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Progesterone-based timed AI protocols for *Bos indicus* cattle I: Evaluation of ovarian function

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ABSTRACT

Three experiments evaluated ovarian dynamics and circulating progesterone (P4) during P4-based protocols initiated with GnRH, estradiol benzoate (EB), or no additional treatment in Nelore (*Bos indicus*) cattle. In Exp 1 (n = 59 cows), a 5-d P4-only protocol (P-5d; D0: P4 implant alone (1g); D5: P4 removal, 0.5 mg estradiol cypionate [EC], 0.526 mg cloprostenol [PGF], and 300 IU equine chorionic gonadotropin [eCG]; D7: 8.4 μg buserelin acetate [GnRH]) was compared to a 9d protocol initiated with EB (EB-9d; D0: 2 mg EB + P4; D9: P4 removal + EC + PGF + eCG), and to a 7d GnRH protocol (G-7d; D0: 16.8 μg GnRH + P4; D6: PGF + eCG; D7: P4 removal + PGF; D9: GnRH). Exp 2 (n = 55 cows) compared G-7d and EB-7d protocols (similar to EB-9d, but D9 treatments were done on D7). Exp 3 (n = 64 heifers) compared EB-7d, G-7d, and P-5d protocols. For all experiments, daily ovarian ultrasonography was done from D0 until 4d after implant withdrawal and blood samples were collected at D0 and first PGF. Follicle dynamics were determined for each individual animal, analyzed within individual experiments, and afterwards combined to determine overall effects of treatments. The protocol that began with GnRH, G-7d, had greater ovulation rate after D0 with subsequently greater number of CL and circulating P4 at time of PGF (52.8%, 1.0 ± 0.1 CL, 4.0 ± 0.4 ng/mL) than for EB protocols (12.1%, 0.4 ± 0.05 CL, 2.0 ± 0.2 ng/mL), or P-5d (2.5%, 0.6 ± 0.09 CL, 2.6 ± 0.3 ng/mL). The G-7d and EB protocols had synchronized follicle wave emergence in 92.1% of animals but with distinct patterns. For the G-7d group, wave emergence occurred earlier in ovulating than non-ovulating animals (1.4 ± 0.2 d vs 2.5 ± 0.4 d). By comparison, most animals in EB-7d or EB-9d (80.3%) displayed atresia of the dominant follicle, followed by wave emergence 2–3 d after EB treatment. In contrast, P-5d protocol synchronized wave emergence in only 30.0% of cows. Nevertheless, no differences among treatments were detected for ovulation at end of the protocol (85.7%). In conclusion, the P-5d protocol did not synchronize follicle wave emergence but produced similar final ovulation, whereas, GnRH and EB protocols had follicle dynamics synchronized by distinct mechanisms that produced differences in CL number and P4 at the time of PGF treatment but similar final ovulation. Based on ovarian function, each of these synchronization methods are promising for use in FTAI, although fertility still needs to be evaluated.

1. Introduction

Artificial insemination (AI) programs in beef cattle primarily utilize fixed-time AI (FTAI) protocols that attempt to synchronize the time of ovulation at the end of the protocol [1,2]. Development of these protocols has been based on an understanding of the reproductive physiology that underlies the normal estrous cycle, particularly the regulation of follicular waves, and the hormonal and follicular dynamics that follow specific hormonal treatments [3–5]. Synchronization of ovulation protocols have been based on three key aspects of ovarian physiology: synchronization of follicle wave emergence using treatments at the

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initiation of the protocol; control of circulating progesterone (P4) concentrations provided by the corpus luteum (CL) or from a P4 implant to regulate growth patterns of the preovulatory follicle (OF), uterine environment, and timing of estrus/ovulation; and finally a method to synchronize the time of ovulation in order to allow FTAI at the end of the protocol.

In many countries, mainly when working with *Bos indicus* cattle, emergence of the follicle wave is synchronized by treatment with estradiol (E2) products, predominantly E2 benzoate (EB), concomitant with the insertion of an intravaginal P4 implant to suppress circulating follicle stimulating hormone (FSH) and luteinizing hormone (LH), and thereby induce atresia of growing follicles followed by a subsequent surge in FSH that induces a new follicular wave [6]. Another strategy to synchronize the emergence of a new follicular wave is treatment with gonadotropin-releasing hormone (GnRH) to induce an LH surge and ovulation of the dominant follicle (DF), followed by an FSH surge that initiates the synchronous emergence of a new follicular wave [7]. However, GnRH-based programs are not widely used in *Bos indicus* cattle and previous studies reported unsatisfactory results [8–12]. Furthermore, there are recent suggestions that P4 released from intravaginal implants or from treatment with injectable P4 may also synchronize the emergence of a new follicle wave without the need for additional hormonal treatments [13].

Emergence of a new follicle wave after treatment with EB and P4 is expected to occur ~3 d after treatment [14–18]. In contrast, treatment with GnRH in the presence of a DF larger than ~9 mm in diameter can induce ovulation within ~28 h with earlier emergence of the follicle wave [19,20]. However, the percentage of animals that ovulate to GnRH treatment is highly variable depending on day of the estrous cycle when GnRH is given, dose of GnRH, functional state of the potentially ovulatory follicle, and circulating P4 concentration at the time of GnRH treatment [21–23]. Daily follicular dynamics and fertility in *Bos indicus* cattle have been reported for EB-based FTAI protocols but much less is known about responses of *Bos indicus* cattle to GnRH-based FTAI protocols. Thus, our study performed specific comparisons of EB and GnRH-based FTAI protocols, as well as the responses induced by another synchronization strategy using only P4 at the start.

The protocol length, time/number of prostaglandin F2α (PGF) treatments, and method for inducing final ovulation have also been varied in previous research trials. For example, the length of treatment with the P4 implant has varied from 5 to 9 d with few detailed comparisons to provide the rationale for a given protocol length. In some experiments, particularly in protocols that are initiated with GnRH, the use of two doses of PGF has been performed to assure complete regression of the CL [24]. This strategy may be particularly important in FTAI protocols that have 7 or fewer days between the initial GnRH and PGF treatments, due to the potential for a younger CL that may be more difficult to regress with a single PGF treatment [25]. Finally, two strategies have generally been used to induce ovulation at the end of the protocol, either treatment with E2 esters to induce an endogenous GnRH/LH surge or treatment with GnRH to directly induce an LH surge. Commonly, E2 cypionate (EC) has been used because of the delayed circulating E2 peak after EC treatment, allowing it to be used concomitant with P4 implant removal, making a more convenient protocol with reduced number of animal handlings [26,27]. It has also been suggested that E2 supplementation may produce a more optimal hormonal environment during the proestrous period (i.e., interval between decrease in P4 and beginning of estrus) [28–31]. Additionally, treatment with GnRH at the time of AI has been used as a convenient strategy to assure ovulation in beef cattle FTAI protocols [32,33].

Based on this previous research on FTAI protocols, we realized that there was a lack of complete information on the ovarian dynamics during different types of protocols that are either commonly utilized in *Bos indicus* cattle (9d protocols initiated with EB) or less commonly utilized in *Bos indicus* cattle but also potentially effective FTAI protocols (7d protocols initiated with EB or GnRH). Thus, three experiments were designed that compared protocols initiated with EB (7d or 9d) and GnRH protocols. In addition, a novel protocol was designed that uses only a P4 implant at the start with no other treatment to synchronize follicular waves. We reasoned that this protocol needed to be of short duration to avoid the atresia of the current DF before the end of the protocol. In two of the experiments this novel, short, P4-only protocol was compared to the more commonly used protocols initiated with GnRH or EB. Thus, the objective of the present study was to evaluate ovarian dynamics and circulating P4 profiles of *Bos indicus* heifers and nonlactating cows during P4-based FTAI protocols differing in initial treatments to synchronize follicle wave emergence (EB, GnRH, or P4 implant only), each strategy with an adjusted length and final treatment to induce ovulation. The two main hypotheses of this research were: 1. The developmental profile of the OF, as well as the CL and circulating P4 patterns, would differ based on treatments that were performed on D0; and 2. Despite differences in ovarian function, all protocols that were evaluated would induce a synchronized ovulation of the OF at the end of the protocol in a high proportion of cows and heifers.

2. Material and methods

The experiments were conducted in a commercial beef farm (Morro do Brumado) located in Itatinga, SP, Brazil. Each of the three experiments had two repetitions. The females were kept on pasture (*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 # 2017.5.1618.11.9).

