Theriogenology 169 (2021) 89–99

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Hormonal combinations aiming to improve reproductive outcomes of *Bos indicus* cows submitted to estradiol/progesterone-based timed AI protocols

Rodrigo L.O.R. Alves [a], Mateus A. Silva [a], Carlos E.C. Consentini [a], Lucas O. e Silva [a], Natália P. Folchini [a], Abraham L. Oliva [d], Alexandre B. Prata [e], José Renato S. Gonçalves [b], Milo C. Wiltbank [c], Roberto Sartori [a, *]

[a] Department of Animal Science, Luiz de Queiroz College of Agriculture, University of São Paulo (ESALQ/USP), Piracicaba, SP, 13418-900, Brazil
[b] "Hildegard Georgina Von Pritzelwiltz" Experimental Station, Londrina, PR, 86010-990, Brazil
[c] Department of Animal and Dairy Sciences, University of Wisconsin-Madison, Madison, WI, 53706, USA
[d] Cuautitlán Higher Education Faculty-UNAM, Cuautitlán Izcalli, 54740, Mexico
[e] GlobalGen Vet Science, Jaboticabal, SP, 14887-244, Brazil

ARTICLE INFO

Article history:
Received 21 January 2021
Received in revised form
10 April 2021
Accepted 13 April 2021
Available online 18 April 2021

Keywords:
TAI
Beef cattle
Fertility
Estrus
GnRH
Synchronization

ABSTRACT

The aim was to study reproductive outcomes of Nelore (*Bos indicus*) cows submitted to a 7-d estradiol (E2)/progesterone (P4)-based timed artificial insemination (TAI) protocol, receiving various combinations of doses and hormones. Primiparous (n = 962) and multiparous (n = 1935) cows were submitted to synchronization (n = 2012) and resynchronization (n = 885 non-pregnant cows at pregnancy diagnosis 30 d after TAI) protocols, following a 2 × 2 × 2 factorial arrangement of eight treatments. At the initiation of the TAI protocol (Day −9), all cows received a 1.0 g intravaginal P4 insert, 2.0 mg E2 benzoate and received (PGF1) or not (PGF0) 0.5 mg cloprostenol sodium (PGF). On Day −2, the P4 insert was removed, all cows received 0.5 mg PGF, 300 IU equine chorionic gonadotropin (eCG) and 0.5 (EC0.5) or 1.0 mg estradiol cypionate (EC1.0). On Day 0, cows were treated (G1) with 8.4 μg buserelin acetate (GnRH) or not (G0), concurrently with TAI. The eight treatments were generated: 1) PGF0-EC0.5-G0 (n = 364), 2) PGF0-EC0.5-G1 (n = 363), 3) PGF1-EC0.5-G0 (n = 363), 4) PGF1-EC0.5-G1 (n = 360), 5) PGF0-EC-1.0-G0 (n = 360), 6) PGF0-EC1.0-G1 (n = 363), 7) PGF1-EC1.0-G0 (n = 361), and 8) PGF1-EC1.0-G1 (n = 363). Pregnancy per AI (P/AI) was greater at first AI compared with resynchronization (58.9 [n = 2012] vs. 54.9% [n = 885]). Presence of CL on Day −9 resulted in more cows expressing estrus (81.3 [n = 680] vs. 67.1% [n = 2033]) and greater P/AI (66.0 [n = 692] vs. 54.9% [n = 2106]). There was no difference in P/AI between cows that received or not PGF on Day -9 (58.7 [n = 1447] vs. 56.6% [n = 1450]). In contrast, PGF tended to increase P/AI of cows with CL on Day −9 (with PGF = 69.1 [n = 375] vs. without PGF = 62.5% [n = 317]). Cows that received 1.0 mg EC expressed more estrus than those treated with 0.5 mg (73.8 [n = 1414] vs. 67.9% [n = 1398]) and had greater P/AI (60.2 [n = 1447] vs. 55.1% [n = 1450]). P/AI was greater in cows treated with GnRH at TAI (59.8 [n = 1449] vs. 55.5% [n = 1448]), particularly in cows that did not show estrus (52.7 [n = 393] vs. 38.1% [n = 420]). Moreover, GnRH on Day 0 increased P/AI in cows with BCS < 3.0 (57.1 [n = 723] vs. 48.6% [n = 698]), in primiparous (50.1 [n = 465] vs. 41.9% [n = 497]) and in cows that received 0.5 mg EC (58.9 [n = 723] vs. 51.3% [n = 727]). In conclusion, 1.0 mg of EC on Day −2 and GnRH at TAI improved P/AI, but the combination of a higher dose of EC and GnRH treatment at AI did not enhance this effect. Furthermore, GnRH improved P/AI especially in *Bos indicus* cows with lower expression of estrus, such as primiparous, thinner cows, and cows treated with 0.5 mg of EC.

1. Introduction

In *Bos indicus* beef cattle, one of the most used timed artificial insemination (TAI) protocol is based on the combination of

* Corresponding author.
E-mail address: robertosartori@usp.br (R. Sartori).

https://doi.org/10.1016/j.theriogenology.2021.04.007
0093-691X/

estradiol (E2) and progesterone (P4), termed E2/P4-based TAI protocols [1,2]. These protocols begin with placement of intravaginal P4 inserts and treatment with an E2 ester, mainly E2 benzoate (EB), to promote the atresia of growing follicles and emergence of a new follicular wave [3,4]. At the time of P4 implant removal, prostaglandin F2α (PGF) is administered to induce luteolysis, along with equine chorionic gonadotropin (eCG) that is used to stimulate the final growth of the pre-ovulatory follicle [5,6]. Different ovulation inducers have been used at the end of E2/P4 TAI protocols such as E2 cypionate (EC), administered at the time of P4 implant withdrawal, EB, administered 24 h after implant removal, or gonadotropin-releasing hormone (GnRH) analogues, administered either before or at the time of TAI [2,7–9]. A recent study [10] reported synchronization of a new follicular wave in 92.4% of Nelore cattle, after treatment with EB and an intravaginal P4 device, and ovulation at the end of the protocol was 84.8%, showing good overall synchronization efficiency with this protocol.

A potential modification on E2/P4-based TAI protocols is treatment with PGF at the beginning of the protocol or before P4 implant withdrawal, in order to diminish P4 during follicle development and/or to increase the length of proestrus [11–14]. Administration of PGF on the first day of synchronization protocols in cyclic beef cattle (*Bos indicus, Bos taurus* and crossbred cows and heifers) promoted either positive effects [12,13,15] or no effects on reproductive outcomes [11,14]. Dadarwal et al. [12] reported greater diameter of preovulatory follicles, more ovulations, larger CL, higher circulating P4 and greater pregnancy per AI (P/AI) in Hereford-cross cows and pubertal heifers treated with PGF at the beginning of a TAI protocol. On the other hand, Sá Filho et al. [14] reported no differences on follicle diameter, time of ovulation, ovulation incidence and P/AI in cyclic Nelore heifers receiving or not PGF on the first day of a TAI protocol. Moreover, Hill et al. [15] reported larger follicles and greater ovulation rate at the beginning of a CO-Synch + CIDR TAI protocol, when *Bos taurus* cows (Angus and crossbred) were presynchronized with PGF 3 d before the initiation of the protocol, although P/AI was not influenced by this treatment.

An additional change in E2/P4-based protocols is altering the dose of EC at the time of P4 implant removal (from 0.5 to 1.0 mg), endeavoring to increase expression of estrus and fertility [16,17]. Torres-Júnior et al. [17] reported better synchronization of ovulations and greater P/AI in *Bos indicus* cows when 1.0 mg of EC was used in TAI protocols with 8 d of P4 insert (55.7 vs. 38.6% for 1.0 vs. 0.5 mg EC, respectively). In contrast, Bosolasco et al. [18], using an E2/P4-based protocol with 7 d of P4 implant reported greater P/AI in crossbred Hereford and Angus cows that received 0.5 mg compared with 1.0 mg EC (60.4 vs. 50.4%).

Another strategy to improve P/AI in beef cattle is treatment with GnRH at the time of TAI [19,20]. Madureira et al. [20] and Prata et al. [19] reported positive effects of GnRH treatment at AI on fertility of Nelore cows either detected or not in estrus at the end of TAI protocols using 0.5 mg EC as final ovulation inducer.

Thus, the main objective of the present study was to evaluate reproductive outcomes of Nelore cows submitted to E2/P4-based TAI protocols, with P4 insert maintained for 7 d, but using different variations of hormonal treatments to: 1) Induce luteolysis by administering PGF at the initiation of the protocol in order to have lower circulating P4 during the protocol, 2) Increase circulating E2 and expression of estrus at the end of the protocol by administering a higher dose of EC at P4 device withdrawal to induce final ovulation, and 3) Increase ovulation and, perhaps, improve timing of ovulation by treating with GnRH at the time of AI. Three main hypotheses were tested: 1) Administration of PGF on Day −9 would increase follicle diameter on Day −2 and at TAI, expression of estrus, and P/AI in cyclic cows, 2) Increasing the dose of EC from 0.5 to 1.0 mg would increase expression of estrus and P/AI, circumventing the need for GnRH at the time of AI, and 3) Administration of GnRH at the time of AI would increase overall P/AI, especially in cows that had not been detected in estrus by the time of AI.

2. Material and methods

2.1. Location

The experiment was conducted in Londrina, Paraná, Brazil, at the Experimental Station "Hildegard Georgina Von Pritzelwiltz" (Figueira Farm) and the data were collected in two consecutive breeding seasons. Nelore cows were kept on pastures of *Brachiaria brizantha*, supplemented with mineral salt and had *ad libitum* access to water. The Animal Research Ethics Committee of "Luiz de Queiroz" College of Agriculture of the University of São Paulo (ESALQ/USP) approved all animal procedures (Protocol # 2018–19).

2.2. Cow management

A total of 2897 suckled Nelore (*Bos indicus*) cows at 59.4 ± 24.1 d postpartum were enrolled. At the beginning of TAI protocols (Day −9), BCS was determined using a 5-point scale [21] with 0.25 increments (1 = emaciated; 5 = obese). Primiparous (n = 962) and multiparous (n = 1935) cows had an average BCS of 2.9 ± 0.01 and 3.0 ± 0.01, respectively. A total of 2012 cows received the first TAI of the breeding season and non-pregnant cows (n = 885) at pregnancy diagnosis 30 d after TAI were randomized to the same experimental design during the resynchronization protocol, considering each non-pregnant cow as a new experimental unit. Discrepancy between the number of resynchronized cows and non-pregnant cows after the first TAI occurred for the following reasons: 1) groups of cows that received first TAI following the farm management (they did not receive experimental treatments) and were entered in the experiment only during the resynchronization protocol; 2) groups of cows that received only one TAI, following the experimental design.

