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Comparative fecal steroid profile during pregnancy, parturition, and lactation between natural fertilization and embryo transfer in ocelots (*Leopardus pardalis*)

Marcel Henrique Blank [a, *, 1], Cristina Harumi Adania [b, 1], William Frederick Swanson [c], Daniel de Souza Ramos Angrimani [a], Marcilio Nichi [a], Marcelo Alcindo de Barros Vaz Guimarães [a], Renato Campanarut Barnabe [a]

[a] Department of Animal Reproduction, School of Veterinary Medicine and Animal Science, University of São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, São Paulo, CEP: 05082-270, SP, Brazil
[b] Brazilian Center for Neotropical Felids Conservation, Av. Emílio Antonon, 1000, Chácara Aeroporto, Jundiaí, CEP: 13212-010, SP, Brazil
[c] Center for Conservation and Research of Endangered Wildlife, Cincinnati Zoo and Botanical Garden, Cincinnati, OH, 45220, USA

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ABSTRACT

Despite the invaluable role that assisted reproduction technologies (ARTs) play in conservation, pregnancy and parturition rates by embryo transfer (ET) are low for most endangered felids. Thus, efforts to expand the knowledge on pregnancy biology and ET are still required. In this context, we examined fecal sex steroid metabolites (i.e., estrogens, glucocorticoids, and progestogens) of eight ocelots submitted to natural fertilization (NF) and ET in 22 pregnancies (19 NF and 3 ET). Fecal samples were collected and assessed for each pregnancy from estrous cycle, pregnancy, and lactation, totaling 155 days. In short, progestogen levels remained high and unchanged ($P < 0.05$) from conception until parturition for females maintained under NF. On the other hand, females submitted to ET exhibited changes ($P > 0.05$) in progestogen levels from conception until parturition, with a significant decrease during pregnancy (480.72 ng/day; $r^2 = 0.81$; $P < 0.0001$). Significant changes between NF and ET also were noted in estrogen levels between the first and last thirds of pregnancy ($P < 0.05$), in which estrogen levels exhibited a negative correlation ($P < 0.01$) between themselves. Regarding glucocorticoids, significant changes ($P < 0.01$) were observed only in the first third of pregnancy between NF and ET, which we believe may be related to the handling for ovarian synchronization and ET. Besides hormonal changes, the pregnancy was more prolonged (2.5 days) and more prone to dystocia in ET than NF. Overall, 24 embryos were transferred into eight females (3/1), with three kittens being born from three distinct deliveries (i.e., 12.5% of embryos and 37.5% of females). Our findings have supported the great potential of production and transfer of long-term frozen embryos in ocelot conservation. However, they reveal possible effects of these biotechnologies on hormonal levels during pregnancy linked with low conception and parturition rates and dystocic cases in felids.

1. Introduction

The crucial role of the steroid hormones such as progestogens, estrogens, and glucocorticoids is evident in the estrous cycle, pregnancy, parturition, and lactation [1,2]. In each phase, these hormones act in a coordinated manner to recognize and maintain gestation and prevent luteolysis [2]. Moreover, they ensure a uterine environment that promotes implantation, placentation, and fetus development, stimulating mammogenesis and lactogenesis over neonatal and postnatal periods [1,2]. Consequently, the monitoring of steroids by noninvasive methods has become an essential tool for assessing ovarian activity and pregnancy biology in felids, highlighting its application in the improvement of ARTs through assessment of follicular development induction and ovulation for artificial insemination, oocyte recovery, and embryo transfer (ET) [3].

* Corresponding author.
E-mail address: marcel.henriqueblank@gmail.com (M.H. Blank).
[1] These authors contributed equally to the research.

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In this sense, cubs of different species (i.e., African wild cat, black-footed cat, caracal, cheetah, fishing cat, Indian desert cat, ocelot, sand cat, serval, and tiger) have been produced from fresh and frozen embryos through intra and inter-specific recipients [4–10]. However, ET in felids has not yet been widely disseminated in conservation programs due to the low pregnancy and birth rates (i.e., less than 20% and 5% of embryos transplanted, respectively) [11]. The lack of species-specific knowledge on their physiology and consequently, their reproductive endocrinology remains the main cause of low birth rates after ARTs are applied in felids [12]. Furthermore, the *in vitro* handling of gametes and embryos together with the ovarian synchronization of recipients are limiting factors in reproduction. In other words, abnormalities observed in kittens produced from cryopreserved embryos are suggestive evidence of changes in epigenetic and gene expression occasioned during *in vitro* embryo production [9]. In fact, several studies have attributed *in vitro* embryo production to several disorders linked to the dam, fetus, and offspring, such as preeclampsia, gestational diabetes, low birth weight, congenital malformation, and potential long-term effects on offspring health [13].

On the other hand, the inconsistency in kitten production from ET may also be a consequence of an altered endocrine environment by exogenous gonadotropin administration for the ovarian synchronization of recipients. In felids, gonadotropin treatments have promoted ovarian hyperstimulation and luteal insufficiency, inducing dysregulation of estrogen and progestogen levels and consequently, limiting the oviductal transport and establishment and maintenance of the embryo in the uterus [14,15]. Furthermore, exposure to a disrupted endocrine environment during pregnancy can result in early embryonic loss, retarded fetal growth, abortions, or stillbirths in a multitude of mammal species [16], including wild felids [8,17–19]. Although the real cause of these disorders remains unclear, noninvasive hormonal monitoring after ET can be a starting point for assessing the effect of ET on pregnancy biology.

To our knowledge, no descriptions have been reported on fecal steroid profiles in ocelots (*Leopardus pardalis*) during pregnancy. Thus, due to the need to characterize normal gestational physiology and the possible effects of ET, this research aimed to describe the longitudinal profiles of steroids following ET and natural fertilization (NF) for the species. Therefore, we believe that new insights on the potential impact of *in vitro* embryo production and ET on pregnancy can be considered through this work, aiming to provide crucial information for improving these procedures in endangered felids.

