follicles reached a plateau later during the evaluation period (3.3 ± 1.0^{a}, 9.0 ± 1.4^{ab}, 15.0 ± 3.0^{b}, and 13.8 ± 1.5^{b} follicles at 24, 48, 72 and 96 h, respectively; P<0.01). In the E-IM cows, no follicle grew over 4 mm, so the diameter of the largest follicle differed between groups from 48 h on (3.1±0.1 vs 5.0±0.4mm, 3.0±0.0 vs 6.8±0.8mm, and 3.0±0.0 vs 8.5±0.2 mm at 48, 72, and 96 h after OPU for treatment and control groups, respectively). There was no difference (P>0.05) in the size of the largest and second largest follicles in the treatment group throughout the evaluation period, while in the control group follicle deviation began at 72 h, when the further dominant and the second largest follicles were 6.8±0.8 and 5.5±0.4 mm in diameter, respectively. In summary, the present results suggest that the lack of physiological FSH stimulus induced by immunization against GnRH does not impair, but delay follicle emergence. The lack of follicular dominance and the slower emergence may partially overcome the expected negative effects of the lack of physiological FSH support in immunized cows, and explain the maintenance of oocyte development potential and the good results of IVEP observed in previously studies (Faria *et al*., 2018). Zoetis, UNB, FAP-DF and CAPES.

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Effects of sire breed and season on in vitro embryo production and subsequent pregnancy rates

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Climatic factors are known to influence reproductive efficiency in cattle. Thus, seasonality shall be considered in the development of breeding strategies to increase the reproductive performance of herds. The aim of this study was to analyze the influence of the season and sire breed in oocyte cleavage rate, blastocyst production and pregnancy rates. These traits were retrospectively analyzed using data from in vitro embryo production (IVEP) performed after OPU (n=3,371) sessions in Girbreed (*Bos indicus*, n=805) donors. The cumulus-oocyte complexes were in vitro matured and then in vitro fertilized with semen of Gir (n=27) or Holstein (n=52) sires in order to produce purebred Gir or F1 (Gir x Holstein) embryos. Data was analyzed by Kruskal-Wallis test followed by Dunn test. The effects of season and sire's breed were considered in the analysis. The highest overall cleavage rate ($P<0.05$) was found during the Spring (60.45±0.76%) and the lowest during the Summer (55.89±0.65%). Higher blastocyst production ($P<0.05$) was found during Spring (25.4±0.7%) than Summer (22.0±0.56%) and Autumn (21.8±0.55%). Fertilization with Holstein semen increased the cleavage rate (58.7±0.47%) compared to Gir semen (56.4±0.55%) but no difference ($P>0.05$) was found for blastocysts production. There was a season x sire breed interaction. Cleavage rate was higher in Winter for F1 embryos than for purebred embryos (61.53±1.08% vs 55.23±1.3%; $P<0.05$). No difference ($P>0.05$) between sire's breed was found for the other seasons. Cleavage rate was lower ($P<0.05$) during Summer and Autumn than during Winter (57.2±1.05% and 57.42±0.81% vs 61.53±1.08%, respectively; $P<0.05$) when fertilization was performed with Holstein semen. No difference ($P>0.05$) among seasons was found when fertilization was performed with Gir semen. There was no difference ($P<0.05$) between sire's breed on blastocyst production within seasons; however, lower ($P<0.05$) blastocyst rate was found during Summer with Gir semen (22.11±.075%) and during Autumn with Holstein semen (21.23±0.71%) than during Spring (26.45±1.3% and 24.9±0.85% for Gir and Holstein semen, respectively). The pregnancy rate of embryos fertilized with Holstein semen (48.56±1.87%) was higher ($P<0.05$) than those fertilized with Gir semen (38.8±2.01%) during the Winter but no difference between sire's breed ($P>0.05$) was found in the other seasons. Pregnancy rate with Gir semen was lower during Winter than during Summer and Autumn (41.79±1.74% vs 44.4±2.13%, respectively; $P<0.05$), whereas no difference ($P>0.05$) between seasons was found with Holstein semen. In conclusion, the effect of season on oocyte competence and further in vitro embryo development and pregnancy rate can be influenced by sire's breed when oocytes are derived from Gir donors. Summer and Autumn are the worst season to produce blastocyst with Gir and Holstein semen, respectively. Interestingly, low pregnancy rate is achieved during Winter with embryos fertilized with Gir semen, even lower than embryos with Holstein semen. The understanding of such differences can be useful to reduce the effect of seasons on in vitro embryo production. Fazendas do Basa, Embrapa, FAPEMIG.

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Ultrasonographic cervical evaluation in Lacaune ewes subjected to transcervical embryo collection

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The degree of completeness and interdigitation of the cervical rings affects cervical passage, which is a necessary step for transcervical embryo collection. This study assessed the use of cervical ultrasonography (US) to select the most suitable animals for transcervical embryo collections. Lacaune ewes (n=24) were synchronous estrus-induced with medroxyprogesterone acetate sponges (60 mg, Progespon®, Syntex, Buenos Aires, Argentina) for nine (n=12) or six days (n=12), 37.5 µg d-cloprostenol i.m. (Prolise®, Tecnopec, São Paulo, Brazil) and 400 IU eCG i.m. (Novormon 5000®, Syntex) 24 h before device removal. After sponge removal, ewes were checked twice daily for estrus detection. At 12 h after the onset of estrus, the cervix was longitudinally evaluated by transrectal US (8.0 MHz, Mindray M5VET®, Shenzen, China), to count the number and disposition of rings to classify the degree of misalignment in three scores: grade I – lined up, grade II – intermediate, and grade III – misaligned (Fonseca, CT 45, Embrapa Caprinos e Ovinos, 2017). Cervical passage was performed at day 7 of estrus cycle by transcervical technique (Fonseca *et al*., Theriogenology, 86:144-151, 2016). Cervical transposition rate was tested by Fisher's Exact test, and the number of rings counted by US or during cervical passage were evaluated by ANOVA and paired t-test at 5% significance, using SPSS Statistics (IBM®Inc., Chicago, USA). Association of variables was evaluated by Pearson correlation. The percentage of ewes for each degree of cervical score on US during estrus was: grade I 20.8% (5/24), grade II 20.8% (5/24) and grade III 54.5% (14/24). Cervical surpass was possible in 100%(5/5) of grade I, 80%(4/5) of grade II and 79.5% (11/14) of grade III ($P<0.05$). The number of rings counted during procedure (6.5±0.2) or by US (6.1±0.1) did not differ ($P>0.05$). However, the association of these variables was poorly correlated ($r=0.20$, $P>0.05$); in 20% the count in US was overestimated and 35% underestimated related to count in procedure. In 45% of ewes, the number of rings was the same in both by US and the procedure, and in 85%, the difference in the number did not exceed one ring. In conclusion, US cervical evaluation allows estimating the number of rings, and the ranking of animals by US cervical misalignment, making possible to select animals suitable for transcervical embryo collection.
Financial Support: EMBRAPA (Project SUPEROV / 02.13.06.026.00.04). CAPES and Vicente M. Munhoz (Cabanha Val di Fiemme).

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Efficient transcervical embryo collection in synchronous estrus-induced Lacaune ewes

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Induction of cervical dilation allows efficient non-surgical embryo recovery, depending on the sheep breed, and thus its usefulness needs to be assessed in each breed. This study checked the efficiency of two estrus induction treatments and the feasibility of transcervical embryo recovery in Lacaune ewes. Ewes (n=28) received medroxyprogesterone acetate sponges (60 mg, Progespon®, Syntex, Buenos Aires, Argentina) for nine (G9, n=14) or six (G6, n=14) days. Both groups received 37.5 µg d-cloprostenol i.m. (Prolise®, Tecnopec, São Paulo, Brazil) and 400 IU eCG i.m. (Novormon 5000®, Syntex) 24 h before sponge removal. Ewes were checked for estrus twice daily and were naturally mated by fertile rams (6:1 ratio) while in estrus. Ultrasonography monitoring (Mindray M5VET®, Shenzen, China - 8.0 MHz) was performed twice daily from sponge removal to ovulation detection and 5 days after to count the number of corpora lutea (CL). Ewes received 37.5 µg d- cloprostenol and 1 mg estradiol benzoate i.m. (Sincrodiol®, OuroFino, Cravinhos, Brazil) 16h before plus 50 IU oxytocin (Ocitocina forte UCB®, São Paulo, Brasil) i.v. 20 min before uterine flushing. Embryo collection was performed at day 6 after ovulation by transcervical technique (Fonseca et al., Theriogenology, 86:144-151, 2016). Qualitative data were tested by chi-square test, and quantitative data were evaluated by ANOVA and t- test at 5% significance, using SPSS Statistics (IBM®Inc., Chicago, USA). Estrous behavior rate was 85.7% for both treatments. There was no difference ($P>0.05$) for interval to estrus and estrus duration, respectively for G9 (37.0 ± 2.3 and 22.0± 2.9 h) *vs* G6 (42.0± 7.0 and 25 ± 2.3 h). Ovulation rate was 100% (G9) and 92.9% (G6) ($P>0.05$). The number of CL was higher ($P<0.05$) in G9 (2.9 ± 0.3) than G6 (1.9 ± 0.3). Overall, cervical transposition was possible in 85.7% (24/28) of ewes and 78.6% (22/28) were successfully collected for embryos. The time to cervical passage was lower ($P<0.05$) in G9 (3.6 ± 0.5) than G6 (6.4 ± 1.2 min). The total procedure time in G9 (23.8 ± 1.8) and G6 (26.5 ± 2.5 min) did not differ ($P>0.05$). In G9 ewes, the number of recovered structures was higher (1.6 ± 0.4 *vs* 0.6 ± 0.2; $P<0.05$) and there was a tendency ($P<0.10$) to recover more viable embryos (1.2 ± 0.4 *vs* 0.4 ± 0.2). Possibly, eCG dosage promoted multiple ovulations (> 2) in 50% of the ewes that ovulated, and two ewes had five ovulations. This is the first report in the literature of transcervical embryo collection in the Lacaune breed. In conclusion, both estrous induction treatments showed a high rate of estrus and ovulation, but the dosage of 400 IU of eCG seems excessive. The protocol for cervical relaxation was efficient to allow the transcervical embryo recovery of Lacaune ewes. CAPES and Vicente M. Munhoz (Cabanha Val di Fiemme). Financial support: EMBRAPA (Project 02.08.02.005.00.04) and Fapemig (Project CVZ-PPM 00201-17).

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Equine chorionic gonadotropin (eCG) impacts the transcriptional profile of genes involved with competence of *cumulus*-oocyte complexes and embryo quality in superstimulated Nelore cows

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The impact of superstimulatory protocols using gonadotropins such as FSH or eCG on competence of *cumulus*-oocyte complexes (COCs) and embryo quality remains unknown. Thus, this study aimed to quantify the mRNA abundance of genes related to quality of COCs and embryos from Nelore cows (*Bos taurus indicus*) submitted to ovarian superstimulation using FSH (FSH group, n=10) or replacement of the last two doses of FSH by eCG (FSH/eCG group, n=10). All animals were slaughtered 12 h after removal of the intrauterine progesterone device. Ovarian antral follicles from superstimulated groups (>9 mm) were aspirated for COC gene expression and *in vitro*-embryo production. Total RNA was extracted and reverse transcribed from each sample (n=3 samples/type); sample were pools of 20 oocytes, their respective *cumulus* cells and a pool of 5 blastocysts (BL). The mRNA abundance of 96 genes related to COC development and 96 genes related to embryo quality were measured by RT-qPCR using the microfluidic platform BioMark™ HD system (Fluidigm®) and the expression of target genes was normalized by geometric mean of three reference genes (*ACTB, GAPDH*, *PPIA*). Bioinformatic analysis was performed using R and Bioconductor software and the gene expression heatmaps were generated using the average of calibration samples to normalize each group. Genes and groups were sorted by hierarchical clustering using the "heatplot" function from the "made4" package. Heatmap analysis successfully clustered similarities and differences between groups and showed a pattern of expression among genes. Means were compared with Student's t-test and differences were considered significant when P=0.05. In the oocytes from the FSH/eCG group, 36 genes involved with lipid metabolism (*ACACA, ACSL3, ACSL6, CPT1B, DGAT1, ELOVL5*, *SCD*), oxidative stress (*CAT, SOD1, TXNRD1*), transcriptional control (*DNMT1, DNMT3A, DNMT3B, HP1*) and cellular development (*BMP15, GDF9, H1FOO, IMPDH1, IFG1R, COX2)* were up regulated. In *cumulus* cells, *CPT1B* and *VCAN* mRNA abundancewas higher in the FSH/eCG group whereas *SREBF1-2*, *ADCY3, NDUFA1, GPX4* and *NPR3* demonstrated a lower abundance. In BLs, *CPT1B*, *SLC2A3* and *BID* was higher in the FSH/eCG group and *SREBF1-2* and *DDIT3* showed a lower abundance. In summary, the present findings suggest that use of FSH combined with eCG could increase the quality and competence of blastocysts and COCs by regulation of genes related to oxidative stress protection, oocyte competence, and especially by modulation of genes involved with lipid metabolism in COCs and bovine embryos. Supported by São Paulo Research Foundation (FAPESP), grants #2011/50593-2, 2012/50533-2, and2013/11480-3.

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Evidence that pregnancy-associated plasma protein A (PAPP-A) plays a role in bovine *in vitro*-embryo production

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The pregnancy-associated plasma protein A (PAPP-A) is able to disrupt IGF's association with IGF binding proteins (IGFPBs) to allow the increase of free IGF. The aim of the present work was to access the effects of PAPP-A during oocyte *in vitro* maturation (IVM) on IGF1 bioavailability, embryo yield, post vitrification survival and transcriptional profile of blastocysts (BLs). The cumulus-oocyte complexes (COCs) from a local abattoir were submitted to IVM for 24 h with TCM199, serum free medium supplemented with PAPP-A (100 ng/mL; P100 group) or not (P0 group). The maturation medium was collected for quantification of free IGF1 by ELISA. The matured COCs were submitted to *in vitro* fertilization followed by *in vitro* embryo culture for 7 d. On day 3 and 7, the cleavage and BL rates were evaluated. On day 7, BLs (3 pools/group; n=5 BLs/pool) were used to analyze the transcriptional pattern of 96 genes and to verify the post vitrification survival. The free IGF1 concentration was transformed to fold change and cleavage and BL rates were calculated as percentage and transformed to arcsine. The mRNA abundance of target genes was normalized by geometric mean of reference genes and data were transformed to fold change. Bioinformatic analysis was performed using R and Bioconductor softwares. Gene expression heatmaps were generated using the average of P0 group levels to normalize each group. Genes and groups were sorted by hierarchical clustering using the "heatplot" function from the "made4" package. The means were compared with t-test using JMP software (SAS Institute Cary, NC). Differences were considered significant when P=0.05. The PAPP-A addition increased by 1.27 fold the free IGF1 concentration in IVM medium but did not impact the cleavage rate or BL yield. Moreover, the rate of post vitrification survival did not differ (P>0.05) after 24 h and was 54% for the P0 group and 43% for the P100 group. The heatmap analysis was able to cluster similarities and differences between groups and also to show the pattern of expression between genes. In BLs, the mRNA abundance of *DMNT3A*, *CASP9*, *CPT2*, *TFAM* and *KRT8* were up regulated in the P100 group. On the other hand, *ATF4*, *IFITM3* and *CASP3* were down regulated in the P100 group. In conclusion, the addition of PAPP-A during IVM provided an increase of free IGF1, and although it did not influenced the BL rate or *in vitro* post vitrification survival, the greater amount of free IGF1 could impact positively the embryo competence by modulation of genes involved with apoptosis, mitochondrial biogenesis, DNA methylation, specially, embryonic lipid metabolism.

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In vitro embryo production in Angus breed with semen sorted for male: effect of semen preparation method

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In Brazil, in vitro embryo production (IVEP) has become the technique of choice in cattle to increase the number of offspring from high-value and genetically superior donors. However, animal breeding programs are a limited market for embryo transfer (ET) services. In the past 10 years, ET numbers in Brazil have increased mainly due to the use of IVEP in association with X-sorted sperm to supply crossbred heifers for dairy production. There is also a great potential for large-scale production of F1 calves in beef, however, there are few reports of the use of Y-sorted sperm for IVEP. In this regard, we hypothesized that semen preparation is a critical step for in vitro fertilization, as observed with X-sorted sperm. The aim of the present study was to evaluate the effect of semen preparation procedure on IVEP, using Y-sorted sperm from Angus sires. Cumulus-oocyte complexes were obtained from ovaries of Nelore cows at the slaughterhouse and in vitro matured in TCM199, following standard procedures. Matured COC were randomly allocated to be in vitro fertilized with a commercially available Y-sorted sperm from an Angus sire prepared either by: a) centrifugation in a Percoll gradient (n = 921 oocytes) or washing with IVF medium (n = 3,112 oocytes). After IVF, the presumed zygotes were cultured in SOF medium at 95% humidity at 38.8°C. A mixed system was used for in vitro embryo culture (IVC), in which the first 72 h used 5.5% CO_2, while the last 96 h of IVC used a defined atmosphere of 5.5% CO_2 and 5% O_2. Cleavage rate, embryo rate at days 7 and 8, and percentage of grade I embryos were analyzed using the Chi-square test. There was no effect of semen preparation method on cleavage rate or on the blastocyst rate and percentage of grade I embryos at D7 (52.0% vs 49.5%, 22.3% vs 20.5%, and 76.8% vs 75.1% for washing with IVF medium and Percoll, respectively; $P>0.05$). However, washing with IVF medium increased the blastocyst rate at day 8 and the overall embryo rate compared to Percoll (8.5% vs 6.4% and 25.6% vs 21.8%, respectively; $P<0.05$). The results show that the preparation method used for Y-sorted Angus sperm has significant effects on IVEP in beef cattle. Thus, large-scale IVEP programs for F1 production in beef cattle should optimize procedures, considering the particularities of each breed and kind of semen used. National Council for Scientific and Technological Development (CNPq) and FAPEMIG.

