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

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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.

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1. Introduction

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

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estradiol (E2) and progesterone (P4), termed E2/P4-based TAI protocols [1,2]. These protocols begin with placement of intra-vaginal P4 inserts and treatment with an E2 ester, mainly E2 ben-zoate (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, adminis-tered 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 treat-ment 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 repro-ductive 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 ovula-tions 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 circu-lating 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* ac-cess 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 preg-nancy 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 man-agement (they did not receive experimental treatments) and were entered in the experiment only during the resynchronization pro-tocol; 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 so-dium (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
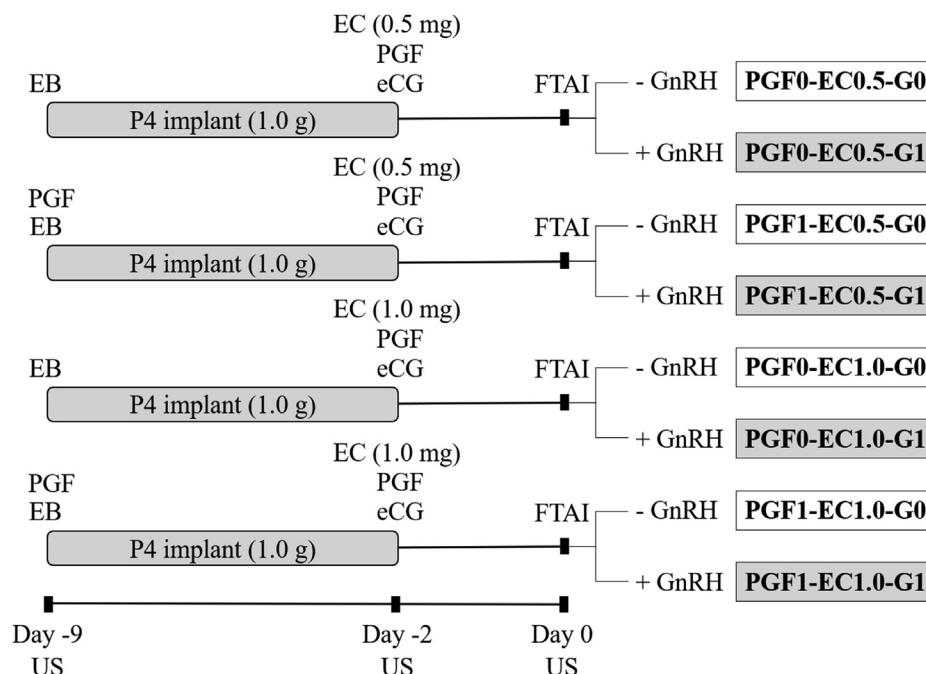
Fig. 1. Schematic diagram of the experimental design following a 2 × 2 × 2 factorial arrangement. The three factors were: 1) cloprostenol sodium (PGF) on Day −9 (with PGF = **PGF1**, or without PGF = **PGF0**); 2) dose of estradiol cypionate (EC) on Day −2 (0.5 EC = **EC0.5**, or 1.0 mg = **EC1.0**); 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 ≤ 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 ≤ 0.05, whereas tendencies were considered when 0.10 ≥ 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 co-efficients provided by the interactive data analysis from SAS and the formula Y = exp (α × X + β)/[1 + exp (α × X + β)], 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/
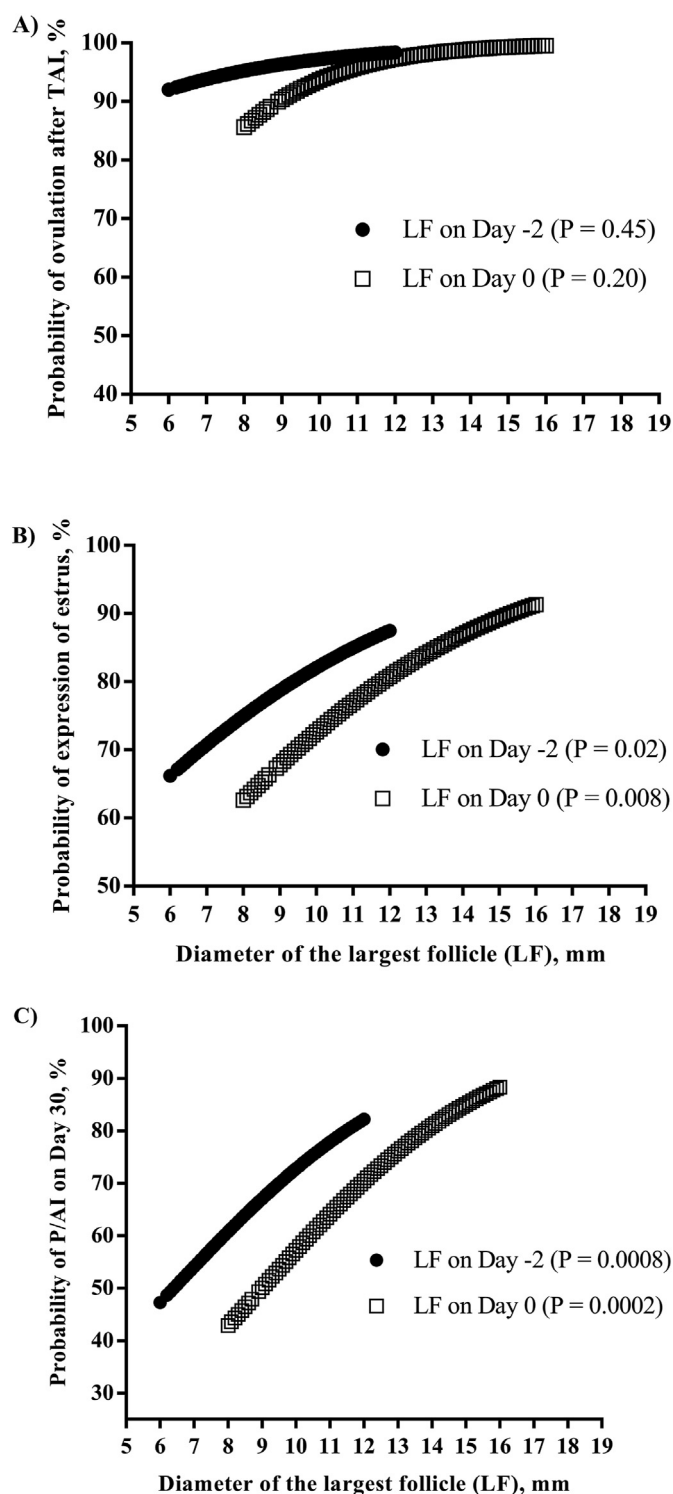