2. Material and methods

All procedures described were reviewed and approved by the Bioethics Committee of the School of Veterinary Medicine and Animal Science, University of São Paulo (protocol number 1519).

2.1. Animals

This research was conducted at the Brazilian Center for Neotropical Felids Conservation located at Jundiaí, SP (23° 11′ 11″ S, 46° 53' 4" W; Licenses IBAMA CTF: 456528, n. process 02027.15609/96–35). Eight female ocelots (*Leopardus pardalis*) were housed in enclosures (38 m^2, 3 m height) enriched with branches, natural vegetation, a hideout, and water *ad libitum*. All ocelots were maintained under the same environmental, nutritional, health, and reproductive management, including pair formation prior to sample collection for minimizing possible effects of pairing on the handling, behavior, and endocrinology. However, males were removed after the detection of pregnancy. Individual information such as identification, age, origin, reproductive history, kitten features, and reproduction technique are summarized in Table 1.

2.2. Sample collection and reproductive stages

With the help of a camera system, individualized fresh feces were collected three times per week except for females submitted to ET, from which the collection was daily. All samples were collected in the morning between 8:00 to 10:00 a.m. to avoid the potential effect of circadian rhythms on the secretion of steroid hormones. Samples were placed in individual plastic bags and stored at −20 °C until analyzed. Females were also monitored daily to register the mating, parturition, and lactation behaviors.

Conception dates were determined from the date of ET (n = 3). For NF, however, because the exact conception date was not known (n = 19), conception was designated as the midpoint between the last sample with a progestogen level equal to or lesser than the baseline (<11401.7 ng/g) and the first sample with a level greater than the mean plus two standard deviations (>21224.3 ng/g) [16]. Then, based on the conception and parturition dates, as well as the duration of the estrous cycle [20], samples were categorized into five stages: estrous (i.e., 18 days pre-conception to conception); first third of pregnancy (i.e., 1–30 days post-conception); second third of pregnancy (i.e., 31–55 days post-conception); last third of pregnancy (i.e., 56 days post-conception to parturition); and lactation (i.e., 1–5 days post-parturition).

2.3. Fecal steroid extraction and enzyme immunoassay

After thawing, fecal samples were manually homogenized, weighed, and extracted. In brief, 0.5 ± 0.25 g of fecal material was mixed in 5 mL of ethanol 90% and placed in conical plastic tubes of 15 mL for homogenization. In comparison, smaller samples (<0.5 g) were placed in tubes containing proportional amounts of solvent (e.g., 0.25 g feces in 0.25 mL of 90% ethanol). These suspensions were vortexed for 40 s, homogenized (20 rpm for 4 h) using a blood rotator (AP 22 Phoenix, Araraquara, SP, Brazil), and then vortexed again for 40 s before centrifugation (400 g for 10 min). Subsequently, supernatants were placed into a clean tube and stored at −20 °C.

Enzyme immunoassay protocols (EIA) were performed by single-antibody enzyme immunoassays adapted from Munro and Stabenfeldt [21] and Young et al. [22]. Each enzyme immunoassay used an antibody monoclonal antiserum progesterone (#425) or polyclonal antiserum estradiol-17b (R4972) or polyclonal antiserum cortisol (R4866); supplied by C.J. Munro (University of California, Davis, CA, USA), labeled with horseradish peroxidase-conjugated (progesterone, estradiol, and cortisol were prepared according to Munro and Stabenfeldt [21] and Young et al. [22] using (progesterone, estradiol, and cortisol) the Sigma-Aldrich (St. Louis, MO, USA) standards).

The adapted assay procedure was as follows: (1) antiserum was diluted at 1:20 for progesterone, 1:100 for estradiol, and 1:50 for cortisol; (2) standards (progesterone 50.000 pg per well with eight serial dilutions, estradiol 20.000 pg per well with seven serial dilutions, and cortisol 200.000 pg per well with seven serial dilutions); and (3) horseradish peroxidase conjugate was used at a dilution of 1:100 for progesterone, 1:500 for estradiol, and 1:100 for cortisol. The progesterone antiserum has been used to monitor luteal function and pregnancy in a variety of felid species and there

Table 1
Individual data for ocelots (*Leopardus pardalis*) utilized in this study: Identification (ID), age, birth origin, breeding history, number of pregnancies, number of kittens per pregnancy, gestation history (natural fertilization vs. embryo transfer), and lactation (natural vs. artificial).

ID	Age (years)	Birth	Breeding	Pregnancy	Kitten	Gestation	Lactation
1	12	Captivity	Yes	1	2	Natural	Natural
				2	1	Natural	Natural
				3	2	Natural	Natural
				4	2	Natural	Natural
				5	Death[c]	Embryo transfer	None
2	8	Captivity	Yes	1	1	Natural	Natural
				2	1	Embryo transfer	Natural
3	8	Captivity	Yes	1	1	Natural	Natural
				2	1	Natural	Natural
				3	Death[c]	Embryo transfer	None
4	6	Captivity	No[a]	1	1	Embryo transfer	Natural
5	5	Captivity	No[a]	1	1[e]	Embryo transfer	Artificial[d]
6	>10	Wild	Yes	1	1	Natural	Natural
				2	1	Natural	Natural
				3	1	Natural	Natural
				4	1	Natural	Natural
				5	Death[c]	Embryo transfer	None
7	6	Captivity	Yes	1	1	Natural	Artificial[d]
				2	Death[b]	Natural	None
				3	1	Natural	Natural
				4	1	Natural	Natural
				5	1	Natural	Natural
				6	Death[c]	Embryo transfer	None
8	9	Wild	Yes	1	1	Natural	Artificial[d]
				2	Death[b]	Natural	None
				3	1	Natural	Natural
				4	1	Natural	Natural
				5	1	Natural	Natural
				6	Death[c]	Embryo transfer	None

[a] Nulliparous female.
[b] Fetus aborted after second third of pregnancy.
[c] Embryo/fetus death during first third of pregnancy.
[d] Breastfeeding not performed by dam.
[e] Kitten delivered by cesarean section.