2.1. Experiment 1

Nonlactating multiparous Nelore (*Bos indicus*) cows (n = 59) with an average body condition score (BCS, scale 1–5 points, using 0.25 increments) 3.0 ± 0.1 and average body weight (BW) 395.5 ± 21.8 kg were randomly assigned to one of three treatment groups (Fig. 1A). The EB-9d (n = 20) group received 2.0 mg EB im (Syncrogen; GlobalGen Vet Science, Jaboticabal, Brazil) and an intravaginal implant with 1.0 g of P4 (Repro neo; GlobalGen Vet Science) on D0. Nine d later (D9), 0.526 mg cloprostenol sodium (PGF; Induscio; GlobalGen Vet Science), 300 IU equine chorionic gonadotropin (eCG; eCGen; GlobalGen Vet Science) and 0.5 mg EC (Cipion, GlobalGen Vet Science) were administered im, concomitant with implant removal. The G-7d (n = 20) group was treated on D0 with 16.8 μg buserelin acetate im (GnRH; Maxrelin; GlobalGen Vet Science) and an intravaginal implant containing 1.0 g of P4. Six d later (D6), cows received 0.526 mg PGF and 300 IU eCG im (double administration of PGF, and earlier eCG in G-groups on D6 was designed to increase luteolysis and follicle growth period). After 24 h (D7), a second PGF was administered and the implant was removed. On D9, cows were treated with 8.4 μg GnRH im. The last group, P-5d (n = 19), received only an intravaginal implant containing 1.0 g P4 on D0. Five d later (D5), 0.526 mg PGF, 300 IU eCG and 0.5 mg EC were administered im, at the same time as implant withdrawal. On D7, a final treatment with 8.4 μg GnRH was performed. All P4 implants used in this experiment have not been previously used.

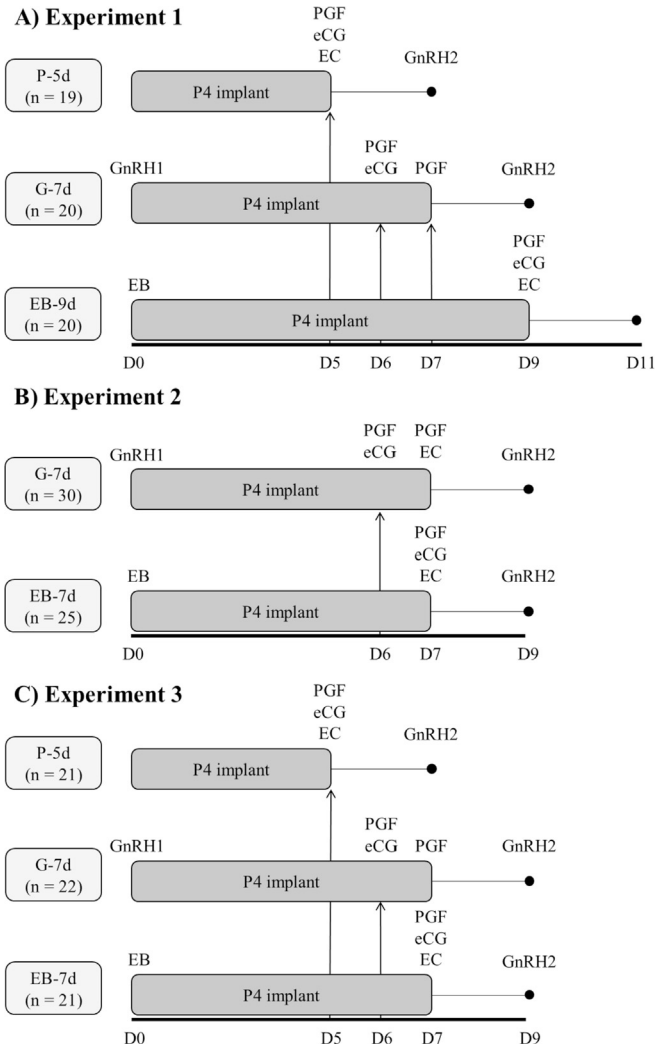

Fig. 1. A) Design of experiment 1 with nonlactating *Bos indicus* (Nelore) cows submitted to three groups: P-5d (n = 19), G-7d (n = 20) or EB-9d (n = 20). Intravaginal implant: 1 g of progesterone (P4); GnRH1: 16.8 μg buserelin acetate; EB: 2 mg estradiol benzoate; PGF: 0.526 mg cloprostenol sodium; eCG: 300 IU; EC: 0.5 mg estradiol cypionate; GnRH2: 8.4 μg buserelin acetate. B) Design of experiment 2 with nonlactating *Bos indicus* (Nelore) cows submitted to two groups: G-7d (n = 30) or EB-7d (n = 25). Intravaginal implant: 1 g of P4; GnRH1: 16.8 μg buserelin acetate; EB: 2 mg estradiol benzoate; PGF: 0.526 mg cloprostenol sodium; eCG: 300 IU; EC: 0.5 mg estradiol cypionate; GnRH2: 8.4 μg buserelin acetate. C) Design of experiment 3 with pubertal *Bos indicus* (Nelore) heifers submitted to three groups: P-5d (n = 21), G-7d (n = 22), or EB-7d (n = 21). Intravaginal implant: 0.5 g of P4; GnRH1: 16.8 μg buserelin acetate; EB: 1.5 mg estradiol benzoate; PGF: 0.526 mg cloprostenol sodium; eCG: 200 IU; EC: 0.5 mg estradiol cypionate; GnRH2: 8.4 μg buserelin acetate. Ultrasound evaluations were performed daily from the start of the protocol (D0) until four d after P4 implant removal and a blood sample was collected on D0 and at the first PGF of each protocol for P4 analysis.

2.2. Experiment 2

Nonlactating multiparous Nelore cows (n = 55) with BCS 3.2 ± 0.1 and BW 418.8 ± 32.9 kg were randomly assigned to one of two groups (Fig. 1B). The EB-7d cows (n = 25) were submitted to the similar protocol as EB-9d from Exp. 1; however, D9 treatments (implant withdrawal; PGF, eCG and EC administration) were carried out on D7. Further, an additional treatment with GnRH (8.4 μg, im) was performed on D9. The G-7d group (n = 30) was similar to G-7d from Exp. 1, but with administration of EC (0.5 mg, im) on D7. All P4 implants used in this experiment have not been previously used.

2.3. Experiment 3

Cyclic nulliparous Nelore heifers (~2 yr old; n = 64) with BCS 3.0 ± 0.1 and BW 336.9 ± 38.9 kg were randomly assigned to one of three groups (Fig. 1C). Heifers from the EB-7d group (n = 21) were treated with 1.5 mg EB im and an intravaginal implant containing 0.5 g P4 (Repro one; GlobalGen Vet Science) on D0. Seven d later (D7), 0.526 mg PGF, 200 IU eCG and 0.5 mg EC were administered im at the same time as implant removal. On D9, heifers were treated with 8.4 μg GnRH im. The G-7d group (n = 22) received 16.8 μg GnRH im and an intravaginal implant with 0.5 g P4. Six d later (D6), heifers received 0.526 mg PGF and 200 IU eCG im. After 24 h (D7), 0.526 mg PGF was administered im and the implant was removed. On D9, heifers were treated with 8.4 μg GnRH im. Finally, the P-5d group (n = 21) received only an intravaginal implant with 0.5 g P4 on D0. Five d later (D5), 0.526 mg PGF, 200 IU eCG and 0.5 mg EC were administered im at the same time as implant withdrawal. On D7, 8.4 μg GnRH was administrated. All the P4 implants used in this experiment have not been previously used.

2.4. Combined data from all three experiments

Data from the three individual experiments were combined in order to provide more consistent information regarding the mechanisms of the synchronization protocols in *Bos indicus* cattle. Thus, data from all treatments were combined to analyze the responses in ovarian dynamics following specific hormonal treatments and the timing of ovulation at the end Fig. 2of each synchronization protocol (P-5d [n = 40]; G-7d [n = 72]; and EB-based [n = 66]). Secondly, females that ovulated to GnRH treatment on D0 of the protocol (Ovulation, n = 38) were compared to those that did not ovulate (No ovulation, n = 34) to investigate patterns of follicular and luteal development and concentrations of circulating P4. In addition, the distribution in emergence of the follicular wave were compared among females that were treated with GnRH at the initiation of the protocol (G-7d) that either ovulated (G-Ovulation, n = 35) or did not ovulate (G-No ovulation, n = 31) after GnRH and also compared to animals that were submitted to the EB-based protocol (EB-based, n = 61).

2.5. Individual ovarian profiles, most frequent and less frequent patterns

Initially, the follicle growth patterns for individual cows (Exp. 1 and 2) and heifers (Exp. 3) were evaluated, excluding any animals that did not ovulate at the end of the protocol. Subsequently, the patterns for all animals were evaluated in order to identify the most frequent patterns of follicle growth during each protocol, as well as to identify the patterns of ovarian dynamics that were less frequent or unusual during each specific protocol in cows or heifers.