Fig. 2. Probability of ovulation after timed artificial insemination (TAI; Panel A; n = 110), expression of estrus (Panel B; n = 366) and pregnancy per AI (P/AI) on Day 30 (Panel C; n = 366) based on the diameter of the largest follicle (LF) on Day −2 and at the time of AI in cows submitted to estradiol/progesterone-based 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).
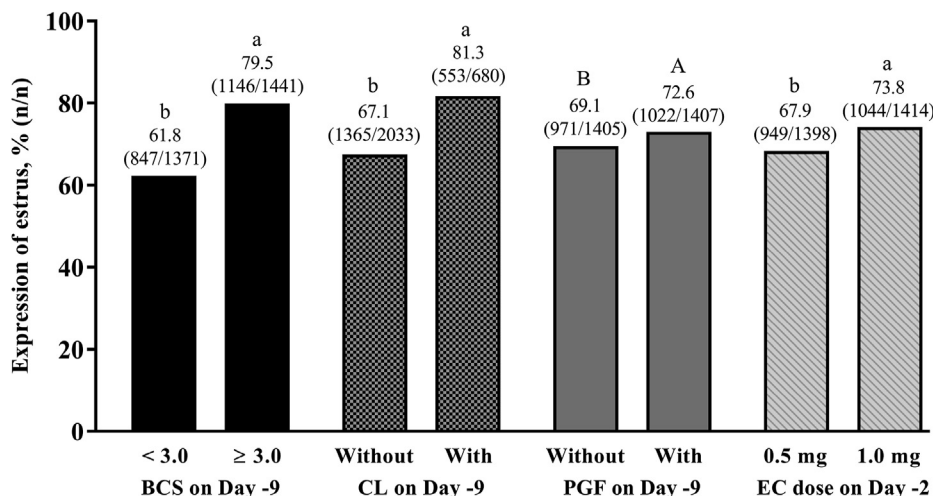
Fig. 3. Expression of estrus of cows submitted to estradiol/progesterone-based TAI protocols based on body condition score (BCS) and presence of corpus luteum (CL) on Day −9, cloprostenol sodium (PGF) treatment on Day −9 and estradiol cypionate (EC) dose at the time of progesterone insert withdrawal (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
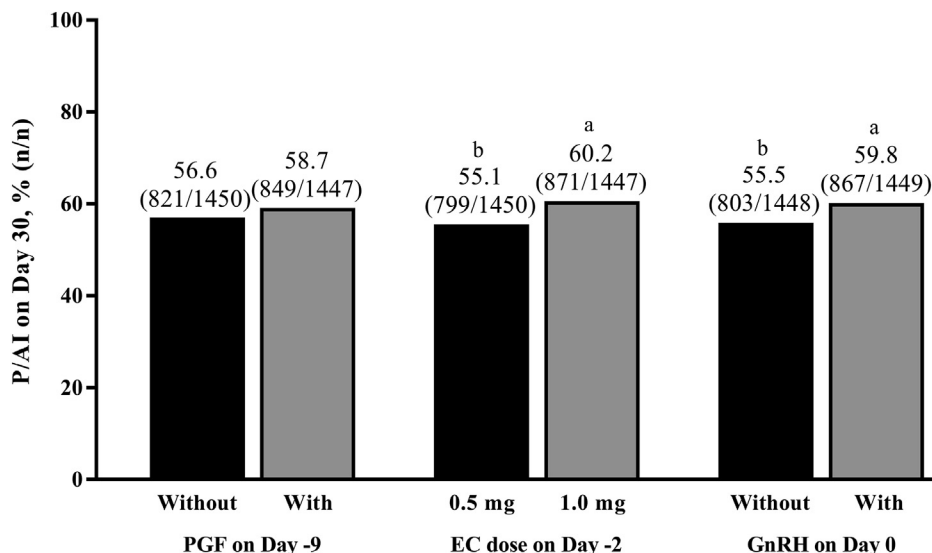
Table 3