A total of 21 and 18 groups of cows (replicates) were used at the first TAI and resynchronization protocol, respectively.

2.3. Experimental design

The experiment used a 2 × 2 × 2 factorial arrangement of eight treatments, as shown in Fig. 1. The three factors were: 1) treatment with PGF on Day −9 (with PGF = **PGF1** or without PGF = **PGF0**); 2) dose of EC on Day −2 (0.5 EC = **EC0.5** or 1.0 mg = **EC1.0**); and 3) treatment with GnRH at the time of AI (with GnRH = **G1** or without GnRH = **G0**). At the initiation of the TAI protocol (Day −9), all cows received a 1.0 g intravaginal P4 insert (Repro neo, GlobalGen vet science, Jaboticabal, Brazil), 2.0 mg EB im (Syncrogen, GlobalGen vet science) and received (**PGF1**) or not (**PGF0**) 0.5 mg cloprostenol sodium (PGF) im (Induscio, GlobalGen vet science). Seven days later (Day −2), the P4 inserts were removed, all cows were treated with 0.5 mg PGF im, 300 IU equine chorionic gonadotropin im (eCG; eCGen, Globalgen vet science) and 0.5 (**EC0.5**) or 1.0 mg (**EC1.0**) of EC (Cipion, GlobalGen vet science). On Day 0 (48 h after insert removal), cows were treated im (**G1**) or not (**G0**) with 8.4 μg buserelin acetate (GnRH; Maxrelin; GlobalGen vet science). The dose of 8.4 μg buserelin was different from the commonly reported 10 μg [22]. This dose was used in several experiments of our lab and provided very good results in terms of ovulation and P/AI [19,20]. Cows were inseminated by one of two technicians using 20 x 10^6 frozen/thawed proven semen from 17 bulls (STGenetics, Indaiatuba, Brazil and Genex, São Carlos, Brazil). Therefore, there were eight resulting

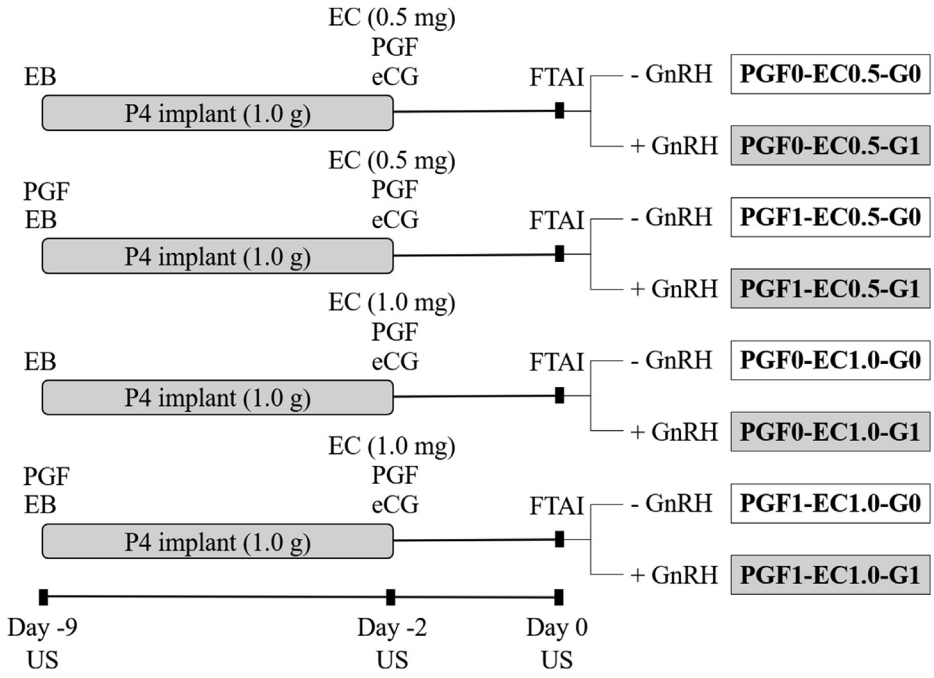

Fig. 1. Schematic diagram of the experimental design following a 2 × 2 × 2 factorial arrangement. The three factors were: 1) cloprostenol sodium (PGF) on Day −9 (with PGF = **PGF1**, or without PGF = **PGF0**); 2) dose of estradiol cypionate (EC) on Day −2 (0.5 EC = **EC0.5**, or 1.0 mg = **EC1.0**); and 3) buserelin acetate (GnRH) at the time of artificial insemination (AI; with GnRH = **G1**, or without GnRH = **G0**). On Day −9, cows received an intravaginal insert containing 1.0 g progesterone (P4), 2.0 mg estradiol benzoate (EB) im and received (**PGF1**) or not (**PGF0**) 0.5 mg PGF im. Seven d later (Day −2), the inserts were removed, all cows were treated with 0.5 mg PGF im, 300 IU equine chorionic gonadotropin (eCG) im, and 0.5 (**EC0.5**) or 1.0 mg (**EC1.0**) EC. On Day 0 (48 h after insert removal), cows were treated im (**G1**) or not (**G0**) with 8.4 μg GnRH and were inseminated.

treatments, according to Fig. 1: 1) **PGF0-EC0.5-G0** (n = 364); 2) **PGF0-EC0.5-G1** (n = 363); 3) **PGF1-EC0.5-G0** (n = 363); 4) **PGF1-EC0.5-G1** (n = 360); 5) **PGF0-EC-1.0-G0** (n = 360); 6) **PGF0-EC1.0-G1** (n = 363); 7) **PGF1-EC1.0-G0** (n = 361); 8) **PGF1-EC1.0-G1** (n = 363). For expression of estrus evaluation, all cows had the base of their tailhead painted with tail-chalk (Walmur, São Geraldo, Brazil) on Day −2 and were checked at the time of AI for the absence of tail-chalk (more than 75% removal), which indicated that the cows expressed estrus before TAI.

2.4. Ultrasound evaluations

Transrectal ultrasound ovarian examinations in B-mode with a 7.5 MHz linear transducer (DP-2200 VET, Mindray, Shenzhen, China) were performed on Days −9 and −2 of the synchronization protocol, in a subgroup of cows, in order to evaluate the presence or absence of CL. Approximately 20% of both primiparous and multiparous cows were randomly submitted to ultrasound examination of the ovaries to evaluate the diameter (mm) of the largest follicle (LF) on Day −2 (primiparous = 223; multiparous = 507) and at the time of AI (primiparous = 218; multiparous = 488). All measurements were conducted by the same operator. Incidence of ovulation at the end of the protocol (n = 696) was calculated by the presence of CL 7 d after AI (Day 7). The percentage of cows with multiple ovulation was calculated as the proportion of cows with two or more CL divided by the number of cows that ovulated.

Pregnancy diagnosis was conducted by the same operator between 30 and 33 d after TAI (Day 30) by transrectal ultrasound by confirming the presence of a viable embryo with a heartbeat. The P/AI was calculated as the proportion of cows pregnant divided by the number of cows inseminated. To analyze embryo/fetal loss, ultrasound evaluation was also performed between 60 and 63 d after TAI (Day 60) in approximately 40% of cows (n = 1063), and only during the second breeding season.

2.5. Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC). Analyses of binomial outcome variables (presence of CL on Day −9, expression of estrus, ovulation after TAI, P/AI and pregnancy losses) were performed using the GLIMMIX procedure fitting a binomial distribution with the Link Logit function. The continuous outcomes (follicle diameter on Day −2 and Day 0) were evaluated using the GLIMMIX procedure with a Gaussian distribution, after testing for normality of residuals according to Shapiro-Wilk test using the UNIVARIATE procedure. In addition, the option ddfm = kenwardroger was included in the model statement to adjust the degrees of freedom for variances.

The analysis was performed as a factorial 2 x 2 x 2 design, in which the model for P/AI at 30 and 60 d after AI and expression of estrus included main effects (with or without PGF on Day −9, 0.5 or 1.0 mg of EC on Day −2, and with or without GnRH on Day 0) and the 2 and 3-way interactions. Moreover, the effects of parity (primiparous or multiparous), number of AI (1st AI or resynch), presence of CL on Day −9 (presence or absence), BCS on Day −9 (<3 or ≥ 3), and expression of estrus (with or without estrus – included only for the P/AI model) were included. All interactions (2-way and 3-way) between treatments and the described outcome variables were also evaluated.

The selection of the model that best fit each outcome variable of interest was performed by finding the model with the lowest value for the Akaike Information Criterion Corrected (AICC) using the stepwise selection procedure that included outcomes and interactions with $P < 0.20$ from the model. In addition, main effects and the factorial interaction of effects were forced into the final model in all analyses.

When interactions were significant ($P \leq 0.05$), the SLICE command in the LSMEANS was used to interpret them. Tukey honest

significant difference post hoc test was performed to determine differences. Significant differences were declared when $P \leq 0.05$, whereas tendencies were considered when $0.10 \geq P > 0.05$. Values are presented as percentage (%; binomial variables). The results of continuous outcome variables are expressed as means ± standard error of the mean.

The LOGISTIC procedure was used for logistic regression to model the probability of expression of estrus, ovulation after TAI and pregnancy on Day 30 according to the diameter of the largest follicle on Day −2 and Day 0 (this analysis was done separately for each day). Logistic regression curves were created using the coefficients provided by the interactive data analysis from SAS and the formula $Y = \exp(\alpha \times X + \beta)/[1 + \exp(\alpha \times X + \beta)]$, where Y = probability of occurrence; exp = exponential; α = slope of the logistic equation; β = intercept of the logistic equation; and X = analyzed outcome. For the probability curves, cutoff points were established for the selection of cows based on the physiology of the estrous cycle, considering the time of follicular wave emergence, 2–5 d after Day −9, and follicular growth rate, 0.5–2.0 mm/d [10,23,24]. Therefore, the cutoff point used on Day −2 ranged from 5.0 mm (minimum value; follicle emerging on Day −4 and growing 0.5 mm/d) to 14.0 mm (maximum value; follicle emerging on Day −7 and growing 2.0 mm/d). In addition, the cutoff point defined on Day 0 ranged from 6.0 mm (minimum value; follicle measuring 5.0 mm on Day −2 and growing 0.5 mm/d) to 18.0 mm (maximum value; follicle measuring 14.0 mm on Day −2 and growing 2.0 mm/d).