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The effect of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on cumulus expansion and glucose metabolism of *in vitro* matured bovine cumulus-oocyte complexes

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Cumulus cells play a pivotal role during oocyte maturation and accomplishment of its developmental competence. During development of bovine ovarian follicles, cumulus cells undergo expansion, which is regulated by various intracellular signaling cascades. It also depends on the synthesis of the extracellular matrix, the main component of which - hyaluronic acid - is synthetized from various compounds includingglucosamine and glucose. Moreover, glucose is utilized for substrates that oocytes can then use for energy production. It was also shown that $PGF_{2\alpha}$ takes part in many reproductive processes. Taking all of the above into consideration, the aim of present study was to examine the role of $PGF_{2\alpha}$ in cumulus expansion, glucose metabolism and expression of genes involved in these processes. Cumulus-oocyte complexes were aspirated from subordinate follicles and matured in vitro in the presence or absence of $PGF_{2\alpha}$ (700 pg/mL) for 24 h. Following maturation, cumulus expansion was visually assessed and cumulus cells were used for gene expression analysis. The mRNA expression of amphiregulin (AREG), epiregulin (EREG), betacellulin (BTC), ADAM metallopeptidase domains (ADAMs), epidermal growth factor-like family receptor (EGFR), tumor necrosis factor alpha-induced protein 6 (TNFAIP6), prostaglandin- endoperoxide synthase 2 (PTGS2), pentraxin 3 (PTX3), hyaluronan synthase 2 (HAS2), glutaminefructose-6-phosphate transaminases (GFPTs), glucose transporters (GLUTs), phosphofructokinase (PFK), and lactate dehydrogenase (LDH) was determined by real-time PCR. The data were analyzed using student's t-test (GraphPad PRISM 6.0. Software). Cumulus expansion was similar in the control and $PGF_{2\alpha}$-treated group. $PGF_{2\alpha}$ stimulated mRNA expression of EREG, EGFR, TNFIP6, PTX3, GLUT4 and PFK and reduced transcript abundance of GLUT1 and LDH. The results suggest that $PGF_{2\alpha}$ supplementation of the maturation medium influences the expression patterns of genes associated with oocyte maturation. Moreover, we suspect that $PGF_{2\alpha}$ might facilitate oocyte maturation in cows. Supported by Grants-in-Aid for Scientific Research from the Polish National Science Centre: 2015/17/B/NZ9/01688 and the grant of KNOW Consortium "Healthy Animal - Safe Food", MS&HE Decision No. 05-1/KNOW2/2015.

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Influence of parity category on conception rate of Holstein females submitted to timed artificial insemination in semiarid conditions

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This study was conducted at the Experimental Station of São Bento do Una (ESSBU/IPA) Pernambuco, Brazil, (Latitude 08 31' 35'' and Longitude 036 27' 34.8"), with the objective to evaluate the influence of category on conception rate of timed artificial insemination (TAI) in Holstein females in semiarid conditions, presenting in the period, mean temperature-humidity index (THI) of 63.5. For the study, 368 cyclic females were used, up to 60 days in milk (DIM), production with an average of 24.8 kg/milk/day, and age ranging from 28 to 108 months for primiparous and pluriparous. The nulliparous females were cyclic at 12 months of age. The animals were kept in semi-intensive system, receiving a diet composed of cactus pear (Opuntia ficus-indica Mill), sorghum silage (Sorghum bicolor (L) Moench) and protein concentrate with 18% of crude protein (CP) besides mineral supplement and water ad libitum. Females used were submitted to gynecological examination by rectal palpation, being randomly distributed into three groups G1 (nulliparous), G2 (primiparous) and G3 (pluriparous). All females of the three groups were treated with an intravaginal device containing 1.9g of progesterone on day 0 (D0) and 2mg of estradiol benzoate (EB). On day 7 (D7) females were treated with 0.530 mg of sodium cloprostenol and 300 IU eCG, and on day 8 (D8) intravaginal devices were removed. Twenty-four hours after device removal (D9), 1mg EB was given and 54 hours after device removal all cows were fixed-time AI at day 10 (D10) with semen from Holstein bulls of a reputable center. All females were subjected to ultrasound examination for pregnancy detection 30 days post-AI. The data were subjected to statistical analyzes carried out by means of computational SAS version 9.1.3. The conception variable was assumed to have a binomial distribution (P = pregnant; N = Not pregnant). Initially a descriptive analysis was performed to observe the percentages of successes and failures and the chi-square test was used to verify if there were significant differences through the FREQ procedure of SAS (2003). The conception rate was 50.2% in G1, 59.6% in G2 and 44.7% in G3, the result of G2 being superior ($P<0.01$) to the other groups. In conclusion, the primiparous presented better conception results with the TAI protocol used. CNPq/FINEP.

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Effect of GnRH on day 5 or 7 on pregnancy rate in dairy cows after AI with sex-sorted sperm

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Reproductive performance in dairy cows after AI with sex-sorted sperm is poor. The aim of this study was to evaluate the effect of a single administration of buserelin acetate (AB), an analogue of the gonadotropin release hormone (GnRH), on pregnancy rate per AI with sex-soted sperm in lactating dairy cows. A total of 162 cows were assigned to one of three treatments. T1 (n=54): Control; T2 (n=53): 0.05 mg AB on day 5 post AI; T3 (n=55): 0.05 mg AB on day 7 post AI. The same technician inseminated cows, 10 to 12 hours after estrus detection and with sex-sorted sperm (Select Sires) from three bulls using the rectal vaginal technique. The animals were observed twice per day for heat detection, and pregnancy diagnosis was done 30 days after AI with a portable ultrasound (ESAOTE, The Netherlands) and a 5.0 MHz transducer. Data were analyzed by Chi square. Pregnancy rate of 9.3%; 30.2% and 16.4% was observed in T1, T2 and T3, respectively, with a significant difference ($P < 0.05$) between T2 compared to T1 and T3 but there was no difference between T1 and T3. The results suggest that the use of GnRH on day 5 improved pregnancy per AI in dairy cows inseminated with sex-sorted sperm.

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Impact of recombinant bovine somatotropin, progesterone, and estradiol benzoate on the ovarian follicular dynamics of *Bos taurus taurus* cows in protocols for synchronization of estrus and ovulation

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The objective of this study was to evaluate the use of recombinant bovine somatotrophin, progesterone (P4), and estradiol benzoate (EB) on ovarian follicular dynamics for the synchronization of estrus and ovulation protocols in crossbred *Bos taurus taurus* cows. Twenty-four non-lactating multiparous cows were randomly divided into two groups: recombinant bovine somatotrophin group (GbST; n = 11) or the control group (GC; n = 13). The GbST group on day zero (d0; start of the study) received an intravaginal device with P4 (1.9 g), EB (1.0 mg, IM), and bST (500 mg, SC); ovarian ultrasonography (US) to examine the follicles and corpus luteum (CL) and transrectal ultrasonography were also performed. On d8, D-cloprostenol (150 μg, IM) was injected, US was carried out, and P4 was removed. On d9, EB (1.0 mg, IM) was injected and on d10 and d15 US was performed. The same protocol was applied to the control group, except bST was not administered on d0. The follicles and CL were evaluated and analyzed by US on d0, d8, d10, and d15. The data were statistically analyzed using the Statistical Analysis System (SAS) with a significance level of 5%. The effects on the groups (GbST and GC) with respect to the follicle size, CL size (F test), and ovulation rate (Fisher test) were evaluated. The GbST group showed a significantly higher follicle diameter (14.5 vs 12.1 mm, $P < 0.03$) on d10, CL diameter (19.7 vs 16.9 mm, $P < 0.01$) on d15, and ovulation rate (90.9% vs 69.2%, $P = 0.09$) compared to the GC group. In conclusion, bST, when used in combination with P4 and EB in timed artificial insemination protocols, significantly increased preovulatory follicle size and the percentage of ovulated cows and promoted a higher follicular growth rate after P4 removal compared to that in the control group. We thank the Pontifícia Universidade Católica do Paraná for the availableness of the structures to the execution of this study.

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Effect of a one-time, strategic donor FSH-treatment on oocyte and embryo production in a commercial buffalo IVP program

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One of the main limitations for the establishment of commercial IVP programs in buffalo resides in the reduced number and competence of OPU-derived oocytes. The objective of this study was to evaluate the effect of a one-time, strategic FSH-treatment in the number of available follicles for aspiration, oocyte quality and subsequent in vitro embryo development. Eleven, mature and fertile Mediterranean and Murrah donors in excellent body condition were available for ovum pick-up; aspirations were conducted during the reproductive season in Argentina. Animals were subjected to two sequential weekly aspirations T0-DFR (initial, unsynchronized complete follicular ablation), T0-control (OPU after 7 days post DFR) and were then treated 180 mg FSH (Folltropin, Vetpharma) distributed in twice daily i.m. injections for 3 days. Subsequent aspirations were conducted 7 or 15 days post FSH treatment (T1-FSH7 and T1-FSH15) and the FSH-residual effect after initial aspiration was also analyzed (T2-Residual). Continuous data was analyzed with ANOVA and Tukey post-hoc comparisons and categorical data was analyzed using Fisher's exact test. All analyses were performed in GraphPad Prism v.7; statistical significance was established at $P<0.05$. There were no significant differences in the average number of oocytes available for puncture between treatment groups (T0 = 8.7±0.5, T0-DFR = 4.7±0.7, T1-FSH7 = 7.6±1.8, T1-FSH15 = 8.0±0.7, T2-Residual = 6.0±1.2) or total oocytes recovered (T0 = 4.9±0.9, T0-DFR = 2.0±0.3, T1-FSH7 = 4.2±1.1, T1-FSH15 = 3.5±0.4, T2-Residual = 1.4±0.4). However, there was a significant improvement in the number of better quality oocytes (grades 1 and 2) in FSH-treated animals (T1-FSH7 = 3.0±0.9 and T1-FSH15 = 1.5±0.5 versus T0 = 0.5±0.3, T0-DFR = 0.9±0.1 and T2-Residual = 0.4±0.1; $P<0.05$). In addition, cleavage (T1-FSH7 = 26%, T1-FSH15 = 71% versus T0 = 7%, T0-DFR = 0 and T2-Residual = 10%; $P<0.05$) and blastocyst rates (T1-FSH7 = 21%, T1-FSH15 = 30% versus T0 = 5%, T0-DFR = 0 and T2-Residual = 0; $P<0.05$) were significantly improved by FHS treatment. Our results indicate one single, strategic FSH treatment results in significant improvements in oocyte quality and embryonic development; these effects are observed when OPU is conducted 7 or 15 days post FSH treatment. Residual effects of FSH treatment do not result in sustained improvement or embryo production after this period. This strategy could have a positive impact on commercial buffalo IVF programs. Universidad Nacional del Nordeste, UNNE, Fecunda Biotecnologia Paraguay y Valdez y Laurentis, Argentina. Universidad Nacional de Colombia, Sede Medellin.

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Down-regulated genes in *in vitro* produced bovine day 8 blastocysts due to an insulin challenge during oocyte maturation

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Insulin is a key metabolic hormone that has mitogen functions during early embryo development. Deviation of its physiological levels occurs in metabolic disorders due to overfeeding or negative energy balance and can be detrimental for oocyte quality. Reproductive disorders are often related to metabolic imbalance. The effect of insulin during oocyte maturation on gene expression of bovine day 8 blastocysts (BC8) by transcriptome analysis was investigated. Abattoir- derived oocytes (n=882) were divided into 3 groups and in vitro matured for 22 h by adding insulin (INS10=10µg/mL; INS0.1= 0.1 µg/mL and INS0= control). This was followed by standard in vitro production up to blastocyst stage. BC8 (n=120) were pooled in groups of 10 and total RNA was extracted (AllPrepDNA/RNA micro kit, Qiagen®) for transcriptomics. Amplified aRNA was hybridized on the Agilent EmbryoGENE-slides in a 2-color dye swap design. An empirical Bayes moderated t-test was applied to search for the differentially expressed transcripts (DET) between control and insulin groups, using the 'limma' package in R (www.r-project.org). DET were defined as having a 1.5 fold-change difference between treatment and control and $P<0.05$. In total, 202 DET were found in INS10 and 142 DET in INS0.1, whereof only 5 respective 4 DET were under-expressed compared to INS0. Thus, as a global pattern, insulin treatment induced an over-expression of most genes. However, some of the under-expressed genes have interesting functions that might be coupled to insulin influence in health and disease as hCTD1 (control of embryo development, involved in cell cycle regulation), UPK1A (mediates signal transduction events that play a role in the regulation of cell development, activation, growth and motility), ERAL1 (depletion of ERAL1 leads to apoptosis, cell death occurs prior to any appreciable loss of mitochondrial protein synthesis or reduction in the stability of mitochondrial mRNA) in INS 10; and ULBP1 (activates PI3K and CD38 immune system with functions in glucose metabolism) in INS0.1. To summarise, although only a few genes were underexpressed after insulin challenge, they demonstrate a potential difference in cellular functions and pathways coupled to mitogenic, mitochondrial or immunological functions of insulin. Thus, under- expression of these genes are potentially related to the pathomechanisms of hyperinsulinemia on the cell level and also involved in reproductive and metabolic disorders. Funded by FORMAS.

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The effect of ceftiofur hydrochloride on pregnancy rates of beef cattle synchronized with intravaginal progesterone implants

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In the past years with the intensification in the livestock sector, attention was given to improve reproductive efficiency to maximize production. The use of estrous synchronization protocols for artificial insemination with fixed time (FTAI) were able to reduce the problems associated with heat detection, maximizing the reproductive efficiency. Studies from our group have identified changes in the vaginal microbiota of cows synchronized with intravaginal device with progesterone and also vaginitis. It is known that the use of antibiotics is a common procedure to treat and to control bacterial infection. Considering that changes in the uterine microbiota may compromise pregnancy, the objective of this work was to evaluate the effect of ceftiofur hydrochloride in beef cattle submitted to FTAI protocol in the pregnancy rates. For this study, 64 adult crossbred beef cows (Angus X Charolais X Hereford) were used. Animals were allocated randomly to two groups (G1 and G2) and further submitted to the same FTAI protocol with the CIDR® implant and 2.0 mL of Estradiol Benzoate (IM) on day 0, followed by the administration of 2.5 mL prostaglandin (IM) on day 7, on day 9, 0.3 mL of ECP® IM and the removal of the implant, on day 11 AI was conducted in all animals by the same veterinarian, using conventional frozen-thawed semen from 2 bulls (Hereford-HF or Maine Anjou-MA). In addition to that, G1 received 2.2 mg/kg of Ceftiofur - Excede® by IM route on day 9 the protocol, but G2 did not receive any additional treatment. Pregnancy detection was conducted in all the cows 60 days after AI. Data were analyzed using a X^2 test. No differences were observed in pregnancy rates among the bulls used. However, cows inseminated with MA Semen and treated with Ceftiofur presented 25.5% more pregnancies compared to the ones non-treated (58.82% MA-G1, 33.33% MA-G2; $P<0.05$). This difference was not observed in the cows inseminated with HF semen. In general, the pregnancy rate of G1 was 57.57% and G2 was 41.93% ($P<0.03$). Our results have shown that the use of antibiotic to modulate the bacterial proliferation in the vaginal microflora of beef cattle was able to reduce the losses of pregnancy by 15.64%. In conclusion, the use of the antibiotic ceftiofur hydrochloride was able to improve the reproductive efficiency of beef cattle submitted to a synchronization protocol.

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Effects of using either estradiol benzoate or GnRH at the beginning of a 7-d P4-based FTAI protocol with or without GnRH at the time of AI in Nelore heifers

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Evaluation of physiology and fertility variables of a 7-d FTAI protocol in *Bos indicus* heifers was the main objective of this study. Moreover, two treatments at the beginning of the protocol [estradiol benzoate (EB) *vs* buserelin acetate (GnRH)], including or not GnRH at the time of AI, were compared. A total of 958 heifers [26.4±2.0 mo old, BCS of 2.9±0.1 (1-5), and body weight of 307.6±22.5 kg; mean±SD] were used. At first, the presence of a corpus luteum (CL) was evaluated by ultrasound. Heifers with CL were assigned to the experimental treatments described below, and heifers without a CL were submitted to a protocol for induction of cyclicity and after 12 d (D0), all heifers, independent of CL presence, were randomly assigned to the treatments: B0 (EB at D0 + no GnRH at AI; n=239), BG (EB at D0 + GnRH at AI; n=246), G0 (GnRH at D0 + no GnRH at AI; n=239), or GG (GnRH at D0 and AI; n=234). On D0, all heifers received a P4 device (0.5 g) for 7 d and were treated with EB (1.5 mg; B) or GnRH (20 µg; G). On D7, concomitant with device withdrawal, all heifers received cloprostenol sodium (PGF; 0.530 mg), estradiol cypionate (EC; 0.5 mg) and had tail-chalk spread on the base of their tailhead. Heifers from the G group also received an extra PGF (24 h before the second PGF) and eCG (200 IU) treatment on D6, whereas B heifers received eCG on D7. At AI (48 h after D7), only BG and GG groups received GnRH (10 µg), and all heifers were checked for estrus. Hormones were donated by Globalgen Vet Science, Jaboticabal, BR. Ultrasound was used to the determine presence of CL (D0, D7 and D16) and diameter of the dominant follicle (DF; D7) and pre-ovulatory follicle (OF; D9). Pregnancy per AI (P/AI) diagnosis was performed 40 d after AI. Statistical analyses were done by GLIMMIX and MIXED of SAS (LSM±SEM; P=0.05). Heifers with CL at first ultrasound represented 15.5% (147/951) of the total. In addition, 77.4% (628/804) ovulated after the induction protocol. After treatment on D0, G heifers ovulated more than B [62.5% (292/467) *vs* 14.2% (68/479)]. Further, more B heifers underwent luteolysis between D0 and D7 [39.4% (146/371)] than G [23.2% (88/379)]. On D7, DF (mm) was greater for G (10.4±0.19; n=54) than B (9.7±0.17; n=68), whereas at AI there was no difference between treatments for OF [mm; 11.8±0.2 (n=54) *vs* 11.6±0.18 (n=68)]. Also, more heifers in G than B were detected in estrus at AI [81.7% (384/470) *vs* 70.7% (343/485)]. Induced heifers detected in estrus at AI had greater P/AI [57.3% (423/726)] than heifers not in estrus [46.9% (111/228)]. There was no effect of CL on D0 (cyclicity) on P/AI, thus data were combined. There was no overall effect of treatments on P/AI [B0=57.3% (137/239), BG=53.7% (132/246), G0=54.2% (130/239), or GG=59.0% (138/234)]. There was also no association between estrus and GnRH at AI on P/AI. Ovulation rate after AI was not different among treatments [91.7% (n=139)], and only one heifer had double ovulation. In summary, there was no improvement in P/AI by treating with GnRH at the time of AI. Moreover, despite producing a larger DF on D7 and more estrus on D9, the GnRH-based protocol had similar P/AI compared to the EB-based *protocol.* FAPESP, CNPq, CAPES, Globalgen, GENEX and Figueira Farm.