2.6. Ultrasound examinations, blood sampling and P4 assay

Transrectal ultrasound ovarian examinations in B-mode with a 7.5 MHz linear transducer (DP-2200 VET, Mindray, Shenzhen, China) were performed daily (24 h interval), from the beginning of the protocol (D0) until 4 d after P4 implant removal. All follicles and CL that had a diameter ≥4 mm and ≥10 mm, respectively, were measured and recorded. Thus, ovulation rate after D0 was determined by the disappearance of the DF and the development of a new CL. Regression of the CL was deemed when there was a CL of ≥14.0 mm diameter and/or circulating P4 concentration ≥1.0 ng/mL at the start of the protocol, but at PGF the same CL was ≤14.0 mm diameter. At the end of the protocol, ovulation rate was determined by disappearance of the OF. The day of follicle wave

emergence was defined by a retrospective evaluation of the OF to the time when it was ~4 mm. Turn-over of the follicular wave was presumed in cases in which a DF emerged between D0 and D5 of the protocol, then this follicle stopped growing or decreased in size (atretic), followed by emergence of a new follicular wave.

Blood samples were collected on D0 and at the first PGF treatment of the synchronization protocols (D5, D6, D7, or D9 depending of the experimental group) by puncture of the jugular vein into evacuated tubes containing heparin sodium (Vacutainer, Dickinson, Franklin Lakes, NJ). Immediately after collection, the tubes were placed on ice and kept refrigerated until processing. Blood samples were centrifuged right after the end of collections at 1700×*g* for 15 min and aliquots of plasma were frozen and stored in duplicates at −20 °C until assayed for P4.

Concentrations of P4 were determined using a solid-phase RIA kit containing antibody-coated tubes and 125I-labeled P4 (Immu-Chem Coated Tube P4 125 RIA Kit, MP Biomedicals, Costa Mesa, CA) validated for bovine plasma in our laboratory as reported [34]. The intra- and inter-assay CVs and the sensitivity were 5.3%, 8.6%, and 0.08 ng/mL, respectively.

2.7. Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC) and all experiments were done in a completely randomized design.

The discrete variables were analyzed by logistic regression using the generalized linear mixed model (GLIMMIX) procedure of SAS, and fit to a binary (percentage of animals with P4 ≥ 1.0 ng/mL on D0, ovulation rate after D0, percentage of the follicle wave emergence between D0 and D5, CL regression between D0 and PGF, and ovulation rate and time of ovulation after the end of the protocol) or exponential distribution (number of CL on D0 and at PGF, and day of follicle wave emergence). Additionally, the option ddfm = kenwardroger was included in the model statement to adjust the degrees of freedom for variances. Continuous variable responses were analyzed using the linear mixed model (MIXED) procedure. All variable responses (plasma P4 concentration on D0 and at PGF, and growth rate and maximum diameter of the DF) were tested for normality of the residuals according to the Shapiro-Wilk test obtained by PROC UNIVARIATE procedure of SAS. When non-normality was detected (this occurred for circulating P4 concentration) data were log transformed. If normality of residuals was still not achieved, nonparametric analysis for ranked transformed data was performed with the RANK procedure of SAS.

The selection of the model that best fit each variable response of interest was performed by finding the model with the lowest value for the *Akaike Information Criterion Corrected (AICC)* using the backward elimination procedure that removed independent variables with P > 0.10 from the model. For all discrete and continuous variables, treatment was considered as a fixed effect and was forced into the final model for all analyses. The tested covariates were repetition of the experiment, BCS and presence of CL on D0. Specifically, during the analyses of combined data (Tables 4 and 5), Experiment and Parity were included in the model to test the effect of these variables, and these effects were not detected. In addition, during one of the analysis of combined data, the variable Ovulation to GnRH on D0 was considered as a fixed effect (Table 5).

Tukey honest significant difference post hoc test for multiple comparisons was performed when the independent variables had more than two levels. Differences were considered significant for P ≤ 0.05, whereas a tendency was designated when P ≤ 0.10 and P > 0.05. The results are expressed as least square means ± standard error of the mean (LSM ± SEM), unless otherwise indicated.

3. Results

3.1. Experiment 1

Results from Exp. 1 are described in Table 1. On D0, all groups had similar number of CL, circulating P4 concentration, and percentage of cows with P4 concentration ≥1.0 ng/mL. Cows from G-7d group had greater ovulation rate after D0 than EB-9d and P-5d (50.0 vs. 5.0 vs. 5.3%), and consequently greater number of CL at PGF compared to EB-9d, but both were not different from P-5d. The percentage of cows that had follicle wave emergence between D0 and D5 was lower for P-5d (26.3%) compared to G-7d and EB-9d groups (90.0%, 18/20 for both groups). The G-7d group tended (P = 0.10) to have earlier follicle wave emergence than EB-9d. Growth rate (mm/d) of the OF was less (P < 0.0001) for P-5d (0.8 ± 0.07) than for G-7d (1.3 ± 0.07) and EB-9d (1.2 ± 0.07). However, the maximum diameter of the OF was similar among groups (P = 0.2). Moreover, ovulation rate at the end of the protocol was similar (P = 0.6) among the three protocols (Table 1). There were also no detectable differences among protocols for the time of ovulation with similar proportion of cows ovulating during the

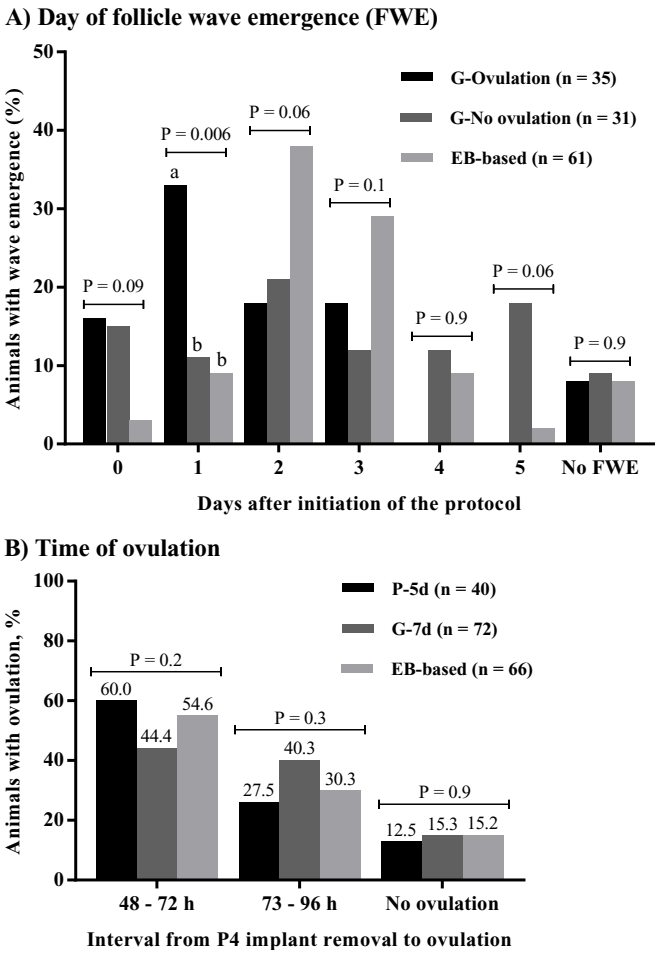

Fig. 2. A) Proportion of Nelore (*Bos indicus*) cows and heifers submitted to a progesterone (P4)-based protocol for synchronization of the ovulation starting with buserelin acetate (GnRH; ovulating or not) or estradiol benzoate (EB) that had follicle wave emergence (FWE) between D0 and D5 of the protocol, or did not have a FWE. B) Proportion of Nelore (*Bos indicus*) cows and heifers submitted to a P4-based protocol with 5-d of P4 implant, or a GnRH-based protocol with 7-d of P4 implant, or an EB-based protocol with 7 or 9-d of P4 implant with ovulation at the end of the protocol between 48 and 72, 73 and 96 h, or no ovulation after P4 implant removal. Both figures (A and B) were based on an analysis of the combined data of all experiments.

Table 1
Ovarian dynamics and circulating progesterone (P4) concentration of nonlactating Nelore (*Bos indicus*) cows from experiment 1 (P-5d: P4-based protocol with 5-d of P4 implant; G-7d: GnRH-based protocol with 7-d of P4 implant and without EC at implant removal; EB-9d: EB-based protocol with 9-d of P4 implant).