3. Results

3.1. Ovarian dynamics

At the initiation of the protocol (Day −9), regardless of number of AI, the proportion of primiparous cows having a CL was less ($P < 0.001$) than that of multiparous cows, as shown in Table 1. At the initiation of the resynchronization protocol, more ($P < 0.001$) cows (38.8% [352/907]) had CL than those at first postpartum AI (18.5% [354/1914]). Also, more cows ($P < 0.001$) with BCS ≥ 3.0 had CL on Day −9 compared with cows with BCS < 3.0 (33.0 [470/1424] vs. 16.9% [236/1397]). Neither administration of PGF on Day −9 nor presence of CL on Day −9 influenced LF diameter on Day −2 (with PGF: 9.4 ± 0.09 mm [n = 255]; without PGF: 9.2 ± 0.09 mm [n = 283]; with CL: 9.4 ± 0.14 mm [n = 125]; without CL: 9.2 ± 0.07 mm [n = 411]), or on Day 0 (with PGF: 12.2 ± 0.12 mm [n = 273]; without PGF: 12.0 ± 0.12 mm [n = 295]; with CL: 12.2 ± 0.18 mm [n = 135]; without CL: 12.0 ± 0.09 mm [n = 431]). In addition, EC dose on Day −2 did not affect ($P = 0.27$) LF diameter on Day 0 (0.5 mg: 12.1 ± 0.12 mm [n = 287]; 1.0 mg: 12.0 ± 0.12 mm [n = 281]). Table 1 also shows that multiparous cows had greater diameter of the LF than primiparous cows, both on Day −2 (insert removal) and on Day 0. Moreover, BCS ≥ 3.0 was associated with a greater ($P < 0.001$) diameter of the LF than BCS < 3.0, on Day −2 (9.8 ± 0.11 [n = 408] vs. 9.1 ± 0.13 mm [n = 322]) and Day 0 (11.8 ± 0.13 [n = 399] vs. 10.7 ± 0.17 mm [n = 307]).

Ovulation rate after Day −9 was low (9.1% [209/2293]), which was expected since EB treatment at the beginning should not induce an ovulation. Moreover, ovulation after Day −9 was similar ($P = 0.87$) in cows with (10.3% [53/515]) and without CL (8.8% [156/1778]). Considering only cows with CL on Day −9, the percentage of cows with CL regression between Days −9 and −2 was greater ($P < 0.001$) in cows that received PGF on Day −9 than cows that did not receive PGF (74.7 [210/281] vs. 53.9% [125/232]).

Overall, ovulation after AI was 89.7% (624/696) and was influenced by parity, BCS and presence of CL on Day −9, GnRH treatment on Day 0 and expression of estrus (Table 2). In contrast, percentage of cows that ovulated was not influenced by treatment with PGF on Day −9 or by the dose of EC on Day −2. There was an interaction ($P = 0.02$) between BCS on Day −9 and treatment on Day −2 on the percentage of cows that ovulated after AI. More cows ($P = 0.02$) with BCS < 3.0 ovulated when 1.0 mg of EC was given than when treated with 0.5 mg of EC (88.1 [140/159] vs. 79.8% [126/158]). Considering cows with BCS ≥ 3.0, there was no difference ($P = 0.24$) on ovulation after AI between 0.5 and 1.0 mg of EC (95.3 [181/190] vs. 93.7% [177/189], respectively).

There was a tendency ($P = 0.10$) for an interaction between expression of estrus and GnRH treatment on ovulation after TAI, in which GnRH treatment increased ($P = 0.006$) ovulation only in cows without expression of estrus (83.6 [56/67] vs. 71.2% [52/73],

Table 2
Ovulation after timed artificial insemination (TAI) based on parity, body condition score (BCS) and presence of corpus luteum (CL) on Day −9, dose of estradiol cypionate (EC) on Day −2, treatment with GnRH at AI (Day 0), and expression of estrus in cows submitted to estradiol/progesterone-based TAI protocols.

Item	Ovulation, % (n/n)	P-value
Parity		
Primiparous	81.7 (165/202)	0.005
Multiparous	92.9 (459/494)	
BCS on Day -9		
<3.0	83.9 (266/317)	0.001
≥3.0	94.5 (358/379)	
CL on Day -9*		
Without CL	86.9 (419/482)	0.02
With CL	95.8 (203/212)	
Treatment on Day -2		
0.5 mg EC	88.2 (307/348)	0.69
1.0 mg EC	91.1 (317/348)	
Treatment on Day 0		
Without GnRH	87.2 (299/343)	0.005
With GnRH	92.1 (325/353)	
Expression of estrus**		
Without estrus	77.1 (108/140)	0.004
With estrus	92.8 (514/554)	

*Some cows were not evaluated by transrectal ultrasound on Day −9.
**Some cows were not evaluated for expression of estrus.

Table 1
Presence of corpus luteum (CL), largest follicle (LF) diameter on Day −2 and at the time of timed artificial insemination (TAI; Day 0) of first AI and resynchronization in primiparous and multiparous cows submitted to estradiol/progesterone-based TAI protocols.

Item	Parity			P-value
	Primiparous	Multiparous	Overall	
CL on Day -9, % (n/n)				
First AI	7.6 (43/565)	23.1 (311/1349)	18.5 (354/1914)	<0.001
Resynchronization	23.6 (86/364)	49.0 (266/543)	38.8 (352/907)	<0.001
Overall	13.9 (129/929)	30.5 (577/1892)	25.0 (706/2821)	<0.001
LF diameter, mm (n)				
Day −2	8.1 ± 0.12 (223)	10.1 ± 0.10 (507)	9.5 ± 0.08 (730)	<0.001
Day 0 (time of AI)	9.7 ± 0.18 (218)	12.0 ± 0.12 (488)	11.3 ± 0.11 (706)	<0.001

with and without GnRH, respectively). In contrast, considering cows that expressed estrus, GnRH did not impact ovulation after TAI (94.0 [267/284] vs. 91.5% [247/270], with and without GnRH, respectively).

In addition, overall multiple ovulation after AI was 8.8% (55/624) and was not influenced by any treatments or variables analyzed.

Fig. 2A shows that the probability of ovulation after AI did not differ based on follicular diameter on either Days −2 (P = 0.45) or 0 (P = 0.20). In contrast, cows that ovulated after AI had larger follicles than cows that did not ovulate, on both Days −2 (9.5 ± 0.15 [n = 189] vs. 7.5 ± 0.51 mm [n = 22]; P < 0.001) and 0 (11.8 ± 0.17 [n = 185] vs. 8.3 ± 0.65 mm [n = 21]; P < 0.001).

3.2. Expression of estrus (by the time of TAI)

Overall, estrus was detected in 70.9% (1993/2812) of the cows and multiparous expressed more estrus (P < 0.001) than primiparous cows (76.9 [1435/1867] vs. 59.1% [558/945]). As shown in Fig. 3, BCS ≥ 3.0 and presence of CL on Day −9 increased (P < 0.001) expression of estrus and cows that were treated with 1.0 mg EC expressed more estrus than cows that received 0.5 mg. Treatment with PGF on Day −9 tended (P = 0.07) to increase expression of estrus (Fig. 3), however, it did not interact (P = 0.28) with presence of CL on Day −9 (cows with CL: with PGF = 83.6 [310/371] vs. without PGF = 78.6% [243/309]; cows without CL: with PGF = 68.4 [676/989] vs. without PGF = 66.0% [689/1044]).

There were interactions for expression of estrus with parity and other factors, such as presence of CL or BCS on Day −9, PGF treatment on Day −9, and dose of EC on Day −2, as shown in Table 3. Presence of CL at the initiation of the protocol increased expression of estrus in both primiparous and multiparous cows, but the effect was much greater (P = 0.04) in primiparous (32.6% increase) than in multiparous cows (12.3% increase). Also, BCS ≥ 3.0 resulted in greater (P < 0.05) expression of estrus in multiparous cows and tended to increase (P = 0.10) expression of estrus in primiparous cows. There was a tendency for an interaction (P = 0.08) of EC dose on Day −2 with parity, indicated by the observation that 1.0 mg of EC increased (P < 0.001) expression of estrus in multiparous, but not (P = 0.4) in primiparous cows (Table 3).

Fig. 2B represents the probability of expression of estrus based on follicular diameter on Day −2 (P = 0.02) or Day 0 (P = 0.008) of the TAI protocol. As the diameter of the LF increased, expression of estrus also increased. Cows that expressed estrus had larger (P < 0.001) follicles than cows that did not express estrus on both Days −2 (9.7 ± 0.10 [n = 537] vs. 8.5 ± 0.16 mm [n = 174]) and 0 (11.7 ± 0.11 [n = 533] vs. 10.1 ± 0.23 mm [n = 172]).

3.3. Pregnancy per AI on Day 30 and Day 60

The overall P/AI on Day 30 was 57.6% (1670/2897) and on Day 60 was 53.2% (566/1063). Overall, pregnancy loss between Days 30 and 60 was 4.9% (29/595) and was not affected by experimental treatments. The P/AI on Day 30 was greater (P = 0.05) at first TAI compared with resynchronization protocols (58.9 [1184/2012] vs. 54.9% [486/885]).

The main effects of treatments on P/AI are represented in Fig. 4. The treatment with PGF on Day −9 did not influence P/AI (P = 0.4). However, 1.0 mg of EC increased P/AI (P = 0.05). Moreover, P/AI was greater (P < 0.001) when cows were treated with GnRH on Day 0.