Expression of estrus (%) of multiparous and primiparous cows submitted to estradiol/progesterone-based timed artificial insemination (TAI) protocols based on presence of corpus luteum (CL) on Day −9, cloprostenol sodium (PGF) treatment on Day −9, body condition score (BCS) on Day −9 and estradiol 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).
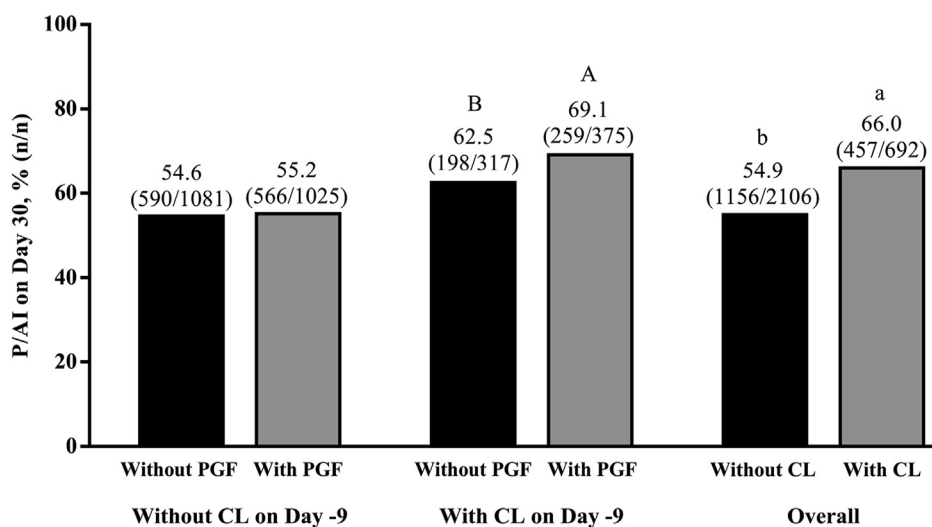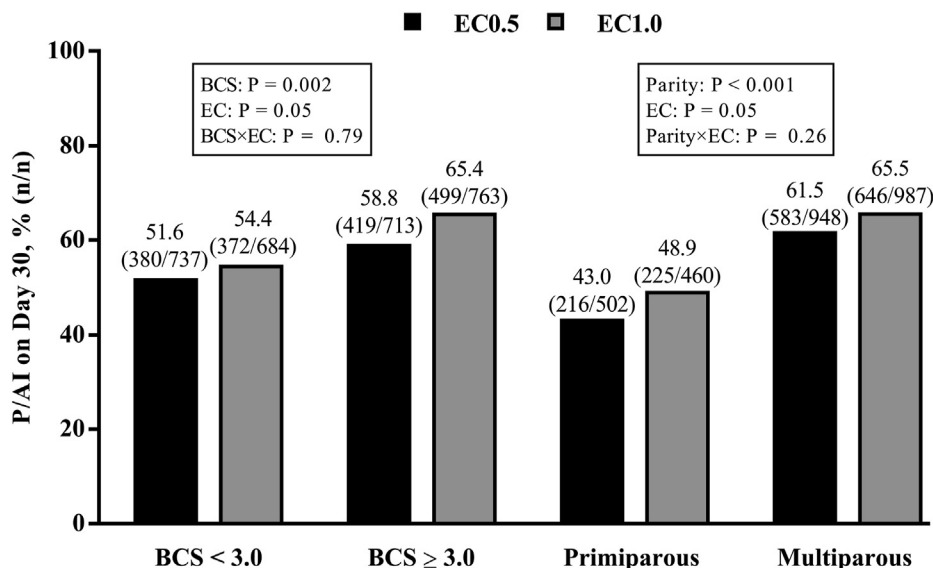

Fig. 5. Pregnancy per artificial insemination (P/AI) 30 d after AI in cows submitted to estradiol/progesterone-based timed AI (TAI) protocols based on presence of corpus luteum (CL) on Day −9 of the protocol, and receiving or not cloprostenol sodium (PGF). [a,b] Effect of presence of CL on Day −9 (P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

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