	P-5d	G-7d	EB-9d	P-value
CL number on D0 (n)	0.7 ± 0.2 (19)	0.7 ± 0.2 (20)	0.7 ± 0.2 (20)	0.9
P4 on D0, ng/mL (n)	3.1 ± 0.9 (19)	2.3 ± 0.7 (19)	1.3 ± 0.5 (16)	0.3
P4 ≥ 1.0 ng/mL on D0, % (n/n)	42.1 (8/19)	42.1 (8/19)	25.0 (4/16)	0.5
Ovulation after D0, % (n/n)	5.3^{b} (1/19)	50.0^{a} (10/20)	5.0^{b} (1/20)	0.005
CL number at PGF (n)	0.7 ± 0.2^{ab} (19)	1.0 ± 0.2^{a} (20)	0.5 ± 0.1^{b} (20)	0.05
P4 at PGF, ng/mL (n)	3.2 ± 0.5^{B} (19)	3.3 ± 0.7^{AB} (19)	2.6 ± 0.6^{A} (17)	0.06
FWE from D0 to D5, % (n/n)	26.3^{b} (5/19)	90.0^{a} (18/20)	90.0^{a} (18/20)	<0.001
Day of FWE (n)[a]	1.6 ± 0.7^{AB} (5)	1.4 ± 0.3^{A} (18)	2.7 ± 0.6^{B} (18)	0.1
Growth rate of the OF, mm/d (n)[b]	0.8 ± 0.07^{b} (18)	1.3 ± 0.07^{a} (18)	1.2 ± 0.07^{a} (17)	<0.001
Maximum diameter of the OF, mm (n)[b]	13.0 ± 0.4 (18)	14.0 ± 0.4 (18)	13.4 ± 0.4 (17)	0.2
Ovulation rate at end of the protocol, % (n/n)	94.7 (18/19)	90.0 (18/20)	85.0 (17/20)	0.6

[a,b]Values in the same row with different superscripts differ (P ≤ 0.05).
[A,B]Values in the same row with different superscripts differ (P > 0.05 and ≤0.1).
Abbreviations: CL, corpus luteum; P4, progesterone; PGF, prostaglandin F2α; FWE, follicle wave emergence; OF, ovulatory follicle.
[a] Analysis of cows with follicle wave emergence between D0 and D5.
[b] Analysis of cows that ovulated at the end of the protocol.

intervals of 48–72 h (54.2% [32/59]) and 73–96 h (33.9% [20/59]) or not ovulating (11.9% [7/59]). Considering only cows that had emergence of a new follicle wave, one from G-7d (5.6% [1/18]), and three from EB-9d group (16.7% [3/18]) had turn-over of the DF during the protocol.

3.2. Experiment 2

Results related to Exp. 2 are described in Table 2. As expected, both groups had similar number of CL, circulating P4 concentrations, and percentage of cows with P4 concentration ≥1.0 ng/mL on D0. The G-7d was greater than the EB-7d group for ovulation rate after D0 (P = 0.02), number of CL at PGF (P = 0.0007) and circulating P4 at PGF (P < 0.0001). In the G-7d group (data not shown), cows that ovulated after D0 (n = 15) compared to cows that did not ovulate (n = 14) had lower (P = 0.009) circulating P4 (2.3 ± 0.8 vs. 5.3 ± 0.9 ng/mL) and a lower (P = 0.05) percentage of cows with P4 ≥ 1.0 ng/mL on D0 (46.7 [7/15] vs. 85.7% [12/14]). The percentage of cows with emergence of a new follicular wave was similar (P = 0.7) for the two protocols (94.5% [52/55]), as well as the day of follicle wave emergence (P = 0.8), growth rate of the OF (P = 0.6), and maximum diameter of the OF (P = 0.4). As expected, cows from G-7d group that ovulated after D0 had earlier (P = 0.03) emergence of the follicle wave compared to cows without ovulation after D0 (1.2 ± 0.3 [n = 14] vs. 2.9 ± 0.8 [n = 14]) and larger (P = 0.02) maximum diameter of the OF (13.7 ± 0.3 [n = 10] vs. 12.3 ± 0.4 [n = 13]). Ovulation rate at the end of the protocol for G-7d and EB-7d was similar (87.3% [48/55]; P = 0.4). Additionally, no differences between protocols were detected for ovulation between 48 and 72 h (43.6% [24/55]), 73 and 96 h (43.6% [24/55]), or percentage of cows that did not ovulate after the end of the protocols (12.7% [7/55]). Among cows with follicle wave emergence, three from G-7d (10.7% [3/28]) and none from EB-7d (0% [0/24]) had turn-over of the DF.

3.3. Experiment 3

Results from Exp. 3 are described in Table 3. On D0, all groups had similar number of CL, circulating P4, and percentage of cows with P4 ≥ 1.0 ng/mL. Ovulation rate after D0 was greater for heifers in G-7d than EB-7d and P-5d groups (P = 0.01). Number of CL at PGF was greater for G-7d compared to EB-7d, but both were not different from P-5d heifers. Circulating P4 concentration at PGF was greater (P < 0.0001) for G-7d compared to the other groups. Heifers with follicle wave emergence between D0 and D5 was lower for P-5d (33.3% [7/21]) compared to G-7d and EB-7d groups (90.7% [39/43]). The day of follicle wave emergence and the growth rate of the OF were similar among protocols, although the maximum diameter of the OF tended (P = 0.07) to be greater for the P-5d than the EB-7d protocol. Ovulation rate at the end of the protocol was similar (P = 0.9) for G-7d, EB-7d, and P-5d (79.7% [51/64]). There was a

Table 2
Ovarian dynamics and circulating progesterone (P4) concentration of nonlactating Nelore (*Bos indicus*) cows from experiment 2 (G-7d: GnRH-based protocol with 7-d of P4 implant and with EC at implant removal; EB-7d: EB-based protocol with 7-d of P4 implant).

	G-7d	EB-7d	P-value
CL number on D0 (n)	0.9 ± 0.2 (30)	1.0 ± 0.2 (25)	0.8
P4 on D0, ng/mL (n)	3.8 ± 0.7 (29)	3.0 ± 0.7 (20)	0.4
P4 ≥ 1.0 ng/mL on D0, % (n/n)	70.6 (19/29)	55.9 (11/20)	0.4
Ovulation after D0, % (n/n)	50.0 (15/30)	20.0 (5/25)	0.02
CL number at PGF (n)	1.1 ± 0.2 (30)	0.4 ± 0.08 (25)	<0.001
P4 at PGF, ng/mL (n)	4.6 ± 0.5 (29)	1.9 ± 0.2 (24)	<0.001
FWE between D0 and D5, % (n/n)	93.3 (28/30)	96.0 (24/25)	0.7
Day of FWE (n)[a]	2.0 ± 0.4 (28)	2.2 ± 0.5 (24)	0.8
Growth rate of the OF, mm/d (n)[b]	1.2 ± 0.04 (23)	1.2 ± 0.05 (23)	0.6
Maximum diameter of the OF, mm (n)[b]	12.9 ± 0.3 (23)	12.5 ± 0.3 (23)	0.4
Ovulation rate at end of the protocol, % (n/n)	83.3 (25/30)	92.0 (23/25)	0.4

Abbreviations: CL, corpus luteum; P4, progesterone; PGF, prostaglandin F2α; FWE, follicle wave emergence; OF, ovulatory follicle.
[a] Analysis of cows with follicle wave emergence between D0 and D5.
[b] Analysis of cows that ovulated at the end of the protocol.

Table 3
Ovarian dynamics and circulating progesterone (P4) concentration of Nelore (*Bos indicus*) heifers from experiment 3 (P-5d: only P4-based protocol with 5-d of P4 implant; G-7d: GnRH-based protocol with 7-d of P4 implant and without EC at implant removal; EB-7d: EB-based protocol with 7-d of P4 implant).