The P/AI on Day 30 for each experimental group was: **PGF0-EC0.5-G0** (50.0% [182/364]), **PGF0-EC0.5-G1** (57.0% [207/363]), **PGF1-EC0.5-G0** (52.6% [191/363]), **PGF1-EC0.5-G1** (60.8% [219/

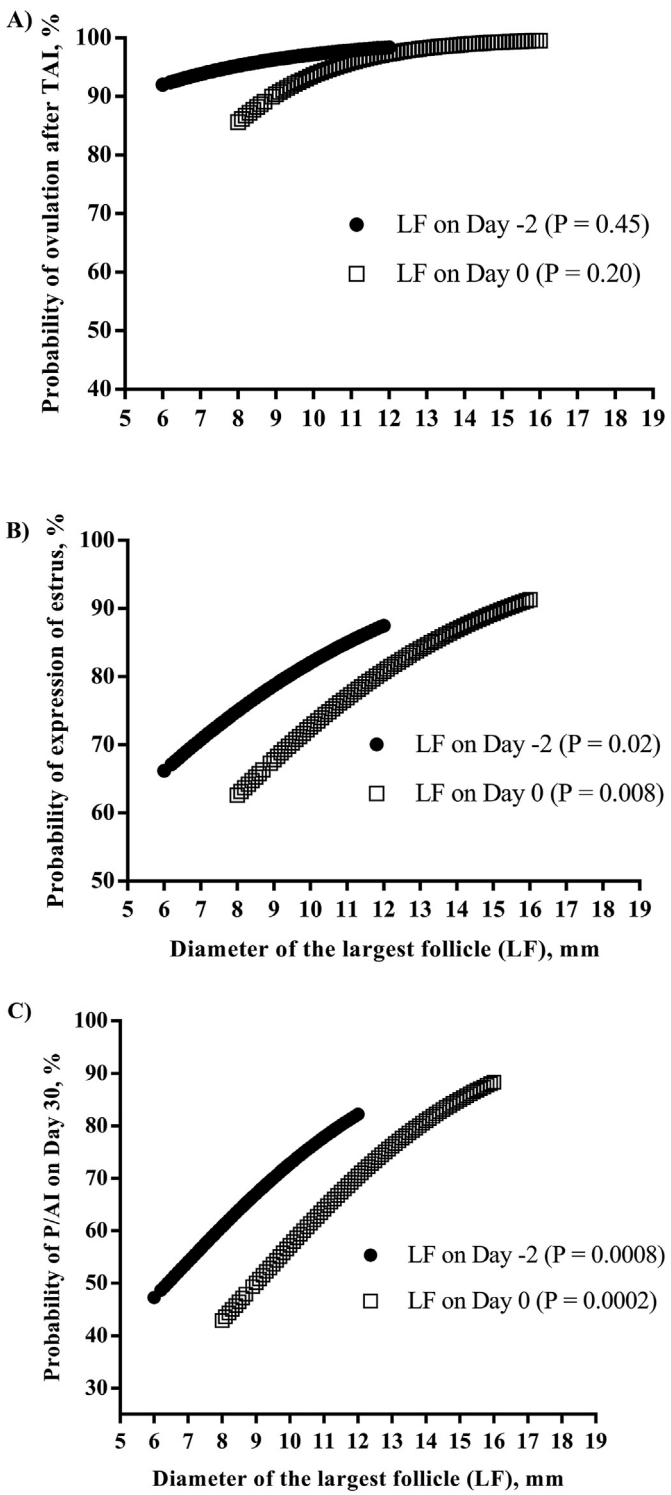

Fig. 2. Probability of ovulation after timed artificial insemination (TAI; Panel A; n = 110), expression of estrus (Panel B; n = 366) and pregnancy per AI (P/AI) on Day 30 (Panel C; n = 366) based on the diameter of the largest follicle (LF) on Day −2 and at the time of AI in cows submitted to estradiol/progesterone-based TAI protocols. Ovulation after AI = 0.7823 + 0.2772 * diameter of the LF on Day −2 (P = 0.45). Ovulation after AI = −1.8356 + 0.4526 * diameter of the LF on Day 0 (P = 0.20). Expression of estrus = −0.5999 + 0.212 * diameter of the LF on Day −2 (P = 0.02). Expression of estrus = −1.3232 + 0.2299 * diameter of the LF on D0 (P = 0.008). P/AI = −1.7473 + 0.2732 * diameter of the LF on Day −2 (P = 0.0008). P/AI = −2.5984 + 0.289 * diameter of the LF on Day 0 (P = 0.0002).

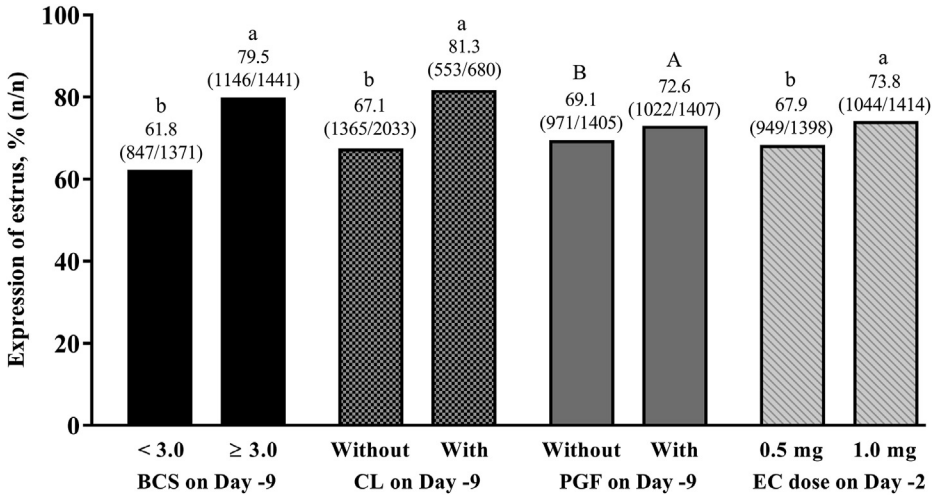

Fig. 3. Expression of estrus of cows submitted to estradiol/progesterone-based TAI protocols based on body condition score (BCS) and presence of corpus luteum (CL) on Day −9, cloprostenol sodium (PGF) treatment on Day −9 and estradiol cypionate (EC) dose at the time of progesterone insert withdrawal (Day −2). [a,b]There were effects of BCS ($P < 0.001$), presence of CL on Day −9 ($P < 0.001$) and EC dose on Day −2 ($P = 0.002$). [A,B]There was a tendency for effects of PGF on Day −9 ($P = 0.07$).

360]), **PGF0-EC1.0-G0** (59.4% [214/360]), **PGF0-EC1.0-G1** (60.1% [218/363]), **PGF1-EC1.0-G0** (59.8% [216/361]), and **PGF1-EC1.0-G1** (61.4% [223/363]).

Cows that expressed estrus had greater ($P < 0.001$) fertility than cows not detected in estrus (62.5 [1235/1976] vs. 45.1% [367/813]). Presence of CL on Day −9 was associated with increased ($P = 0.001$) P/AI, and PGF tended ($P = 0.07$) to increase P/AI only in cows with CL on Day −9 (Fig. 5).

There were no interactions ($P = 0.79$) of EC dose on Day −2 (0.5 or 1.0 mg) and BCS on Day −9 on P/AI (Fig. 6). However, cows with BCS ≥ 3.0 had greater ($P = 0.002$) P/AI than cows with BCS < 3.0 (62.2 [918/1476] vs. 52.9% [752/1421]). In addition, there was no interaction ($P = 0.26$) between parity (primiparous or multiparous) and EC dose (Fig. 6) on P/AI, but multiparous cows had greater ($P < 0.001$) P/AI than primiparous cows (63.5 [1229/1935] vs. 45.8% [441/962]).

There were interactions for P/AI with GnRH treatment on Day 0 and other factors, such as BCS on Day −9, parity and expression of estrus, as shown in Table 4. Treatment with GnRH on Day 0 increased ($P < 0.001$) P/AI in cows with BCS < 3.0, but there was no difference ($P = 0.11$) in cows with BCS ≥ 3.0. Also, GnRH treatment on Day 0 increased ($P = 0.003$) P/AI in primiparous but did not change P/AI in multiparous cows. Moreover, cows that did not express estrus had greater ($P < 0.01$) P/AI when GnRH was given on Day 0, but no effect ($P = 0.46$) of GnRH was observed in cows that expressed estrus.

Table 4 also shows a tendency ($P = 0.10$) for an interaction on P/AI on Day 30 between dose of EC on Day −2 and GnRH on Day 0. Thus, GnRH on Day 0 increased ($P < 0.001$) P/AI in cows that received 0.5 mg EC on Day −2, but did not influence P/AI in cows receiving 1.0 mg EC.

When only cows that ovulated after TAI were included in the analysis of P/AI on Day 30, there was no effect ($P = 0.44$) of GnRH treatment at TAI or interaction ($P = 0.84$) between this factor and expression of estrus (cows without expression of estrus = 55.4 [31/56] vs. 53.9% [28/52]; cows with expression of estrus = 67.4 [180/267] vs. 62.8% [155/247], with and without GnRH, respectively).

Fig. 2C presents the probability of P/AI on Day 30 based on diameter of the ovulatory follicle on Days −2 and 0, showing P/AI increased as the diameter of the LF increased on Day −2 ($P < 0.001$) and on Day 0 ($P < 0.001$). Pregnant cows on Day 30 had larger ($P < 0.001$) follicles on both Days −2 (9.9 ± 0.10 [n = 434] vs. 8.8 ± 0.14 mm [n = 296]) and 0 (12.0 ± 0.11 [n = 422] vs. 10.2 ± 0.19 mm [n = 284]) compared with non-pregnant cows.

4. Discussion

With a high number of cows for evaluation of fertility and ovarian dynamics, this study shows the importance of adjustments in hormone treatments within TAI protocols and describes the interactions of these adjustments with several variables, such as parity, BCS, expression of estrus, cyclicity status, and ovulation after

Table 3
Expression of estrus (%) of multiparous and primiparous cows submitted to estradiol/progesterone-based timed artificial insemination (TAI) protocols based on presence of corpus luteum (CL) on Day −9, cloprostenol sodium (PGF) treatment on Day −9, body condition score (BCS) on Day −9 and estradiol cypionate (EC) dose on Day −2.

Item	Expression of estrus, % (n/n)				P-value		
	Primiparous		Multiparous				
CL on Day -9	**Without**	**With**	**Without**	**With**	**Parity**	**CL**	**Parity × CL**
	56.4[b] (436/773)	74.8[a] (95/127)	73.7[b] (929/1260)	82.8[a] (458/553)	<0.001	<0.001	0.04
PGF on Day -9	**Without**	**With**	**Without**	**With**	**Parity**	**PGF**	**Parity × PGF**
	58.6 (290/495)	59.6 (268/450)	74.8 (681/910)	78.8 (754/957)	<0.001	0.07	0.13
BCS on Day -9	**< 3.0**	**≥ 3.0**	**< 3.0**	**≥ 3.0**	**Parity**	**BCS**	**Parity × BCS**
	53.8[Y] (272/506)	65.1[X] (286/439)	66.5[y] (575/865)	85.8[x] (860/1002)	<0.001	<0.001	<0.001
EC dose on Day -2	**0.5 mg**	**1.0 mg**	**0.5 mg**	**1.0 mg**	**Parity**	**EC**	**Parity × EC**
	58.4 (289/495)	59.8 (269/450)	73.1 (660/903)	80.4 (775/964)	<0.001	0.002	0.08

[a,b]Effect of CL on Day −9 within parity ($P < 0.05$).
[x,y]Effect of BCS on Day −9 within parity ($P < 0.05$).
[X,Y]Effect of BCS on Day −9 within parity ($P = 0.10$).