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Murrah buffalo antral follicle count in ovaries from a slaughterhouse

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The study of antral follicle count (AFC) is currently widespread in bovine animals to obtain parameters for identifying the best females in terms of reproductive efficiency. The parameters related to AFC for buffaloes are still unknown. Therefore, the objective of the present study was to determine parameters of AFC for the ovaries of bubalina species from the slaughterhouse, in view of the significant increase in the production of these animals. According to the AFC of the female, it is ranked as the number of follicles present in a pair of ovaries, thus resulting in three groups: high, medium or low AFC. For the study, a pair of ovaries was obtained from buffaloes (n = 26) collected in the slaughterhouse of São José do Rio Preto and transported in a thermal container at 36°C with 0.9% saline solution. These were identified (left/right) and kept in a water bath until the AFC was determined by macroscopic visualization. We measured the diameter of the largest follicle antral found in ovary with a Trident® ruler. The antral follicles = 1 mm (visible to the naked eye) were counted to obtain the high (G-high), average (G-average) and low (G-low) AFC groups. Statistical analysis for comparison between the AFC of high, medium and low groups was performed using Fisher test, with 5% level of significance through the Statistical Programme Action 3.1 R version 3.0.2 (Campinas, SP, Brazil). For comparison between the AFC of the right and left ovaries we used t-test using the same statistical programme referred to above. The parameters obtained for AFC of G-high was = 19, for G-average = 18, and for G-low = 8 follows follicles, with the number of animals classified in the groups: n = 7; n = 11 and n = 8, respectively. On comparison between the buffaloes with high, medium and low AFC, there was a significant difference. Regarding the comparison between the AFC of the right and left ovaries between G-average and G- high, there was no significant difference. But we observed a significant difference when comparing right and left ovaries for animals of G-low ($P < 0.05$). Therefore, we find that with the few antral follicles Murrah buffaloes presented in the ovaries, resulting in smaller differences for high, medium and low AFC. In addition, the buffaloes with low AFC showed a difference in follicular number between the ovaries, suggesting that there is an ovary with greater follicular recruitment and possibly functionality. We thank the veterinary hospital Halim Atique for giving up the space to do the work.

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Plasma and acrossomal integrity of bull spermatozoa cryopreserved with iodixanol

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The oxidative stress has been reported one of the most important factor contributing to sperm quality and fertility reduction. Recently, iodixanol showed to protect bull, ram, buffalo and equine sperm membranes when added to freezing extender, either by altering the formation of ice crystals, or by preventing reactive oxygen species generation. Therefore, this study was carried out to verify the effects of addition of iodixanol as a cryoprotectant for bovine semen freezing on post-thaw plasma and acrossomal membrane integrity (PAMI), translocation of phosphatidylserine (TPS) and plasma membrane destabilization (PMD). Thus, we compared a commercial freezing medium (CFM; BotuBOV®, Botupharma, Botucatu, Brazil) with 7% glycerol (C; control), CFM with 3.5% glycerol plus 3.5% iodixanol (GI), and CFM with 7% iodixanol (I). After routine sperm assessment (motility, concentration and morphology), the ejaculates (n=18) from three Nellore bulls were extended according to the groups previously described. The samples were filled in 0.5 straws, cooled for 5h at 4°C, frozen in a programmable freezer machine (Digitcool, IMV, L'Aigle, France) and stored in liquid nitrogen until evaluation. Plasma and acrossomal membrane integrity were simultaneously assessed using Propidium Iodide and FITC-PSA probes, respectively, while TPS was identified by Annexin V and PMD by YO-PRO-1. All sperm samples were analyzed by flow cytometry (BD LSR; Fortessa, Becton Dickinson, Mountain View, USA). Sperm parameters were expressed percentage (%) for the total number of cells. ANOVA and Tukey's test were used for statistical analysis (six replicates), with $P<0.05$ taken as significant. Higher MPAI was observed for CO (56.7 ± 2.1) and GI (57.1 ± 1.3) than for I (33.5 ± 2.9). Furthermore, GI (53.3 ± 1.6) showed higher percentage of spermatozoa without TPS than CO (45.6 ± 2.4) and both than I (32.5 ± 2.9). For PMD, the I group (12.6 ± 1.7) exhibited lower rate of membrane without destabilization than GI (42 ± 2.5) and CO (62.4 ± 2.7), which were significantly different. In conclusion, freezing medium with iodixanol as cryoprotectant was not able to effectively conserve the membranes integrity of bull spermatozoa as well as tests using fluorescent probes (data not showed) observed that it was not able to control oxidative stress as expected. However, the association of ioxidanol and glycerol is positive by reducing the concentration of glycerol which is toxic to spermatozoa. CAPES, Tairana AI Station and Botupharma, Brazil.

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Effects of moxibustion during the mid-luteal phase on blood flow to the corpus luteum in cattle

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It is known that moxibustion improves the fertility of the dairy cattle. However, the mechanisms of the effects of moxibustion are not clear. To investigate the physiological effects of moxibustion on ovarian function of the cow, the effects of moxibustion on the function of corpus luteum (CL) was evaluated. In the present study, the changes in luteal blood flow (LBF) and plasma progesterone (P_4) concentration in the mid-luteal phase and $PGF_{2\alpha}$-induced regression phase were examined after moxibustion treatment. Non-lactating Holstein-Friesian cows (n=3) were applied a series of control experiments (control group, without moxibustion treatment), and thereafter, in another estrous cycle, moxibustion treatment was conducted (moxa group). Soybean paste (miso, 30g each) were placed on nine specific acupoints on the back skin of the cow. Dried mugwort (moxa, 2.5g each) were put on miso and burnt for 20 min. Moxibustion treatment was performed on 5 days from day 9 to day 13 of the estrous cycle (ovulation = day 1). After moxa on day 13, $PGF_{2\alpha}$ was injected to induce luteal regression. To evaluate the size of the CL (CLS) and LBF, transrectal color Doppler ultrasonography examination was carried out before moxibustion treatment on day 9, on day 13 and just before $PGF_{2\alpha}$ injection (0 h), and at 1, 2, 4, 8 h after injection. Examination of CLS by transrectal ultrasonography was carried out continuously at 12, 24, 36, and 48 h after injection. To assess the change of LBF, the ratio of the colored area to the area of the maximum diameter of CL was used. Blood samples were collected at each time point, and plasma P_4 concentrations were measured by EIA. Data were analyzed using repeated measures ANOVA followed by Dunnett's multiple-comparison test. There was no significant effect of moxa during the mid-luteal phase on CLS, LBF, or plasma concentrations of P_4. A decrease in CLS was observed in both groups at 4 h after $PGF_{2\alpha}$ injection. At 1 h compared with 0 h, LBF significantly increased in both groups but no significant difference was found between moxa and control. A decreasing ratio of the changes in LBF from 1 h after $PGF_{2\alpha}$ injection was significantly larger at 4 h compared with 2 h in the moxa group, but not in the control group. A decrease in P_4 concentrations was observed in both groups at 8 h. There was no significant difference in P_4 concentrations between the two groups at all examination time points. An earlier decrease in LBF observed in the moxa group may indicate that moxibustion influenced vasoconstriction or vasodilation and changed the LBF after $PGF_{2\alpha}$ injection. In contrast, moxibustion may have no effects to decrease of plasma P_4 level after $PGF_{2\alpha}$ injection. These results suggest that the changes in responsiveness of LBF to $PGF_{2\alpha}$ by moxibustion may affect to the function of CL, and this effect may contribute to improving fertility in the cow.

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Blastulation timing affects the blastocyst development and its secretion of extracellular vesicles

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Blastocyst formation is an essential event in preimplantation development. An early blastulation has been related to a better synchrony of embryo development and hence better embryo quality. The normal embryo development needs a constant crosstalk with the environment. This interaction is mediated by molecules and extracellular vesicles (EVs) secreted by the embryo. The nature of this molecules and vesicles may vary depending on embryo stage and quality. Thus, a better understanding of the embryo secretome during blastulation time can help in improving the selection accuracy of competent embryos. To examine the effect of blastulation time on blastocyst development and secretion of EVs, cattle ovaries were collected at an abattoir and transported within 2 h to the laboratory. Cumulus-oocyte-complexes (COCs) were recovered and matured in four-well dishes (25 to 30 COCs per 500 µL well) for 22-23 h. Oocytes were fertilized using frozen-thawed semen that was used before in other in vitro fertilization procedures with good blastocyst production (control bull). Presumptive zygotes were in vitro cultured (IVC) in groups in four-well plates (25 to 30 zygotes per 500 µL well) using SOFaa culture medium at 39°C under 5% CO2, 5% O2 and 90% N2 until morula stage (day 5 post IVF). At day 5, morulae were selected and cultured individually in 96-well plates in SOFaa medium depleted of EVs (SOFdep) and monitored for blastulation at day 6.5 (early) and 7.5 (late) post fertilization. At day 7.5, blastocyst diameter was measured and culture media from individual blastocysts was collected and EVs were isolated and analyzed using a nanoparticle tracking analysis. Comparison analyses between early (n=189) and late (n=174) blastulation were performed using ANOVA, the non-parametric U Mann-Whitney test, and clustering analysis and principal component analysis to discriminate groups according to blastulation time. Statistical analysis was performed with SAS system V8 for windows (SAS Institute Inc). Early blastulation rate (day 6.5) was 16.2% (222/1371) while late blastulation rate (day 7.5) was 21.3% (292/1371), showing a significant difference (P = 0.0046). Embryos derived from early blastulation (day 6.5) were larger at day 7.5 (163.7±22.6 vs 154.0±18.6 µm; P = 0.0001), secreted a greater number of vesicles (28.7±18.2 vs 23.4±9.0 x 10^8 per mL; P = 0.0001) and these particles were larger (108.4±25.9 vs 99.3±19.0 nm; P = 0.0001), compared to those derived from Day 7.5 blastocysts. However, there was no correlation between blastocyst diameter at day 7.5 and characteristics of secreted EVs (size, r = 0.25, P = 0.0001; and concentration, r = 0.040 and P = 0.007), independently of blastulation time. Clustering analysis of populations of EVs did not allow discrimination between embryos of early and late blastulation. In conclusion, blastulation time affects the diameter of bovine embryos as well as the characteristics of populations of EVs secreted by the embryo during the period from morula to blastocyst stage. Work supported by Grants Fondecyt 1170310 and Corfo 17Cote-72437, Government of Chile.

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Extracellular vesicle-depleted culture media improves quality of in vitro produced bovine embryos

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Extracellular vesicles (EVs) are currently considered a mechanism of cell communication. The EVs are secreted into the extracellular environment by different cell types including embryos, and can be identified *in vivo* in different biological fluids as well as *in vitro* in embryo culture medium. Usually, media used for *in vitro* culture of embryos are supplemented with serum or other protein sources that favor cell proliferation and development. These protein sources also may contain EVs, including microvesicles and exosomes that in principle can be internalized by embryonic cells. The aim of this study was to determine whether the absence of EVs in culture media could affect in vitro bovine embryo development. Several variables were evaluated, including embryo developmental rate up to blastocyst stage, blastocyst quality (based on morphological criteria of the IETS) and gene expression. Bovine embryos were produced by in vitro fertilization (IVF). Cumulus-oocyte-complexes (COC's) were recovered from slaughterhouse ovaries and in vitro matured for 22-23 h in four-well dishes (25 to 30 COCs per 500 μL well), and fertilized with commercial frozen-thawed semen previously proved for IVF. Embryos were *in vitro* cultured in groups (25 embryos/well in 4-well plates) in global® total® medium (GTc) or global® total® EV-depleted medium (GTd) at 39°C under 5% CO2, 5% O2, and 90% N2. Presumptive zygotes were randomly assigned to experimental (GTd) or control (GTc) groups. Medium was depleted by ultrafiltration (centrifugal filter devices 100 kDa, Amicon) for 15 min at 3000 RPM. EV populations from GTd and GTc were analyzed using a nanoparticle tracking analysis (NTA). Blastocyst rate and quality was determined at day 7 and 8 after IVF. At day 8, blastocysts total cell number was analyzed. Also, expression analysis of 9 genes (OCT4, SOX2, NANOG, BCL2, CDX2, GATA6, TP1, BAX and CASP3) was performed by RT-PCR; ACTB was used as the housekeeping gene. Data were analyzed with Wilcoxon non-parametric test using InfoStat program. The ultrafiltration protocol eliminated 98% of culture media EVs (original culture media 2.54 $x10^{10}$ particles per mL versus depleted culture media $4.4x10^{8}$ particles per mL). Blastocyst rate at day 7 was 18.7% (37/198) and 13.5% (27/201) in GTc and GTd, respectively, whereas at day 8 it was 22.7% (45/198) and 19.4% (39/201) in GTc and GTd respectively; no statistical differences were observed for blastocyst rate at day 7 or at day 8 ($P>0.05$). It seems that depleted media delay embryo development since at day 7 there were more early blastocysts (54% vs 48% for GTc and GTd, respectively; $P>0.05$). However, no statistical differences were observed for blastocyst stage and total cell count at day 8. However, morphological quality of blastocysts derived from GTd was significantly higher at day 8 ($P=0.03$) and the expression of CASP3 was significantly lower in these embryos ($P=0.0024$). The results indicate that the absence of EVs from protein sources added to culture media improves bovine *in vitro* embryo development by increasing blastocyst quality and perhaps reducing apoptosis at day 8. Supported by FONDECYT, Chile (1170310).

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Interference of dairy production on oocyte recovery and embryo conversion rate of donnors from Gyr and crossbred (Holstein x Gyr) dairy cows

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Considering the impact of cattle farming in the country's economy, it is important to emphasize the relevance of research aimed at improving the existing techniques and the development of new technologies for the industry. This study was developed at In Vitro Brasil S/A®, a company headquartered in the city of Mogi Mirim, in the state of São Paulo, in Brazil, where data from follicular aspirations performed in a farm in the city of Passos, in the state of Minas Gerais, were collected. A total of 118 oocyte donor cows were used, selected according to their genetic merit. The animals belonged to the following breeds: 43 Gyr (*Bos taurus indicus*), 60 half-blood Gyr-Holstein (1/2 *Bos taurus taurus* x 1/2 *Bos taurus indicus*) and 15 5/8 Gyr-Holstein (5/8 *Bos taurus taurus* x 3/8 *Bos taurus indicus*). Data were analyzed using the Kruskal-Wallis test (Statisticsx9®), a non-parametric analysis of variance for samples that are non-dependent on oocyte recovery and embryo conversion data. The ANOVA test was also used, which is an analysis of variance for different dairy production rates and its interference in the oocyte recovery and embryo conversion. Data related to oocyte recovery, embryo production and percentage of oocyte-embryo conversion are reported, comparing the milk production of those animals. According to the statistical analysis, the Gyr breed presented lower oocyte recovery than the other breeds ($P<0.05$); however, the blastocyst conversion rate was higher, despite not presenting statistical difference in the amount of produced embryos ($P>0.05$). There was no statistical difference in relation to milk production among the animals, despite the numerical variation ($P>0.05$). Nevertheless, a progression in the oocyte recovery and embryo production was observed, according to the increase in the lactation rate in the Gyr and half-blood Gyr-Holstein. On the other hand, the 5/8 Gyr-Holstein donors presented a regression in the oocyte recovery and embryo conversion according to the increase in milk production. This study provided unpublished data on the lactation interference in oocyte and embryo production in the assessed breeds. Universidade Paranaense - Unipar and In Vitro Brasil®.