is a cross-relativity with several progesterone metabolites [23]. Cross-reactivity for estradiol antiserum was 100% with estradiol 17β, 3.3% with estrone, 1.0% with testosterone, 0.8% with progesterone, and less than 0.1% with the other steroids tested, including estrone sulfate, cortisol, corticosterone, and androstenedione. Cortisol antiserum was cross-reactive with cortisol 100%, prednisolone 9.9%, prednisone 6.3%, cortisone 5%, and <1% with corticosterone, desoxycorticosterone, 21-deoxycortisone, testosterone, androstenedione, androsterone, and 11-desoxycortisol (C.J. Munro Ph.D., pers. comm.). Two internal controls of high and low concentrations were tested on all assays for all antibodies (progesterone, estradiol, and cortisol). For both intra and inter-assays, coefficients of variation (CVs) did not exceed 15%; when any plate showed a CV higher than 15%, the plate was processed again. Parallel displacement curves were obtained between serially diluted pooled fecal samples and the progesterone, estradiol, and cortisol standard curves.

2.4. Laparoscopic oocyte recovery and in vitro fertilization

The embryos used in this study were produced by Dr. William F. Swanson, whose protocol for oocyte recovery and IVF are described in Swanson et al. [24]. In short, behaviorally anestrous or interestrus females were synchronized using 400 IU of equine chorionic gonadotropin (eCG) and 200 IU of human chorionic gonadotropin (hCG), administered intramuscularly at 84 h apart, respectively. At 25–32 h after hCG administration, females were submitted to oocyte aspiration using laparoscopic equipment constituted by Verres needle, trocar/cannula, an atraumatic grasping forceps, fiberoptic cable, and light source. Mature follicles (≥2 mm) were aspirated using a 22-gauge needle with aspiration pressure (~1.5 mm Hg) provided by a vacuum pump. Follicular contents were collected into pre-warmed (37 °C) tubes containing Ham's F-10 (HF10) medium supplemented with 0.011 mg/mL pyruvate, 0.284 mg/mL glutamine, 5% fetal bovine serum (FBS), and 40 IU/mL heparin [25]. Recovered oocytes were washed in a petri dish containing 3 mL HF10 and subsequently rinsed three times through three consecutive drops (100 μL) of HF10 overlaid with mineral oil. Then, good quality oocytes (i.e., dark, uniformly pigmented with an expanded cumulus, and distinct corona radiata) were placed in 90 μL drops (5–10 oocytes per drop) of HF10 supplemented with 5% FBS. In each drop, 5×10^3 mobile spermatozoa were added using 10 μL of the same medium. Immediately after insemination, embryos were cultured (38 °C, 5% CO_2 in air) for between 30 and 45 h in HF10 medium until the 2–8 cell cleavage stage.

2.5. Embryo cryopreservation

Embryos were cryopreserved using a slow and controlled rate cooling procedure, where they were firstly equilibrated (10–15 min at 22 °C) in cryoprotectant solution (1.5 M ethylene glycol (EG), 0.125 M sucrose (S), 10% dextran 70, and 10% FBS in HF10). Subsequently, embryos were loaded into 0.25 straws and placed into a freezing machine (model CL-863, Cryologic, Victoria, Australia), refrigerated at a rate of 2.0 °C/min until 6.0 °C. After 10 min of equilibrium at 6.0 °C, the cooling rate was then restarted at 0.3 °C/min until −30 °C, and after a new equilibration of 10 min, embryos were finally stored in liquid nitrogen (−196 °C).

2.6. Embryo transfer

Before ET, estrogen levels were analyzed to establish the estrous cycle stage for each female. Thus, ocelots in estrus (n = 4) were synchronized with two doses of porcine luteinizing hormone (pLH, 3000 IU, intramuscular, with 13 h apart). In contrast, ocelots in interestrus (n = 4) were synchronized using 400 IU of eCG and 3000 IU of pLH administered intramuscularly 85 h apart. Regardless of synchronization protocol, approximately 50 h after the last dose of pLH, ocelots were anesthetized for laparoscopic oviductal embryo transfer (LO-ET). There was an evaluation of the reproductive tract for any potential pathology or abnormalities and the presence of ovarian follicles and corpora lutea (CL). After confirming the absence of any pathology and the presence of recent ovulation (i.e., CL) sites, embryos were immediately thawed in the air at room temperature (22 °C) for 2 min and washed three times in HF10 (37 °C) to remove the cryoprotectant. For LO-ET, the oviductal infundibulum was stabilized with an atraumatic grasping forceps (diameter 5 mm) and the cranial uterine horn cannulated with an intravenous catheter (20G, 32 mm length). Then, using a polyethylene tubing (PE10), three long-term frozen (~7years) embryos (2–8 cells) were deposited into one oviduct of each female (n = 8) using slight air pressure from an attached 1 mL syringe.

2.7. Pregnancy determination and monitoring

Approximately 21 d after ET, recipient ocelots were submitted to transabdominal ultrasonography (Aloka SSC 500, Tokyo, Japan) using a 7.5 MHz linear array transducer. Thus, after confirming the pregnancy, recipients were housed individually until the end of the study. Subsequently, from 60 d after ET, recipients were monitored by video recording and frequent visual observation until the parturition date.

2.8. Statistical analysis

Twenty-two events (pregnancy, parturition, and lactation) were categorized into two groups: natural fertilization (NF) and embryo transfer (ET). Thus, baselines for each fecal steroid were calculated separately between groups using an iterative process in which values that exceeded two standard deviations (SD) (i.e., progestogens or glucocorticoids) and 1.5 SD (i.e., estrogens) above the mean were excluded [20,26]. The average was then recalculated, and the elimination process was repeated until no values exceeded 2SD and 1.5SD above the mean. The average of the remaining values was considered as the baseline for those hormones. In contrast, values greater than twice the baseline were defined as a peak. As sample collections were not concurrent, they were normalized by the parturition date of each pregnancy, which was described as Day 0. Thus, samples collected every five days were pooled, and hormonal means were determined using a minimum of six samples for each interval of five days.

