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Progesterone-based timed AI protocols for *Bos indicus* cattle II: Reproductive outcomes of either EB or GnRH-type protocol, using or not GnRH at AI

Guilherme Madureira [a, c], Carlos E.C. Consentini [a], Jéssica C.L. Motta [a, c], Jéssica N. Drum [a, c], Alexandre B. Prata [a, c], Pedro L.J. Monteiro Jr. [a, c], Leonardo F. Melo [a], José Renato S. Gonçalves [b], Milo C. Wiltbank [c], Roberto Sartori [a, *]

[a] Department of Animal Science, Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo, Av. Pádua Dias, 11, Piracicaba, SP, 13418-900, Brazil
[b] Experimental Station Hildegard Georgina Von Pritzelwitz, Londrina, PR, 86010-990, Brazil
[c] Department of Animal and Dairy Science, University of Wisconsin-Madison, 1675 Observatory Drive, Madison, WI, 53706, USA

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ABSTRACT

The aim of these experiments was to study ovarian dynamics and fertility of *Bos indicus* beef cattle submitted to 7-d progesterone (P4)-based fixed-time AI (FTAI) protocols using different hormonal treatments. In Exp. 1, 2 yr old Nelore heifers (n = 973) were randomly assigned to one of four treatments: EB-0 (estradiol benzoate, EB on D0 and no GnRH at AI), EB-G (EB on D0 and GnRH at AI), G-0 (GnRH on D0 and no GnRH at AI), or G-G (GnRH on D0 and at AI). On D0, heifers received an intravaginal P4 implant (0.5 g) for 7 d and EB (1.5 mg) or GnRH (16.8 μg). On D7, the P4 implant was withdrawn and heifers received cloprostenol (PGF; 0.5 mg) and estradiol cypionate (EC, 0.5 mg). Heifers in G groups also received PGF and eCG (200 IU) on D6, whereas EB heifers received eCG on D7. At FTAI on D9, only EB-G and G-G groups received GnRH (8.4 μg). In Exp. 2, Nelore cows (n = 804) received the same treatments (EB-0, EB-G, G-0, or G-G) using a 1.0 g P4 implant, 2.0 mg EB, and 300 IU eCG. Effects were considered significant when $P \leq 0.05$. After treatment on D0, G had more ovulations than EB in heifers (60.3 [287/476] vs. 12.7% [63/497]) and cows (73.7 [83/112] vs. 24.4% [28/113]). Luteolysis after D0 was greater in EB than G in heifers (39.2 [159/406] vs. 20.0% [77/385]) and cows (25.5 [14/55] vs. 1.6% [1/64]). Heifers in G had larger follicles (mm) than EB on D7 (10.3 ± 0.2 vs. 9.2 ± 0.2) and at AI (11.9 ± 0.2 vs. 11.3 ± 0.2). Cows had larger follicles in G than EB on D7 (11.0 ± 0.3 vs. 9.9 ± 0.3) but not at AI. More estrus was observed in G than EB for heifers (80.3 [382/476] vs. 69.6% [346/497]) and cows (67.6 [270/400] vs. 56.2% [227/404]). There was no interaction between D0 and D9 treatments on pregnancy per AI (P/AI) in heifers (EB-0: 56.7 [139/245], EB-G: 53.6 [135/252], G-0: 52.6 [127/241], and G-G: 57.5% [135/235]). However, cows from EB-G had greater P/AI than EB-0 (69.5 [142/204] vs. 60.2% [120/200]), whereas P/AI for G-0 (62.7% [127/203]) was similar to G-G (60.9% [120/197]). In heifers, there was no interaction of GnRH at AI with estrus, however, cows that did not display estrus had greater P/AI if they received GnRH at AI (GnRH = 59.1 [91/154] vs. No GnRH = 48.2% [78/162]). Thus, protocols initiated with EB or GnRH for *Bos indicus* heifers and cows had differing ovarian dynamics but similar overall fertility, enabling their use in reproductive management programs. Treatment with GnRH at time of AI increased fertility in some instances in *Bos indicus* cows but not in heifers.

1. Introduction

Synchronization protocols that allow for incorporation of fixed-time AI (FTAI) have become an important part of strategies to improve reproductive management in cattle operations in many parts of the world, including the USA and Brazil [1–3]. These

* Corresponding author.
E-mail addresses: gmadureira@usp.br (G. Madureira), robertosartori@usp.br (R. Sartori).

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strategies allow for controlled breeding seasons, increased reproductive efficiency, and improved genetic progress [4]. In beef cattle operations, FTAI protocols have been tailored to the hormones that are approved for use in specific countries and that match the management style of the operation and the physiology of the animals that are being bred. Programs for FTAI in *Bos indicus* cattle have generally utilized estradiol (E2) products, such as E2 benzoate (EB), although many countries with *Bos indicus* cattle do not currently have approval for the use of E2 products in FTAI protocols. It is generally assumed that E2 protocols are more effective in *Bos indicus* beef cattle than protocols that are initiated with gonadotropin-releasing hormone (GnRH), although direct comparisons of these protocols are needed. Therefore, this study compared ovarian responses and fertility in FTAI protocols that are initiated with EB or GnRH, both in association with an intravaginal progesterone (P4) implant, in *Bos indicus* heifers and cows.