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Contribution of breed and equine chorionic gonadotropin on the collection of oocytes and in vitro embryo production in young goats during the breeding season

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The gonadotropins which have been used in the treatment of superovulation in goats are porcine or ovine follicle-stimulating hormone (FSH), equine chorionic gonadotropin (eCG) or a combination between FSH and eCG in regime known as "one-shot" (FSH + eCG). The use of eCG in goats, opens the possibility of collecting and maturing oocytes from superior does to accelerate genetic progress in this species. The objective of this study was to determine the effect of breed and eCG on ovarian response and *in vitro* embryo production from young goats. Twenty-nine goats (15 Alpine, 6 Nubian and 8 Saanen) were assigned into 3 treatments of eCG (T1: 0 IU; T2: 500 IU and T3: 1000 IU). The cumulus-oocyte-complexes (COC) were classified in three categories (grade I, II and III) according to their homogeneity morphology of the cytoplasm and compaction of the cumulus cells. Number of follicles before and after ultrasound was analyzed with a linear model that included the effect of breed, dose of eCG and their interaction. The retrieved oocytes, quality and the embryos production were analyzed using the Chi-square test ($\chi 2$) to test significant differences between breeds and cGG doses. Alpine goats showed the largest amount and size of follicles ($P = 0.003$). An effect of eCG dose 24 hours post treatment ($P<0.05$) was presented, being superior for the goats of T2. The aspiration rate of COC was 34%. The COC quality was zero or low results of Grade I and II, increasing the rate of nudes, obtaining the highest number ($P = 0.003$) in the Saanen goats; the same difference was found ($P = 0.02$) in oocytes grade III in the T2 and T3, with a 42.5 and 37.9% respectively. *In vitro* embryo production was in the Alpine goats (80.0 % of IVF/segmentation; $P = 0.003$). Embryo production was greatest for T2 (69.2%; $P = 0.004$). T3 goats had higher percentage of morula stage (66.6; $P = 0.030$). Therefore, it is concluded that treatment with eCG affected the ovarian status, quality and quantity of embryos according to the breed evaluated. To CONACYT, for the support of scholarship granted to the first author within the program of masters in animal science Agronomy-Veterinary Graduate of the UANL. Also, for the support program for scientific and technological research, PAICYT-UANL-CT249-15, for the financing of the project. To Dr. Juan Francisco Villarreal Arredondo, for his infinite support to carry out this project.

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Is the laterality of the corpus luteum and the dominant follicle of Wave 1, and the GnRH administration 5 d after insemination, relevant for pregnancy establishment in heifers?

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Laterality between corpus luteum (CL) and dominant follicle (DF) of Wave 1 may affect pregnancy establishment in cattle (Miura *et al*., 2015. Development of the first follicular wave dominant follicle on the ovary ipsilateral to the corpus luteum is associated with decreased conception rate in dairy cattle. J Dairy Sci, 98:318-321), with lower fertility reported when CL and DF were in the same ovary (ipsilateral). In addition, although the positive effect of GnRH administration during Wave 1 to improve pregnancy rate is contradictory, the interaction of this treatment with the laterality of CL/DF has not been yet evaluated. This experiment was carried out to evaluate the relationship among the laterality of the CL and the DF of Wave 1, and its interaction with the administration of GnRH 5 d after FTAI, with pregnancy rate in heifers. Hereford and Angus crossbreed heifers (n = 467) with body condition score 4.4 ± 0.1 (Mean ± SEM, 1 to 8 scale) received a hormonal treatment for FTAI. The program consisted of the administration of an intravaginal device containing progesterone (DIB 0.5 g, Zoetis, Argentina) for 6 d and 2 mg of estradiol benzoate (Gonadiol, Zoetis) at device insertion. One dose of 500 µg of cloprostenol (Ciclase DL, Zoetis) and 300 IU of eCG (Novormon, Zoetis) administered im at device removal, and 100 µg of gonadorelin acetate (GnRH; Gonasyn GDR, Zoetis) administered im at FTAI performed 48 to 72 h after device removal. The laterality of the CL was determined by ultrasonography (WED-9618V, Well.D) at the moment of insemination to define the ovary containing the preovulatory follicle, and confirmed by the presence of the subsequent CL 5 d later. Laterality of the DF of Wave 1 was determined 5 d after insemination, and heifers were allocated into four experimental groups according to the laterality of the CL and the DF of Wave 1 (ipsi or contralateral when both were or not in the same ovary). Half of the herd received one dose of GnRH in a 2 X 2 factorial design. Pregnancy diagnosis was determined by ultrasonography 30 d after FTAI. Data were analyzed by GLMM and presented as mean ± SEM. Ovulation rate (heifers ovulated/treated) was 97.4% (455/467). Pregnancy rate from ovulated females (70.3%, 320/455) was not different between heifers with CL/DF ipsi or contralateral (70.3%, 175/249 and 70.4%, 145/206; respectively, P = NS), and between heifers with or without GnRH (68.7%, 158/230 and 72.0%, 162/225; respectively, P = NS). No interaction between laterality of CL/DF and GnRH treatment 5 d after FTAI was found (P = NS). In conclusion, the presence of DF of Wave 1 in the ipsi or contralateral ovary to the CL does not affect pregnancy establishment, and the administration of GnRH 5 d after insemination does not improve fertility in *Bos taurus* beef heifers.

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Influence of estrus expression in a fixed-time AI protocol on reproductive performance

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The ability to induce ovulation of a dominant follicle with an injection of GnRH facilitated the development of fixed-time AI protocols. However, it has been established that cattle need to experience elevated concentrations of progesterone, a drop in progesterone, and a rise in estradiol to establish timing for changes in uterine gene expression. Animals that exhibit estrus prior to fixed-time AI have greater pregnancy success compared to animals that are induced to ovulate without expressing estrus. Thus, the objective of this study was to determine the impact of estrus expression prior to fixed-time AI on timing of when pregnancy occurred. Data were collected on 4,499 cows (Bos taurus beef animals ages 13 months to 13 years) in 31 different herds. All animals were synchronized using an injection of GnRH at insertion of an intravaginal progesterone device, an injection of prostaglandin F2α at device removal, and an injection of GnRH at time of AI. Estrus expression was determined at time of AI based on activation of estrus detection patches. Bulls remained separated for 10 days after AI, and fetal age was determined by transrectal ultrasonography. Animals were grouped as having conceived to AI, or into each of the possible return estrous cycles (cycle 1-d 10 to 31, cycle 2-d 32 to 53, or cycle 3-d 54 to 75, and cycle 4 after d 75). Data were analyzed using the GLIMMIX procedure of SAS with herd as a random variable. Animals that exhibited estrus before AI had increased ($P < 0.01$) pregnancy success to AI (64 ± 1.3% vs 45 ± 1.6%, respectively) and during the entire breeding season (93 ± 0.7% vs 89 ± 1.1%, respectively) compared to animals that did not exhibit estrus. Of the animals that did not conceive to AI but did conceive during the breeding season, more animals that exhibited estrus before AI conceived during cycle 1 ($P < 0.01$; 46 ± 2% vs 34 ± 3%). However, more animals that did not exhibit estrus conceived in cycle 2 ($P = 0.02$; 46 ± 2% vs 39 ± 2%) and 4 ($P = 0.01$; 6 ± 1% vs 3 ± 1%). There was no difference in cycle 3 ($P = 0.15$; 11 ± 1% and 13 ± 2% for estrus and no estrus, respectively). Overall, animals that exhibited estrus before AI but did not conceive to AI conceived earlier in the breeding season compared to animals that did not exhibit estrus ($P < 0.01$; cycle 1.71 ± 0.04 vs cycle 1.93 ± 0.04). There was no difference ($P = 0.74$) in embryonic loss between groups (2.3 ± 0.3% and 2.1 ± 0.4% for estrus and no estrus, respectively). Thus, the ability to induce ovulation is critical for fixed-time AI, but animals that do not exhibit estrus prior to fixed-time AI had decreased AI conception rates, decreased breeding season pregnancy success, and the animals that did conceive did so later in the breeding season compared to animals that did exhibit estrus. USDA is an equal opportunity provider and employer.

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Efficacy of Doppler ultrasonography to detect non-pregnant Nelore cows and heifers submitted to three timed-AI in 48 days

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The present study aimed: 1) to evaluate reproductive performance using Color Doppler ultrasonography for detection of luteolysis in non-pregnant cows and heifers 22 days after timed-AI (TAI); and 2) to compare the use of Doppler and B (brightness; gray scale) modes for detection of pregnancy. Suckling Nelore cows (n = 174) and heifers (n =161) were submitted to a TAI progesterone/estradiol-based protocol. Animals received one progesterone intravaginal device (CIDR, Zoetis) on D13 (D0 = first TAI). On D22, CIDR were withdrawn and animals were evaluated by transrectal ultrasonography (Z5, Mindray), using B and color Doppler modes to measure the luteal area and blood perfusion. Animals with a corpus luteum (CL) = 25% of color signals indicating blood perfusion in the luteal area were diagnosed as non-pregnant (n = 141). Non-pregnant animals received estradiol cypionate (1 mg; ECP, Zoetis), dinoprost tromethamine (25 mg; Lutalyse, Zoetis) and eCG (200 IU for heifers and 300 IU for cows; Novormon, Zoetis) to induce ovulation, and a second TAI was performed on D24. On D37, inseminated animals were resynchronized as previously described. A third TAI was done on D48 in non-pregnant cows detected by Doppler ultrasonography on D46 (n = 80), and in animals where pregnancy loss was observed after first TAI (n = 30). For B-mode evaluation, animals with a CL < 2cm^2 were considered non-pregnant and pregnancy predictions were compared with the Doppler mode (gold standard) in each category (heifer or cow). Pregnancy was confirmed 37 days after fisrt (D37), second (D61) and third (D85) TAI by detection of an embryo vesicle. Binomial data was evaluated by Fisher's Exact test using PROC FREQ and by logistic regression using PROC GLIMMIX of SAS. Parametric data was evaluated by ANOVA using PROC GLM. Considering all females, pregnancy diagnoses on D22 were in agreement between B and Doppler modes in 91.7% (410/447) of the cases. Accuracy, sensitivity, specificity, negative predictive value and positive predictive value were, respectively, 94.9%, 91.7%, 99.1%, 90.1%, and 99.3% for cows, and 88.3%, 78.2%, 99.1%, 81.2%, and 98.8% for heifers. The majority of incorrect results resulted from 8.3% and 21.8% of false-negatives in cows and heifers, respectively. The calculated kappa value indicated very good (0.897) and good (0.768) agreements between both methods, respectively, for cows and heifers. Luteal area (cm^2) was greater (P < 0.05) in cows (2.79 ± 0.05) than in heifers (2.47 ± 0.03) diagnosed as true-positive. Pregnancy loss at day 37 was greater (P < 0.05) for heifers (19.5%, 23/119) than cows (9.6%, 13/135). Accumulative pregnancy rate after three TAI was 85.1% (148/174) for cows and 72.7% (117/161) for heifers. In conclusion, the resynchronization on D13 associated with use of early detection of luteolysis on D22 by Doppler ultrasonography results in desirable pregnancy rates after three TAI in 48 days. The single use of CL size accessed my B-mode ultrasonography for detection of luteolysis results in a high rate of false-negative results and lower accuracy compared to Doppler imaging, especially in heifers. Authors thank: FAPESP (2015/10606-9; 2016/23964-3); Zoetis; Qualitas Melhoramento Genético; DPS Mindray (Adriane), Fazenda Bela Vista (Silvio).

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Effect of supplementation of α-tocopherol in superovulated Dorper ewes

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The efficiency of a multiple ovulation and embryo transfer (MOET) program depends on the number of good quality embryos obtained, and some studies suggest that supplementation with antioxidants can help to improve embryo quality. The objective of the study was to evaluate the effect of α-tocopherol supplementation on embryo quality of a MOET program in Dorper ewes. In total, 20 Dorper ewes were superovulated, from which 10 ewes were treated with 1000 IU of α-tocopherol given im 60 h before sponge removal, while the other 10 Dorper ewes were not treated (0 IU). The ewes were synchronized with intravaginal sponges containing 20 mg fluorogestone acetate (FGA) for 12 d and on the 10th d they were superovulated with a purified source of follicle stimulating hormone. Estrus was detected with teaser rams, and ewes in estrus were inseminated by laparoscopy 18 h after estrus onset with 4 doses of fresh semen containing $100x10^6$ spermatozoa each. Only semen from a single ram and of proven fertility was used. Embryo recovery was attempted 7 d after estrus by laparotomy. Ovulation rate, recovery rate, fertilization rate and embryo quality were measured. The results were analyzed using the least-squares procedures (Proc GLM of SAS) in case of continuous variables (ovulation rate, mean number of transferable embryos, mean number of unfertilized eggs). In the case of fertilization rate and recovery rate, data were analysed as binomial events using a generalized linear model (Proc GENMOD of SAS) with a logit link function, with ewe considered as the experimental unit. Fertilization rate was higher ($P<0.05$) in ewes treated with α-tocopherol (59.26%) compared to non-treated ewes (40.74%). However, there was no effect ($P>0.05$) of α-tocopherol administration on ovulation rate (15.40 vs. 11.20), embryo recovery rate (58.62 vs. 41.38%), mean number of transferable embryos (4.60 vs. 2.85) or unfertilized eggs (1.80 vs. 3.43) for α-tocopherol and vs non-treated ewes, respectively. In conclusion, the administration of α- tocopherol only improved the fertilization rate of superovulated ewes under the conditions of the study. The authors acknowledge the support of Univesidad Autónoma Chapingo for the conduction of the study.

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Characterization and control of the oocyte population for ovum pick up in Nellore donors

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Current strategies for *in vitro* embryo production (IVP) include OPU on random day of estrous cycle. Oocytes recovered are heterogeneous in chromatin configuration, oocyte-cumulus coupling, transcriptional activity and developmental competence. Since oocyte *in vitro* maturation (IVM) should promote synchrony between nuclear and cytoplasmic maturation, this heterogeneity may underlie the low efficiency of current IVP strategies. This study aimed to characterize oocyte population obtained by OPU and to develop a protocol to synchronize oocytes destined to IVP in Nellore. In experiment 1, 14 Nellore cows were subjected to two treatments in crossover design: 1) OPU at random day (Control); 2) aspiration of follicles >2mm at random day (D0), two IM injections of FSH (Folltropin; 24+16mg) 10h apart on D2, OPU from follicles >2mm on D5 (ASP/FSH-40). In experiment 2, 12 Nellore cows were subjected to the same treatments as in experiment 1, except for reduction of FSH dose (Folltropin; 12+8mg) (ASP-FSH/20). At OPU, antral follicles were counted and classified as small (3-5mm), medium (6-8mm) and large (>8mm). Oocytes were counted, fixed and stained with Hoechst 33342 then examined by fluorescence microscopy to be classified according with chromatin condensation and germinal vesicle configuration in GV0, GV1, GV2, GV3 and GVBD (Lodde *et al.,* 2007). Data were arcsine transformed and groups compared by paired T test ($P<0.05$). Total number of follicles aspirated at OPU was not affected by treatment in any experiments. In experiment 1, 285 follicles were aspirated in Control group and 306 in Asp/FSH-40 group, while in experiment 2, total numbers 468 and 463 follicles were aspirated in Control and ASP-FSH/20 groups, respectively. However, in experiment 1, ASP-FSH/40 treatment reduced percentage of small follicles ($P=0.02$). Percentages of small, medium and large follicles were 67.6*, 24.1 and 8.3 in Control, and 50*, 35.4 and 14.6 in ASP-FSH/40, respectively. Distribution of oocytes in different categories of chromatin compaction was only affected by treatment in experiment 2, with ASP-FSH/20 reducing the percentage of GV3 oocytes ($P=0.01$). In experiment 1, percentages of GV0, GV1, GV2, GV3, GVBD and degenerated oocytes in Control group were 1.34, 0.25, 39.06, 46.37, 0.12 and 12.86 respectively (n=177), while they were 0, 4.14, 40.49, 38.94, 0 and 16.42 in ASP-FSH/40 respectively (n=195). In experiment 2, percentages of GV0, GV1, GV2, GV3, GVBD and degenerated oocytes were 0, 1.95, 47.55, 40.47*, 0 and 10.03 in Control group respectively (n=267), while they were 0, 4.22, 49.53, 30.50*, 0 and 15.75 in ASP-FSH/40 respectively (n=265). In conclusion, oocytes recovered by OPU at a random day in Nellore were mostly at intermediate (GV2) or advanced (GV3) stage of chromatin compaction. Since these stages have been associated with higher developmental competence, this finding may help to explain the suitability of the Nellore breed to IVP. Protocols combining antral follicle aspiration and low doses of FSH to stimulate follicle recruitment may further homogenize the oocyte population for OPU in Nellore. However, further improvement of ASP-FSH protocols and fine-tuning with IVM culture system are needed before their real impact on IVP outcomes can be determined. FAPESP, CAPES.

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Differences in the endometrial response to age-matched long and short conceptuses during early pregnancy in cattle

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Bovine embryos undergo a prolonged post-hatching development during which they undergo dramatic morphological change (elongation process), passing sequentially from a spherical to a filamentous-shaped structure. Inadequate elongation of the conceptus results in lower production of interferon tau (IFNT), maybe contributing to the inability to maintain the corpus luteum and thus pregnancy loss. Significant gaps in our knowledge of the complex biological mechanisms governing conceptus elongation remain. We have previously reported significant variation in conceptus length amongst conceptuses recovered from the same uterine environment following multiple embryo transfer that may be related to an inherent lack of developmental competency. Others have reported differences in gene expression between conceptuses recovered on Day 15 of gestation, based on their length. Here, we combined in vitro production of bovine blastocysts, multiple embryo transfer techniques and a conceptus-endometrial explant co-culture system to study the dialogue between an advanced (long) and retarded (short) conceptus and the maternal endometrium on Day 15 of pregnancy. We hypothesized that differences in endometrial response between long and short age-matched conceptuses could be either dependent or independent of IFNT. Seven long (mean length ± SEM 25.4 ± 5.7 mm) and six short (1.8 ± 0.3 mm) Day 15 conceptuses recovered from recipient heifers following the transfer of Day 7 blastocysts (10 per recipient), were individually placed on top of endometrial explants (from uteri recovered from heifers in the late luteal phase) and then co-cultured for 6 h in one mL of RPMI medium. Additional explants were cultured with medium containing 100 ng/mL of recombinant ovine IFNT (IFNT; n = 6) or in media alone (Control; n = 6). Total RNA was isolated from explant cultures and analyzed by RNA-Seq. Exposure of endometrium to IFNT, a large conceptus or a small conceptus altered ($P < 0.05$) the expression of 491, 498 and 230 transcripts, respectively, compared with control endometrium. Further analysis revealed three categories of differentially expressed genes (DEGs) compared to the control: (i) genes commonly responsive to exposure to IFNT and conceptuses irrespective of size (n = 223); (ii) genes commonly responsive to IFNT and long conceptuses only (n = 168) that are over-represented by metabolism, cell-cell adhesion, receptor-mediated endocytosis, and mitochondrion organization functions, and (iii) genes induced by the presence of a conceptus but independent of IFNT (n = 108) - of these genes, the vast majority (n = 101) were exclusively induced by long conceptuses. Functional analysis revealed that regulation of molecular function, magnesium ion transmembrane transport, clathrin coat assembly, and beta-amyloid metabolism were the principal gene ontologies associated with these DEG. In conclusion, bovine endometrium responds differently in terms of its gene expression signature to embryos of varying size, in both an IFNT-dependent and independent manner, suggesting that these differences in communication between a long and short conceptus and the endometrium may be critical for embryo survival. Funded by Irish Department of Agriculture, Food and The Marine through the Research Stimulus Fund (Grant number: 13/S/528).

