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# GnRH34 with or without estradiol cypionate in timed AI in *Bos indicus* beef cows

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ABSTRACT

Two experiments were performed to evaluate the effects of GnRH treatment on the fertility of suckled Nelore beef cows treated with an estradiol/progesterone (E2/P4)-based protocol for timed artificial insemination (TAI). Experiment 1 focused on determining the effects of estradiol cypionate (EC) on ovulation in TAI cows treated with GnRH 34 h after removal of the intravaginal P4 device (IPD). Suckled cows (n = 26) were treated with 2 mg estradiol benzoate (EB) and IPD containing 1 g P4. After 8 days, IPDs were removed, and all cows were treated with 150 μg of d-cloprostenol (prostaglandin F2 alpha analog) and 300 IU of equine chorionic gonadotropin (eCG), then separated into two treatment groups consisting of cows who received 1) saline 0.9% i.m. (GnRH34 group) or 2) 0.6 mg i.m. of EC (EC-GnRH34 group). On day 9 (05:00 p.m.), all cows were given GnRH (10.5 μg of buserelin acetate) i.m. No differences were observed between the groups ($P > 0.05$) in the time of ovulation after IPD removal or in the proportion of cows ovulating. Experiment 2 focused on determining the effects of GnRH34 along with or in the absence of EC on day 8 on pregnancy per AI (P/AI) in postpartum beef cows. Cows (n = 981) were treated similarly to those in Experiment 1, but an additional group, the EC-GnRH48 group, was included, in which cows received EC on day 8 whereas those that did not show estrus received GnRH at TAI. Thus, in this experiment, groups consisted of GnRH34 (n = 322), EC-GnRH34 (n = 335), and EC-GnRH48 (n = 324). A higher rate of estrus expression was observed in cows treated with EC following IPD removal (EC-GnRH34: 69%, EC-GnRH48: 64.8%) than in cows in the GnRH34 group (45.6%). No difference in P/AI was observed between the treatment groups ($P = 0.45$), but P/AI in cows in the EC-GnRH34 group (64.2%) tended to be greater ($P = 0.1$) than in cows in the GnRH34 group (58%). In summary, although ovulation synchrony did not differ among the groups, P/AI in cows treated with EC and GnRH 34 h after IPD removal tended to be greater than in cows treated solely with GnRH; this was most likely due to a shorter proestrus/estrus period, considering the lower proportion of cows that displayed estrus in the GnRH34 group. Finally, given that P/AI did not differ between the EC-GnRH34 and EC-GnRH48 groups, our results suggest that, for cows not displaying estrus, administration of EC at the time of IPD removal followed by treatment with GnRH 48 h afterward represents the most cost-efficient TAI strategy for South American Zebu-based beef operations.



## 1. Introduction

Controlling the estrous cycle of suckled beef cows with exogenous hormones based on estradiol/progesterone (E2/P4) treatment

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facilitates the use of artificial insemination (AI) in South American beef production systems. In addition, the development and growing adoption of timed AI (TAI) programs have significantly increased the use of AI in Brazil over the past 20 years; at present, it is estimated that ~25% of beef cows in the country are now enrolled in TAI programs each year [1].

Ovulation is typically induced via administration of a low dose of estradiol ester following intravaginal P4 device (IPD) removal (usually within 24 h) in TAI protocols [2], depending on the type of estradiol ester used. Estradiol cypionate (EC) is often administered at the time of IPD removal to reduce animal handling [3,4]. Suckled *Bos indicus* beef cows treated with 0.5 mg or 1 mg EC at the time of IPD removal ovulated approximately 78 h and 71 h after administration, respectively [5]. Although EC is widely used to induce ovulation in TAI cows in South American beef operations, the results of a recent study of suckled *B. indicus* beef cows indicated that injection of EC at the time of IPD removal followed by GnRH administration 34 h later (i.e., EC-GnRH34) increased ovulation synchrony and pregnancy per AI (P/AI) relative to EC treatment alone [6]. Furthermore, the authors noted that administration of GnRH 14 h before TAI increased P/AI in cows regardless of estrus expression between IPD removal and TAI, suggesting that cows treated with the EC-GnRH34 protocol had a high P/AI because of optimal synchrony between the time of ovulation and TAI, considering that AIs were performed 14 h after GnRH.

Administration of GnRH induces a preovulatory luteinizing hormone (LH) peak within 2 h [7] and ovulation within 28–30 h [8]. Cedeño et al. [9] demonstrated that GnRH administered 8 h before TAI improved P/AI in suckled *B. taurus* beef cows that did not show estrus. Similarly, GnRH injected at TAI was also shown to increase P/AI in suckled *B. indicus* beef cows that failed to show estrus [10]. However, concurrent treatment with GnRH and AI did not enhance P/AI in beef cows that showed estrus [11–14]. Therefore, the use of GnRH concurrently with TAI (i.e., EC-GnRH48) has become a commonly used approach by veterinarians in South America for treating *B. indicus* cows that do not display estrus. Although previous studies have examined the effects of GnRH after (34 h or 48 h) IPD removal in tropical beef cows [6,10,11,15], how GnRH influences E2/P4-based protocols when administered alone 34 h after IPD removal has not been tested. In light of the current trend towards reducing the use of estradiol in livestock, and considering that GnRH treatment alone decreases exposure to estradiol during proestrus/estrus [16,17], the impacts of EC and GnRH treatment on ovulation induction and fertility after TAI require further investigation.