Protocols that are initiated with a progestogen treatment, such as intravaginal P4 implants, and E2 esters (mainly EB), are termed E2/P4-based protocols and employ distinct physiology to initiate a follicular wave [5] compared to GnRH-based protocols [6,7]. Treatment with EB and P4 at the initiation of the protocol causes suppression of the circulating gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), leading to inhibition of the current follicular wave, and subsequently induces a surge in FSH that initiates a new follicular wave [8,9]. Recent physiological experiments from our lab using daily ovarian ultrasonography compared E2/P4-based and GnRH-type protocols [10], and demonstrated that protocols initiated with EB produce synchrony in emergence of the new follicular wave resulting in subsequent ovulation of a single dominant follicle at the end of the protocol in *Bos indicus* heifers and cows. In contrast, during the same study the use of GnRH at the initiation of the protocol generated an immediate LH/FSH surge, rather than the gonadotropin suppression produced by EB treatment, and produced ovulation in ~53.0% of the animals. Nevertheless, both methods for initiating the protocol produced synchronized emergence of a new follicular wave (from D0 to D5 of the protocol) in a high percentage of animals (92.4% [61/66] for GnRH, and 91.4% [66/72] for EB), and induced high ovulation rate at the end of the protocol (~90%).

Ovulation to GnRH at the initiation of the protocol requires an LH surge of sufficient magnitude to initiate ovulation, as well as the presence of a dominant follicle with ovulatory capacity (i.e. LH receptors in the granulosa cells) [11–13]. It is known that the magnitude of the LH surge can be negatively influenced by circulating P4 and positively influenced by increasing the GnRH dose [14]. In addition, the presence of younger corpus luteum (CL) at the time of P4 implant removal in the GnRH-initiated protocol necessitated the use of two PGF treatments to assure complete CL regression by the end of the protocol [15–17]. Thus, since these two types of protocols (EB- and GnRH-based) have specific mechanisms of action, studies employing FTAI are needed to compare fertility outcomes between them.

Expression of estrus prior to AI has been associated with increased fertility [18–20]. Cattle that express estrus are more likely to ovulate to the protocol because the hormonal environment that induces estrus (high circulating E2 in the absence of P4) is also likely to induce a GnRH surge from the hypothalamus and an LH surge from the pituitary [21], and have a hormonal environment that properly supports endometrial function, conceptus growth, and pregnancy maintenance [22,23]. One strategy that has been employed during FTAI protocols, particularly in animals that do not express estrus prior to AI, is to give GnRH at the time of AI to assure that all animals have an LH surge, theoretically reducing the percentage of animals with no ovulation or delayed ovulation, and improving fertility outcomes in animals not displaying estrus [24,25].

Thus, the main objectives of this study were to further explore the physiology of EB- and GnRH-type FTAI protocols, and to compare fertility of these protocols in *Bos indicus* heifers and cows. This research employed P4-based FTAI protocols using 7 days of treatment with an intravaginal P4 device, as this strategy has previously been found to yield satisfactory pregnancies per AI (P/AI) [24,26]. In addition, the effect of GnRH treatment at the time of AI was investigated in these two types of protocols. Our hypotheses were: 1) Using EB or GnRH at the beginning of a P4-based protocol would result in different follicle and luteal dynamics; 2) Both EB- and GnRH-type protocols would produce similar P/AI; 3) Administration of GnRH at the time of AI would increase P/AI in females that were not detected in estrus by the time of AI.

2. Material and methods

These experiments were conducted at the Experimental Station "Hildegard Georgina Von Pritzelwiltz", located in Londrina, PR, Brazil. All females were kept on pasture (*Brachiaria brizantha*), were supplemented with mineral salt, and had *ad libitum* access to water. The Animal Research Ethics Committee of "Luiz de Queiroz" College of Agriculture of University of São Paulo (ESALQ/USP) approved all procedures involving heifers and cows (Protocol # 2017.5.1618.11.9).

2.1. Experiment 1

A total of 973 nulliparous heifers were used and averaged 26.0 ± 2.0 mo old with body condition score (BCS) of 3.0 ± 0.01 (1–5 scale) and average body weight of 307.1 ± 22.6 kg. At initiation, all heifers greater than 265.0 kg were evaluated by ultrasound for the presence of a corpus luteum (CL). Heifers with CL were assigned to one of four experimental treatments, described below, and heifers without a CL were submitted to a protocol for induction of cyclicity (D-24: insertion of a 7-d used intravaginal P4 implant with 0.5 g [Repro one, GlobalGen Vet Science, Jaboticabal, Brazil]; D-12: P4 implant withdrawal and administration of 0.5 mg E2 cypionate im [EC; Cipion, GlobalGen Vet Science]). After 12 d (D0), all heifers, regardless of CL presence, were randomly assigned to the treatments (Fig. 1): EB-0 (EB at D0 + no GnRH at AI; n = 245), EB-G (EB at D0 + GnRH at AI; n = 252), G-0 (GnRH at D0 + no GnRH at AI; n = 241), or G-G (GnRH at D0 and at AI; n = 235). On D0, all heifers received a new P4 implant with 0.5 g P4 (Repro one, GlobalGen Vet Science) and EB-groups were treated with 1.5 mg EB im (Syncrogen, GlobalGen Vet Science), whereas G-groups were treated with 16.8 μg buserelin acetate im (GnRH; Maxrelin, GlobalGen Vet Science). On D7, all heifers had their P4 implant removed and received 0.530 mg cloprostenol sodium im (PGF; Induscio, GlobalGen Vet Science), 0.5 mg EC, and had tail-chalk applied on the base of their tailhead. Heifers from G-groups also received an extra PGF and 200 IU eCG im (ECGen, GlobalGen Vet Science) treatment on D6, 24 h before the procedures done on D7. Thus, eCG treatment was on D6 in G-groups and D7 in EB-groups. At AI (48 h after P4 implant withdrawal), EB-G and G-G groups received 8.4 μg GnRH, whereas all heifers were checked for estrus. In order to make it clear, within the text, when groups are denominated EB, it means that cattle were treated with EB on D0 and PGF, EC, and eCG on D7. When groups are called G, it means they were treated with GnRH on D0, PGF and eCG on D6, and PGF and EC on D7.