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Effect of time of gamete co-incubation on *in vitro* production of bovine embryos

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In vitro production of bovine embryos has become a widespread technology of assisted reproduction implemented in cattle breeding in Bazil. The aim of this study was to evaluate the effect of duration of gamete co-incubation on embryo production. The experiment was conducted at the laboratory of animal breeding and reproduction in the experimental station of the Agronomic Institute of Pernambuco (IPA) in the city of Arcoverde. For the study, 319 oocytes were aspirated from antral follicles (3-8 mm) of ovaries from recently slaughtered cows, and then each 20-25 oocytes were matured for 24 hours in drops of 100 microliters of TCM199 medium. After maturarion, the oocytes were divided into three groups acording to the co-icubation time: T1 (10 hours-105 oocytes); T2 (14 hours -113 oocytes) and T3 (18 hours-101 oocytes) co-incubation, respectively, and were fertilized by $2*10^6$ spermatozoa. Spermatozoa were obtained from frozen-thawed commercial semen collected from one bull (Alta Genetics Brazil). Thawed spermatozoa were washed in a discontinuous gradient of 45/90% Percoll using centrifugation at 700 g for 10 min. The pellet was resuspended with washing medium TALP and centrifuged once again at 700 g for 5 min. The final pellet was resuspended with *in vitro* fertilization (IVF) medium. After the IVF, drops of 100 μL of IVF medium were covered with mineral oil. After IVF, the presumptive zygotes were washed and removed from fertilization wells at 10, 14 or 18 hours post-IVF and then cultured in SOF medium. The incubation was at 38.5°C, with 5% CO2, and 95% of humidity. The cleavage rate was observed 48 hours after IVF; and 7 days after IVF, the blastocyst rate was observed. Statistical analyses were performed using the SAS software package version 9.1.3, using the PROC FREQ procedure and the Chi-square test. The cleavage rates were 38.0%; 41.5%; 45.6% in T1, T2 and T3 respectively, and the co-incubation time of the gametes did not influence the cleavage rate ($P > 0.05$). Similarly, no differences were observed in relation to the number of initial blastocysts, blastocysts, expanded blastocysts and hatched blastocysts ($P > 0.05$). Although studies of other research groups have shown good results with a short period of co-incubation of the gametes, in this study the lowest blastocyts rate was produced after 10 hours of co-incubation. The rates of embryos produced were 17,14, 23,89, and 28,71% ($P < 0.005$). Although co-incubation for only 10 hours presented a smaller number of embryos produced, we believe that more studies must be done, because there is a strong influence of the semen on IVF of cattle. Our results have shown that in commercial production of embryos, it is possible to maximize the use of semen, since we have an interval of 4 hours of co-incubation without negatively affecting the production of embryos. References: Effect of spermatozoa concentration on *in vitro* production of bovine embryos. Al Shebli Wasim[1], Santos Filho A.S[2], Bartolomeu C.C[3], Guido S.I.[2] Anim. Reprod., v.14, n.3, p.734, Jul./Sept. 2017.

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Improvement on the composition of sequential culture media based on the bovine content of oviductal and uterine fluids

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The *in vitro* production (IVP) of bovine embryos aims to mimic the female reproductive tract in order to generate blastocysts more similar to those produced *in vivo*. However, the concentration of nutrients available in a dynamic system such as the *in vivo* may be greater than that required for a static *in vitro* system. In fact, in rats, the development of zygotes at reduced amounts of nutrients in the culture medium increased blastocyst quality and rates. The aim of this work is to develop a sequential culture media – named embryonic culture supplementation (ECS) – based on the content of energy substrates and amino acids present in the composition of bovine oviduct (ECS1) and uterus (ECS2) fluids. Also, we intend to verify if, *in vitro*, a reduced concentration of such substrates could be beneficial to embryo development and quality. For this, we used the salt-based composition of SOF (Synthetic Oviduct Fluid) supplemented with 8 mg/mL of Bovine Serum Albumin (BSA) as the base for *Conventional control group* (SOFaa: supplemented with 2% essential amino acids, 1% nonessential amino acids, 1.5 mM glucose) and both culture media (*ECS1 and ECS2* supplemented with glucose, pyruvate, lactate and amino acids according to the composition of oviductal and uterus fluid). We prepared ECS1 and ECS2 supplemented with 100% (ECS 100), 75% (ECS 75), 50% (ECS 50) and 25% (ECS 25) of substrates concentration to evaluate the effect of reduced supplementation on *in vitro* culture (IVC). To determine the efficiency of these new culture media, blastocysts were produced *in vitro* by conventional protocols. At the time of IVC, presumptive zygotes were transferred to *ECS1 100, ECS1 75, ECS1 50, ECS1 25* and *SOFaa*. At Day 4 (D4), cleavage rates were assessed and embryos from all ECS1 groups were transferred to their correspondent ECS2 where they remained until blastocyst (D8) and hatching (D9) evaluation. All data were analyzed by Student t test (n = 469 oocytes/3 replicates). Preliminary data show that the reduction of nutrients availability does not affect cleavage and blastocysts rates when compared to control group, respectively in ECS 25 (75.8 ± 6.6 and 27.4 ± 6.7), ECS 50 (85.7 ± 4.5 and 34.8 ± 10.5), ECS 75 (82.1 ± 4.8 and 25.8 ± 7.5), ECS 100 (84.2 ± 4.5 and 20 ± 1.2) and SOFaa (80.6 ± 2.8 and 22 ± 4.7). However, the hatching rate is significantly higher in groups ECS50 (48.2 ± 5.7; P = 0.05) and ECS75 (73.5 ± 8.5; P = 0.01) when compared to SOFaa (12.1 ± 12.1). Blastocysts from the ECS 50 presented higher speed of development and better morphology (subjective evaluation). In conclusion, the formulated sequential culture media is not only able to support embryo development to blastocyst, but the reduction in energy substrates and amino acids concentration seems to be beneficial for blastocyst quality. Despite these promising results, the molecular and biochemical analysis are being conducted to verify a possible improvement in viability. Center of Natural and Human Sciences, Universidade Federal do ABC, Santo André, SP, Brazil.

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Transcervical is more efficient than surgical embryo collection in Brazilian hair sheep

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This study compared the efficiency of either transcervical (TC) or surgical (laparotomy; LP) embryo collection in hair sheep. Santa Inês ewes (n = 27) were subjected to a short-term protocol for estrus synchronization and superovulation. Both cervix passage attempt and laparoscopic *corpora lutea* (CL) count were performed 12 to 24 h before embryo collection. Depending on the success of cervical passage, the ewes were collected by either TC (n = 16) or LP (n = 11). The cervical dilation protocol was applied in all ewes and consisted of 120 µg cloprostenol i.m and 100 µg estradiol benzoate i.v. (diluted in 2.5 mL of saline + 2.5 mL of ethanol), both given 12 h before, and 100 IU oxytocin i.v. 15 min prior to cervical passage attempt. All ewes were sedated with 0.1 $mg.kg^{-1}$ acepromazine and 0.2 $mg.kg^{-1}$ diazepam i.v. and then received an epidural injection with 2.0 $mg.kg^{-1}$ ketamine. The TC collection was performed using a circuit closed system (Circuito Embrapa for goats/sheep embryo recovery). LP collection was carried out using the same sedation procedures but with anesthetic induction with 4 $mg.kg^{-1}$ propofol and 0.1 $mg.kg^{-1}$ diazepam i.v. and maintenance with 3% isoflurane. Uterine flushing recovery was aided by a foley catheter and an urethral probe. Behavioral aspects such as time to standing and to eat after each procedure were recorded. Heart rate (HR) and rectal temperature (RT) were measured at 10 moments: before fasting, before sedation, during the procedure, immediately after the procedure, and 1, 3, 6, 12, 24 and 48 h after the procedure. Normal data were compared by one-way ANOVA followed by Student's t-test or Tukey's test. Non-normal data were analyzed by Kruskal-Wallis test followed by Student Newman-Keuls. the LP procedure took longer than TC (31.6 ± 14.3 *vs* 24.5 ± 6.5 min; $P < 0.05$). The uterine flushing recovery (99.2 *vs* 91.9%) and embryo recovery (60.5 *vs* 37.1%) were greater for the TC than LP method ($P < 0.05$). Compared with the LP group, TC ewes had a higher RT during (37.0 *vs* 36.4°C; $P < 0.05$) and immediately after (36.9 *vs* 35.7°C; $P < 0.05$) the procedures. HR was higher in TC when compared to LP group immediately after collection (101.8 *vs* 88.0 bpm; $P < 0.05$). However, HR was higher ($P < 0.05$) in the LP than TC group at 12 h (94.2 *vs* 79.8 bpm) and 24 h (108.7 *vs* 88.3 bpm) after the procedures. Behavioral aspects were not different between techniques ($P > 0.05$). This study demonstrated the overall superior efficiency of TC embryo collection in Brazilian hair sheep. FAPERJ, CNPq (400785/2016-1) and Embrapa (02.13.06.026.00.02).

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Factors that affect embryo transfer conception rate in lactating dairy cows

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Embryo transfer could be used to produce more calves from high genetic merit cows and also could increase fertility of cows with high milk production. The objective of this study was to evaluate the effects of donor category (dry or milking cow); embryo category (*in vivo* produced fresh or frozen); and synchronism (6, 7 or 8) on conception rate following embryo transfer (ET) in lactating Holstein cows. Data from 8241 ET performed from 2007 until 2017, in a single commercial dairy farm were analyzed. Cycling lactating Holstein cows, producing 42.64 ± 7.2 kg milk/d, treated with Presynh-Ovsynch or after natural heat received ET 6 to 8 days later. The embryos were produced *in vivo* in non-lactating or lactating dairy cows and were transfered fresh or frozen. Only embryos grade 1 or 2 were transfered fresh or frozen. Pregnancy was diagnosed 35 days after estrus. The variable conception rate was analyzed by multiple logistic regression at SAS program and the model included effects of donor category, embryo category, synchronism, and interactions. The effect of donor category was not detected ($P = 0.395$). The conception rate for embryos produced in non-lactating cows was 50.92% (3122/6131) and in lactating cows 49.62% (1047/2110). But the conception rate was affected by embryo category ($P < 0.001$). Transfer of fresh embryos resulted in 56.08% (2311/4121) of conception rate and frozen embryos 45.10% (1858/4120). Effects of synchronism were detected in conception rate ($P < 0.001$). The ET at 6 days after estrus resulted in 48.10% (695/1445) of conception rate, at 7 days 49.85% (2748/5513) and at 8 days 56.59% (726/1283). No interaction between the variables was detected. The transfer of fresh embryos is an important tool to increase the probability of conception of lactating Holstein cows. Despite of lower conception rate after transfer of frozen embryos, for the establishment of an embryo transfer program and to achieve a good reproduction efficiency, the farm should have a reserve of frozen embryos, because the cows that will receive embryos are set 7 days before, and sometimes the production of fresh embryo is less than what is needed, and for a more successful program, embryo transfer should be performed at 8 days after estrus. Ruann and Maddox Dairy.

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Sertoli cell-mediated differentiation of bovine fetal mesenchymal stem cells into germ cell lineage in an *in vitro* co-culture system

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Due to their abundant source and high differentiation potential, mesenchymal stem cells (MSC) may be suitable candidates for in vitro gamete derivation. Nevertheless, germ cell differentiation requires endocrine and auto/paracrine regulation in a specific environment, as well as direct cell-to-cell interactions provided by the somatic cells of the testis. Sertoli cells (SC) play an essential role by forming niches for germ cells providing essential factors for germ cell (GC) differentiation. The aim of the present study was to evaluate the effect of co-culture of SC on bovine fetal MSC (bfMSC) differentiation into germ cell lineage. Sertoli cells were isolated from bull testis and characterized by quantification of biomarker wilms tumor 1 (WT1) expression and androgen binding protein (ABP) mRNA levels using flow-cytometry (FC) and quantitative-PCR (Q-PCR) analyses. bfMSC were isolated from bone marrow (BM-MSC) and adipose (AT-MSC) tissue and were co-cultured with SC for 21 days. Bovine testis samples (positive controls), fibroblasts (negative controls), bfMSC-SC co-cultures, bfMSC and SC were analyzed for expression of housekeeping genes β-ACTIN and GAPDH, pluripotent genes OCT4 and NANOG and male germ cell genes DAZL and PIWIl2 by Q-PCR and FC. High levels of WT1 and ABP mRNA were quantified in bovine SC and testes samples; however, no transcripts of these genes were detected in bovine fibroblasts. Moreover, a high ($P < 0.05$) proportion (85.9 ± 3.9%) of SC were positive for WT1. OCT4 mRNA levels were higher ($P < 0.05$) in cocultures of SC with BM-MSC and AT-MSC at Day 14 compared to monocultures of SC, BM-MSC and AT-MSC. Moreover, a higher ($P < 0.05$) proportion of cells positive for Oct4 was detected in cocultures of SC and BM-MSC (71.2 ± 0.9%) compared to SC monoculture (4.4 ± 0.7%). The proportion of cells positive for Nanog was higher ($P < 0.05$) in cocultures of SC with BM-MSC compared to monocultures of SC and BM-MSC. Levels of mRNA of DAZL were upregulated ($P < 0.05$) in coculture of SC with AT-MSC at Day 14 compared to monocultures of SC and AT-MSC. However, at Day 21, DAZL mRNA levels were reduced ($P < 0.05$) in coculture of SC and AT-MSC. Dazl expression was higher ($P < 0.05$) in monocultures of AT-MSC (57.2 ± 0.4%) and cocultures of SC with AT-MSC (54.2 ± 3.4%) compared to monocultures of SC (36.8 ± 1.7%) and BM-MSC. Levels of mRNA of PIWIL2 were higher in monocultures of SC and AT-MSC, and in cocultures of SC with BM-MSC compared to Day 14. Moreover, PIWIL2 mRNA levels were upregulated in coculture of SC with AT-MSC at Day 14 compared to Day 21 and monocultures of SC and AT-MSC. These data indicate that coculture with SC may increase the proportion of cells expressing Oct4 and Nanog suggesting an advance of BM-MSC into a pluripotent state. Moreover, the cell-to-cell interaction with SC may mediate activation of GC-specific genes inducing differentiation into the GC lineage. Supported by Fondecyt grant 1161251, Government of Chile.

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Nuclear Maturation of bovine oocytes submitted to intrafollicular transfer of immature oocytes (IFIOT)

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The objective of the present study was to evaluate nuclear maturation status of bovine cumulus oocyte complexes (COCs) submitted to different periods of *in vivo* maturation after intrafollicular transfer of immature oocytes (IFIOT). For IFIOT, animals were synchronized by administrating an intramuscular injection of 2.0 mg estradiol benzoate and inserting an intravaginal progesterone-releasing device on Day 0. On D8 Progesterone device was removed and an intramuscular injection of PGF2α was administered. On Day 9 those heifers presenting one dominant follicle (≥ 10 mm) received an injection of estradiol benzoate and the IFIOT. COCs were aspirated from slaughterhouse ovaries (2-8 mm diameter follicles), selected and injected into the dominant follicle in groups of 50. To evaluate maturation, the injected follicles were aspirated at 16 h (n = 171), 24 h (n = 104) and 30 h (n = 144) after IFIOT. For control, COCs were in vitro maturated for 0 h (CONT0, n = 125), 16 h (CONT16, n = 122), 24 h (CONT24, n = 126) or 30 h (CONT30, n = 133). During the handling period all COCs were kept in follicular fluid. After maturation period, oocytes from all groups were denuded, fixed and stained with lacmoid. Oocytes were classified according to meiotic stage as germinal vesicle (GV), germinal vesicle break down (GVBD), metaphase I (MI), anaphase I (AI), telophase I (TI) and metaphase II (MII). Data of nuclear maturation were analyzed by Chi-square test ($P < 0.05$). At 0 h all of the COC were at in GV stage. On the CONT group the majority of the oocytes were in MI at 16 h (45.6%) and in MII at 24 h (96.8%) and 36 h (94.05). However, a different kinetics ($P < 0.05$) was observed for all IFIOT groups in every maturation time. Higher percentage ($P < 0.05$) of oocytes were at GVBD stage at 16 h (45.6%) than at 24 (10.6%) and 30 h (13.2%) after IFIOT. In contrast, MI stage was observed in a lower percentage ($P < 0.05$) of oocytes in the IFIOT16 (24.6%) compared to IFIOT 24 (57.6%) and 30 (57.6%). The percentage of oocytes that completed maturation and were at MI was different ($P < 0.05$) for all IFIOT groups being 0.6% for ITFOI16, 3.8% for IFIOT 24 and 12.5% for IFIOT30. These results suggest that IFIOT induces a delay in nuclear maturation in at least 8 h. In addition, 30 h of *in vivo* maturation is not sufficient for the oocyte to reach MII stage, if the IFOIT is performed 24 h after the removal of progesterone device.
Acknowledgements: CNPq, FAPDF, Embrapa.