	P-5d	G-7d	EB-7d	P-value
CL number on D0 (n)	0.6 ± 0.1 (21)	0.6 ± 0.1 (22)	0.5 ± 0.1 (21)	0.8
P4 on D0, ng/mL (n)	2.1 ± 0.5 (21)	2.4 ± 0.5 (22)	2.9 ± 0.6 (21)	0.8
P4 ≥ 1.0 ng/mL on D0, % (n/n)	57.1 (12/21)	59.1 (13/22)	57.1 (12/21)	0.9
Ovulation after D0, % (n/n)	0.0[b] (0/21)	59.1[a] (13/22)	9.5[b] (2/21)	0.01
CL number at PGF (n)	0.48 ± 0.1[ab] (21)	0.82 ± 0.2[a] (22)	0.25 ± 0.1[b] (21)	<0.001
P4 at PGF, ng/mL (n)	1.9 ± 0.3[b] (21)	3.8 ± 0.7[a] (22)	1.6 ± 0.4[b] (21)	<0.001
FWE from D0 to D5, % (n/n)	33.3[b] (7/21)	90.9[a] (20/22)	90.5[a] (19/21)	<0.001
Day of FWE (n)[a]	2.1 ± 0.4 (7)	2.3 ± 0.2 (20)	2.2 ± 0.2 (19)	1.0
Growth rate of the OF, mm/d (n)[b]	1.0 ± 0.1 (16)	1.1 ± 0.1 (18)	1.1 ± 0.1 (16)	0.4
Maximum diameter of the OF, mm (n)[b]	13.4 ± 0.4[A] (16)	12.8 ± 0.3[AB] (18)	12.2 ± 0.4[B] (16)	0.07
Ovulation rate at end of the protocol, % (n/n)	81.0 (17/21)	81.8 (18/22)	76.2 (16/21)	0.9

[a,b]Values in the same row with different superscripts differ (P ≤ 0.05).
[A,B]Values in the same row with different superscripts differ (P > 0.05 and ≤0.1).
Abbreviations: CL, corpus luteum; P4, progesterone; PGF, prostaglandin F2α; FWE, follicle wave emergence; OF, ovulatory follicle.
[a] Analysis of heifers with follicle wave emergence between D0 and D5.
[b] Analysis of heifers that ovulated at the end of the protocol.

Table 4
Ovarian dynamics and circulating progesterone (P4) concentration of Nelore (*Bos indicus*) heifers and cows submitted to three treatments (P-5d: only P4-based protocol with 5-d of P4 implant; G-7d: GnRH-based protocol with 7-d of P4 implant; EB: EB-based protocol with 7 or 9-d of P4 implant) from all experiments (combined data).

	P-5d	G-7d	EB	P-value
Ovulation rate after D0, % (n/n)	2.5[b] (1/40)	52.8[a] (38/72)	12.1[b] (8/66)	<0.001
Regression of the CL between D0 and PGF, % (n/n)	22.2[b] (4/18)	26.3[b] (10/38)	58.8[a] (20/34)	0.01
CL number at PGF (n)	0.6 ± 0.09[b] (40)	1.0 ± 0.1[a] (72)	0.4 ± 0.05[b] (66)	<0.001
P4 at PGF, ng/mL (n)	2.6 ± 0.3[b] (40)	4.0 ± 0.4[a] (70)	2.0 ± 0.2[b] (62)	<0.001
FWE from D0 to D5, % (n/n)	30.0[b] (12/40)	91.7[a] (66/72)	92.4[a] (61/66)	<0.001
Day of FWE (n)[a]	1.9 ± 0.6 (12)	1.9 ± 0.2 (66)	2.4 ± 0.3 (61)	0.5
Growth rate of the OF, mm/d (n)[2]	0.9 ± 0.05[b] (35)	1.2 ± 0.03[a] (61)	1.2 ± 0.04[a] (56)	<0.001
Maximum diameter of the OF, mm (n)[2]	13.0 ± 0.3 (35)	13.1 ± 0.2 (61)	12.7 ± 0.2 (56)	0.4
Ovulation rate at end of the protocol, % (n/n)	87.5 (35/40)	84.7 (61/72)	84.8 (56/66)	0.9

[a,b]Values in the same row with different superscripts differ (P ≤ 0.05).
Abbreviations: CL, corpus luteum; P4, progesterone; PGF, prostaglandin F2α; FWE, follicle wave emergence; OF, ovulatory follicle.
[a] Analysis of animals that ovulated at the end of the protocol.

Table 5
Ovarian dynamics and circulating progesterone (P4) concentration of Nelore (*Bos indicus*) heifers and cows submitted to GnRH treatments (16.8 μg buserelin acetate on Day 0 of the synchronization protocol and insertion of an intravaginal P4 implant) from all experiments (combined data) based on whether they ovulated or not to the GnRH treatment at the beginning of the protocol.

	Ovulation	No ovulation	P-value
CL number on D0 (n)	0.7 ± 0.1 (38)	0.8 ± 0.1 (34)	0.8
P4 on D0, ng/mL (n)	2.4 ± 0.5 (38)	3.6 ± 0.6 (32)	0.09
P4 ≥ 1.0 ng/mL on D0, % (n/n)	52.6 (20/38)	62.5 (20/32)	0.4
CL number at PGF (n)	1.3 ± 0.2 (38)	0.7 ± 0.1 (34)	0.01
P4 at PGF, ng/mL (n)	4.1 ± 0.5 (38)	3.8 ± 0.5 (32)	0.5
FWE from D0 to D5, % (n/n)	92.1 (35/38)	91.2 (31/34)	0.9
Day of FWE (n)[a]	1.4 ± 0.2 (35)	2.5 ± 0.4 (31)	0.03
Growth rate of the OF, mm/d (n)[b]	1.2 ± 0.04 (31)	1.2 ± 0.04 (28)	0.6
Maximum diameter of the OF, mm (n)[b]	13.5 ± 0.3 (31)	12.8 ± 0.3 (28)	0.08
Ovulation rate at end of the protocol, % (n/n)	86.8 (33/38)	82.4 (28/34)	0.6

Abbreviations: CL, corpus luteum; P4, progesterone; PGF, prostaglandin F2α; FWE, follicle wave emergence; OF, ovulatory follicle.
[a] Analysis of animals with follicle wave emergence between D0 and D5.
[b] Analysis of animals that ovulated at the end of the protocol.

tendency (P = 0.10) for greater percentage of heifers ovulating during the 48–72 h interval and fewer percentage ovulating during the 73–96 h interval for P-5d (75.3 and 7.3%, respectively) compared to G-7d group (41.4 and 35.7%, respectively), with EB-7d having an intermediate percentage of ovulations during 48–72 and 73–96 h intervals (53.1 and 20.2%). Additionally, percentage that did not ovulate to the protocols was similar among treatments (20.3% [13/64]). Considering heifers that had follicle wave emergence, one from EB-7d (5.3% [1/19]) and none from the other groups (G-7d: 0% [0/20]; P-5d: 0% [0/7]) had turn-over of the DF during the protocol.

3.4. Combined data from all three experiments

A comparison was done between the EB-9d and EB-7d protocols (data not shown). The only detectable difference was a larger maximum diameter of the OF for the longer protocol (EB-9d, 13.4 ± 0.4 vs. EB-7d, 12.4 ± 0.3 mm; P = 0.02). There were no detectable differences (P > 0.1) in ovulation rate, CL number at PGF, P4 at PGF, percentage of cows with follicular wave emergence (D0 to D5), day of wave emergence, or ovulation rate at the end of the protocol, and therefore results for EB-9d and EB-7d were combined into a single EB group for comparisons to the P-5d and the G-7d

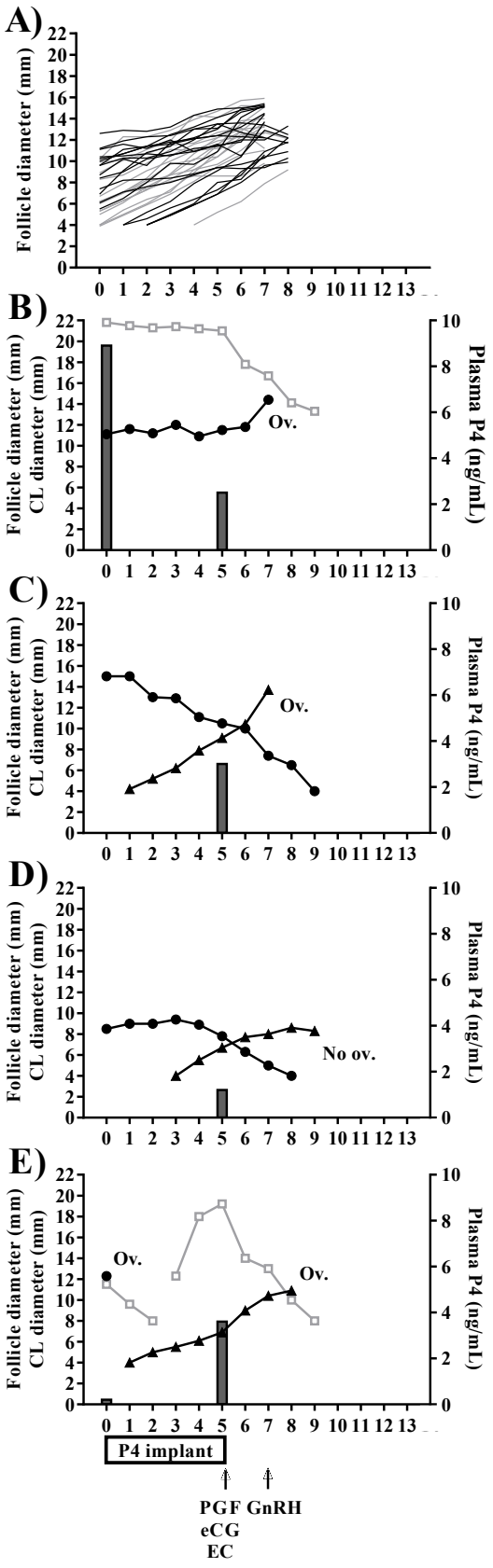

Fig. 3. A: Individual patterns for P-5d (only females that ovulated at the end of a P4-based protocol with 5-d of P4 implant). Gray lines represent pubertal heifers and black lines represent nonlactating cows. B: Representative cow with most frequent pattern observed for P-5d protocol (70.0%; 28/40). C, D and E: Representative cows with unusual patterns observed for P-5d. Corpus luteum (gray lines with open squares), follicular development (first follicle [black line with solid circle], second follicle [black line with solid triangle] and third follicle [black line with solid square]) and P4 concentration (gray column at D0 and at PGF administration) are illustrated.