2.2. Experiment 2

A total of 804 Nelore cows were used and averaged 3.0 ± 0.01 (1-5 BCS scale), and 67.2 ± 23.1 d postpartum for the lactating cows. The majority of them were multiparous (62.7%; n = 504; BCS 2.9 ± 0.01), ~¼th were primiparous (23.4%; n = 188; BCS 3.0 ± 0.02),

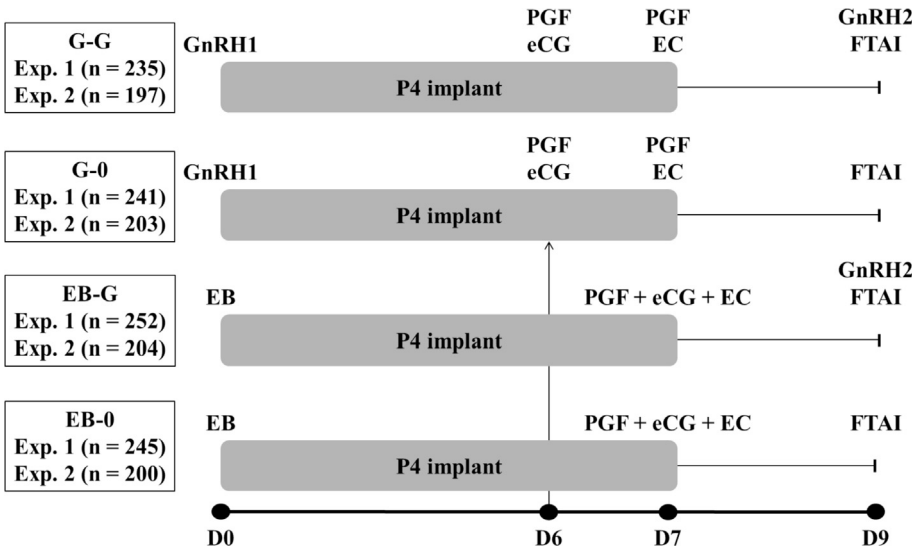

Fig. 1. Experimental design from Exp. 1 and Exp. 2 in *Bos indicus* beef cattle (Nelore heifers and cows, respectively) submitted to a progesterone (P4)-based fixed-time AI protocol initiated with buserelin acetate (GnRH1; 16.8 μg), or estradiol benzoate (EB; 1.5 mg for heifers and 2.0 mg for cows), and receiving GnRH2 (G-G or EB-G; 8.4 μg) or not (G-0 or EB-0) at the time of AI. All groups had an intravaginal P4 implant inserted on D0 (heifers: 0.5 g; cows: 1.0 g) and removed on D7 and were treated with PGF and estradiol cypionate (EC; 0.5 mg) on D7. Treatment with equine chorionic gonadotropin (eCG; heifers: 200 IU; cows: 300 IU) was on D6 in protocols initiated with GnRH and on D7 in protocols initiated with EB. Cloprostenol treatments (PGF; 0.530 mg) were on D6 and D7 in protocols initiated with GnRH and on D7 in protocols initiated with EB. Ultrasound evaluations were performed for CL presence at D0, D6, D7, and D16, and for diameter of the largest follicle on D7 and D9 in a subset of animals.

and the remaining were nonlactating cows that had calved at least once (13.9%; n = 112; BCS 3.3 ± 0.02). During this study, most of the cows were receiving their first AI of the breeding season (n = 579), whereas the remaining cows were at their second opportunity to conceive by FTAI (n = 225). All cows were submitted to the same experimental treatments as described for heifers (Fig. 1), with some adjustments in hormone doses: on D0, a new intravaginal P4 implant with 1.0 g (Repro neo, GlobalGen Vet Science) was used and 2.0 mg of EB was administered im rather than the 1.5 mg used for heifers. The dose of eCG for cows was 300 IU im. Thus, treatments were: EB-0 (EB at D0 + no GnRH at AI; n = 200), EB-G (EB at D0 + GnRH at AI; n = 204), G-0 (GnRH at D0 + no GnRH at AI; n = 203), or G-G (GnRH at D0 and at AI; n = 197).

In both experiments, heifers and cows were inseminated by one of three technicians using 20×10^6 frozen/thawed sperm from six proven Nelore sires (Genex, São Carlos, Brazil).

2.3. Ultrasound examinations

For all heifers (n = 973; Exp. 1), transrectal ultrasound examinations of the ovaries in B-mode using a 7.5 MHz linear transducer (DP-2200 VET, Mindray, Shenzhen, China) were performed on D0 and D6 for G-groups, and on D0 and D7 for EB-groups to evaluate the presence of CL. Also, approximately 15.0% of heifers from EB (n = 84) and G-groups (n = 71) were evaluated on D7 and D9 for diameter of the largest follicle (mm). During Exp. 2, only cows that were being submitted to the second FTAI (n = 225) were evaluated on D0 for presence of CL, but all cows (n = 804) were evaluated on D6 (G-groups) and D7 (EB-groups) for CL presence. Similar to Exp. 1, approximately 10.0% of cows from EB (n = 42) and G-groups (n = 43) were evaluated on D7 and D9 for diameter of the largest follicle. All follicle measurements were performed by the same operator.