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Effects of ovarian synchronization associated with a two-step IVM strategy on *in vitro* embryo production in cattle

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Suboptimal *in vitro* conditions for oocyte maturation uncoupling nuclear and cytoplasmic maturation is a major challenge to increased efficiency of *in vitro* fertilization (IVF) in cattle. In addition, oocytes obtained by ovum pick up (OPU) are heterogeneous with respect to chromatin configuration, transcriptional profile and developmental competence. We have recently developed a synchronizing protocol including ultrasound-guided follicle aspiration and FSH treatment that allows the recovery of a more homogenous population of oocytes, most of them at an intermediate stage of chromatin compaction. This study aimed to assess the association of this synchronizing protocol with a two-step *in vitro* maturation (IVM) strategy previously tested in our laboratory on *in vitro* embryo production in cattle. Twenty-four non-lactating Holstein cows were subjected to two treatments in a "cross-over" design with a 30-d interval between replicates. The Control treatment was OPU at a random day followed by the farm IVM protocol with culture for 24 hin a medium containing 0.5 mg/mL FSH and 10% bovine fetal serum. Cows in the Treatment group had all follicles larger than 3 mm aspirated at a random day considered as day 0, received two IM injections of FSH (Folltropin; 56mg) 12 h apart on day 2, and were subjected to OPU on day 5. Cumulus-oocyte complexes (COCs) were cultured for 9 h in a pre-IVM medium containing 100 ng/mL NPPC and intrafollicular levels of FSH and steroids, and then 24 h in a serum-free medium containing intrafollicular concentrations of FSH and 100 ng/mL AREG (compositions described in Soares *et al.* 2017). After IVM, COCs from both groups were submitted to the farm protocol for IVF and *in vitro* culture (IVC). After 8 d of IVC, embryo production was assessed by total blastocyst rate in relation to the number of oocytes subjected to IVM and the percentage of viable embryos (those morphologically selected for freezing and later transfer) in relation to total blastocysts. Data were arcsine transformed and groups compared with the Student's t-test. Control and Treatment groups did not significantly differ for either blastocyst rate or percentage of viable embryos. Blastocyst rates were 28.9 ± 3.95% (n = 416 oocytes) and 17.84 ± 2.15% (n = 428 oocytes) for Control and Treatment groups, respectively, whereas rates of viable blastocysts were 61.87 ± 7.18% and 69.85 ± 7.06%, respectively. In conclusion, protocols for ovarian synchronization still need to be fine-tuned with embryo culture methods to significantly impact on *in vitro* embryo production in cattle. Nevertheless, the association of ovarian synchronization with a serum-free two step IVM system provided embryo outcomes comparable to those provided by a regular IVM protocol using serum and a supraphysiological concentration of FSH. CAPES, FAPESP, Prof. José Luiz Moraes Vasconcelos, União Química.

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Superstimulation prior to *ovum pick-up* to improve *in vitro* embryo production in buffalo donors

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The aim of this study was to evaluate follicular population and oocyte and embryo production of buffalo donors submitted to superstimulation with FSH prior to *ovum pick-up* (OPU) and *in vitro* embryo production (IVEP). A total of 54 buffalo donors (18 heifers, 15 primiparous and 21 multiparous) was randomly allocated to one of two groups (Control or FSH), in a *cross-over* experimental design. All animals received an intravaginal P4 device (1.0 g) plus EB (2.0 mg, intramuscular [im]) at a random stage of the estrous cycle (Day 0). Buffalo donors in the Control group received no further treatment, whereas buffalo donors in the FSH group received a total dosage of 200 mg of p-FSH on Days 4 and 5 in four decreasing doses 12 h apart (57, 57, 43, and 43 mg). On Day 7, the progesterone device was removed, and OPU was conducted in both groups. Data were analyzed by the GLIMMIX procedure of SAS 9.3®. There was no difference between groups (P = 0.53) regarding the total follicles aspirated, however, the FSH treatment increased (P < 0.001) the proportion of large (> 10 mm; FSH = 16.2% and Control = 2.0%) and medium-sized (6-10 mm; FSH = 36.3% and Control=6.1%) follicles available for the OPU procedure. The viable oocyte rate was greater in buffalo donors treated with FSH compared to Control group (Heifers = 58 vs. 50%; Primiparous = 56 vs. 47%; Multiparous = 57 vs. 50% respectively; P = 0.03). Also, buffalo donors treated with FSH had a higher blastocyst rate (Heifers = 34 vs. 17%; Primiparous = 28 vs. 27%; Multiparous = 32 vs. 24%; P = 0.03) and embryo yield per OPU-IVEP session (Heifers = 3.7 ± 0.7 vs. 1.8 ± 0.5; Primiparous=2.7 ± 0.8 vs. 2.4 ± 0.6; Multiparous=2.6 ± 0.7 vs. 2.0 ± 0.5; P = 0.07). These results provide evidence that superstimulation with FSH increased the proportion of medium-sized follicles available for the OPU procedure. Consequently, the treatment also enhanced the proportion of viable oocytes for culture and resulted in greater blastocyst rates and embryo yield per OPU-IVEP session in buffalo. It was concluded that superstimulation with FSH prior to OPU increased the IVEP efficiency in buffalo donors.

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A pre-maturation with C-type natriuretic peptide plus estradiol prolongs meiotic arrest in juvenile goat oocytes

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In vitro embryo production (IVP) could be an important assisted reproductive technology to rapidly disseminate desirable genetic traits in goat breeds. Oocyte competence is a key factor for the success of IVP, which is dependent on synchronizing both nuclear and cytoplasmic maturation. Oocytes retrieved from antral follicles are arrested at the germinal vesicle (GV) stage, but immediately resume meiosis spontaneously. Furthermore, oocytes from juvenile goats have low embryo development due to the small size of the follicles from which they come, with an unknown grade of atresia that make them unpredictable for IVP. Previous studies in other livestock species have reported that a prolonged meiosis inhibition by implementing a 'pre-maturation' period, with IBMX (a general phosphodiesterase inhibitor) or C-type natriuretic peptide (CNP; which physiologically maintains the oocyte in GV), improves oocyte competence. The aim of the present study was to investigate the impact of CNP +/- estradiol on meiosis of oocytes from juvenile goats (2 months old), as the first step to develop a pre-in vitro maturation (pre-IVM) system that could improve their competence. Ovaries from juvenile goats were collected at a local slaughterhouse. Cumulus oocyte complexes (COCs; N = 668) were recovered by slicing follicles in TCM-199 medium with HEPES, NaHCO3, and heparin plus 500 µM IBMX (to avoid GV breakdown during oocyte recovery). Morphologically selected COCs were incubated for 6 or 8 h in a pre-IVM medium at 38.5ºC and 5% CO_2. The pre-IVM medium (control group) was: TCM-199 with 100 µM cysteamine and 0.4 % (w/v) bovine serum albumin. In experiment 1, pre-IVM medium was supplemented with different doses of CNP (50 nM, 100 nM and 200 nM). In experiment 2 pre-IVM medium contained the same doses of CNP plus 10 nM estradiol (E2; 50 nM CNP + E2, 100 nM CNP + E2, 200 nM CNP + E2 and an E2-only group). After pre-IVM oocytes were fixed in ethanol:acetic (3:1) and stained with 1% orcein in 45% acetic acid for nuclear assessment. Twelve oocytes were assessed per group per time point in 3 replicates. Data was analyzed with two-way ANOVA followed by Tukey's post-hoc test. The GV rate between control and experimental treatments were not different in experiment 1. In contrast, in experiment 2, 200 nM CNP plus E2 had a higher GV rate ($P < 0.05$) at 6 h (75.4%) compared to the control group (27.8%) and the E2 group (31.6%). No significant differences were observed with the other concentrations of CNP (50 nM 48.7%, 100 nM 50.2%). However, the increased GV rate with 200 nM + E2 at 6 h was ameliorated by 8 h (44.4%) compared to the control group (25.4%). In other species, CNP can inhibit GVBD by itself, but the addition of E2 enhances the effect of CNP by increasing the expression of the CNP receptor (NPR2). Our results imply that basal NPR2 expression in juvenile goat oocytes is low but with the addition of E2 enables CNP to transiently block nuclear maturation. In conclusion, we have developed a protocol for blocking the nuclear maturation using CNP plus E2 in juvenile goat oocytes. More studies are required to understand the mechanisms of action of these molecules and to assess if this pre-IVM protocol improves the developmental competence of juvenile goat oocytes. Funding: Spanish MINECO (AGL2017-85837-R) and Spanish MECD (FPU14/00423).

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Effect of CRISPR/Cas9 microinjection on development and mutation rate of sheep emrbyos

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The objective was to evaluate the effect of zigote cytoplasmic microinjection on development and mutation rate of *in vitro* produced sheep embryos. Ovaries were collected from a slaughterhouse and oocytes were recovered and selected for *in vitro* maturation. Only good quality oocytes were incubated for embryo production by *in vitro* maturation, fertilization and culture as previously described (Menchaca *et al*., 2016. Anim Reprod, 13:273-278). The time of insemination was defined as Day 0. Microinjection of presumptive zygotes was performed into the cytoplasm with CRISPR/Cas9 system 16-18 h after fertilization. A total of 983 presumptive zygotes were divided in different experimental groups: Buffer (n = 323), Protein (n = 347), RNA (n = 313) and a control group without microinjection (n = 146). Microinjection of Buffer group was done with buffer alone (10 mM Tris, 0.1 mM EDTA); in the Protein group it was performed with 100 ng/µL of sgRNA85, 100ng/µL of sgRNA258 and 50 ng/µL of Cas9 protein diluted in injection buffer (20 mM Hepes, 150 mM KCl); while the RNA group was microinjected with 7.5 ng/µL of sgRNA85, 7.5 ng/µL of sgRNA258 and 20ng/µL of Cas9 mRNA diluted in injection buffer (10 mM Tris, 0.1 mM EDTA). After microinjection, the zygotes were mantained under cultutre conditions with 5% CO_2, 5% O_2, 90% N_2 at 39°C in a humidified atmosphere until Day 8. On Day 6, DNA from morulae/blastocyst obtained from the Protein group (n = 11) and the RNA group (n = 9) was extracted and analyzed by Sanger sequencing to detect mutations at the gene level. Cleavage rate on Day 3 (2 to 8-cell embryos/microinjected zygotes) and development rate on Day 6 (morulae and blastocysts/microinjected zygotes) were evaluated and the results were expressed as Mean±SEM. Statistical analysis was performed by generalized linear mixed models and differences were considered significant at a level of $P < 0.05$. Cleavage and development rate were lower in microinjected than control zygotes (Buffer 60.7 ± 4.6 and 23.2 ± 2.1, Protein 65.5 ± 3.3 and 28.0 ± 2.5, RNA 62.9 ± 4.0 and 22.3 ± 3.5, Control 79.4 ± 2.9 and 43.9 ± 5.4, respectively; $P < 0.05$). There was no difference between microinjected groups (Buffer, Protein and RNA; P = NS). As a result of the sequencing analysis, none of the Protein microinjected embryos resulted in mutation whereas 53.85% (7/13) of RNA microinjected embryos analyzed resulted in mutation ($P < 0.05$). The current experiment demonstrated that microinjection into zygotes negatively affected the cleavage and development rate of *in vitro* produced embryos, independently of the use of Buffer, Protein or RNA. Furthermore, the greater efficiency for mutation induction was obtained with Cas9 RNA microinjection for CRISPR/Cas9 system.

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Relations between plasma anti-muellerian-hormone (AMH) concentrations, fertility performance and influencing factors in Holstein-Friesian heifers

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An independent prognostic biomarker for fertility would be worthwhile in cattle breeding. Some recent publications described the measurement of the ovarian reserve as an interesting candidate. One specific marker of the ovarian reserve is the Anti-Muellerian-Hormone (AMH). However, results between the existing studies in this field are inconsistent. The aim of this study was to investigate, whether plasma AMH levels could be used in German Holstein heifers as a predictive and independent biomarker for their fertility as heifer or later as dairy cow under the conditions of milk production. Therefore, blood samples, animal related data and fertility parameters from 926 German Holstein heifers (age of 16 ± 2.3 month) were collected on five farms. Their plasma AMH concentration was measured by using a human, but for bovines validated AMH-ELISA-Kit (DSL-10-144400). Fertility parameters and performance data of the first lactation could be obtained and analyzed subsequently for 672 of the tested heifers. For statistical analysis correlations and linear regressions were calculated between the AMH levels and fertility/performance data. Differences between animal groups (different farms, numbers of insemination, months of pregnancy) were tested by ANOVA. To validate the applicability of AMH as an independent biomarker, environmental and animal related influencing factors on the AMH concentration were analyzed in a first step. No significant correlation between AMH and farm, age, weight, height, BCS, progesterone level and early pregnancy status at blood sampling was detected. Also the later milk yield, milk fat or milk protein showed no statistical significant correlation to the AMH level as heifer ($r < 0.1$, $P > 0.1$). In a second step relationships between AMH and fertility were examined, but the statistical analysis revealed also no significant correlation between AMH levels and the acquired fertility parameters during the observation period (age at first insemination, calving age, number of inseminations, days to first post-partum insemination, period from first to successful insemination, days open, conception rates; $r < 0.1$, $P > 0.1$). The results support the perception that AMH plasma levels of heifers could be considered as mainly independent of environmental and animal related influencing factors, but the results showed also clearly that a single assessment of the AMH plasma concentration in Holstein-Friesian heifers is not suitable as biomarker to predict fertility under the conditions of German milk production. This does not exclude a causal biological relationship between ovarian reserve and fertility, which may become apparent in other sampling schedules, production systems, breeds or the use of other assessment methods of the ovarian reserve.

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Prostacyclin PGI_2 modulates *in vitro* maturation of bovine cumulus-oocyte complexes

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Cumulus cells are essential for the proper proceeding of the bovine oocyte maturation. During maturation this germ cell acquires adequate developmental competence, cumulus cells undergo expansion, taking active part in the glucose metabolism, which is the energy substrate for the oocyte. The objective of the present study was to examine the effect of prostaglandin (PG) I_2 on cumulus expansion and glucose uptake as well as expression of genes involved in these processes. Cumulus-oocyte complexes (COCs; n = 10 for control and examined groups, replied 8 times) were obtained by aspiration from subordinate ovarian follicles (of the diameter less than 6 mm) and matured *in vitro* in presence or absence of PGI_2 (500 pg/mL) for 24 h. Following maturation, cumulus cells were separated from oocytes and used for gene expression analysis (amphiregulin - AREG, epiregulin - EREG, betacellulin - BTC, metalloproteinase family member - ADAM, epidermal growth factor receptor - EGFR, tumor necrosis factor alpha induced protein 6 - TNFAIP6, prostaglandin endoperoxide synthase 2 - PTGS2, pentraxin - 3-PTX3, hyaluronian synthase 2 - HAS2, glutamine-fructose-6phosphate transaminase- GFPT, phosphofructokinase - PFK, lactate dehydrogenase- LDH; Real-Time PCR). The data from Real-Time PCR were analysed by Miner PCR Software Algorithm with GAPDH as the reference gene. The obtained results were analyzed using student T test (GraphPad PRISM 6.0. Software). Although cumulus expansion did not vary in the examined groups of COCs, PGI_2 simulated mRNA expression of ADAM, PTGS2, GLUT1 and GLUT4 and reduced transcript abundance of CTSS and CTSK. The data obtained in the present study suggest that supplementation of the maturation medium with PGI_2 might enhance bovine oocyte maturation *in vitro* via the modulation of cumulus expansion and glucose metabolism in cumulus cells. Supported by Grants-in-Aid for Scientific Research from the Polish National Science Centre: 2015/17/B/NZ9/01688 and by the grant of KNOW Consortium "Healthy Animal - Safe Food", MS&HE Decision No. 05-1/KNOW2/2015.

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Effect of successive ovum pick up on follicular development, and oocyte quality and quantity, in a commercial, buffalo (*Bubalus bubalis*) *in vitro*, embryo production program

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The first buffalo calf born after *in vitro* embryo production (IVP) was in 2004 and despite the fact that many research teams are working to improve buffalo embryo production, there are very few commercial IVP programs for buffalo. In addition, although researchers have attempted applying methods used in cattle to buffaloes, there are species differences in follicular size and number, quantity and quality of oocytes, embryo development, and the need to use specially designed medium that make IVP in buffalo of lower efficiency than in cattle. The aim of this study was to evaluate the effect of short term, repeated ovum pick up (OPU) on follicular number and quality of oocytes in a commercial buffalo IVP program. The study was conducted in Corrientes Argentina during the breeding season of 2017. Twenty mature and fertile Mediterranean and Murrah donors, body condition score 3/5 to 4/5 with no anatomical abnormalities were available for OPU, and were subjected to 3 sequential, weekly transvaginal, ultrasound guided aspirations, using a vacuum pump at 40 mm/Hg pressure attached to an 18-g needle. Antral follicle count (AFC) was performed before aspiration. Oocytes were recovered from follicular fluid and graded from I to IV according number of granulosa cells and cytoplasm. Follicle number, grade and quantity of oocytes were recorded, and data was analyzed using paired Student t test and Pearson correlation coefficients. Statistical significance was established at $P < 0.05$. Fifty-nine OPU were performed, and the time for each averaged 9 min. Five hundred follicles were punctured, and 260 oocytes were recovered (52%); of those, 9%, 20%, 34%, 35% were Grade I, II, III, IV, respectively. There were no differences in AFC (mean 7.2 per buffalo). There were differences in the number of follicles aspirated within the third (7.8) aspiration compared with the first (8.8) and the second (8.4). No correlations were found between number of follicles and recovered oocytes. Our results show that, in buffalos, the number of ovarian follicles declines after 21 days of aspiration, which has also been reported by others. Because buffalo females tend to produce lower quality oocytes than cattle, all oocytes recovered after OPU are used for embryo production. Although it may be necessary to increase the sample size to find correlations between follicle numbers and oocyte recovery, this research contributes with more information for commercial OPU buffalo programs to improve the efficiency of IVP. Rincon del Madregon Cabaña, UNNE, Universidad Nacional de Colombia.