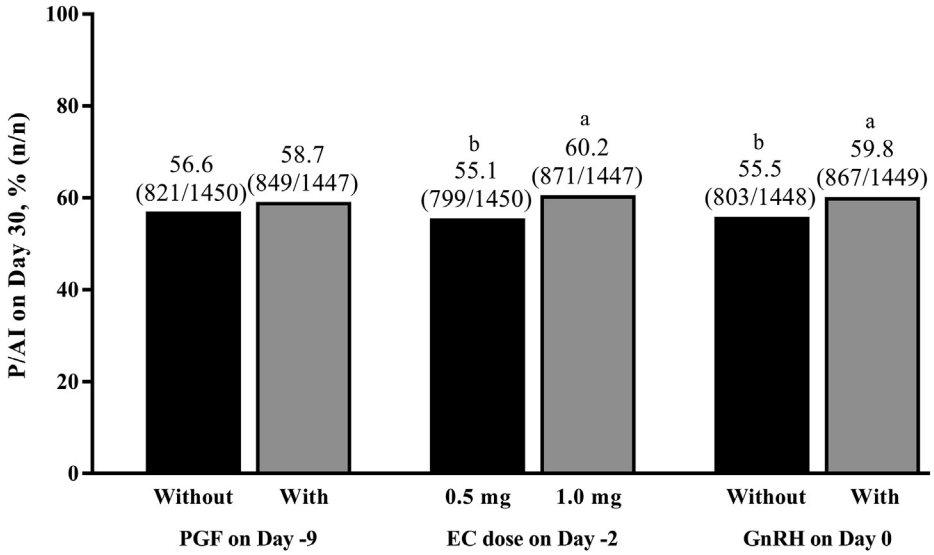

Fig. 4. Pregnancy per artificial insemination (P/AI) 30 d after AI of cows submitted to estradiol/progesterone-based timed AI (TAI) protocols, with or without cloprostenol sodium (PGF) on Day −9, using either 0.5 or 1.0 mg of estradiol cypionate (EC) at the time of progesterone insert removal (Day −2), and with or without buserelin acetate (GnRH) at TAI. [a,b]Difference between cows that received 0.5 vs. 1.0 mg of EC on Day −2 ($P = 0.05$), and received or not GnRH at the time of AI ($P < 0.001$). No differences between PGF treatment on Day −9 (with or without PGF; $P = 0.4$).

AI. Moreover, this study has shown that P/AI can be improved by > 10% points when optimized treatments are administered instead of using the traditional protocol (no PGF on Day −9, 0.5 mg EC on Day −2 and no GnRH at AI).

More cows with greater BCS and with CL at the initiation of the TAI protocol expressed estrus and subsequently had greater P/AI. It is well known that resumption of cyclicity is affected by many factors including nutritional status, suckling, parity, change in body weight after calving, and BCS, and also that these factors can impact reproductive performance [25–30]. Our results corroborate the data reported by Ayres et al. [31] that described a lower percentage of cows with CL at the initiation of a synchronization protocol (42 d postpartum) when the BCS was < 3.0, compared with cows with BCS > 3.0 (33.8 vs. 43.4%). In the present study, more cows with greater BCS had CL on Day 0, and this was associated with a greater expression of estrus and P/AI, confirming previous reports that described earlier resumption of cyclicity and better reproductive performance when beef cows gained weight or lost less BCS in the postpartum period and had greater BCS at the initiation of the protocol [28,29,32–35].

As previously reported, primiparous beef cows have longer postpartum anestrus periods and lower fertility than multiparous cows [32,36]. In our study, a greater percentage of multiparous cows had a CL at the beginning of the protocol compared with primiparous cows, consistent with the results of Dimmick et al. [37], that described a longer interval between parturition and first ovulation in primiparous than multiparous cows (112 vs. 46 d). Guedon et al. [38] also reported that the first postpartum ovulation occurred earlier in multiparous than primiparous beef cows (7.7 vs. 9.9 wk).

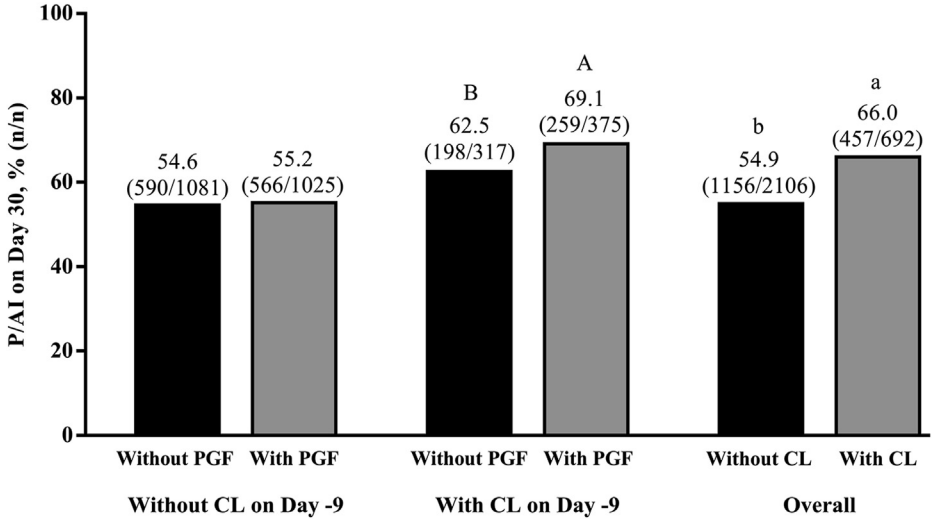

Fig. 5. Pregnancy per artificial insemination (P/AI) 30 d after AI in cows submitted to estradiol/progesterone-based timed AI (TAI) protocols based on presence of corpus luteum (CL) on Day −9 of the protocol, and receiving or not cloprostenol sodium (PGF).
[a,b] Effect of presence of CL on Day −9 ($P < 0.001$). [A,B]Effect of PGF administration on Day −9, considering cows with CL ($P = 0.07$).

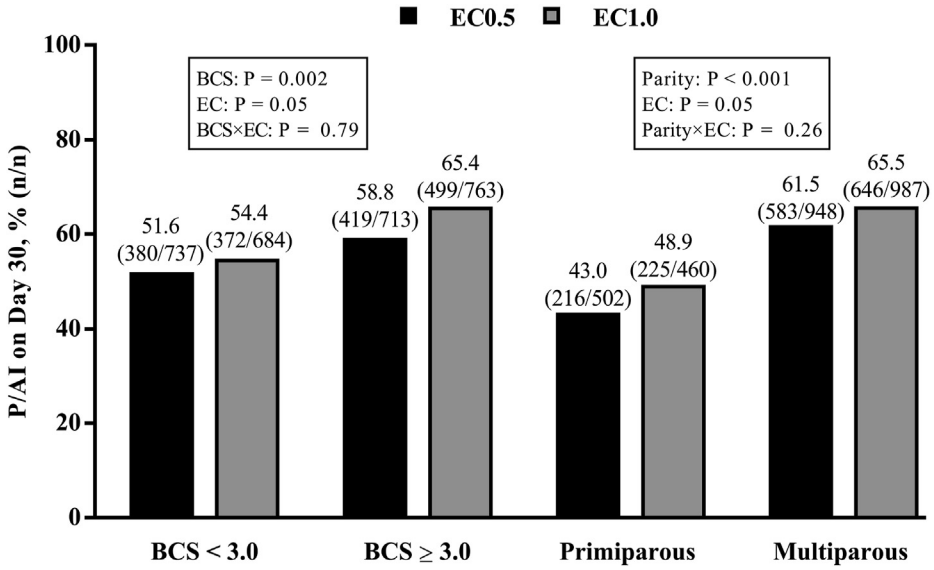

Fig. 6. Pregnancy per artificial insemination (P/AI) 30 d after AI in cows submitted to estradiol/progesterone-based timed AI (TAI) protocols based on body condition score (BCS) on Day −9, parity and dose of estradiol cypionate (EC) at the time of progesterone insert removal (Day −2).

In our study, the diameter of the LF at P4 device removal and at AI influenced expression of estrus, ovulation incidence and P/AI, as observed previously [32,39]. Several negative impacts on reproductive outcomes may occur by reducing the size of the ovulatory follicle, such as lesser circulating E2 during proestrus [40] leading to an inappropriate environment in the uterus and oviduct [41–43], decreased expression of estrus [39], fewer ovulations after TAI [32], development of smaller CL with lesser P4 secretion [44–47], and worse P/AI [47,48]. In fact, Vasconcelos et al. [44] generated smaller ovulatory follicles in lactating dairy cows and observed lower circulating E2 prior to ovulation, decreased CL volume and less circulating P4 concentrations in the following diestrus, as well as decreased P/AI compared with cows with bigger ovulatory follicles. In the present study, multiparous cows with greater BCS had larger follicles on Days −2 and 0 than those of cows with lesser BCS and all primiparous cows, which resulted in greater expression of estrus, ovulation after AI and P/AI. These results are consistent with previous findings, that described greater probability of expression of estrus and P/AI when cows had larger follicles before TAI [32,39,48]. Despite larger follicles before TAI, the probability of ovulation after TAI was not affected by follicle diameter on Days −2 and 0. Lack of difference in ovulatory response may have occurred because cutoffs for follicle diameter that we used during these studies excluded cows with too small of follicles that are less likely to ovulate. In addition, even without effects on probability of ovulation based on follicle diameter, cows that ovulated after TAI had larger follicles on Days −2 and 0, confirming the positive effect of follicular diameter on the risk of ovulation. The impact of parity and BCS classes on P/AI may be explained by differences in cyclicity status at initiation of TAI protocol and the subsequent differences in diameter of ovulatory follicles and expression of estrus.