Additionally, hormonal levels between NF and ET were compared during each reproductive stage: estrous cycle, first third of pregnancy, second third of pregnancy, and last third of pregnancy, and lactation. All data were evaluated using the SAS System for Windows (SAS Institute Inc., Cary, NC, USA) and are presented as mean value ± SEM. Variables were initially tested to determine variance homogeneity and data normality (Guided Data Analysis – SAS System) for multiple regression and analysis of variance (ANOVA). Whenever one of these assumptions was not respected, data were transformed using a logarithmic scale. The interaction effect of steroid levels between groups (i.e., NF and ET) and reproductive stages were evaluated by general linear models procedures (PROC GLM – SAS). Differences between groups were analyzed using a *t*-test (PROC TTEST – SAS) to compare two groups and Fisher's test (LSD) for multiple treatments. The significance level used for a given response variable was $P < 0.05$. Finally, a linear regression was performed using hormone concentration as the dependent variable and days of pregnancy as the independent variable. Slope comparisons between groups were made using GraphPad Prism 8.0 (GraphPad Software Inc) with confidence intervals of 95% and $P < 0.05$.

3. Results

3.1. In vitro embryo production

The embryos used in this study were produced by Dr. William F Swanson, and data on oocyte recovery and embryo production are reported in Swanson et al. [24]. In short, after 24 procedures using 20 females, the mean ± SEM number of ovarian follicles was 9.9 ± 1.7. Overall, the oocyte recovery rate was 94.96% (i.e., 226 oocytes from 238 follicles), with 46.4% of them (n = 105) being of high quality (i.e., very dark cytoplasm, uniformly pigmented with an expanded cumulus, and distinct corona radiata), 25.7% (n = 58) of intermediate quality (i.e., nonuniform (mosaic) dark cytoplasm, surrounded by a nonexpanded cumulus, and intact corona radiata), and 27.9% (n = 63) of low quality (i.e., degenerated oocytes with morphological defects and pale cytoplasm, the cumulus cells are absent, and corona radiata and zona pellucida damaged). Furthermore, *in vitro* fertilization rates were significantly ($P < 0.05$) higher using the high quality oocytes (57.1%) than the intermediate (9.9%), and low quality (0%) ones. Finally, a total of 64 embryos were produced and cryopreserved for future transfers.

3.2. Ovarian synchronization and embryo transfer

After ovarian synchronization, all females showed at last one fresh CL, where the mean number of follicles (0 and 3.0 ± 1.5) and CL (1.0 ± 0.0 and 3.8 ± 1.8) were not statistically different between groups (i.e., estrus and interestrus, respectively). However, females at interestrus exhibited a greater pregnancy rate (50%) than those in estrus (25%). Overall, 38% of recipients became pregnant, of which 13% of transplanted embryos were born.

3.3. Gestation length

From 19 gestations in six females assessed in the NF condition, the mean gestation length was 81.2 ± 0.4 days (±SEM, range: 79–83 d, n = 19). On the other hand, embryo recipients with kitten births (n = 3) exhibited a mean gestation length of 83.7 ± 0.3 days (±SEM, range: 83–84 d). One female had dystocia, requiring a cesarean section to deliver a single healthy offspring 83 days after the ET.

3.4. Hormone analysis

Fecal steroid profiles during the estrous cycle (i.e., ovarian synchronization for ET group), and pregnancy and lactation phases for NF and ET are depicted in Fig. 1. Overall, greater levels of progestogens were noted during the luteal phase with a gradual decline until parturition. However, there was a more evident decline in the ET group than NF, where peaks of progestogens were observed only during the first third of pregnancy, declining to around baseline (<24472.64 ng/mL) from the late second third of gestation (Fig. 1D). Meanwhile, peaks of progestogens were observed throughout pregnancy in the NF, where hormonal levels always remained above baseline (<11401.7 ng/mL) until parturition (Fig. 1A). Similarly, significant ($P < 0.05$) changes in progestogen