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Birth of fresh or vitrified CRISPR/Cas9 microinjected sheep embryos transferred on Day 3 or Day 6

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The objective was to evaluate the pregnancy outcomes of fresh or vitrified CRISPR/Cas9 microinjected sheep embryos transferred on Day 3 or Day 6 of *in vitro* development. A total of 501 microinjected embryos were randomly assigned to three experimental groups; fresh embryos on Day 3 (n = 120), fresh embryos on Day 6 (n = 75) or vitrified embryos on Day 6 (n = 306). Embryos were obtained by *in vitro* fertilization following the procedure routinely used in our laboratory (Menchaca *et al*., 2016. Anim Reprod, 13:273-278). Microinjection of the CRISPR/Cas9 system was performed into the cytoplasm of presumptive zygotes 17 h after fertilization (Day 0) using a specific CRISPR/Cas9 design for a knock out model, and the embryos were *in vitro* cultured until vitrification or transfer. Vitrification/warming were performed using Cryotop method in embryos on Day 6 (Santos-Neto *et al*., 2015. Cryobiology,70:17-22). Fresh 8 to 12-cell embryos on Day 3 (five embryos per ewe) and fresh or vitrified morulae/blastocysts embryos on Day 6 (two-three embryos per ewe) were transferred into synchronized Corriedale breed recipients. For both stages, embryo transfer was performed by laparoscopy placing embryos into the cranial side of the ipsilateral uterine horn to the corpus luteum. Pregnancy rate (number of pregnant ewes/total transferred ewes) and embryo survival rate on Day 30 of gestation (number of embryos on Day 30/total transferred embryos) were determined by ultrasonography, and birth rate (total born lambs/viable embryos on Day 30) was registered at delivery. Statistical analysis was performed by GLMM. Pregnancy rate was greater for recipients receiving fresh embryos on Day 3 and Day 6 compared with those vitrified on Day 6 (54.2%, 13/24; 48.0%, 12/25; versus 22.0%, 24/109, respectively; $P < 0.05$). Embryo survival on Day 30 of fresh embryos transferred on Day 3 (14.2%, 17/120) did not differ from those transferred on Day 6 (21.3%, 16/75; P = NS), while it was lower with vitrified embryos on Day 6 (10.5%, 32/306; $P < 0.05$) if compared with those transferred fresh on Day 6. Birth rate was similar between groups (fresh embryos on Day 3, 88.2%, 15/17; fresh embryos on Day 6, 75.0%, 12/16; vitrified embryos on Day 6, 87.5%, 28/32; P = NS). These preliminary results suggest that the transfer of microinjected fresh embryos allows greater pregnancy rate when compared to vitrified embryos. Since survival rate of embryos transferred on Day 3 and Day 6 was similar, the advantage of a reduced period of in vitro culture for early embryo transfer deserves further investigation. This information has implications for the generation of CRISPR/Cas9 edited animals in livestock.

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Oocyte quality and *in vitro* embryo production of aged Nellore cows selected for fertility

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Decreased fertility with maternal aging has been well documented in cattle. The main causes of the low fertility in aged cows are attributed to the poor quality of the oocytes and to depletion of the number of oocytes, due to endocrine disturbances and exhaustion of the ovarian follicular reserve, respectively. However, in some circumstances, cows older than the age in which they are regularly discarded from the herd can still ovulate regularly a good-quality oocyte and give birth to healthy calves. The present study aimed to evaluate the oocyte number and quality of aged and young cows, submitted to ovum pickup (OPU). Lactating Nellore cows (*Bos indicus*) with a history of annual calving and ages of 17 to 20 years (n=12) and 8 to 10 years (n=15), were treated with a progesterone vaginal device (PD) containing 1 g of progesterone and injected, by IM route, with 2 mg estradiol benzoate and 0,5 µg of cloprostenol. Five days later, the PD was removed and the oocytes were recovered by OPU. A second OPU was done five days later in all cows. The number and quality of recovered cumulus-oocyte complexes (COCs) were registered. The quality of immature COCs was classified as good (viable) and poor (not viable), according the appearance of cumulus, compaction and granulation of ooplasm. The COCs were matured in TCM plus FSH and 10% estrous cow serum. After fertilization, presumptive zygotes were co-cultured with cumulus cells to assess developmental rates to blastocyst. Data were analyzed by Chi-square and Anova tests. In total, 826 oocytes were recovered, of which 510 (mean = 42.5 ± 7.4) were from aged cows and 316 (mean = 21.1 ± 6.7) from young cows, respectively ($P < 0.05$). Although the proportion of morphologically viable oocytes was not different among groups (59.8% and 55.7%, respectively ($P > 0.05$), more viable oocytes were recovered from aged than young cows (37.7 ± 6.8 vs 15.0 ± 6.1), respectively ($P < 0.05$). More total oocytes were recovered in the first OPU than in the second OPU in both aged (28.9 ± 5.4 and 12.6 ± 3.1) and young (15.6 ± 4.9 and 5.8 ± 2.4) cows ($P < 0.05$). Similarly, the number of viable oocytes was higher in the first than in the second OPU (older = 25.4 ± 4.8 and 12.3 ± 2.3; young = 11.7 ± 4.3 and 3.3 ± 2.1), respectively ($P < 0.05$). Three aged cows older than 20 years did not produce oocytes at the two OPUs. There were significant differences among groups ($P < 0.05$) in cleavage rate (aged = 75.7%, and young = 95.4%) and blastocyst formation (aged = 26.9% and young = 39.2%). The mean number of blastocysts per cow was 3.1 ± 0.3 and 2.9 ± 0.3 for aged and young cows, respectively ($P > 0.05$). In conclusion, although the oocyte quality appears to be lower in aged cows, the higher number of oocytes produced by aging cows, selected for fertility, makes embryo production no different from young cows. Furthermore, the age threshold at which cows stop producing oocytes seem to be around 20 years.

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Differences in transcriptomic data from preovulatory follicles of buffalo *(Bubalus bubalis)* and (*Bos indicus*) cattle: A meta-analysis

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For years investigating the differences in reproduction between species has been restricted to describing the differences in clinical or biological parameters evaluated at different levels including gene expression using "omics," but very few publications have reported this information to understand the source of the differences or to analyze their influence on the reproductive performance of the species. Despite that buffalo and cattle belong to the bovine family, it has been reported that buffalo has a smaller ovary size with less number of primordial and antral follicles and higher incidence of atresia compared with cattle. This may provide a possible explanation for the reduced response in buffaloes to ovarian follicular hyperstimulation, low recovery of embryos and oocytes and low number of transferable embryos. The ovulatory process in mammals follows the same developmental pattern, the meiotically arrested oocyte must grow inside the follicle until ovulation, and if two closely related species have the same pattern in their estrus cycle, it can be deduced that the physiological and the molecular events are also similar. The aim of this paper was to compare the gene expression pattern of the granulosa cells of preovulatory follicles of buffalo and cattle, based on the available and comparable data reported in PubMed GSE39589 and GSE11312. In both experiments RNA was extracted, converted to cDNA, evaluated using Affymetrix Gene- Chip Bovine Genome Arrays, which contained 24,128 probe sets. Genes with =2 fold change as cut-off for identification where considered differentially expressed. The comparison of relative expression of the top 15 up- regulated genes from buffalo and cattle were very different, only 3 genes (20%) PLAT (plasminogen activator tissue), STAR (steroidogenic acute regulator, similar to Steroidogenic acute regulatory protein), F2RL- 1 (Coagulation factor II (thrombin) receptor-like 1) were expressed in the two species. It is very important to note the relative expression of the genes was very different, 9.7 vs 17.5, 8.7 vs 3.4, and 7.7 vs 5.3 fold for PLAT, STAR AND F2RL-1, respectively. All the shared genes are not related specifically with follicle development and are not considered as markers of ovulation. Although the two species are very close bovines and with the simplicity of the analysis, it seems that each one has its own different pathways leading to the same endpoint; i.e., ovulation. More sophisticated bioinformatic tools will be necessary to analyze the data to compare directly the data sets to construct a theory to explain the differences observed in the field. Asociación Colombiana de Criadores de Búfalos.

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Histone deacetylase inhibitor during pre-maturation (PIVM) and/or *in vitro* maturation (IVM) of bovine oocytes: effect on transcript levels of histone acetylation related genes

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Pre-maturation (PIVM), a period that precedes *in vitro* maturation (IVM), has been reported as a means to provide oocytes additional time to acquire competence and increase their developmental potential. The rationale behind PIVM approaches is to allow mRNA and protein accumulation within the ooplasm before meiotic resumption. However, for mRNA transcription to occur oocyte chromatin should be in A permissive or hyperacetylated status. The objective of the present study was to determine if the presence of a histone deacetylase inhibitor during PIVM and/or IVM could affect the expression profile of genes involved in the histone acetylation/deacetylation processes in bovine oocytes. Cumulus oocyte complexes (COCs) obtained from slaughterhouse ovaries were submitted to a PIVM using 100 nM of C-type natriuretic peptide (NPPC), in the presence or absence of a deacetylase inhibitor (500 nM of scriptaid). Grade 1 and 2 COCs were distributed into 5 groups: T1- IVM for 22 h; T2- PIVM for 6 h and IVM for 22 h; T3- PIVM with Scriptaid for 6 h and IVM for 22 h; T4- PIVM for 6h and IVM with Scriptaid for 22 h; and T5- PIVM with Scriptaid for 6h and IVM with Scriptaid for 22 h. For gene expression analysis, oocytes from all groups were collected at 0h of IVM and PIVM, 6 h of PIVM and at 22h of IVM. Levels of transcripts for genes coding for enzymes involved in acetylation (HAT1 and KAT2A) and deacetylation (HDAC1 and HDAC3) of histones were determined by qPCR, using the constitutive gene PPIA for normalization. Total RNA was extracted from 3 pools of 20 oocytes from each treatment. Data were analyzed by ANOVA, and the means compared by Tukey test ($P < 0.05$). Expression of all genes studied was similar among treatments at any of the different time points evaluated. However, when the profile of the genes during PIVM and IVM was analyzed it was observed that transcript levels for HAT1 in the control group decreased during maturation, being lower at 22 h compared to 0 h ($P < 0.05$). A different profile was noted when Scriptaid was present during either PIVM, IVM or both, since no decrease in transcript levels for the HAT1 gene was observed during maturation, but rather the levels were similar to those at the beginning of maturation ($P > 0.05$). For the other genes no alterations were observed during PIVM and/or IVM ($P > 0.05$). In conclusion, the presence of a histone deacetylase inhibitor during PIVM and/or IVM affected the transcript level of HAT1, preventing its decrease that occurs during *in vitro* maturation of bovine oocytes. Financial support: FAP-DF, CNPq, Embrapa.

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Heritability and genetic correlations for scrotal circumference at different ages in Brahman bulls raised under tropical conditions

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Beef herds in the tropics are based on the utilization of *Bos indicus* and its crosses bred naturally. Genetic progress has been traditionally restrained by many factors including social, climatic, nutritional and management, among others. Besides, there is little selection of sires through variables correlated to their profitability, such as scrotal circumference (SC). This fact justifies attention to and research on those valuable characteristics as selection criterion to benefit genetic improvement in those systems. This paper aimed to determine the heritability index and genetic correlations for SC at different ages in Brahman bulls, to establish its use as a robust selection criterion at early ages in sires raised under tropical conditions. Heritability index (h^2) and genetic correlations (r^g) for SC at different ages were determined in 485 full blood Brahman bulls distributed in 8 herds from the North dry Pacific region of Costa Rica. Monthly SC measurements were performed by the same operator from 7 to 24 months of age. The genetic estimates were determined with a bivariate random regression animal model (SAS, ver 9.3, 2010). The model considered the fixed effects of herd, year and season of birth, nutritional plane, body weight, age at weaning and calving number of the mother. In addition, the random permanent effect of environment and animal additive genetic effect were considered. The genealogy data base included 3000 animals distributed in 7 ancestors´ generations. The average h^2 index for SC was 0.58 (range 0.48-0.72), being highest at 20 months of age. In addition, SC h^2 was higher in the period comprising 14 to 20 months of age compared to younger ages. The h^2 index found in this study in Brahman bulls is higher than previous reports in crossbred zebu sires in Australia (Brahman and Sahiwal x Shorthorn; h^2=0.40 and 0.45 at 18 and 24 months respectively; Fordyce et al., 1996. Aust J Exp Agric, 36:9-17). In contrast, they are lower than those published for most *Bos taurus* breeds raised under sub-tropical climate. Coulter for instance (Coulter *et al*., 1976. J Anim Sci, 43:9-12), reported average h^2=0.68 in Holstein bulls under conditions of an experimental station. The SC r^g coefficients obtained in this study in Brahmans aged 7 to 24 months ranged from 0.43 to 1. Furthermore, r^g among yearling and bulls 18, 20, 22 and 24 months of age were higher (0.95, 0.92, 0.89 and 0.84 respectively) than those obtained among 7 months-old bulls and the same age range (0.43, 0.43, 0.44 and 0.45). These findings indicate that selection of Brahman sires by their SC can be performed as early as 12 months of age with high confidence (>80%) of their SC when adults (24 months). In addition, the lower h^2 and r^g for SC obtained among bulls <11 months and older ages (20-24 months), suggest that the initial post weaning is a stressful and adaptation period in Brahman steers. This fact should be considered by breeders and practitioners when choosing the right age for selection of prospective Brahman sires. This study was funded by the Andrology Section-UNA.

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The embryo regulation of the immune system in day 14 endometrium is affected by the level of nutrition

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The effects of the presence of conceptus and undernutrition on the endometrium transcriptome at Day 14 of the oestrous cycle or pregnancy were investigated. Adult Rasa Aragonesa ewes were allocated to one of two nutritional treatment groups: control, fed to maintenance requirements and undernourished, fed at 0.5-fold of daily requirements for maintenance. Sheep uterus transcriptome profiles were evaluated using an ovine oligonucleotide microarray in control and undernourished cyclic and pregnant ewes (n=4 each). Microarray data were analyzed with a mixed model using Proc MIXED (SAS Inst.). Functional bioinformatics analyses were performed using the Dynamic Impact Approach and Ingenuity Pathway Analysis. The upregulation of genes belonging to immune system pathways were consistent with the presence of an embryo regardless the nutritional treatment, underscoring their importance for pregnancy maintenance. Cytosolic DNA-sensing, RIG-I-like receptor signaling, and Toll-like receptor signaling pathways were upregulated in pregnant ewes. The presence of the embryo stimulated gene expression of the Interferon-induced helicase C domain 1 by 3.4-fold (control ewes) or 2-fold (undernourished ewes), which modulates local immune cells in the endometrium during pregnancy. Similarly, citokine *CXCL10* was upregulated by pregnancy with 6- (control) or 3- (undernourished) fold change, and is one classical interferon stimulated gene with biological effects on trophectoderm growth and adhesion in ruminants. While cytokine *CXCL12* (essential role in communication between trophoblast cells and the maternal endometrium) and Toll like receptor 7 (by acting in the trophectoderm influences conceptus development and IFNT production) were upregulated in control pregnant ewes, they remained unchanged in undernourished pregnant ewes. Activation of nuclear factor kappa B (3-fold change in undernourished pregnant vs. cyclic ewes) enhances uterine receptivity and development of conceptus during establishment of pregnancy. It is noteworthy that despite the similar flux among these pathways between control and undernourished ewes, the impact was lower in undernourished pregnant vs. cyclic animals, probably associated with a lower fold change in immune related genes, which could be associated with embryo losses after maternal recognition of pregnancy. Few genes of the immune system were affected according to nutritional treatment in pregnant ewes. In undernourished pregnant ewes, immune-related genes with known increased expression during pregnancy (CD180 molecule, Complement 5 and MYD88: myeloid differentiation primary response 88) were downregulated in undernourished vs. control pregnant ewes. CD180 is a TLR4 accessory protein that potentiates its action, and TLR4 through MYD88 induce activation of cytokines production involved in modulate the embryo-maternal tolerance. Indeed, C5 compensates for the decreased adaptive immunity observed in normal pregnancy, and is aimed to protect the mother and fetus from antigens. Clearly, downregulation of these important genes in undernourished ewes could be detrimental for successful uterine development, a scenario that agrees with the embryo mortality occurring after the period of maternal recognition of pregnancy.