Here, we hypothesized that ovulation synchrony in cows treated with GnRH 34 h after IPD would be similar to that of cows treated with the EC-GnRH34 protocol (Experiment 1), and have a similar P/AI to cows treated with the EC-GnRH34 and EC-GnRH48h protocols (Experiment 2). Thus, the primary objectives of this study were to compare ovulation synchrony in cows undergoing GnRH34 treatment with EC and in cows undergoing GnRH34 treatment without EC, and to compare the fertility of cows treated with these protocols with the fertility of cows administered with EC-GnRH48, the most common approach used by veterinarians under TAI conditions.

## 2. Material and methods

The Committee for Ethics in Animal Experimentation of the Brazilian Agricultural Research Corporation (Embrapa) approved all procedures performed in the experiments described in this manuscript (Protocol 02/2022).

### 2.1. Experiment 1

Experiment 1 compared ovulation synchrony between Nelore beef cows treated with GnRH34 associated or not with EC treatment.

This study was conducted at the experimental research farm of Embrapa Rondônia (Brazilian Agricultural Research Corporation, Rondônia, Brazil; $08^{0}48'12"$S, $63^{0}50'56"$W). The predominant climate in this region is Am (Köppen Classification). The mean annual air temperature is 26 °C and the mean annual rainfall is 2,095 mm.

Twenty-six suckled multiparous Nelore (*B. indicus*) cows, 45–70 days postpartum (DPP), 4–7 years old (y), weighing 450–600 kg, with a 2.75–3.5 of body condition score (BCS; range 1–5, where 1 = emaciated and 5 = obese) were used. Cows were housed in an outdoor grazing system (*Brachiaria brizantha* pasture) with *ad libitum* access to trace mineral salts and water.

The cows received an IPD (1 g progesterone, Primer®, Agener União, Embu-Guaçu, Brazil) and 2 mg intramuscular (i.m.) of estradiol benzoate (EB; RicBE®, Agener União, Embu-Guaçu, Brazil) at the beginning of the protocol (day 0). The IPDs were removed on Day 8, and all cows received 0.39 mg i.m. of cloprostenol sodium (PGF-analog; Estron®, Agener União, Embu-Guaçu, Brazil), and 300 IU i.m. of equine chorionic gonadotropin (eCG; ECGON®, Biogenesis Bagó, Curitiba, Brazil). On Day 8, cows were homogeneously assigned, according to DPP, age, and BCS (mean ± SD), to one of two treatments: 1) 0.5 mL of Saline 0.9% (GnRH34 group, n = 13, 51.2 ± 10.5 DPP, 5.6 ± 1 y old, and 3.0 ± 0.26 BCS), or 2) 0.6 mg i.m. of estradiol cypionate (EC-GnRH34 group, n = 13; Cipiotec®, Agener União, Embu-Guaçu, Brazil, 49.3 ± 7.7 DPP, 5.9 ± 0.9 y old, and 3.1 ± 0.26 BCS). On Day 9, all cows received 10.5 μg i.m. of buserelin acetate (GnRH; Gonaxal®, Biogenesis Bagó, Buenos Aires, Argentina) 34 h after IPD removal.

The status of the ovulatory follicle was followed by transrectal ultrasonography (Mindray® M5 VET® equipped with 5 MHZ linear probe) at 12 h intervals from IPD removal to ovulation. Ovulation was defined as the disappearance (from one scanning session to the next) of a previously identified follicle greater than or equal to 8 mm in diameter. Once the disappearance of the preovulatory follicle (POF) was detected, the time of ovulation was considered the midpoint between the last two ultrasonic scanning sections. Cows that did not ovulate 120 h after IPD removal were considered non-responders to the synchronization treatment.

Nine days after IPD removal, both ovaries were scanned and the location of the corpus luteum (CL) was recorded for each animal. Subsequently, short color-flow mode (CFM) videoclips (7 s duration) were recorded. The luteal tissue area (LTA), CL blood flow (CLBF), and adjusted CLBF (CLBF: LTA ratio) were calculated as shown elsewhere [6].

Blood samples were collected, nine days after IPD removal by caudal venipuncture into 10 mL tubes containing EDTA (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Blood samples were centrifuged (1500×*g* for 15 min), and plasma was harvested and stored at −20 °C. Plasma progesterone concentrations were determined using a chemiluminescence assay (ADVIA Centaur Systems Progesterone Kit; Siemens, Ref. 01586287; sensitivity of 0.21 ng/mL), with an intra- and inter-assay coefficients of variation below to 12%.