One of the advantages of using E2/P4-based TAI protocols is that these protocols can induce cyclicity in postpartum anovular cows [1]. Presence of the P4 insert, providing sub-luteal concentrations of P4 (cows without CL), leads to an increased LH pulse frequency, stimulating follicular growth in anestrous animals [49–51], and ovulation by the end of the protocol [35]. In the current study, the percentage of cows with CL was greater at the initiation of the resynchronization protocol than at initiation of the first TAI protocol. Nevertheless, the observation that most of the cows did not have a CL at the initiation of the resynchronization protocol (61.2%), especially primiparous cows (76.4%), indicates that cows remain in an anovular condition even after the luteal phase that follows the induction of ovulation after a TAI protocol. Thus, the use of strategies for resynchronization of ovulation using TAI protocols that induce ovulation can be particularly important during reproductive programs for beef cattle, in order to increase number of breedings that occur compared to using only bulls for rebreeding and thereby increase the percentage of cows pregnant early in the breeding season.

Ovulation at the end of the protocol is obviously a critical determinant of fertility in TAI programs [2]. All protocols in the present study generated large percentages of ovulation after AI (89.7%), consistent with previous studies that reported final ovulation ranging from 80 to 94% [32,39,48]. Ovulation risk was greater in cows treated with GnRH at the time of AI than in controls, thus explaining why GnRH improved fertility in the study. In addition, many of the other factors that improved P/AI also

Table 4
Pregnancy per AI (P/AI) on Day 30 for cows submitted to estradiol/progesterone-based timed artificial insemination (TAI) protocols based on body condition score (BCS) on Day −9, parity, estradiol cypionate (EC) dose on Day −2, expression of estrus, and GnRH treatment at the time of AI (Day 0).

Variable	GnRH treatment[1]		P-value*		
	Without	With	T	V	T × V
BCS on Day -9					
<3.0	48.6[b] (339/698)	57.1[a] (413/723)	0.001	0.002	0.05
≥3.0	61.9 (464/750)	62.5 (454/726)			
Parity					
Primiparous	41.9[b] (208/497)	50.1[a] (233/465)	0.001	<0.001	0.05
Multiparous	62.6 (595/951)	64.4 (634/984)			
EC dose on Day -2					
0.5 mg	51.3[b] (373/727)	58.9[a] (426/723)	0.001	0.05	0.1
1.0 mg	59.6 (430/721)	60.7 (441/726)			
Expression of estrus					
Without	38.1[b] (160/420)	52.7[a] (207/393)	0.001	<0.001	0.003
With	62.4 (607/972)	62.5 (628/1004)			

[1]Treatment was GnRH at the time of AI (with or without).
*T = GnRH treatment effect; V = variable effect; T × V = treatment by variable interaction.

increased percentage of cows ovulating to the protocol such as presence of CL on Day −9, BCS on Day −9, and expression of estrus at the end of the TAI protocol. Another observation was the incidence of double ovulation of 8.8% found in our experiment, similar to the 8.0% reported in previous studies [20] using Nelore cows submitted to E2/P4-based TAI protocols with P4 inserts maintained for 7 d. In addition, the overall pregnancy loss was 4.9%, which is similar to what has been previously reported [31].

Greater concentrations of P4 suppress LH pulse frequency and negatively impact follicular growth, ovulation efficiency, and fertility in beef cattle [12,13,52–55]. Carvalho et al. [13] described lower circulating P4 concentrations in cycling beef heifers receiving PGF on Day −9 of an E2/P4-based TAI protocol, resulting in greater diameter of the dominant follicle and greater ovulation incidence at the end of the protocol (78.8 vs. 54.0%). Therefore, our first hypothesis was based on the idea that the administration of PGF at the initiation of the protocol would induce luteolysis in cyclic cows, resulting in greater follicular diameter and better fertility outcomes. This hypothesis was partially supported since more cows regressed their CL (when present on Day −9) if treated with PGF and there was a tendency for an effect of PGF on Day −9 on expression of estrus, although no effects were detected on P/AI or on follicle diameter. In contrast, considering only cows that had CL at the initiation of the TAI protocol, PGF treatment tended to increase P/AI (69.1 [259/375] vs. 62.5% [198/317]). This result can be partially explained by the high proportion of cows without CL at the initiation of the protocol (>70%). Sá Filho et al. [14] also did not detect differences in follicle diameter, ovulation incidence and P/AI between cycling Nelore heifers receiving or not PGF on the first day of an E2/P4-based TAI protocol. Moreover, Surjus et al. [11] detected no effect on expression of estrus or P/AI in Nelore heifers treated or not with PGF on the first day of an E2/P4-based TAI protocol. However, Dadarwal et al. [12] reported larger follicles at the time of AI and greater P/AI in Hereford-cross cows and pubertal heifers receiving PGF at the initiation of the synchronization protocol.

Expression of estrus before AI is associated with greater P/AI in both primiparous and multiparous beef cows [20,32,56]. Sufficient concentrations of E2 to initiate estrus is related to the likelihood of ovulation, because elevated E2, in the absence of P4, induces a GnRH surge and subsequent LH surge, essential for ovulation. In TAI protocols, different ovulation inducers can be used with similar results for synchronization of ovulation, expression of estrus and P/AI, and some adjustments in terms of type and doses of inducers have been tested [2,7,8,17,57]. Torres-Júnior et al. [17] compared two inducers of ovulation (0.5 or 1.0 mg of EC, administered at the time of P4 implant removal, and EB, administered 24 h later) in Nelore cows submitted to an E2/P4-based TAI protocol with P4 insert maintained for 8 d, and reported no differences in follicle diameter (24 h after P4 insert removal) or percentage of cows ovulating to the protocols. Furthermore, 1.0 mg of EC resulted in shorter interval to ovulation than 0.5 mg (71.1 ± 3.6 vs. 78.0 ± 3.5 h), better synchronization of ovulation (ovulation in a shorter time interval) and greater P/AI [17]. In contrast, as previously mentioned, Bosolasco et al. [18], using an E2/P4-based TAI protocol with P4 insert maintained for 7 d, reported greater P/AI in postpartum multiparous crossbred Hereford and Angus cows when 0.5 mg of EC was administered compared with 1.0 mg of EC. Furthermore, in the same study [18], the authors reported a shorter interval to ovulation for 1.0 compared with 0.5 mg EC (58.7 ± 2.7 vs. 66.7 ± 2.5 h) and an interaction between dose of EC and insemination time on P/AI. When 1.0 mg of EC was administered, P/AI was greater if TAI was performed 46–50 h after P4 implant removal compared with 52–56 h (54.0 [564/1045] vs. 46.7% [467/999]).

In this context, our second hypothesis was that treating cows with 1.0 mg of EC and inseminating 48 h after P4 implant withdrawal would increase expression of estrus and P/AI, reducing the requirement for GnRH at the time of AI. In fact, 1.0 mg EC increased expression of estrus (73.8%) and P/AI on Day 30 (60.2%), with similar results on fertility compared with cows that received GnRH, supporting our hypothesis. However, a higher dose of EC did not alter either the diameter of the LF at the time of AI nor the ovulation frequency, consistent with the results of Torres-Júnior et al. [17]. Thus, in the present study, 1.0 mg of EC may have promoted a more synchronized ovulation (in a shorter period), due to a greater stimulation of the pre-ovulatory LH surge. In addition, the greater pharmacological E2 dose probably produced a more adequate uterine environment after TAI, providing better support for fertilization and early embryo development [16,41,58,59].

Our third hypothesis was supported because simultaneous treatment with GnRH at TAI increased overall P/AI. Besides, since GnRH increased P/AI of cows that did not express estrus by the time of AI, this treatment improved fertility of groups of cows that had lower incidence of expression of estrus, such as primiparous, thinner cows, and cows treated with 0.5 instead of 1.0 mg of EC on Day −2. These results could be justified by an increased ovulation after AI (without GnRH: 87.2 [299/343] vs. with GnRH: 92.1% [325/353]), especially in cows without estrus expression (without GnRH: 71.2 [52/73] vs. with GnRH: 83.6% [56/67]). In addition, although not evaluated in the present study, it is very likely that the GnRH treatment provided a better synchronization of ovulation, preventing a delayed occurrence of a spontaneous LH surge, optimizing the time of TAI in relation to ovulation [60–63]. With this in mind, we performed an analysis using only cows that ovulated after TAI, in which there was no effect of GnRH treatment at TAI or interaction between this factor and expression of estrus (cows without expression of estrus = 55.4 [31/56] vs. 53.9% [28/52]; cows with expression of estrus = 67.4 [180/267] vs. 62.8% [155/247], with and without GnRH, respectively). In addition, possibly due to the decrease in the number of animals in this analysis, the effect of expression of estrus on P/AI was also not detected, despite 11% points greater P/AI in cows expressing estrus. Similarly, two recent studies from our laboratory reported improved fertility outcomes when GnRH treatment was included at the time of AI. Madureira et al. [20], after submitting Nelore cows to TAI protocols with P4 insert maintained for 7 d, reported that treatment with GnRH at TAI improved P/AI of cows that did not express estrus by the time of AI (59.1 vs. 48.2%). Prata et al. [19] compared protocol duration in Nelore cows and treatment or not with GnRH at TAI. Protocol duration did not influence P/AI, but GnRH treatment had a positive effect on fertility. In experiment 1 [19], GnRH tended to increase overall P/AI of cows that did not express estrus, although in experiment 2 this tendency was found only in cows that expressed estrus, contrasting with our findings, since we did not find an effect of GnRH treatment on P/AI in cows that expressed estrus.

In conclusion, larger follicles at the time of P4 insert removal and at TAI was associated with greater expression of estrus, likelihood of ovulation, and P/AI. Treatment with PGF at the initiation of TAI protocols has a potential benefit on fertility outcomes in cows with CL. Administration of 1.0 of EC at P4 implant withdrawal improved expression of estrus and P/AI, reducing the need for GnRH treatment at TAI. Finally, GnRH at the time of AI improved P/AI, especially in cows that did not express estrus, such as primiparous, thinner cows (BCS < 3.0), and cows treated with 0.5 mg of EC.

CRediT authorship contribution statement

Rodrigo L.O.R. Alves: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Project administration. **Mateus A. Silva:** Methodology, Investigation. **Carlos E.C. Consentini:** Conceptualization, Methodology, Formal analysis,

Writing – original draft. **Lucas O. e Silva:** Conceptualization, Methodology, Investigation. **Natália P. Folchini:** Conceptualization, Methodology, Investigation. **Abraham L. Oliva:** Methodology, Investigation. **Alexandre B. Prata:** Visualization, Investigation. **José Renato S. Gonçalves:** Visualization, Investigation. **Milo C. Wiltbank:** Conceptualization, Methodology, Writing – review & editing. **Roberto Sartori:** Funding acquisition, Conceptualization, Methodology, Writing – review & editing, Supervision.