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Dynamic changes in bovine endometrial stem cells throughout estrus cycle and postparturition

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We isolated and characterized bovine endometrial mesenchymal stem cells (beMSCs) during follicular (FP), early luteal (ELP) and late luteal (LLP) phases of the estrous cycle and after parturition in healthy cows (PPHE) and in cows with subclinical and clinical endometritis (PPSE and PPCE). Estrous cycle stages were confirmed by plasma P4 and 17ß estradiol measured by RIA, and health status by cytological and microbiological diagnosis. Characterization of putative beMSCs was based on fibroblast-like morphology, adherence to plastic and proliferation and colony formation at low density (cloning efficiency; CE) and also by expression of embryonic and MSC markers at mRNA (OCT4, NANOG, SOX2, and CD44, CD117 via RT-qPCR) and protein (Oct4, Sox2, and Cd44) levels. For the latter we used immunohistochemistry in fixed tissues and/or Western blot (WB) for both tissue and cells. We also evaluated the ability of isolated cells to differentiate into chondrogenic(C), adipogenic(A) and osteogenic(O) lineages after 7 and 14 days of induction, as measured by lineage-specific staining (Alcian blue, Oil Red and Alizarin Red, respectively) and by specific gene (AGGRECAN and SOX9 for C, PAPF1 for A, and SPARC and RUNX2 for O) and protein expression (Aggrecan for C, Ppar? for A, and Sparc for O) expression via RT-qPCR and WB. Cell lines were derived from endometrial biopsies by enzymatic digestion and cultured in supplemented DMEM-F12 (10% FCS, 1X AAM solution, 1mM sodium pyruvate, 2mM L-glutamine) at 5% CO_2, 39°C and full humidity. Results are shown separately for estrous cycle (group1) and post- partum (group2) or mixed. Statistical analysis of RT-PCR and CE was conducted using Kruskal–Wallis nonparametric test. All statistical analyses were tested for a=0.05. Putative beMSCs isolated from all groups showed fibroblast-like morphology and adherence to plastic. The ability to differentiate into C, A, and O mesodermal lineages after 7 and 14 days was not homogeneous among the samples: ELP cells did not differentiate and PPCE did not differentiate to the A lineage as judged by staining and further confirmed by individual analysis of specific differentiation markers. In all groups, cells proliferated and formed colonies when plated at low density. The best CE was in ELP cells (0.61±0.08; group1) and PPHE (0.64±0.1; group2), whereas PPCE displayed the lowest CE (0.08±0.1) of both groups. Expression of markers (RNA): both groups expressed with varying levels SOX2, OCT4, CD44, and CD117 both in cells and tissue, being higher in the latter. NANOG was not detected at all. Protein: Sox2 in all groups, Oct4 and Cd44 only in group1. All analyses were statistically significant. Seemingly, there are distinct populations of stem/progenitor cells in cow endometria during the estrous cycle, puerperium and endometritis. This detailed characterization of beMSCs had not been published earlier. Funding: Fondecyt1110642 and Conicyt national doctoral scholarship N°21150425, Government of Chile.

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A Comparative analysis of calcium channels in holstein and hanwoo (korean cattle) in the duodenum and kidney

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Transembrane calcium (Ca^{2+}) channels such as transient receptor potential cation channel subfamily V members 5 and 6 (Trpv5/6), Na^{+} /Ca^{2+} exchanger 1 (Ncx1) and plasma membrane Ca^{2+}-transporting ATPase1 (Pmca1b) are known to play an important role in maintaining homeostasis and metabolizing Ca^{2+} ions. Trpv5 and Trpv6 play an important role in Ca^{2+}absorption to the cell, while Ncx1 and Pmca1b play a role in Ca^{2+} excretion from the cell. Holstein is known to provide higher milk production than other cattle breeds but in this respect, it has higher susceptibility to hypocalcaemia that is a risk factor for many of calcium-related diseases such as milk fever. In contrary, Hanwoo (Korean cattle) is relatively strong in the calcium-related diseases. The hypothesis of this study is the differently expressed calcium transport genes in duodenum and kidney results in an increased prevalence of calcium related diseases such as milk fever. Genetic background (or breed) will influence transcript abundance of calcium transport genes in the duodenum and kidney. Expression of Trpv5/6, Ncx1 and Pmca1b was analyzed by realtime polymerase chain reaction (Realtime-PCR), Western blot analysis, and immunohistochemistry. Data were expressed as means ± standard deviations and were analyzed by using a nonparametric one-way ANOVA followed by the Tukey post hoc test (n = 12 per each group). In dairy cows (Holstein), Trpv5 mRNA was greater in kidney and duodenum than in kidney and duodenum of Hanwoo cows. Conversely, Pmca1b mRNA was less in Holstein cows than in kidney from Hanwoo, but no difference in Trpv6 or Ncx1 mRNA expression was observed in duodenum and kidney between the two breeds. Protein expression showed similar patterns in Hanwoo and Holstein cows to those of mRNA expression data. Localization of calcium transporter genes were identified in the glomerulus, proximal and distal convoluted tubules expressed TRPV5, 6, Pmca1b and Ncx1 in the kidney. These four calcium transport genes may play an important role in bovine duodenum, and kidney. But the difference between Holstein and Hanwoo, which show different gene expression patterns, may be helpful in studying diseases associated with calcium metabolism and to develop estartegies to prevent milk fever. This work was supported by a National Research Foundation of Korea (No. 2017R1A2B2005031) grant funded by the Korean government.

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Similarities in endometrial transcriptomic profile between high producing and anestrous dairy cows

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High milk production (HMP) dairy cows present a profound increase in feed intake which has been associated with higher steroid metabolism and lower concentration of plasma P_4 and E_2 compared with medium milk production (MMP) cows. Low P_4 may alter endometrial gene expression, reducing the capacity of the uterus to support conceptus development. However, the effect of low P_4 concentrations on endometrial gene expression is not well known yet. The aim of this study was to compare endometrial gene expression among HMP, MMP, superovulated (SO) and anestrous (ANE) cows during diestrus using RNA-seq technology. Grazing lactating dairy cows (~50 DIM) without any clinical disease were allocated to four groups: HMP (~10,000 kg/305d, n=3), MMP (~7,500 kg/305d, n=3), ANE (follicles <8 mm, without CL, <0.3 ng/mL P_4; n=3) and SO (P_4 =90 ng/mL; n=3). Cows from HMP, MMP and SO groups were synchronized with a Presynch-Ovsynch protocol. The SO cows also received a superovulation protocol. Blood was obtained on days 0, 4 and 9 of the estrous cycle to measure P_4 and E_2 concentrations by chemiluminescence (Immunoanalyzer Elecsys and Cobas e, Roche). Endometrial samples were collected using a biopsy instrument on day 9 and stored at -80ºC until RNA extraction. The cDNA libraries for RNA-seq were constructed using the TruSeq Stranded mRNA and sequenced using the HiSeq 1500 platform 2x150pb (Illumina Inc. CA, USA). Resulting sequence reads were aligned to the bovine reference genome (bosTau8), using the Rsubread package (R-software). Multidimensional scaling (MDS) was performed to determine similarities between samples according to gene expression. Differentially expressed genes (DEG, FDR<0.05) in HMP compared to MMP, and in ANE compared to SO, were determined by genewise statistical analysis (EdgeR package). The resulting up or down-regulated DEG were compared by Pearson's chi-square test to determine relatedness between the same significant genes determined in both HMP vs MMP and ANE vs SO (overlapping DEG). Enrichment analysis of the significant overlapping genes was performed with DAVID database. The MDS analysis showed that samples from MMP and SO cows clustered apart from HMP and ANE cows' samples. The numbers of upregulated DEG were 353 and 897, while down- regulated DEG were 324 and 764, for HMP versus MMP and ANE versus SO, respectively. There were 124 overlapping genes for the up-regulated DEG ($P=1.8x10^{-88}$) and they were enriched for MHC Class I (P=0.0000002), innate immunity ($P=3.3x10^{-8}$) and inflammatory response ($P=4.7x10^{-6}$). For the down-regulated DEG, the 40 overlapping genes ($P=1.8x10^{-5}$) were enriched for the GnRH signaling pathway (P=0.01), oxytocin signaling pathway (P=0.03) and calcium signaling pathway (P=0.004). In conclusion, HMP cows in diestrus and ANE cows present a similar endometrial transcriptomic profile showing enriched with up-regulated genes involved in the immune system and inflammatory response. This work was supported by a PICT 2014-0414 grant to LVM, by UNLP Incentive Program V11/230 grant to RLS and by ARPECOL grant to RLS. Keywords: RNA sequencing, dairy cows, milk yield, endometrial expression.

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Expression of immunological markers by bovine endometrial stem cells after priming with PGE_2

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The aim of this research was to characterize, at the mRNA level, the response of bovine endometrial mesenchymal stem cells (beMSCs) to an inflammatory illness-like environment after priming with PGE_2. For this, we used previously tested beMSC lines from the late luteal phase of the estrous cycle, which showed an altered expression of immune response genes after PGE_2 priming (Lara *et al*., 2017. Stem Cells International. Doi.org/10.1155/2017/4297639). All cell lines were cultured in DMEM-F12 medium supplemented with 10%FCS,1X AAM solution, 1 mM sodium pyruvate and 2 mM L-glutamine, at 5%CO_2, 39°C and 100% humidity. The beMSCs showed fibroblastoid-like morphology, adherence to plastic, and the ability to differentiate into chondrogenic(C), adipogenic(A) and osteogenic(O) mesodermal lineages when treated for 7 and 14 days with lineage specific inducers. The expression of immunological markers was measured after priming of beMSC cultures with 0 (mock), 1, 3 or 10 μM of PGE_2, pH 6.8 (Caymann Chemical) in the presence of serum. All experiments were repeated three times. After 28 hours of priming, the cells were scraped, and subjected to RT-PCR. For this, RNA extraction and synthesis of cDNA was performed using EZNA Total RNA Kit I (Omega Biotek) and M-MLV Reverse Transcriptase enzyme (Thermo Fisher), respectively. We used DDCT RT-qPCR, to measure the relative expression of the following genes: IL6, IL10, PGES, TNFa, IFNg, TLR2, TLR4 and COX2; ACT B expression was used as housekeeping gene. Multiple comparison statistics was performed comprising fold change of expression of individual genes in all cell lines and doses of PGE_2 priming. For this, ANOVA and Fisher LSD post-hoc test were used. Values of $P<0.05$ were considered as statistically significant. After priming with PGE_2, statistically significant, dose dependant over expression was found among all evaluated dosages in relation to untreated, for all genes except COX2. For IL6, expression was different for all dosages except 1 μM of PGE_2 and for TNFa at 10μM. In conclusion, priming of beMSC with PGE_2 induces a shift in the expression of important inflammation-related genes which can be associated to the response observed in uterine disease. Apparently 1 μM is sufficient to induce such response. These results confirm at the individual gene level our previous transcriptomic findings. No such data are available in the literature for beMSC. Our findings might have an impact on the development of PGE2-based strategies to manage inflammation in uterine disease such as endometritis in cows. Funding: CORFO-INNOVA national funding for undergraduate thesis code EM.TES-67609, Government of Chile.

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Abductive analysis of metabolic interactions in bovine mammary epithelial cells

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Studying the regulation and interactions of different pathways of amino acid (AA), carbohydrate (CHO), lipid, and energy (E) metabolism in mammalian cells is important to understand the mechanisms of milk protein synthesis. An abductive type analysis was carried out, to provide an approximation to a description of the possible metabolic relationship of the proteome in an in vitro model of bovine lactocytes. Primary bovine mammary epithelial cells (PBMEC) were cultured for 6 days after reaching confluence in lactogenic DMEM. Following lysis of cells, cellular total protein concentration was measured by BCA (Pierce®). Extracts of total protein were subjected to label-free, quantitative proteomics using liquid chromatography separation and mass spectometry analysis (ISOQuant®). To study the metabolic pathway, a multi-fasta file was created with all protein sequences and submitted to BlastKOALA in KEGG. KEGG pathways (n=44) were grouped into metabolism of AA, CHO, lipids and E. Using the KEGG results, a data matrix was created with the metabolic pathways as columns, the proteins identified within these pathways as rows, and the values of the proteins as absent or present. To better understand the dynamics underlying the metabolic pathways, and as a first data exploration, a P-value matrix of chi-squared distances was calculated and a pictogram was created including values equal to or less than 0.1. To eliminate proteins and metabolic pathways that were not significant ($P>0.05$), multiple correspondence analysis was used, and the R^2 of each metabolic pathway was calculated to determine its relevance in the analysis. An algorithm was designed that iterative conduct multiple correspondence analysis and select significant variables between each iteration, eliminating routes that were not significant to the first dimension or component (protein synthesis, PS). This procedure was performed until there were no metabolic pathways to eliminate. Finally, a cluster analysis was performed using the resulting data matrix from which the possible simultaneous relationships between the proteins were detected. The groups of routes corresponding to the metabolism of AA and of CHO, represent the greatest number of interactions, 19 and 26 respectively. The algorithm took five iterations until the number of non-significant metabolic pathways was equal to zero. The metabolism of tryptophan was the route that explained most of PS variance (70%), while the biosynthesis of arginine explained the least (20%). The cluster analysis resulted in 9 groups of proteins (n=36) that possibly interact in 9 pathways: ascorbate and aldarate metabolism; fatty acid degradation; glycerolipid metabolism; alanine, aspartate and glutamate metabolism; valine, leucine and isoleucine degradation; lysine degradation; arginine biosynthesis; histidine metabolism; and tryptophan metabolism. We conclude that the PBMEC model is a useful tool to study metabolic interactions in the complex milk protein synthesis pathway. Acknowledgement to University of Antioquia for granting ZT R-C a postdoctoral commission 2016-17; to the College of Agriculture and Life Sciences David R. and Margaret Lincicome Endowment at Virginia Tech for partial support of ZT R-C as Visiting Scientist, and to Dr. Loor for cell donation.

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Effect of maternal ability of Corriedale and Corriedale Pro ewes on lamb growth

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Maternal ability of ewes of two biotypes and its effect on growth and development of their lambs were evaluated under grazing conditions in Uruguay. The biotypes used were Corriedale (C) and Corriedale PRO (C-PRO). The latter is a prolific biotype composed of 25% Finnish Landrace (FL), 25% East Friesian (EF) and 50% C. All the lambs were C-PRO, either from C-PRO ewes sired by rams from the same biotype or C ewes sired by 50% FL and 50% EF rams. From 132 ewes, 205 lambs were born (117 male and 88 female lambs); 52 ewes were single bearing (34 C-PRO and 18 C) and 80 were twin bearing (41 C-PRO and 39 C). The ewes were kept on native pasture and 45 days before parturition twin bearing ewes (as diagnosed by ultrasound) were moved to a cultivated pasture for the rest of the experimental period. Lamb weight was recoded at birth (BirthWt) and on average at days 26, 49 and 62 after lambing had peaked (LW1, LW2 and LW3, respectively) and at weaning (WW, day 110). Statistical analysis for BirthWt included the main effects of ewe biotype (C, C-PRO), ewe age (2, 3, or more than 3 years), sex of lamb (male, female), number of fetuses gestated (NFG; one, two) and ewe biotype*NFG. For LW1, LW2, LW3 and WW the effects included in the model were ewe biotype, ewe age, sex of lamb, lamb age in days at recording (LAR) as covariate, NFG*number of lambs reared (NLR; one, two) and ewe biotype*NFG*NLR. For BirthWt, ewe biotype*NFG interaction was statistically significant ($P<0.05$), with higher BirthWt in lambs from C-PRO ewes gestated as singles. Significant effects on LW1, LW2 and LW3 were sex (higher weights for male lambs), NFG*NLR interaction and LAR. Lambs gestated and reared as singles had the highest body weight, followed by lambs gestated as twins but reared as singles, and last the lambs gestated and reared as twins. Ewe age affected only LW2. Ewe biotype*NFG*NLR had a significant effect on WW, with lambs gestated and reared as singles from both biotypes heavier than twin lambs reared as twins or singles. Ewe biotype only affected BirthWt in favor of C-PRO ewes, but not any of the other weights, which suggests no differences in milk production between biotypes in spite of the EF component of C-PRO. Twins had lower BirthWt than singles, which was maintained throughout weaning. The NLR affected initial weights (LW1 and LW2); twins reared as singles had higher weights than when reared as twins, however, no differences were found in LW3 and WW. Authors kindly thank the Refugio stud and the Echeverría family for the use of their flock.

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Quantification of PAG genes in semen of high and low fertility sires using droplet digital PCR

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Substantial variation in pregnancy rates and pregnancy loss exist among sires that are deemed satisfactory in traditional semen fertility evaluations. Developing techniques to identify or predict the field fertility of a sire can help improve sire selection and reproductive efficiency. The objective of this experiment was to evaluate genomic PAG profiles in semen samples from sires classified as having high (n = 3) or low (n = 3) pregnancy loss in a previous experiment (Franco et al., 2018, JAS, 96, 632-640). Extraction of DNA was performed using the Chelex-100 method described by Manuja et al., (2010) and absolute quantification of gene copies was performed using QX100™ Droplet Digital™ PCR System (ddPCR; Bio-Rad, Pleasanton, CA). A pool of DNA collected from 2 Holstein bulls were included in each gene experiment for data normalization. Approximately 16,000 droplets were generated in each well using droplet generator (Bio-Rad QX200) and cycled droplets were read individually with the QX200 droplet-reader (Bio-Rad). Reads were analyzed with QuantaSoft droplet reader software 1.3.2.0 (Bio-Rad) where concentration estimates were based on the fraction of droplets which amplification has occurred. It was modeled as a Poisson distribution and the normalization of the abundance of the query sequence to the control sample. PAG genes from modern group (PAGs 6, 7, 16, 18, 19, 20, 21) and ancient group (PAGs 2, 8) were analyzed and difference in concentration were calculated using PROC GLM on SAS 9.4. Overall, *Bos indicus* sires (Nelore) had numerically increased concentrations of all genes analyzed, but only PAG 21 was statistically different (3.2 vs 1.8 copies/uL; P = 0.06) when compared to *Bos taurus* sires. No statistical difference in individual genes was observed between sires classified as high or low pregnancy loss (P = 0.15). Additionally, there was no statistical difference when genes were grouped by phylogenetic groups. However, it was interesting to observe variance among groups, where ancient PAGs genes were higher in high pregnancy loss samples while modern PAG genes were increased in low pregnancy loss samples (P = 0.1). These differences could be explained by the fact that the assay used to classify the sires was developed targeting modern PAG genes. Although a low number of samples prevented statistical significance from being achieved, variation in PAG gene copy number between sires encourages further exploration of the PAG profile in sires to address differences in fertility and pregnancy loss.

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