protocols (Table 4). There was a greater ($P < 0.0001$) ovulation rate after D0 treatment for the GnRH protocol than for the EB or P-5d protocols resulting in a greater number of CL ($P < 0.0001$) and circulating P4 ($P < 0.0001$) for G-7d compared to P-5d or EB. In addition, EB protocol induced more regression of the CL between D0 and the first PGF ($P = 0.01$) compared to G-7d and P-5d. Day of wave emergence was similar among protocols. Growth rate of the OF was slower ($P < 0.0001$) for P-5d than for GnRH or EB protocols, but there was a similar percentage of cows that ovulated at the end of the protocol.

Additionally, a comparison was made for the GnRH-treated groups during the three experiments to determine differences in animals that ovulated ($n = 38$) compared to animals that did not ovulate ($n = 34$) to the GnRH treatment on D0 (Table 5). There was a tendency ($P = 0.09$) for greater circulating P4 on D0 in cows that did not ovulate although there was no difference in CL number on D0 ($P = 0.8$) or percentage of cows with P4 $\geq$ 1.0 ng/mL ($P = 0.4$). As expected, there was a greater ($P = 0.01$) number of CL at time of PGF for cows that ovulated compared to cows that did not ovulate (1.3 ± 0.2 vs. 0.7 ± 0.1 CL/cow), although, surprisingly there was no difference in P4 at PGF ($P = 0.5$). The percentage of animals with follicle wave emergence between D0 and D5 was very high (91.7% [66/72]) and similar ($P = 0.9$) between groups. However, the animals that ovulated compared to those that did not ovulate had earlier ($P = 0.03$) emergence of the follicular wave (1.4 ± 0.2 vs. 2.5 ± 0.4 d) and tended ($P = 0.08$) to have a larger OF (13.5 ± 0.3 vs. 12.8 ± 0.3 mm).

The timing of follicular wave emergence was compared between the EB and GnRH protocols with animals from the GnRH protocol divided into animals that ovulated or did not ovulate to the initial GnRH (Fig. 2A). The animals that ovulated to GnRH had earlier follicular wave emergence, as evidenced by greater ($P = 0.006$) percentage of animals with emergence on day 1 than the other groups and all animals having emergence on days 0–3 and none on days 4–5 after treatment. In contrast, EB-treated animals had synchronized wave emergence primarily on days 2 and 3 with lower percentage of animals with wave emergence on day 0 ($P = 0.09$), 1 ($P = 0.006$), and 5 ($P = 0.06$). The distribution of follicular wave emergence was similar (~15%/d) for all days after treatment in animals treated with GnRH that did not ovulate to the protocol (Fig. 2A). There was a similar percentage of animals with no wave emergence (8.0%; [11/138]) for the three groups.

The timing of ovulation at the end of the protocol was compared for the three types of protocols (P-5d, G-7d, and EB-based) in Fig. 2B. There were no detectable differences ($P > 0.1$) between groups in the percentage of animals that ovulated during the two intervals (48–72 or 73–96 h) or that did not ovulate to the protocols (13.5% [24/178]). Nevertheless, analyzing the timing of ovulation within a protocol for animals that ovulated to the protocol indicated a greater ovulation during the 48–72 h interval than the 73–96 h interval for cows treated with the P-5d ($P = 0.003$) and EB-based ($P = 0.01$) protocols but no difference ($P = 0.9$) for the GnRH group. Although not shown, comparison of GnRH-treated animals that ovulated or did not ovulate to GnRH1 indicated no difference in timing of final ovulation during the 48–72 h (Ovulation: 54.6% [18/33] vs. No ovulation: 50.0% [14/28]; $P = 0.7$) and 73–96 h interval (Ovulation: 45.6% [15/33] vs. No ovulation: 50.0% [14/28]; $P = 0.7$).

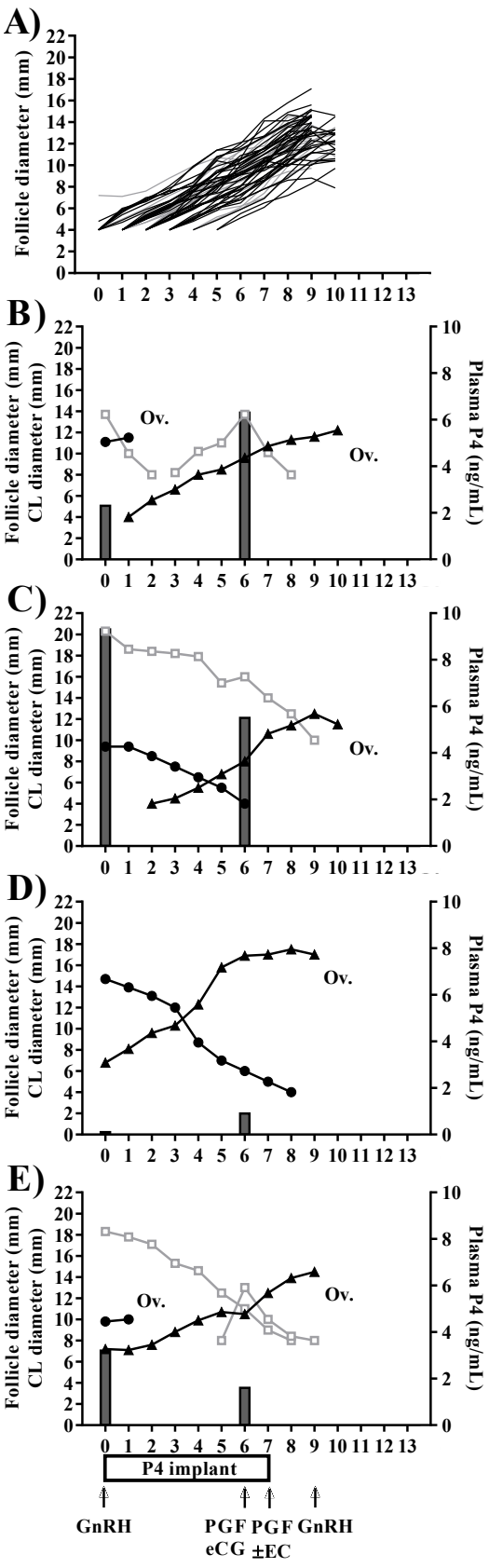

Fig. 4. A: Individual patterns for G-7d (only females that ovulated at the end of a GnRH-based protocol with 7-d of P4 implant). Gray lines represent pubertal heifers and black lines represent nonlactating cows. B: Representative cow for most frequent pattern observed for G-7d protocol with animals ovulating after GnRH treatment (48.6%; 35/72). C. Representative cows for second most frequent pattern observed with G-7d with cows not ovulating to GnRH but having synchronized wave emergence (43.1%; 31/72). D and E: Unusual patterns observed for G-7d. Corpus luteum (gray lines with open squares), follicular development (first follicle [black line with solid circle], second follicle [black line with solid triangle] and third follicle [black line with solid square]) and P4 concentration (gray column at D0 and at PGF administration) are illustrated.

3.5. Individual ovarian profiles, most frequent and less frequent patterns

The OF growth profiles for each individual cow and heifer that ovulated at the end of the synchronization protocol are shown for P-5d (Fig. 3A), G-7d (Fig. 4A), EB-7d (Fig. 5A1) and EB-9d treatments (Fig. 5A2) to graphically illustrate the timing, growth rates, and synchrony of emergence of the OF for each synchronization strategy.