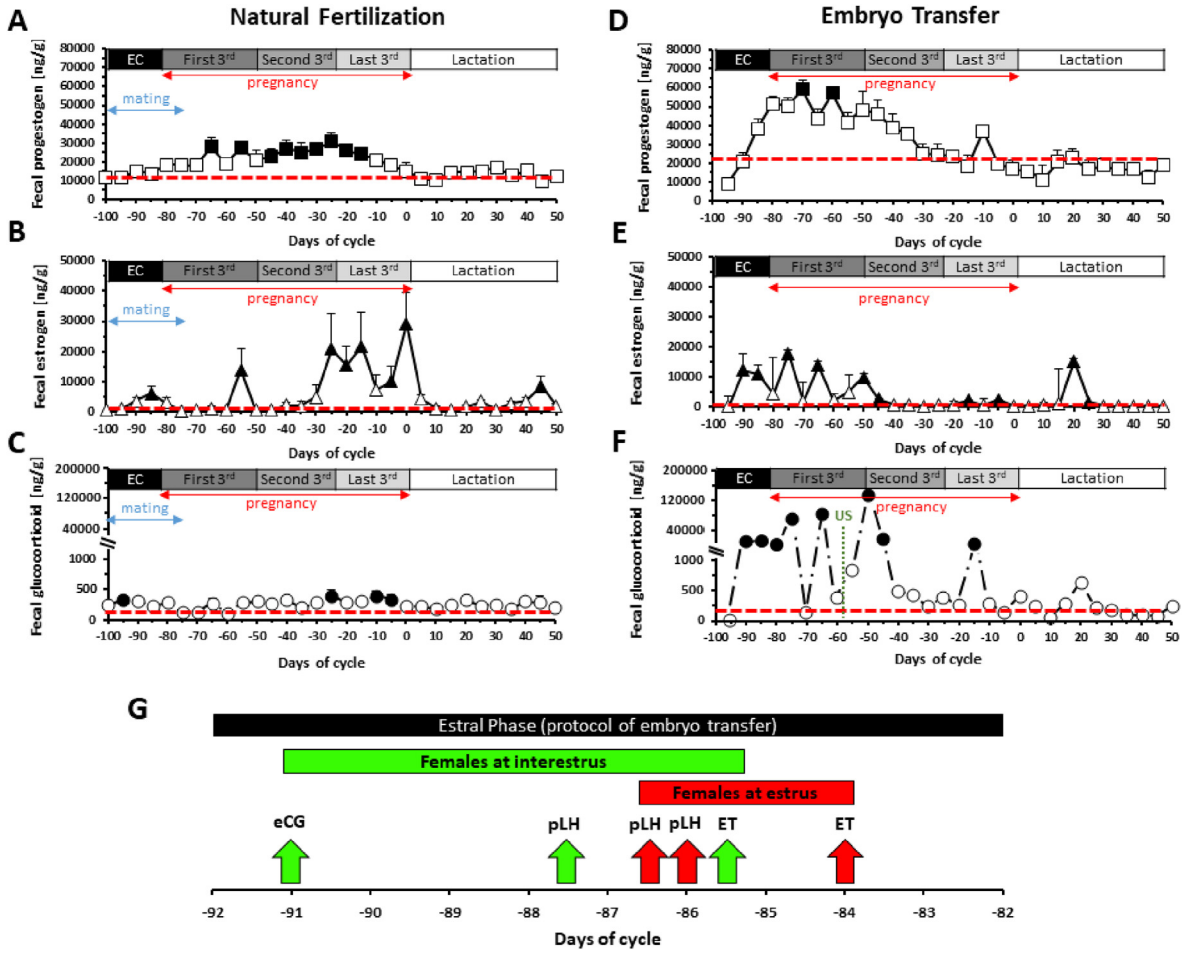

Fig. 1. Longitudinal fecal steroid profile during estrous cycle (EC), first third of pregnancy (First 3rd), second third of pregnancy (Second 3rd), last third of pregnancy (Last 3rd), and lactation (Lactation) in ocelots. Left panels represent the steroid profile in natural fertilization represented by letters A, B, and C, which denote progestogen, estrogen, and glucocorticoid levels, respectively. Right panels represent the steroid profile in embryo transfer represented by letters D, E, and F, which denote progestogen, estrogen, and glucocorticoid levels, respectively. Red dotted lines illustrate the baseline calculated for each hormone and group (natural fertilization and embryo transfer), whereas peaks are represented in black (squares, triangles, and circles) for each metabolite. The blue arrow indicates mating period and red arrow indicates pregnancy period, while US indicates ultrasound date. Day 0 indicates parturition. The panel G illustrates the recipient synchronization protocol for females at interestrus and estrus phases, while ET indicates the embryo transfer time. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

levels between NF and ET during pregnancy were determined by linear regression (Fig. 2). From conception to parturition date, there was a significant decrease in daily progestogen levels (480.72 ng/day; $r^2 = 0.81$; $P = < 0.0001$) with ET. On the other hand, the daily reduction of progestogen levels was no significant throughout pregnancy for the NF group (32.30 ng/day; $r^2 = 0.03$; $P = 0.5071$). Furthermore, changes in progestogen levels were observed among reproductive stages (i.e., estrous cycle, first third, second third, and last third of pregnancy, and lactation), for both NF and ET by interaction analysis (Table 2). There were significant changes in progestogen levels during the first third of pregnancy between females exposed to NF and those submitted to ET (Fig. 3; $P < 0.001$).

In NF, ovulation was detected by the peak of estrogen during mating in the estrous cycle. Although estrogen peaks occurred in the first and second thirds of pregnancy, they were more frequent in the last third of pregnancy with a maximal value before parturition (Fig. 1B). On the other hand, females exposed to ET exhibited high levels of estrogens, with most of the peaks during hormonal synchronization (i.e., estrous cycle) and the first third of pregnancy. Meanwhile, the last third of pregnancy was characterized by decreased estrogen levels with a reduced number of peaks before parturition (Fig. 1E). These opposite estrogen profiles were analyzed by linear regression, in which there were significant changes in the estrogen levels during pregnancy between NF and ET ($P < 0.05$). In short, females submitted to NF exhibited a daily increased rate of estrogen levels of 196.73 ng/day, whereas ET showed a daily decreased rate of 112.58 ng/day (Fig. 4). Furthermore, changes between groups and pregnancy phases were found by interaction analyses (Table 2), in which estrogen levels were significantly different between NF and ET in the first and last thirds of pregnancy (Fig. 5).

Overall, glucocorticoid levels were higher in the ET group than NF, principally during the estrous cycle and the first third of pregnancy. Though glucocorticoid levels had not shown great changes during pregnancy in NF, the increase of levels and the number of peaks were more frequent from the late second third of pregnancy until near to parturition (Fig. 1C). Conversely, glucocorticoid peaks in ET were observed mainly in the first third until the early second third of pregnancy, decreasing to baseline (<195.92 ng/ml) from the mid second third of pregnancy until parturition (Fig. 1E). Similar to estrogen, linear regression of glucocorticoids showed an opposite relation of glucocorticoid levels between groups. However, there were no statistical differences (Fig. 6; $r^2 = 0.19$; $P = 0.07$ and $r^2 = 0.15$; $P = 0.11$, respectively). On the other hand, an interaction