Seven d after FTAI (D16), ultrasound evaluation was performed in a subset of the heifers from Exp. 1 (n = 173) and cows from Exp. 2 (n = 313) to check for CL presence, in order to determine the percentage of animals that ovulated to the protocols.

Pregnancy diagnosis was performed by ultrasonography between 30 and 35 d after FTAI.

2.4. Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC) and both experiments were performed as completely randomized 2 × 2 factorial designs.

Discrete responses of measured variables were analyzed using the generalized linear mixed model (GLIMMIX) procedure, and fit to a binary (ovulation rate after D0, luteolysis between D0 and PGF, expression of estrus, pregnancy per AI [P/AI], and ovulation rate at the end of the protocol) or exponential distribution (CL number at PGF). Continuous variable responses were analyzed using the linear mixed models (MIXED) procedure. All variable responses (diameter of the largest follicle on D7 and D9) were tested for normality of the residuals using the Shapiro-Wilk statistic method obtained by the PROC UNIVARIATE procedure of SAS.

Selection of the model that best fit each variable of interest was determined 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. Treatments were considered fixed effects and the tested variables were: BCS and CL on D0, semen batch, bull and inseminator. In addition, estrus was considered a fixed effect during the analysis related to the effect of GnRH at AI.

Differences were considered significant for $P \leq 0.05$, whereas a tendency was designated when $P \leq 0.10$ and $P > 0.05$. Mean comparisons were performed by the adjusted Tukey test. The results are expressed as least squares means ± standard error of the mean (LSM ± SEM), unless otherwise indicated.

3. Results

3.1. Experiment 1

At the beginning of the breeding season, 14.9% (145/973) of heifers were considered cycling (presence of CL at the first

ultrasound evaluation). After the protocol for induction of cyclicity, 78.0% (646/828) of the induced heifers had a CL on D0 of the FTAI protocol.

Results regarding ovarian dynamics are presented in Table 1. As expected, G groups had much greater percentage of heifers that ovulated after D0 (60.3 vs. 12.7%; $P < 0.001$) and greater number of CL at PGF (1.1 vs. 0.5; $P < 0.001$) compared to EB (n = 973). Also, more heifers underwent luteolysis between D0 and PGF when the protocol was initiated with EB than G (39.2 vs. 20.2%; $P < 0.001$). The size of the largest follicle on D7 and D9 of the protocol was greater for heifers receiving G than EB. In addition, more heifers from G than EB were detected in estrus after P4 implant withdrawal (80.3 vs. 69.6%; $P < 0.001$). In spite of these differences, the percentage of heifers that ovulated after D9 of the protocol was above 90% in both groups and did not differ between groups. Also, no differences were detected for double ovulation after AI ($P = 0.9$; G groups: 0 [0/74], and EB groups: 2.4% [2/84]).

The P/AI were similar between treatments on D0 (EB vs. GnRH), and treatments on D9 (No GnRH vs. GnRH; Fig. 2A). However, there was an effect of cyclicity ($P = 0.01$), with P/AI greater in heifers that were cyclic prior to the induction protocol (62.0%; 90/145) and in heifers with a CL present after the induction protocol (56.8%; 367/646) than in heifers without a CL after the induction protocol (46.2%; 84/182). There was no interaction between expression of estrus and treatment with GnRH at the time of AI on P/AI (Fig. 3A), however, heifers that expressed estrus had greater P/AI than those that did not show estrus (57.4 [428/745] vs. 48.1% [110/228]). Finally, GnRH treatment at the time of AI did not alter the fertility of heifers without estrus that were submitted to each type of protocol (EB or GnRH-based; Fig. 4A).

3.2. Experiment 2

Results on ovarian dynamics and expression of estrus in cows (Exp. 2) are presented in Table 2. There was a much greater percentage of cows that ovulated to GnRH than EB (73.7 vs. 24.4%; $P < 0.001$). In addition, over 25% of the cows from the EB groups underwent luteolysis between D0 and PGF compared to less than 2% in the G groups ($P = 0.004$). These two responses resulted in a much greater number of CL at PGF in G than in EB (1.1 vs. 0.3; $P < 0.001$). Moreover, on D7, the diameter of the largest follicle was greater in the G than EB-groups, whereas there was no difference in follicle diameters at time of AI (D9). In addition, more cows from the G-groups were detected in estrus than in the EB-groups (67.6 vs. 56.2%; $P < 0.001$). However, there were no differences in percentage of cows that ovulated to the protocol, which was high in both groups (average of 93.3%). There was also no difference in percentage of cows that double ovulated to the protocols ($P = 0.9$; G groups: 8.2 [12/147], and EB groups: 7.6% [11/145]).

Regarding fertility, EB-G had greater P/AI than EB-0 ($P = 0.05$), but G-0 and G-G did not differ from one another (Fig. 2B). There was an effect of parity ($P = 0.03$) where multiparous (64.5%; 325/504) and nonlactating cows (69.8; 78/112) had greater P/AI than primiparous cows (55.3%; 104/188). There was a tendency ($P = 0.1$) for an interaction between expression of estrus and GnRH treatment at AI (Fig. 3B) where GnRH at AI had a positive effect in cows that did not express estrus ($P = 0.05$; 10.9% absolute increase; 22.6% relative