Acknowledgments

The authors thank the staff of Experimental Station "Hildegard Georgina Von Pritzelwiltz", located in Londrina, PR, Brazil for the use of their animals and facilities, and GlobalGen vet science for providing the hormones. The first author was supported by a scholarship from the São Paulo Research Foundation (FAPESP, São Paulo, Brazil; Grant # 2018/14723-8). The coauthors were supported by scholarships from the National Council for Scientific and Technological Development (CNPq, Brasília, Brazil), the Coordination for the Improvement of Higher Education Personnel (CAPES, Brasília, Brazil), or from FAPESP. This study was supported by FAPESP (Grant # 2018/03798-7).

References

[1] Baruselli PS, Reis EL, Marques MO, Nasser LF, Bo GA. The use of hormonal treatments to improve reproductive performance of anestrous beef cattle in tropical climates. Anim Reprod Sci 2004;82–83:479–86. https://doi.org/10.1016/j.anireprosci.2004.04.025.

[2] Bo GA, Baruselli PS. Synchronization of ovulation and fixed-time artificial insemination in beef cattle. Animal 2014;8:144–50. https://doi.org/10.1017/S1751731114000822.

[3] Bo GA, Adams GP, Nasser LF, Pierson RA, Mapletoft RJ. Effect of estradiol valerate on ovarian follicles, emergence of follicular waves and circulating gonadotropins in heifers. Theriogenology 1993;40:225–39.

[4] Bo GA, Adams GP, Pierson RA, Tribulo HE, Caccia M, Mapletoft RJ. Follicular wave dynamics after estradiol-17β treatment of heifers with or withot a progesten implant. Theriogenology 1994:1555–69.

[5] Souza ALB, Segui MS, Kozicki LE, Weiss RR, Abreu A, Bertol MAF, Oliveira DMA. Impact of equine chorionic gonadotropin associated with temporary weaning, estradiol benzoate, or estradiol cypionate on timed artificial insemination in primiparous Bos indicus cows. Braz Arch Biol Technol 2016;59:1–7. https://doi.org/10.1590/1678-4324-2016150389.

[6] Souza AH, Viechnieski S, Lima FA, Silva FF, Araújo R, Bo GA, Wiltbank MC, Baruselli PS. Effects of equine chorionic gonadotropin and type of ovulatory stimulus in a timed-AI protocol on reproductive responses in dairy cows. Theriogenology 2009;72:10–21. https://doi.org/10.1016/j.theriogenology.2008.12.025.

[7] Sales JNS, Carvalho JBP, Crepaldi GA, Cipriano RS, Jacomini JO, Maio JRG, Souza JC, Nogueira GP, Baruselli PS. Effects of two estradiol esters (benzoate and cypionate) on the induction of synchronized ovulations in Bos indicus cows submitted to a timed artificial insemination protocol. Theriogenology 2012;78:510–6. https://doi.org/10.1016/j.theriogenology.2012.02.031.

[8] Bo GA, de la Mata JJ, Baruselli PS, Menchaca A. Alternative programs for synchronizing and resynchronizing ovulation in beef cattle. Theriogenology 2016;86:388–96. https://doi.org/10.1016/j.theriogenology.2016.04.053.

[9] Geary TW, Whittier Pas JC, Thrift FA, Dolezal SL. Effects of a timed insemination following synchronization of ovulation using the ovsynch or co-synch protocol in beef cows. Prof Anim Sci 1998;14:217–20. https://doi.org/10.15232/S1080-7446(15)31832-5.

[10] Madureira G, Motta JCL, Drum JN, Consentini CEC, Prata AB, Monteiro PLJ, Melo LF, Alvarenga AB, Wiltbank MC, Sartori R. Progesterone-based timed AI protocols for Bos indicus cattle I: evaluation of ovarian function. Theriogenology 2020;145:126–37. https://doi.org/10.1016/j.theriogenology.2020.01.030.

[11] Surjus R, Prata AB, Costa Jr O, Lima JCO, Serafim L. Influence of PGF at the first day and/or GnRH at the time of AI during a shorter FTAI protocol in Nelore heifers. Anim Reprod 2018;15:341 (abstract).

[12] Dadarwal D, Mapletoft RJ, Adams GP, Pfeifer LFM, Creelman C, Singh J. Effect of progesterone concentration and duration of proestrus on fertility in beef cattle after fixed-time artificial insemination. Theriogenology 2013;79:859–66. https://doi.org/10.1016/j.theriogenology.2013.01.003.

[13] Carvalho JBP, Carvalho NAT, Reis EL, Nichi M, Souza AH, Baruselli PS. Effect of early luteolysis in progesterone-based timed AI protocols in Bos indicus, Bos indicus × Bos taurus, and Bos taurus heifers. Theriogenology 2008;69:167–75. https://doi.org/10.1016/j.theriogenology.2007.08.035.

[14] Sá Filho MF, Marques MO, Gimenes LU, Torres-Júnior JRS, Batista EOS, Carvalho JPB, Baruselli PS. Prostaglandin treatment at the onset of norgestomet and estradiol-based synchronization protocols did not alter the ovarian follicular dynamics or pregnancy per timed artificial insemination in cyclic Bos indicus heifers. Anim Reprod Sci 2014;144:1–5. https://doi.org/10.1016/j.anireprosci.2013.11.008.

[15] Hill SL, Perry GA, Mercadante VRG, Lamb GC, Jaeger JR, Olson KC, Stevenson JS. Altered progesterone concentrations by hormonal manipulations before a fixed-time artificial insemination CO-Synch + CIDR program in suckled beef cows. Theriogenology 2014;82:104–13. https://doi.org/10.1016/j.theriogenology.2014.03.008.

[16] Sá Filho MF, Gonella-Diaza AM, Sponchiado M, Mendanha MF, Pugliesi G, Ramos RS, Andrade SCS, Gasparin G, Coutinho LL, Goissis MD, Mesquita FS, Baruselli PS, Binelli M. Impact of hormonal modulation at proestrus on ovarian responses and uterine gene expression of suckled anestrous beef cows. J Anim Sci Biotechnol 2017;8:1–14. https://doi.org/10.1186/s40104-017-0211-3.

[17] Torres-Júnior JRS, Penteado L, Sales JNS, Sá Filho MF, Ayres H, Baruselli PS. A comparison of two different esters of estradiol for the induction of ovulation in an estradiol plus progestin-based timed artificial insemination protocol for suckled Bos indicus beef cows. Anim Reprod Sci 2014;151:9–14. https://doi.org/10.1016/j.anireprosci.2014.09.019.

[18] Bosolasco D, Oliveira RN, Cuadro F, Meikle AMA. Effect of estradiol cypionate on time of ovulation, luteal function and pregnancy rate in protocols for FTAI in beef cattle. Proc 30th Annu Meet Brazilian Embryo Technol Soc 2016;(Abstract):432.

[19] Prata AB, Madureira G, Robl AJ, Ribeiro HS, Sagae M, Elias MCV, Pimenta C, Barrios J, Hartmman D, Schneider AA, Sandoval GAF, Wiltbank MC, Sartori R. Progesterone-based timed AI protocols for Bos indicus cattle III: comparison of protocol lengths. Theriogenology 2020;152:29–35. https://doi.org/10.1016/j.theriogenology.2020.04.020.

[20] Madureira G, Consentini CEC, Motta JCL, Drum JN, Prata AB, Monteiro PLJ, Melo LF, Gonçalves JRS, Wiltbank MC, Sartori R. Progesterone-based timed AI protocols for Bos indicus cattle II: reproductive outcomes of either EB or GnRH-type protocol, using or not GnRH at AI. Theriogenology 2020;145:86–93. https://doi.org/10.1016/j.theriogenology.2020.01.033.

[21] Houghton PL, Lemenager RP, Hendrix KS, Moss GE, Stewart TS. Effects of body composition, pre-and postpartum energy intake and stage of production of energy utilization by beef cows. J Anim Sci 1990;68:1447–56.

[22] Chenault JR, Kratzer DD, Rzepkowski RA, Goodwin MC. LH and FSH response of Holstein heifers to fertirelin acetate, gonadorelin and buserelin. Theriogenology 1990;34:81–98. https://doi.org/10.1016/0093-691X(90)90579-I.

[23] Cavalieri J, Coleman C, Rodrigues H, Macmillan KL, Fitzpatrick LA. The effect of timing of administration of oestradiol benzoate on characteristics of oestrus, timing of ovulation and fertility in Bos indicus heifers synchronised with a progesterone releasing intravaginal insert. Aust Vet J 2002;80:217–23. https://doi.org/10.1111/j.1751-0813.2002.tb10817.x.

[24] Prata AB, Drum JN, Melo LF, Araujo ER, Sartori R. Effect of different chorionic gonadotropins on final growth of the dominant follicle in Bos indicus cows. Theriogenology 2018;111:52–5. https://doi.org/10.1016/j.theriogenology.2018.01.011.

[25] Butler WR. Nutritional effects on resumption of ovarian cyclicity and conception rate in postpartum dairy cows. BSAP Occas Publ 2001;26:133–45. https://doi.org/10.1017/s0263967x00033644.

[26] Crowe MA, Diskin MG, Williams EJ. Parturition to resumption of ovarian cyclicity: comparative aspects of beef and dairy cows. Animal 2014;8:40–53. https://doi.org/10.1017/S1751731114000251.

[27] De Castro T, Ibarra D, Rodriguez M, Valdez L, Benquet N, Rubianes E. Resumption of postpartum ovarian cyclicity after different suckling manipulation treatments in primiparous beef cows. Anim Prod Sci 2011;51:111–4. https://doi.org/10.1071/AN10108.

[28] Richards MW, Spitzer JC, Warner MB. Effect of varying levels of postpartum nutrition and body condition at calving on subsequent reproductive performance in beef cattle. J Anim Sci 1986;62:300–6. https://doi.org/10.2527/jas1986.622300x.

[29] Richards MW, Wettemann RP, Schoenemann HM. Nutritional anestrus in beef cows: body weight change, body condition, luteinizing hormone in serum and ovarian activity. J Anim Sci 1989;67:1520–6. https://doi.org/10.2527/jas1989.6761520x.