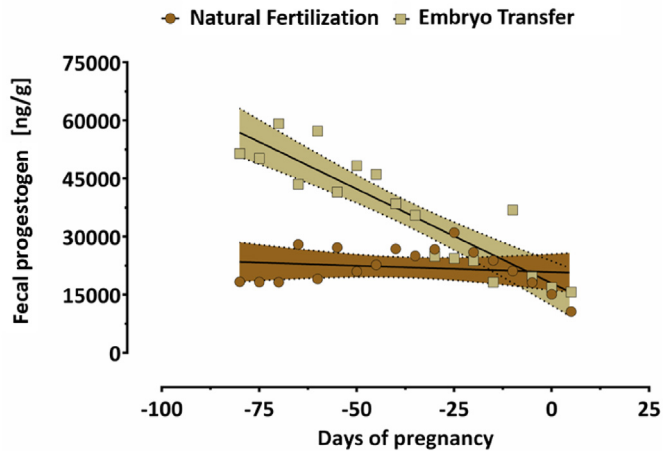

Fig. 2. Scatter plot of fecal progestogen during pregnancy and 5 days after parturition between natural fertilization and embryo transfer. The parturition date is defined as Day 0. The solid black line is linear regression, while the dotted lines represent a confidence interval of 95%. Predicted mean progestogen concentrations are represented by a solid black line decreasing at a rate of 32.30 ng/day ($y = -32.68 \times ^2 + 20834$; $r^2 = 0.02798$; $P = 0.51$) for natural fertilization and 480.72 ng/day ($y = -486.4 \times ^2 + 17960$; $r^2 = 0.8084$; $P < 0.0001$) for embryo transfer. A statistical difference was found between slopes with $P < 0.0001$.

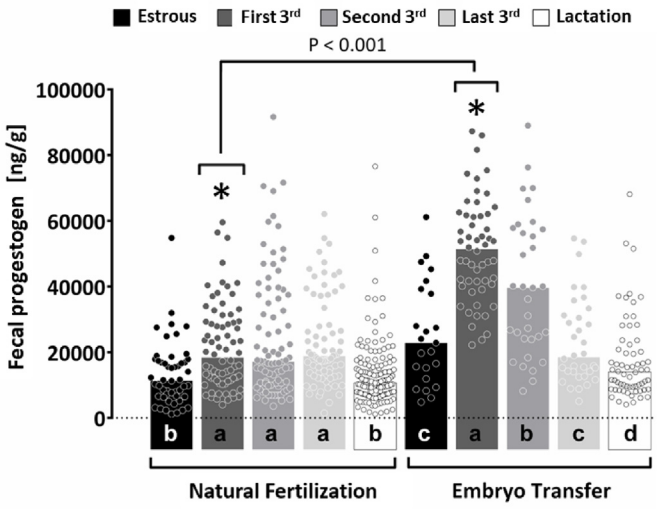

Fig. 3. Effects on progestogen levels between natural fertilization and embryo transfer ($P < 0.01$). Different subscripts indicate significant differences among reproductive stages (estrous cycle, first third, second third, and last third of pregnancy, and lactation) within group and asterisks indicate significant differences of the reproductive stages between natural fertilization and embryo transfer.

between groups and pregnancy phases was found for glucocorticoids (Table 2), where statistical differences in the first third of pregnancy were found between NF and ET (Fig. 7). Moreover, the glucocorticoid levels were statistically higher in the last third of pregnancy in NF (Fig. 7; $P < 0.05$).

4. Discussion

This study provides essential data on fecal steroid concentrations (i.e., estrogens, progestogens, and glucocorticoids) for pregnant ocelots submitted to NF and ET. Intriguingly, steroid levels exhibited an intergroup variation, suggesting a negative effect of ET on steroid levels during pregnancy. Moreover, the present study provides new insights on *in vitro* embryo production and ovarian synchronization for ET in *Leopardus pardalis*. Overall, the ET protocol used is an acceptable method for ocelots, where 38% of females used (3/8 females) became pregnant, resulting in three kittens being born (i.e., 13% of embryos transplanted; 3/24). This rate is greater than those already postulated for other felids, whose overall pregnancy rate is less than 20% after ET [11], whereas the birth rate is less than 5% of transplanted embryos [4,7,9,27–29].

Despite these encouraging results, one kitten needed assistance at parturition after an emergency C-section due to dystocia. Moreover, ocelots submitted to ET showed a gestation deviation of 2.5 days longer compared to those exposed to NF. There is a common understanding that the use of ARTs can promote many consequences for the embryo, dam, and their offspring, such as preeclampsia and diabetes [30], abnormal placental development [13,31], extended gestation [32], and dystocia [33,34]. Although the reason for the development of these complications remains uncertain, for Pope et al. [9], the decrease of embryo survival and increase of phenotypic abnormalities in kittens are presumably linked to epigenetic and gene expression changes induced during cryopreservation/thawing of the embryos. Moreover, the lower capacity of implantation and development of these embryos could originate during *in vitro* production steps (i.e., follicular aspiration, oocyte maturation, fertilization, and embryo culture) [35,36], or subsequently to ET, due to ovarian synchronization of recipients by gonadotropins [37].

Concerning progestogens, high levels were observed from the luteal phase until the parturition date in pregnancies from NF. Similar profiles were reported in domestic cats [38] and

Table 2
Probability value of the interaction effect between groups (natural fertilization vs. embryo transfer) and phases (estrous cycle/ovarian synchronization, first third of pregnancy, second third of pregnancy, last third of pregnancy, and lactation) on fecal steroid profiles (progestogens, estrogens, and glucocorticoids) in ocelot (Leopardus pardalis) females.

Variables	Group	Phase	Group vs. Phase
Progestogen ng/ml feces	<0.0001	<0.0001	**<0.0001**
Estrogen ng/ml feces	0.8390	0.0026	**<0.0001**
Glucocorticoid ng/g feces	0.0806	0.0003	**0.0010**

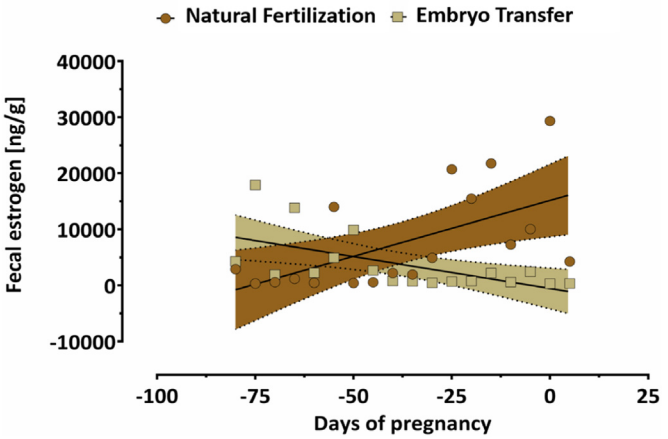

Fig. 4. Scatter plot of fecal estrogen during pregnancy and 5 days after parturition between natural fertilization and embryo transfer. The parturition date is defined as Day 0. The solid black line is linear regression, while the dotted lines represent a confidence interval of 95%. Predicted mean estrogen concentrations are represented by a solid black line increasing at a rate of 196.73 ng/day ($y = 199 \times ^2 + 15134$; $r^2 = 0.3551$; $P = 0.0091$) for natural fertilization and decreasing at a rate of 112.58 ng/day ($y = -113.9 \times ^2 - 558$; $r^2 = 0.3636$; $P = 0.0081$) for embryo transfer. A statistical difference was found between slopes with $P < 0.0003$.