As shown in Fig. 3, the P-5d protocol was based on maintenance of the growing DF until the end of the protocol and not on synchronization in emergence of a new follicular wave. The most common profile is illustrated by one cow (Fig. 3B) with maintenance and subsequent ovulation of the largest follicle that was present at the beginning of the protocol. A similar pattern was observed in 70.0% (28/40) of the animals treated with P-5d and most of the cows with this pattern ovulated at the end of the protocol (92.9% [26/28]). Some unusual patterns were also observed. For example, some animals had atresia of the DF during the protocol and emergence of a new follicular wave (27.5% [11/40]). Ovulation of the new DF occurred in most cows (72.7% [8/11]) as illustrated in Fig. 3C, although some cows did not ovulate the new DF as illustrated in Fig. 3D with emergence of the follicular wave later in the protocol. One cow (2.5% [1/40]) ovulated near the start of the protocol with subsequent synchronized emergence of the ovulatory follicular wave (Fig. 3E).

In GnRH-based protocols, although most animals had synchronized emergence of the ovulatory follicular wave (91.7% [66/72]), there were two common patterns based on ovulation or no ovulation to the GnRH treatment. The most frequent pattern (48.6% [35/72]) is illustrated by the cow in Fig. 4B with ovulation after D0 and emergence of a new follicular wave during the first two d of the protocol. Most of the cows with this pattern ovulated the new DF at the end of the protocol (88.6% [31/35]). The next most common pattern (43.1% [31/72]) is illustrated by the cow in Fig. 4C with lack of ovulation to the GnRH treatment but emergence of a new follicular wave during the first five d of the protocol. Most of these cows ovulated (83.9% [26/31]) this new DF at the end of the protocol. Some of the unusual patterns are also illustrated. Some cows (4.2% [3/72]) that did not ovulate to the GnRH also did not have synchronized emergence of a new follicle wave as illustrated in Fig. 3D, and 66.7% (2/3) of cows with this pattern had ovulation at the end of the protocol. Furthermore, a few animals (4.2% [3/72]) had ovulation at the start of the protocol but did not have emergence of a new follicle wave as illustrated for one cow in Fig. 4E.

After EB treatment there was generally a delay in emergence of the new follicular wave (EB-7d and EB-9d; Fig. 5). The most frequent pattern (80.3% [53/66]) is illustrated in Fig. 5B1 and B2 with atresia of the DF, followed by follicle wave emergence 2–3 d after the beginning of the protocol, and subsequent ovulation of this new DF (84.9% [45/53]). The next most common pattern (12.1% [8/66]) is illustrated in Fig. 5C1 and 5C2 with ovulation near the beginning of the protocol and subsequent emergence of a new follicular wave. All of these cows (8/8) ovulated the new DF at the end of the protocol. A few animals (7.6% [5/66]) did not have wave emergence during the first 5 d of the protocol, as illustrated in Fig. 5D1 and 5D2, with 60.0% (3/5) of these cows ovulating at the end of the protocol. A few animals (6.6% [4/61]) had a new follicular

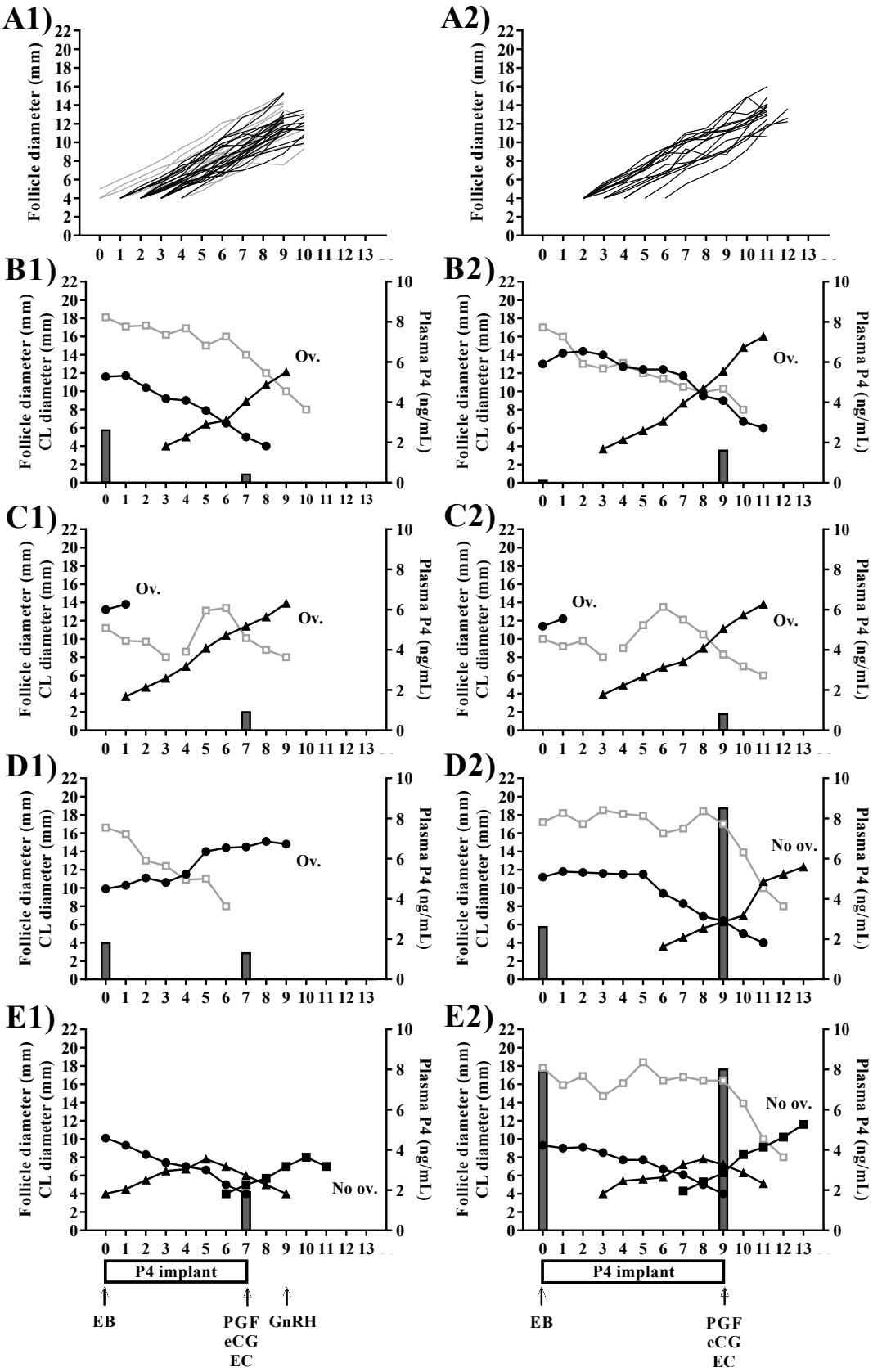

Fig. 5. A1: Individual patterns for EB-7d (only females that ovulated at the end of an EB-based protocol with 7-d of P4 implant). A2: Individual patterns observed for EB-9d. Gray lines represent pubertal heifers and black lines represent nonlactating cows. Representative cows with most frequent pattern observed for EB-7d (B1) or EB-9d (B2) protocols (80.3%; 53/66). Unusual patterns observed for EB-7d (C1, D1, and E1) or EB-9d (C2, D2, and E2). Corpus luteum (gray lines with open squares), follicular development (first follicle [black line with solid circle], second follicle [black line with solid triangle] and third follicle [black line with solid square]) and P4 concentration (gray column on D0 and at PGF administration) are illustrated.

wave with subsequent atresia of the new DF before the end of the protocol, and emergence of a subsequent new follicular wave late in the protocol, as illustrated in Fig. 5E1 and 5E2. Only 25.0% (1/4) of these animals ovulated at the end of the protocol. In addition, regression of the CL that was present at the start of the protocol occurred during the protocol in 58.8% (20/34) of animals that received the EB protocols.

4. Discussion

This study provided unique insights into ovarian function during protocols that synchronize ovulation in beef cattle by performing daily ultrasound evaluations in a total of 178 *Bos indicus* beef cattle. Previous studies have described the follicular dynamics and physiology that follow EB treatments at the initiation of E2/P4-based timed AI protocols in both *Bos taurus* and *Bos indicus* beef cattle [14–18]. However, this manuscript is the first report of direct comparisons of the ovarian dynamics in *Bos indicus* cattle during P4-based protocols that are initiated with either EB, GnRH, or only the intravaginal P4 implant. There were clear differences in the average values for follicular and luteal dynamics among the different types of protocols. Moreover, some of the most revealing information on ovarian dynamics was best distinguished by combining the data from the three experiments and by evaluating the follicle growth patterns in individual animals. These in-depth, physiological results will be important to consider in designing and testing future protocols for synchronizing ovulation in *Bos indicus* cattle.