[30] Tanaka T, Arai M, Ohtani S, Uemura S, Kuroiwa T, Kim S, Kamomae H. Influence of parity on follicular dynamics and resumption of ovarian cycle in postpartum dairy cows. Anim Reprod Sci 2008;108:134–43. https://doi.org/10.1016/j.anireprosci.2007.07.013.

[31] Ayres H, Ferreira RM, Torres-Júnior JRS, Demétrio CGB, Sá Filho MF, Gimenes LU, Penteado L, D'Occhio MJ, Baruselli PS. Inferences of body energy reserves on conception rate of suckled Zebu beef cows subjected to timed artificial insemination followed by natural mating. Theriogenology 2014;82:529–36. https://doi.org/10.1016/j.theriogenology.2014.04.026.

[32] Sá Filho OG, Meneghetti M, Peres RFG, Lamb GC, Vasconcelos JLM. Fixed-time artificial insemination with estradiol and progesterone for Bos indicus cows II: strategies and factors affecting fertility. Theriogenology 2009;72:210–8. https://doi.org/10.1016/j.theriogenology.2009.02.008.

[33] Humblot P, Grimard B, Ribon O, Khireddine B, Dervishi V, Thibier M. Sources of variation of post-partum cyclicity, ovulation and pregnancy rates in primiparous Charolais cows treated with norgestomet implants and PMSG. Theriogenology 1996;46:1085–96.

[34] Meneghetti M, Vasconcelos JLM. Calving date, body condition score, and response to a timed artificial insemination protocol in first-calving beef cows. Arq Bras Med Vet e Zootec 2008;60:786–93. https://doi.org/10.1590/S0102-09352008000400002.
[35] Wiltbank MC, Gümen A, Sartori R. Physiological classification of anovulatory conditions in cattle. Theriogenology 2002;57:21–52.
[36] Wiltbank JN. Research needs in beef cattle reproduction. J Anim Sci 1970;31:755–62.
[37] Dimmick MA, Gimenez T, Spitzer JC. Ovarian endocrine activity and development of ovarian follicles during the postpartum interval in beef cows. Anim Reprod Sci 1991;24:173–83. https://doi.org/10.1016/S0378-4320(05)80001-6.
[38] Guedon L, Saumande J, Desbals B. Relationships between calf birth weight, prepartum concentrations of plasma energy metabolites and resumption of ovulation postpartum in Limousine suckled beef cows. Theriogenology 1999;52:779–89.
[39] Sá Filho MF, Crespilho AM, Santos JEP, Perry GA, Baruselli PS. Ovarian follicle diameter at timed insemination and estrous response influence likelihood of ovulation and pregnancy after estrous synchronization with progesterone or progestin-based protocols in suckled Bos indicus cows. Anim Reprod Sci 2010;120:23–30. https://doi.org/10.1016/j.anireprosci.2010.03.007.
[40] Sá Filho OG, Thatcher WW, Vasconcelos JLM. Effect of progesterone and/or estradiol treatments prior to induction of ovulation on subsequent luteal lifespan in anestrous Nelore cows. Anim Reprod Sci 2009;112:95–106. https://doi.org/10.1016/j.anireprosci.2008.04.006.
[41] Binelli M, Pugliesi G, Hoeck VV, Sponchiado M, Ramos RS, Oliveira ML, França MR, D'Alexandre FL, Mesquita FS, Membrive CMB. The role of proestrus on fertility and postovulatory uterine function in the cow. Anim Reprod 2014;11:246–53.
[42] Geisert RD, Zavy MT, Biggers BG, Garrett JE, Wettemann RP. Characterization of the uterine environment during early conceptus expansion in the bovine. Anim Reprod Sci 1988;16:11–25. https://doi.org/10.1016/0378-4320(88)90003-6.
[43] Li S, Winuthayanon W. Oviduct: roles in fertilization and early embryo development. J Endocrinol 2017;232:R1–26. https://doi.org/10.1530/JOE-16-0302.
[44] Vasconcelos JLM, Sartori R, Oliveira HN, Guenther JG, Wiltbank MC. Reduction in size of the ovulatory follicle reduces subsequent luteal size and pregnancy rate. Theriogenology 2001;56:307–14. https://doi.org/10.1016/S0093-691X(01)00565-9.
[45] Pfeifer LFM, Leal SCB, Schneider A, Schmitt E, Corrêa MN. Effect of the ovulatory follicle diameter and progesterone concentration on the pregnancy rate of fixed-time inseminated lactating beef cows. Rev Bras Zootec 2012;41:1004–8. https://doi.org/10.1590/S1516-35982012000400024.
[46] Echternkamp SE, Cushman RA, Allan MF. Size of ovulatory follicles in cattle expressing multiple ovulations naturally and its influence on corpus luteum development and fertility. J Anim Sci 2009;87:3556–68. https://doi.org/10.2527/jas.2009-2049.
[47] Oosthuizen N, Cooke RF, Schubach KM, Fontes PLP, Brandão AP, Oliveira Filho RV, Colombo EA, Franco GA, Reese S, Polher KG, Lamb GC. Effects of estrous expression and intensity of behavioral estrous symptoms on variables associated with fertility in beef cows treated for fixed-time artificial insemination. Anim Reprod Sci 2020;214. https://doi.org/10.1016/j.anireprosci.2020.106308.
[48] Santos MH, Ferraz Junior MVC, Polizel DM, Barroso JPR, Miszura AA, Martins AS, Bertoloni AV, Oliveira GB, Pires AV. Decreasing from 9 to 7 days the permanence of progesterone inserts make possible their use up to 5 folds in suckled Nellore cows. Theriogenology 2018;111:56–61. https://doi.org/10.1016/j.theriogenology.2018.01.017.
[49] Rhodes FM, Burke CR, Clark BA, Day ML, Macmillan KL. Effect of treatment with progesterone and oestradiol benzoate on ovarian follicular turnover in postpartum anoestrous cows and cows which have resumed oestrous cycles. Anim Reprod Sci 2002;69:139–50. https://doi.org/10.1016/S0378-4320(01)00141-5.
[50] Stock AE, Fortune JE. Ovarian follicular dominance in cattle: relationship between prolonged growth of the ovulatory follicle and endocrine parameters. Endocrinology 1993;132:1108–14. https://doi.org/10.1210/endo.132.3.8440173.
[51] Savio JD, Thatcher WW, Badinga L, de la sota RL, Wolfenson D. Regulation of dominant follicle turnover during the oestrous cycle in cows. Reproduction 1993;97:197–203.
[52] Abreu FM, Geary TW, Silva MAC, Cruppe LH, Mussard ML, Madsen CA, Martins C, Bridges GA, Harstine BR, Day ML. Role of progesterone concentrations during early follicular development in beef cattle: II. Ovulatory follicle growth and pregnancy rates. Anim Reprod Sci 2018;196:69–76. https://doi.org/10.1016/j.anireprosci.2018.06.011.
[53] Martins T, Peres RFG, Rodrigues ADP, Pohler KG, Pereira MHC, Day ML, Vasconcelos JLM. Effect of progesterone concentrations, follicle diameter, timing of artificial insemination, and ovulatory stimulus on pregnancy rate to synchronized artificial insemination in postpubertal Nellore heifers. Theriogenology 2014;81:446–53. https://doi.org/10.1016/j.theriogenology.2013.10.020.
[54] Bergfeld EG, Kojima FN, Wehrman ME, Cupp AS, Peters KE, Mariscal V, Sanchez T, Kittok RJ, Garcia-Winder M, Kinder JE. Frequency of luteinizing hormone pulses and circulating 17β-oestradiol concentration in cows is related to concentration of progesterone in circulation when the progesterone comes from either an endogenous or exogenous source. Anim Reprod Sci 1995;37:257–65. https://doi.org/10.1016/0378-4320(94)01341-I.
[55] Grant JK, Abreu FM, Hojer NL, Fields SD, Perry BL, Perry GA. Influence of inducing luteal regression before a modified controlled internal drug-releasing device treatment on control of follicular development. J Anim Sci 2011;89:3531–41. https://doi.org/10.2527/jas.2011-3852.
[56] Meneghetti M, Sá Filho OG, Peres RFG, Lamb GC, Vasconcelos JLM. Fixed-time artificial insemination with estradiol and progesterone for Bos indicus cows I: basis for development of protocols. Theriogenology 2009;72:179–89. https://doi.org/10.1016/j.theriogenology.2009.02.010.
[57] Colazo MG, Kastelic JP, Mapletoft RJ. Effects of estradiol cypionate (ECP) on ovarian follicular dynamics, synchrony of ovulation, and fertility in CIDR-based, fixed-time AI programs in beef heifers. Theriogenology 2003;60:855–65. https://doi.org/10.1016/S0093-691X(03)00091-8.
[58] Hawk HW. Sperm survival and transport in the female reproductive tract. J Dairy Sci 1983;66:2645–60. https://doi.org/10.3168/jds.S0022-0302(83)82138-9.
[59] Madsen CA, Perry GA, Mogck CL, Daly RF, MacNeil MD, Geary TW. Effects of preovulatory estradiol on embryo survival and pregnancy establishment in beef cows. Anim Reprod Sci 2015;158:96–103. https://doi.org/10.1016/j.anireprosci.2015.05.006.
[60] Pursley JR, Silcox RW, Wiltbank MC. Effect of time of artificial insemination on pregnancy rates, calving rates, pregnancy loss, and gender ratio after synchronization of ovulation in lactating dairy cows. J Dairy Sci 1998;81:2139–44. https://doi.org/10.3168/jds.S0022-0302(98)75790-X.
[61] Day ML. State of the art of GnRH-based timed AI in beef cattle. Anim Reprod 2015;12:473–8.
[62] Motta JCL, Madureira G, Silva LO, Alves RLOR, Silvestri M, Drum JN, Consentini CEC, Prata AB, Pohler KG, Wiltbank MC, Sartori R. Interactions of circulating estradiol and progesterone on changes in endometrial area and pituitary responsiveness to GnRH. Biol Reprod 2020:1–15.
[63] Hill SL, Grieger DM, Olson KC, Jaeger JR, Dahlen CR, Crosswhite MR, et al. Gonadotropin-releasing hormone increased pregnancy risk in suckled beef cows not detected in estrus and subjected to a split-time artificial insemination program. J Anim Sci 2016;94:3722–8. https://doi.org/10.2527/jas.2016-0582.