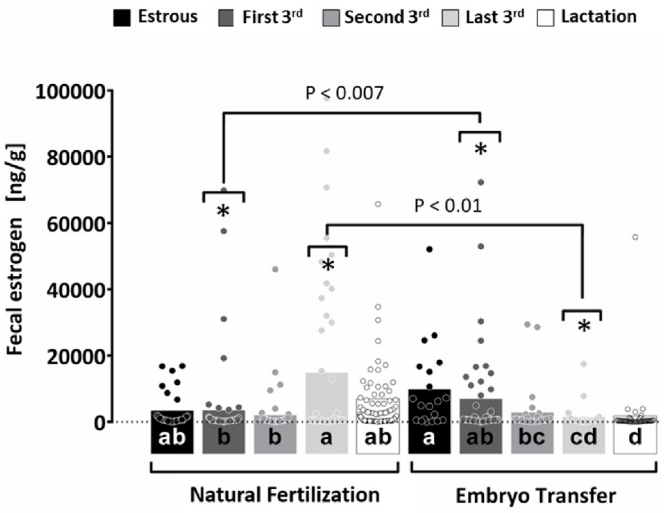

Fig. 5. Effects on estrogen levels between natural fertilization and embryo transfer (P < 0.01). Different subscripts indicate significant differences between reproductive stages (estrous cycle, first third, second third, and last third of pregnancy, and lactation) within groups and asterisks indicate significant differences of the reproductive stages between natural fertilization and embryo transfer.

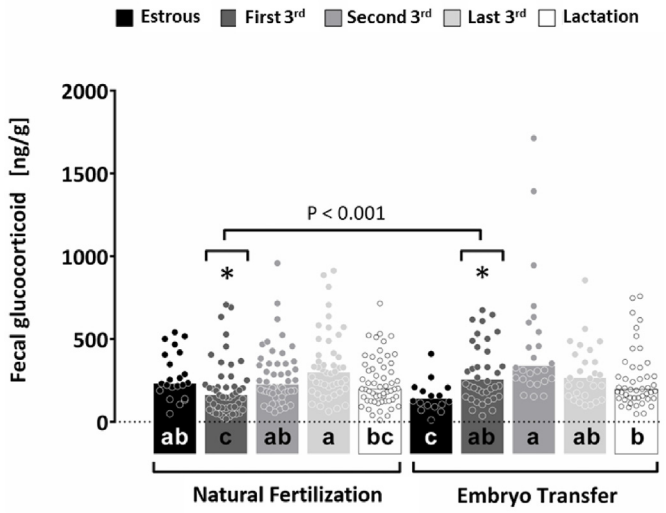

Fig. 7. Effects on glucocorticoid levels between natural fertilization and embryo transfer (P < 0.01). Different subscripts indicate significant differences between reproductive stages (estrous cycle, first third, second third, and last third of pregnancy, and lactation) within groups and asterisks indicate a significant difference of the reproductive stages between natural fertilization and embryo transfer.

nondomestic felids such as the Pallas' cat [39], clouded leopard [3], tiger [40], snow leopard [41], and lion [42]. Conversely, females exposed to ET exhibited progestogen peaks only during the first third of pregnancy with a substantial decrease of progestogen levels 20 days after conception. A similar profile was reported in nonpregnant cats, where the CLs are confronted by luteolytic processes (approximately 21 days after mating [43]), leading to loss of steroidogenic capacity and ultimately, a decrease in the synthesis and secretion of progestogen [44,45]. On the other hand, the luteal regression is late for pregnant cats, where histomorphological signs of regression are found only 38–39 days after conception [46]. From this moment, the progestogen is synthesized mainly by the placenta in felids [47], and any issue linked to it or cells that originate it may induce a decrease of progestogen level throughout pregnancy.

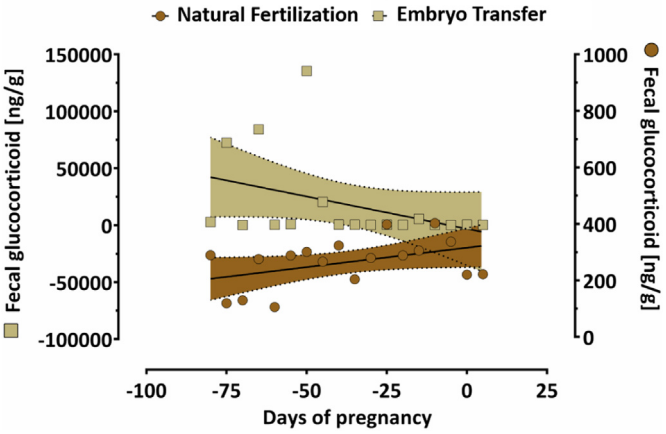

Fig. 6. Scatter plot of fecal glucocorticoids during pregnancy and 5 days after parturition between natural fertilization and embryo transfer. The parturition date is defined as Day 0. The solid black line is linear regression, while the dotted lines represent a confidence interval of 95%. Predicted mean glucocorticoid concentrations are represented by a solid black line increasing at a rate of 1.36 ng/day ($y = 1.377 \times ^{2} + 316$; $r^2 = 0.1874$; $P = 0.0728$) for natural fertilization and decreasing at a rate of 558.58 ng/day ($y = -565.1 \times ^{2} - 3094$; $r^2 = 0.1534$; $P = 0.1080$) for embryo transfer. A statistical difference was not found between slopes with $P = 0.10$.