The first hypothesis of our study was clearly supported since P4-based protocols that began with EB, GnRH, or only P4 produced distinct profiles of follicular and luteal growth and circulating P4 concentrations. One important observation was that treatments at the initiation of the protocol, either EB or GnRH, are essential to successfully synchronize emergence of a new follicular wave, as evidenced by high synchronization with either treatment (>90%) and low synchronization of follicular wave emergence (30%) in animals that received only P4 at the beginning of the protocol. It seems unlikely that P4 alone synchronized emergence of a new follicular wave; whereas, EB and GnRH were both very effective initiators of a synchronized ovulation protocol. This contrasts with the previous results of Cavalieri [13] that reported that two intravaginal P4 implants induced follicle wave emergence by atresia of the DF in 23 *Bos indicus* heifers. However, each P4 implant used by Cavalieri contained 3.12 g P4 and circulating P4 reached a peak of ~50 ng/mL [13], which would be much greater than circulating P4 concentrations achieved during our experiments.

Treatment with EB produced particularly consistent results with >80% of animals having atresia of the largest follicle and synchronized emergence of the new follicular wave at 2.4 d, on average, after EB treatment. Previous studies have reported similar results, although combining results from previous studies with daily ultrasound after EB treatment [14–18] indicated a later time of wave emergence (4.0 ± 0.9 [n = 431]) than observed in our study. Perhaps different criteria for timing of follicular wave emergence accounts for this difference in timing as we used retrospective evaluation to determine the first day that the future DF was observed at 4 mm or more. Nevertheless, the distribution of emergence in our study showed that >70% of animals treated with EB had follicular wave emergence on day 2 or 3 after EB treatment. Previous studies have shown that the synchronization of follicular wave emergence occurs because of a rapid suppression of FSH after EB treatment with a subsequent rebound in circulating FSH that induces the synchronized follicular wave emergence [35,36].

Treatment with GnRH produced consistent emergence of a synchronized follicular wave but there were two distinct patterns that combined to produce this synchrony. About half the cows (38/72) had ovulation and wave emergence around 1 d after treatment (1.4 d average); whereas, the other half of the synchronized animals did not have ovulation but still displayed synchrony of follicular wave emergence but a little over 1 d later (2.5 d), on average. The synchrony after GnRH-induced ovulation has been previously described [7,37], with an initial LH/FSH surge that peaks about 2 h after GnRH treatment and a second FSH surge that peaks near 24 h after GnRH treatment, termed the periovulatory FSH surge [38,39]. The periovulatory surge produced a clear synchrony in follicular wave emergence, mostly on day 1 after GnRH treatment in animals that ovulated. In contrast, GnRH-treated animals that did not ovulate had synchronized wave emergence (91.2%; 31/34) but the distribution of follicular wave emergence was evenly divided over all 5 d after GnRH treatment (Fig. 2A). The average of 2.5 and standard deviation of 2.23 supports the possibility that random chance is determining the time of wave emergence after ovulating animals are removed from the group. Alternatively, GnRH treatment, in the absence of ovulation, may have increased synchrony of wave emergence given that cows that received only P4 did not have a clear synchrony in wave emergence. After wave emergence, growth rate of the dominant follicle was similar for cows that ovulated or did not ovulate to the GnRH treatment (1.2 mm/d), although ovulating animals tended to have a larger OF, most likely due to the earlier time of wave emergence in ovulating compared to non-ovulating animals.

The percentage of animals that ovulated after GnRH treatment (52.8%) was similar in our study to previous studies with beef cattle (52.3% [204/390]) [9,12,40,41]. Buserelin acetate was used in this study which has about 10-fold greater potency compared to Gonadorelin [42,43]. Although high circulating P4 can suppress the GnRH-induced LH surge [44,45], treatment with a greater dose of GnRH can increase the magnitude of the LH peak, even in the presence of high circulating P4, and this can increase the ovulatory response after GnRH [21,22]. In addition, some ovulation was observed after EB (12.1%; 8/66) and this tended ($P = 0.08$ by one-tailed Fisher's exact test) to be greater than after P4 treatment alone (2.5%; 1/40) suggesting that EB treatment may induce ovulation in some cows even when an intravaginal P4 implant is being inserted [46,47].

A second aspect of Hypothesis 1 was that the patterns of CL development and circulating P4 would vary between protocols, and this idea was also supported by the results of these experiments. The number of CL and the circulating P4 concentrations were greater at the time of PGF in the GnRH protocol compared to protocols that used EB/P4 or only P4 at the beginning of the protocol. This is logical due to greater ovulation incidence following the GnRH treatment compared to the other protocols. In addition, treatment with EB on D0 of the protocol has been found to induce luteolysis [46,47] and this was also observed in about 58.8% (20/34) of the EB-treated animals in our study. Consistent with this idea, animals treated with only P4 had intermediate circulating P4 concentrations and number of CL at the time of PGF. Within the GnRH-treated group, animals that ovulated had greater number of CL and circulating P4 concentration at PGF compared to animals that did not ovulate, as previously observed [48].

Our second hypothesis was supported since either GnRH-, EB- or just P-based protocols induced final ovulation of the OF in 85.4% (152/178) of the animals, with no detectable differences among protocols in percentage ovulating, time of ovulation, or size of the OF. This result is especially important for the P-5d protocol, because it is a novel and shorter duration protocol for *Bos indicus* cattle, which does not require EB or GnRH on D0. In fact, it has been reported in *Bos taurus* that extended exposure to P4 can improve follicle synchrony by extending follicle age but compromised

fertility [48,49]. Other studies in *Bos taurus* have also shown that persistent follicles result in poor embryo quality and reduced fertility [50–54]. However, the fertility that will be obtained using the P-5d protocol in *Bos indicus* is unknown. Despite the fact that the OF in 70% of the animals in this protocol emerged outside of the 5 d window that we evaluated, preliminary studies in our laboratory did not show a reduction in fertility in Nelore (*Bos indicus*) cows that ovulated persistent follicles during an experiment with precise manipulation of follicle age (Sartori et al., unpublished).

Although the most common individual follicle growth patterns were consistent with the pharmacologic interventions, there were also some unusual patterns that were noteworthy. For example, some unusual asynchronous animals were observed in P-5d due to the short length of the protocol. Some animals had wave emergence late in the protocol (Fig. 3D) and therefore the follicle did not ovulate to the GnRH treatment, probably due to lack of sufficient LH receptors on the largest follicle [19,20]. In addition, an animal that ovulated at the beginning of the protocol did not appear to completely regress the CL at the end of the protocol which is likely to reduce fertility if FTAI occurs in elevated circulating P4 [55]. For G-7d and EB-based protocols, the two most common patterns for each protocol accounted for more than 90% of the animals with any observed asynchrony due to recovery of a subordinate follicle after GnRH-induced ovulation of a larger follicle (Fig. 4E) as observed previously in lactating cows [20], lack of follicular wave emergence after EB or GnRH treatment (Figs. 4D and 5D1), or wave emergence late in the protocol (Fig. 5E1 and 5E2). These patterns provide detailed information to future researchers on the precise abnormalities that can occur during different types of FTAI protocols since published patterns for individual *Bos indicus* cattle are limited.

In conclusion, the present study has described the physiology that underlie the synchrony that is observed with P4-based protocols. Importantly, despite distinct patterns of ovarian function, each of these different synchronization strategies appear to be promising for FTAI in beef cattle due to the synchronized ovulation at the end of the protocols. Particularly noteworthy, the P-5d protocol did not synchronize follicle wave emergence but produced a synchronized ovulation rate and OF size at the end of the protocol similar to the G-7d and EB-P4 protocols that clearly synchronized follicular wave emergence. Future studies are needed to determine the fertility to a FTAI at the end of these protocols.

CRediT authorship contribution statement

Guilherme Madureira: Conceptualization, Methodology, Investigation, Writing - original draft. **Jéssica C.L. Motta:** Conceptualization, Methodology, Investigation, Writing - original draft. **Jéssica N. Drum:** Visualization, Investigation. **Carlos E.C. Consentini:** Conceptualization, Methodology, Investigation, Writing - original draft. **Alexandre B. Prata:** Visualization, Investigation. **Pedro L.J. Monteiro:** Visualization, Investigation. **Leonardo F. Melo:** Visualization, Investigation. **Amanda B. Alvarenga:** Investigation, Formal analysis. **Milo C. Wiltbank:** Funding acquisition, Conceptualization, Methodology, Writing - review & editing. **Roberto Sartori:** Funding acquisition, Conceptualization, Methodology, Writing - review & editing.

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