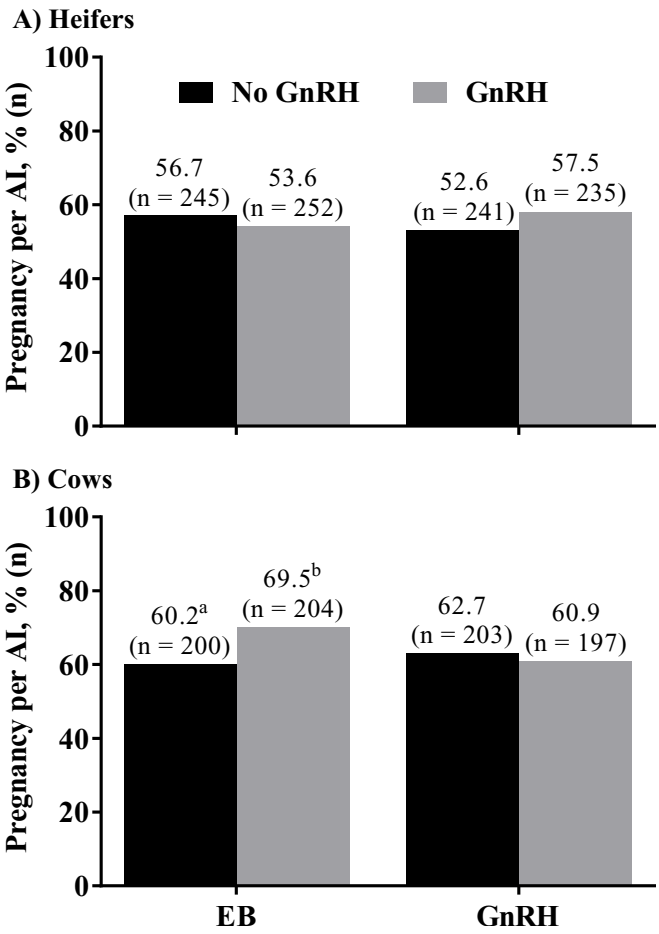

Fig. 2. Pregnancy per AI (P/AI) in *Bos indicus* beef cattle (Nelore heifers [A] and cows [B]) submitted to a 7-d progesterone (P4)-based FTAI protocol initiated with estradiol benzoate (EB; termed EB-based) or buserelin acetate (GnRH; termed GnRH-based). Moreover, animals were randomly assigned to receive GnRH or no GnRH at the time of AI. In heifers, there were no effects of treatments on D0 ($P = 0.9$) or D9 ($P = 0.8$) and no interactions between D0 and D9 treatments ($P = 0.2$). However, in cows there was a tendency for an interaction between treatments on D0 and D9 ($P = 0.1$), and the administration of GnRH at AI increased the P/AI of EB-based protocol ($P = 0.05$). $^{a,b}P < 0.05$.

Table 1
Ovarian dynamics and estrus expression of Nelore (*Bos indicus*) heifers (Exp. 1) submitted to 7-d progesterone (P4)-based FTAI protocols initiated with buserelin acetate (GnRH; 16.8 μg) or estradiol benzoate (EB; 1.5 mg).

	GnRH[a](n = 476)	EB[b](n = 497)	*P*-value
Ovulation incidence after D0, % (n/n)	60.3 (287/476)	12.7 (63/497)	<0.001
Luteolysis between D0 and PGF, % (n/n)	20.0 (77/385)	39.2 (159/406)	<0.001
CL number at PGF (n)	1.1 ± 0.06 (476)	0.5 ± 0.02 (497)	<0.001
Diameter of largest follicle on D7, mm (n)	10.3 ± 0.2 (71)	9.2 ± 0.2 (84)	<0.001
Diameter of largest follicle on D9, mm (n)	11.9 ± 0.2 (71)	11.3 ± 0.2 (84)	0.01
Expression of estrus, % (n/n)	80.3 (382/476)	69.6 (346/497)	<0.001
Ovulation incidence after D9, % (n/n)	90.2 (74/82)	92.3 (84/91)	0.6

[a] GnRH (D0: intravaginal P4 implant [0.5] and 16.8 μg buserelin acetate [GnRH]; D6: 0.526 mg cloprostenol [PGF] and 200 IU equine chorionic gonadotropin [eCG]; D7: P4 implant removal, PGF, and 0.5 mg estradiol cypionate [EC]; D9: ±8.4 μg GnRH).
[b] EB (D0: intravaginal P4 implant and 1.5 mg estradiol benzoate [EB]; D7: P4 implant removal, PGF, EC, and eCG; D9: ±8.4 μg GnRH).