In this context, similar to what we observed with progestogen levels in ocelots, Collier et al. [48] demonstrated differences in placental steroid metabolism between pregnancies conceived naturally and those arising from ARTs, where the steroids produced by the placenta were significantly lower in murine pregnancies by ET. However, although there was a substantial decrease of progestogen levels throughout gestation in embryo recipient ocelots, differences in progestogen levels were noted only in the first third of pregnancy between NF and ET, probably due to ovarian hyperstimulation by gonadotropin treatment. On the other hand, since two of three embryo recipients were primiparous, the absence of changes in progestogen levels at late stages of pregnancy between NF and ET can be the linked to the fact that primiparous women tend to exhibit greater progestogen levels than multiparous throughout gestation [49] than the absence of any hormonal dysfunction for ET females.

Concerning estrogens, despite peaks being observed during estrous and the first third of pregnancy, hormonal levels declined to baseline after mating, returning to increase substantially after the late second third of pregnancy until parturition with NF. In nondomestic felids, different profiles of estrogens can be observed during pregnancy. For instance, the clouded leopard, lion, and tiger do not display significant changes of estrogen levels from conception until parturition [40,42,50]. In contrast, the black-footed cat, cheetah, fishing cat, Pallas cat, and sand cat exhibit a gradual increase in estrogen from mid or late pregnancy until parturition [39,51–53], similar to that found in ruminants such as sheep and cows. In this case, the increase of estrogen in ruminants originates mainly from the placenta by the action of 17α-hydroxylase (P450c17), a key enzyme in estrogen synthesis, regulated by the activity of fetal adrenal glands as a consequence of the fetal pituitary–adrenal axis maturation [54]. However, this mechanism is still highly uncertain in felids.

The greatest estrogen peaks were observed after ovarian stimulation and the first third of pregnancy in ET. The sustained elevation of estrogen levels after gonadotropin treatment has been associated with the development of ancillary follicles resulting

from ovarian hyperstimulation [14]. Although there are several characteristics that can promote ovarian hyperstimulation (i.e., weight, age, health status, and reproductive status; see more in Thongphakdee et al. [11]), the overdose can be an overpowering factor, mainly in species such as ocelots that require a higher eCG dose than other felids [55,56]. Compared to our findings, Graham et al. [15] showed that follicular stimulation through eCG is dose-dependent, where overdose increased ovarian hyperstimulation and consequently, estrogen concentrations in tigers. Moreover, they reported distinct hormone patterns between naturally fertilized tigers and eCG stimulated tigers.

Notwithstanding, estrogen levels displayed a significant decrease from the first third until the last third of pregnancy, with substantial changes compared to NF between the first and the last thirds of pregnancy (Fig. 5). Thus, we believe that the low estrogen level in the last third of pregnancy could be linked to dystocia found in ET. In cows, low estrogen levels in the last third of pregnancy could interfere with the parturition progress, leading to dystocia [57]. However, dystocia in domestic cats is unusual, where less than 6% of litters by NF exhibit dystocic cases [58]. On the other hand, abortions and dystocic cases are relatively common in felids exposed to ET [7,17,59–62].

Concerning glucocorticoids, females submitted to NF exhibited an increase in hormonal levels from the second to the last third of pregnancy, whereas no change was noted in ET. Edwards and Boonstra [63] noted two distinct glucocorticoid profiles during pregnancy in mammals. However, most mammals increase glucocorticoids in late pregnancy, as described in ocelots in this study. Other carnivores such as *Alopex lagopus* and *Spotted hyaenas* also exhibit a substantial increase of glucocorticoids from the second to the last third of gestation [64,65]. Though it cannot be universally assigned to all species, the rise of glucocorticoid levels during pregnancy can have several origins, driven by dam energetic needs and by changes in dam estrogen or progesterone levels [63], as well as by the development of the fetal pituitary–adrenal axis and placenta [54,66,67]. In this sense, the unchanged glucocorticoid levels during pregnancy in the ET group could be related to both dam physiological dysfunctions, an undeveloped placenta and embryo pituitary–adrenal axis. However, we cannot ignore that the absence of hormonal changes during pregnancy, specifically in the last third of pregnancy, may also be associated with ongoing stressful events that occurred in the early phases (i.e., ovarian stimulation, ET, and pregnancy confirmation by ultrasound) than lack of glucocorticoids in the last third of the pregnancy. Furthermore, most embryo recipient females were nulliparous (Table 1), which, eventually, are more susceptible to stress during gestation than parous females [41,68].

5. Conclusion

This study presents a data set on the normal biology of pregnancy in ocelots and shows the effect of ET on dam endocrinology, as well as its possible effect on the dam–fetus complex. Despite the kitten births by ET, females submitted to assisted reproduction exhibited significant hormonal changes throughout pregnancy, as well as an increase of gestation time and dystocia, presumably associated with *in vitro* production and ET. In this sense, considering the effect of *in vitro* production on both embryo and placenta development (e.g., DNA methylation and damage to extraembryonic tissue), we believe that hormonal changes induced by assisted reproduction may be one of the main causes of low conception and parturition rates in Felidae. Finally, we hope that this study brings new insights and approaches that could be explored to improve the breeding of felids and ultimately, their conservation.

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CRediT authorship contribution statement

Marcel H. Blank: Design of the study, Statistical analysis, Interpretation of data and Writing. **Cristina H. Adania:** Conception and Design of the study and Acquisition of data. **William F. Swanson:** embryo production and trasnferring. **Daniel S.R. Angrimani:** Analysis of data and Revising the article. **Marcilio Nichi:** Analysis of data and Revising the article. **Marcelo A.B.V. Guimarães:** Conception and design of the study. **Renato C. Barnabe:** Conception and design of the study.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2022.01.026.

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