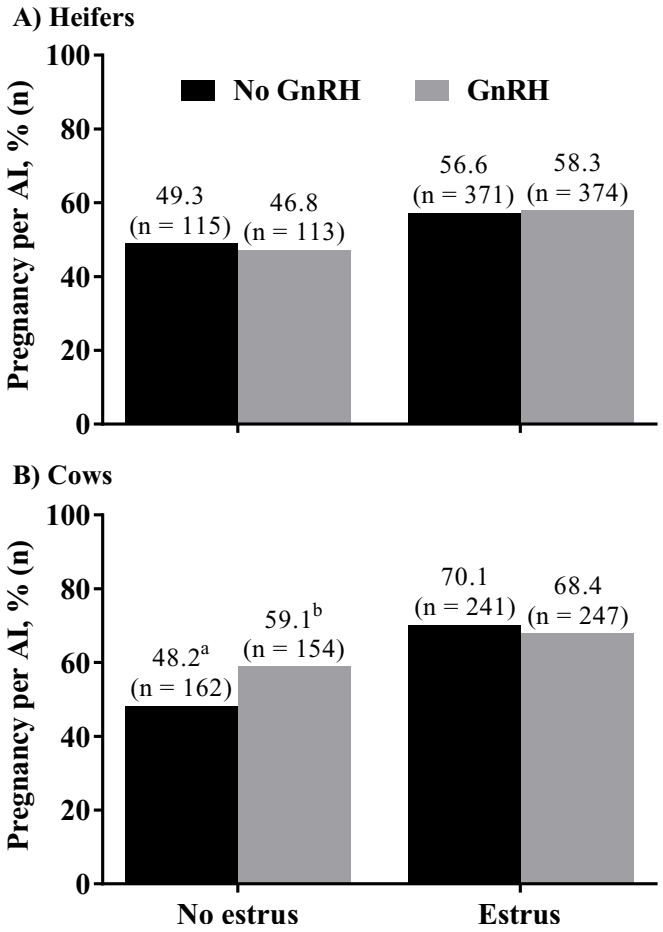

Fig. 3. Pregnancy per AI (P/AI) divided by whether animals expressed estrus and/or received GnRH treatment at the time of AI in *Bos indicus* beef cattle (Nelore heifers [A] and cows [B]) submitted to the 7-d progesterone (P4)-based fixed-time AI protocols. In heifers, there was no main effect of GnRH treatment at AI ($P = 0.9$) and no interaction between expression of estrus and GnRH treatment ($P = 0.6$); however, heifers that expressed estrus had greater P/AI compared to heifers without expression of estrus ($P = 0.01$). In cows there was a tendency for an interaction between expression of estrus and GnRH treatment ($P = 0.08$); furthermore, cows that did not express estrus but received GnRH at AI had greater P/AI compared to cows without estrus that did not receive GnRH ($P = 0.05$). $^{a,b}P < 0.05$.

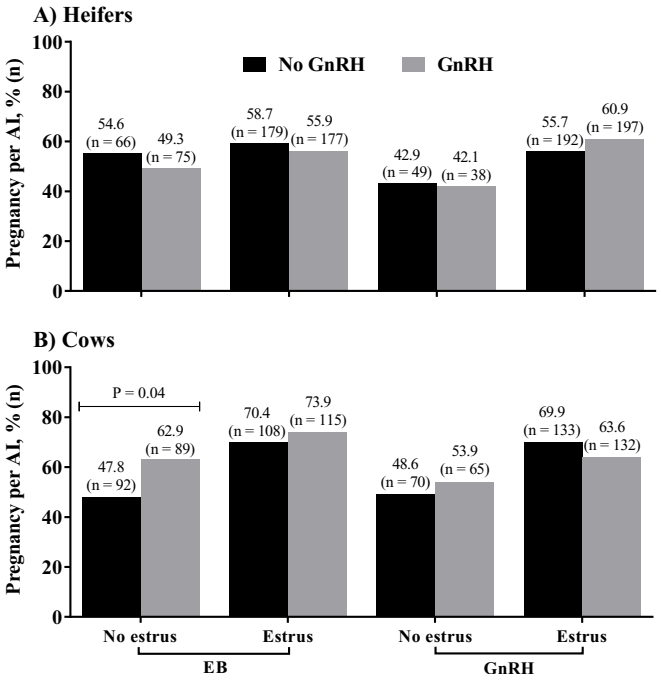

Fig. 4. Pregnancy per AI (P/AI) in *Bos indicus* beef cattle (Nelore heifers [A] and cows [B]) submitted to the 7-d progesterone (P4)-based fixed-time AI protocol. Expression of estrus and type of protocol based on the treatment at the start (estradiol benzoate [EB; termed EB-based] or buserelin acetate [GnRH; termed GnRH-based]) were associated with the GnRH treatment at the time of AI. In heifers, there was no main effect of GnRH treatment at AI based on the expression of estrus during EB or GnRH-based protocol ($P = 0.6$). In addition, cows that were submitted to the EB-based protocol and did not express estrus were positively affected by the GnRH treatment at the time of AI.

increase [10.9/48.2]), but had no effect at AI in cows that expressed estrus. Cows that were detected in estrus had greater P/AI compared to those that did not express estrus (69.1 [337/488] vs. 53.5% [169/316]; $P < 0.001$). Lastly, analyzing each type of protocol separately (EB or GnRH), GnRH at AI only increased P/AI in cows receiving the EB-based protocol that did not express estrus ($P = 0.04$) but did not increase fertility of cows submitted to the GnRH protocol (Fig. 4B).

4. Discussion

Bos indicus cattle represent a major part of the global cattle industry, particularly in tropical regions of the world [27]. Many reproductive management programs utilize FTAI protocols and these can be of particular benefit in *Bos indicus* cattle due to delayed puberty and post-partum acyclicity, extensive management conditions that make AI after estrus impractical, and the major economic and genetic advantages from cross-breeding using *Bos taurus* genetics.

The current study compared FTAI protocols that were initiated with EB, as is traditionally done in many countries with *Bos indicus* cattle, to protocols initiated with GnRH, which are utilized, even in *Bos indicus* cattle, in countries that do not have approval for the use of EB in FTAI protocols. In heifers, the results were impressive, with P/AI greater than 55% (55.6%; 541/973). The heifers in this study were 24 mo of age but less than 15% had a CL at the first ultrasound examination, consistent with previous reports that environmental and genetic factors cause low cyclicity in Nelore heifers [28,29]. Additionally, consistent with previous reports [30,31], a 12-d induction protocol using an intravaginal P4 implant was effective in inducing cyclicity with almost 80% of previously non-cycling heifers having a CL at the time of FTAI protocol initiation. Even the heifers that were not induced to cycle had acceptable fertility (46.2%; 84/182), although lower than the remarkable fertility (57.8%; 457/791) observed in heifers that had a CL at initiation of FTAI protocol. High fertility was also observed in *Bos indicus* cows that received FTAI after similar protocols (64.1%; 507/804). These overall results demonstrate that simple FTAI protocols with the need for handling the animals on only three occasions (EB protocols) or FTAI protocols that utilize GnRH can result in high fertility in *Bos indicus* heifers and cows.

Although this study did not provide the in-depth follicular dynamics that were provided by daily ultrasound in our previous physiological experiments [10], there were still considerable data that supported our first hypothesis that different physiology is involved in FTAI protocols that are initiated with EB compared to GnRH. Thus, many responses were quite different based on the treatment at the start of the protocol including: percentage of cows that ovulated after D0, luteolysis between D0 and PGF, number of CL at PGF, diameter of the largest follicle on D7 and D9, and expression of estrus before AI.

The greater ovulation rate after GnRH administration on D0 of

Table 2
Ovarian dynamics and expression of estrus in Nelore cows (Exp. 2) submitted to 7-d progesterone (P4)-based FTAI protocols initiated with buserelin acetate (GnRH; 16.8 μg), or estradiol benzoate (EB; 2.0 mg).

	GnRH[a](n = 400)	EB[b](n = 404)	*P*-value
Ovulation incidence after D0, % (n/n)	73.7 (83/112)	24.4 (28/113)	<0.001
Luteolysis between D0 and PGF, % (n/n)	1.6 (1/64)	25.5 (14/55)	0.004
CL number at PGF (n)	1.1 ± 0.06 (400)	0.3 ± 0.02 (404)	<0.001
Diameter of largest follicle on D7, mm (n)	11.0 ± 0.3 (43)	9.9 ± 0.3 (42)	0.01
Diameter of largest follicle on D9, mm (n)	13.0 ± 0.3 (43)	12.7 ± 0.3 (42)	0.5
Expression of estrus, % (n/n)	67.6 (270/400)	56.2 (227/404)	<0.001
Ovulation incidence after D9, % (n/n)	93.6 (147/157)	93.0 (145/156)	0.8

[a] GnRH (D0: intravaginal P4 implant [1.0 g] and 16.8 μg buserelin acetate [GnRH]; D6: 0.526 mg cloprostenol [PGF] and 300 IU equine chorionic gonadotropin [eCG]; D7: P4 implant removal, PGF, and 0.5 mg estradiol cypionate [EC]; D9: ±8.4 μg GnRH).
[b] EB (D0: intravaginal P4 implant and 2.0 mg estradiol benzoate [EB]; D7: P4 implant removal, PGF, EC, and eCG; D9: ±8.4 μg GnRH).

the protocol was expected and clearly observed in both Exp. 1 and 2 (~67%). A larger dose of GnRH was used in this study (16.8 μg of buserelin acetate) than typically utilized (10 μg) based on results showing that high circulating P4 is extremely inhibitory to the LH surge in Nelore cattle [32]. In *Bos taurus* cattle, it has been shown that a greater dose of GnRH produces a greater LH surge, even in the presence of high circulating P4 concentrations, and this can increase the percentage of animals that ovulate to the GnRH treatment [14,33]. In addition to the use of an adequate GnRH dose, the ovulation incidence of over 60% in heifers and almost 75% in cows indicates that a high percentage of these females had a follicle with ovulatory capacity at the time of GnRH treatment [34,35]. In heifers, the induction protocol ended 12 d prior to the FTAI protocol and therefore, heifers that were induced to have a CL after the induction protocol (n = 646) would likely be on D7-10 of the estrous cycle, a time of high ovulation incidence in GnRH-treated dairy cows [36]. Also, non-pregnant cows that were beginning the rebreeding protocol on Day 28−32 after previous AI, would also be expected to be on D7-10 of the estrous cycle. Thus, the combination of an elevated dose of GnRH and the stage of the cycle at the initiation of the protocol resulted in a high ovulation response in both heifers and cows in this study. Ovulation was 3 (cows) to 5 (heifers) times greater after GnRH than after EB treatment.

As expected, heifers and cows from the G-groups had a greater number of CL at PGF than animals in the EB-groups. This result can be attributed to the greater ovulation incidence in females receiving GnRH at the beginning of the protocol rather than EB, and to the lesser percentage of cows that underwent luteolysis between D0 and PGF in the G-groups compared to the EB-groups. During both experiments more luteolysis occurred between D0 and PGF for the EB-group, especially during Exp. 1 with almost 40% of EB-treated heifers undergoing luteolysis prior to PGF. It is likely that the high circulating E2 concentrations after EB treatment on D0 activated the E2 receptors, probably E2 receptor α that is present on uterine endometrial cells initiating a premature luteolytic process [37]. In sheep, for example, administration of 0.750 mg EB on D9 of the estrous cycle caused an increase in endometrial oxytocin receptors and subsequent luteolysis [38]. In addition, Robinson et al. [39] reported an increase in E2 receptor α mRNA in the luminal epithelium and epithelial cells of the superficial glands during the bovine luteal phase. Other studies have also reported an elevated luteolysis incidence in protocols initiated with EB compared to GnRH in *Bos taurus* cattle [40,41].

A larger dominant follicle was observed on D7 of the protocols initiated with GnRH compared to EB in both Exp. 1 and 2. It is well known that larger ovulatory follicles have been associated with greater fertility during FTAI protocols in *Bos indicus* cattle [42]. The larger size of the dominant follicle is likely related to two factors. First, the high ovulation incidence after D0 in GnRH-treated females, leads to an earlier emergence of the ovulatory follicular wave compared to the gonadotropin suppression and delayed wave emergence that occurs in EB/P4-treated cattle [10]. Second, the FTAI protocols initiated with GnRH also had treatment with eCG and PGF 1 d earlier (D6) than in the EB FTAI protocol. Thus, the earlier decrease in P4 could drive an increase in endogenous LH pulses and this effect, combined with the earlier exogenous eCG treatment, could have also contributed to the larger follicle size in the GnRH groups [43,44]. Nevertheless, the difference in follicle size between the two groups was less dramatic on D9 with only a 0.6 mm difference in diameter in heifers and no significant difference between GnRH and EB in cows (Exp. 2). Thus, the GnRH protocol used in this experiment likely provided an advantage in follicle growth time and follicle stimulation, although the differences became less dramatic during the final 2 d of the protocol in the low P4 and high gonadotropin environment of the proestrous/estrous period prior to AI. Our previous physiologic study that used daily ovarian ultrasound [10] also found earlier emergence of the ovulatory follicle wave during GnRH-based 7-d FTAI protocols in Nelore heifers and cows that ovulated after GnRH administration, consistent with results in *Bos taurus* dairy cattle [45].

Expression of estrus by the time of AI was also greater in heifers and cows from G-groups compared to EB-groups. This is likely related to the larger dominant follicles on D7 in the G-groups, which would be expected to produce greater circulating E2 between D7 and D9 of the FTAI protocol [46], and could stimulate more animals to express estrus prior to AI [47]. In addition, the earlier treatment with PGF could lead to a lower circulating P4 concentration during the proestrous period, even though circulating P4 at the time of PGF treatment should be greater in the GnRH-treated than EB-treated animals. Earlier decrease in circulating P4 could also stimulate development of the dominant follicle [43]. Thus, over 10% points more GnRH-treated females showed estrus than EB-treated females. However, all animals were treated with EC on D7, including animals that received GnRH, and this is likely to have increased the percentage of animals that showed estrus prior to AI, as shown previously [48,49]. Treatment with EC was found to increase expression of estrus most dramatically in heifers that ovulated smaller follicles [48].

All of these protocols produced a high ovulation incidence after D9, which is important because ovulation at the end of the protocol is critical for fertility. Thus, FTAI protocols initiated with either GnRH or EB can produce final ovulation efficiency of over 90%, and high fertility. In addition, no differences were detected between G and EB-groups for double ovulation. As previously observed [10], both synchronization strategies (with GnRH or EB) promoted emergence of a new follicle wave with sufficient time prior to eCG treatment to not have double ovulation, likely due to the dominant follicle deviation/selection process already having occurred [50,51].

The second hypothesis was related to fertility outcomes of conventional EB or GnRH-adapted protocols, and our results clearly

demonstrate that a P4-based protocol starting with GnRH, associated to an eCG administration 1 d before the P4 implant removal and two PGF treatments 24 h apart can achieve similar P/AI in *Bos indicus* cattle compared to the high fertility obtained with P4-based protocols starting with EB. Thus, GnRH-based protocols can be an effective alternative strategy for synchronization programs in *Bos indicus* cattle, especially in countries in which E2-based treatments are not available. However, it should be mentioned that the GnRH protocol required an additional animal handling in order to optimize luteolysis [15–17].

Finally, our third hypothesis was partially supported, that GnRH treatment at the time of AI would increase fertility during P4-based 7-d FTAI protocols. Interestingly, the increase in fertility due to GnRH treatment was only observed in cows that were treated with EB at the start of the protocol and not in cows treated with GnRH at the initiation of the FTAI protocol. A stimulatory effect of GnRH at the time of AI was not observed in either type of FTAI protocol in heifers. The lack of an effect of GnRH in heifers may be due to the high expression of estrus in heifers in this experiment, where more than 80% of GnRH-treated heifers expressed estrus and almost 70% of EB-treated heifers. These heifers likely had an endogenous GnRH/LH surge, associated with estrus, prior to the GnRH treatment at AI. The use of EC on D7 may have contributed to the high expression of estrus [48,49]. Nevertheless, even in heifers that did not express estrus, there was no indication of a stimulatory effect of GnRH on fertility. In contrast, there was an improvement in P/AI of over 10% points due to GnRH treatment in cows that did not express estrus. This could be due to an increase in ovulation efficiency, although in the subset of cows evaluated in this study we did not observe this effect (No GnRH: 88.2% [75/85] vs. GnRH: 90.5% [57/63]). Moreover, GnRH treatment may prevent a delayed LH surge and ovulation producing a more optimal timing of ovulation and AI [52]. Thus, treatment with GnRH at the time of AI can improve fertility in certain situations, particularly in FTAI protocols that have reduced expression of estrus and smaller dominant follicles at the end of the protocol.

In summary, P4-based 7-d FTAI protocols that were initiated with either GnRH or EB produced high P/AI in *Bos indicus* heifers and cows. The different protocol strategies (EB vs. GnRH) resulted in interesting differences in follicle size, CL number during protocol, and expression of estrus, but both had adequate synchronization of the preovulatory follicular wave, size of the ovulatory follicle, ovulation efficiency at the end of the protocol, and P/AI. Importantly, this study demonstrated that similar fertility can be obtained using an adequately designed FTAI protocol with GnRH as obtained with EB-based protocols. Additionally, use of GnRH at the time of AI can be a strategy to enhance fertility in certain situations in *Bos indicus* cattle.

CRediT authorship contribution statement

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

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