### 2.2. Experiment 2

Experiment 2 was designed to compare the P/AI between the GnRH34, EC-GnRH34, and EC-GnRH48 protocols in postpartum

Nelore beef cows. The EC-GnRH48 protocol was included as a third group to compare the GnRH34 and EC-GnRH34 treatments with a conventional protocol that uses GnRH at TAI 48 h after IPD removal but only in cows that did not display estrus from IPD removal to TAI (Sa Filho, 2014).

This study was conducted on two commercial farms in the Amazon biome. The animals were maintained in a grazing system (*Brachiaria brizantha*) with *ad libitum* access to trace mineral salts and water. This study used 981 suckled Nelore cows (214 primiparous and 767 multiparous) with BCS ranging from 2.5 to 4. The TAI protocol (similar to Experiment 1) was initiated between 30 and 120 days postpartum. The BCS of the cows was also evaluated using the VetScore device, which classifies the BCS of cows included in TAI programs as low (L), adequate (A), or excessive (E) [18]. In addition to the groups in Experiment 1, the EC-GnRH48 group was included in Experiment 2. For this group, cows received 0.6 mg i.m. of EC on Day 8 and cows that did not display estrus between IPD removal and TAI, on Day 10, received a dosis of GnRH (10.5 μg i.m. of buserelin acetate, Gonaxal®, Biogenesis Bagó, Buenos Aires, Argentina) at that time. The groups used in this study were the GnRH34 (n = 322), EC-GnRH34 (n = 335), and EC-GnRH48 (n = 324) groups. The distribution of cows according to category, BCS, and calving–TAI interval is shown in Table 1.

On Day 8, all cows were painted with a chalk marker on the sacrocaudal region to identify animals that displayed estrus. At TAI, estrus was evaluated, and deemed to have occurred in cows without a tail-head chalk mark (>75% paint loss). All cows were inseminated 48 h after IPD removal. Frozen/thawed semen from six sires was used for TAI and equally distributed between treatments. The cows included in this study were not exposed to sires after TAI. The pregnancy status was assessed by ultrasonic examination 30 days after TAI. Pregnancy was determined by visualizing embryonic vesicles and embryos.

### 2.3. Statistical analyses

All statistical analyses were performed using SAS software (SAS Institute, Cary, NC, USA). In Experiment 1, single-point outcome variables (e.g., time of ovulation, follicle growth, diameter of the POF, LTA, CLBF, adjusted CLBF, and plasma progesterone concentration) were analyzed using ANOVA (PROC GLIMMIX; SAS Inst. Inc., Cary, NC). The ovulation rate was analyzed using the Chi-square test.

In Experiment 2, the occurrence of estrus and P/AI was analyzed using logistic regression. The variables initially included in the model were treatment, farm, sire, BCS (LAE scale), category (primiparous or multiparous), and calving-TAI interval (early, <40 d; middle, 41–60 d; late, > 60 d). These variables were considered fixed effects. The variables farm, sire, BCS, and calving-TAI interval had no significant effect on the occurrence of estrus and P/AI, and were therefore excluded from the model. Logistic regression was used to analyze the effects of treatment, category, and their interactions on the occurrence of estrus and P/AI. Another round of analyses was performed to evaluate the effect of treatment on P/AI according to the occurrence of estrus (yes/no). Therefore, the effect of treatment on P/AI was evaluated using the chi-square test for each estrus status (yes/no).

Results are expressed as means ± SEM or as percentages. In all analyses, differences were considered significant when $P \leq 0.05$, and a probability between 0.051 and 0.1 was considered a trend.

**Table 1**
Number of cows in each category, BCS, and average of calving-TAI interval according to groups in Experiment 2.

| | GnRH34 | EC-GnRH34 | EC-GnRH48 |
|---|---|---|---|
| Category | | | |
| Primiparous | 69 | 72 | 73 |
| Multiparous | 253 | 263 | 251 |
| BCS by Vetscore[a] | | | |
| Red | 31 | 34 | 40 |
| Green | 278 | 290 | 269 |
| Yellow | 13 | 11 | 15 |
| Farm | | | |
| 1 | 238 | 244 | 242 |
| 2 | 84 | 91 | 82 |
| Calving - TAI interval | 71,88 ± 6,55 | 72,51 ± 6,60 | 73,61 ± 6,74 |

[a] Devices used to classify the BCS of cows subjected to TAI protocols into red (low), green (adequate), and yellow (excessive).

## 3. Results

### 3.1. Experiment 1

The ovarian responses observed in Experiment 1 are summarized in Table 2. No differences in the time of ovulation, proportion of cows ovulating, or POF diameter were detected between the groups ($P > 0.05$). However, cows in the EC-GnRH34 group had a higher follicle growth rate than cows in the GnRH34 group ($P = 0.04$). The distribution and percentage of cows that ovulated after IPD removal in the GnRH34 and EC-GnRH34 groups are shown in Fig. 1. In both groups, most animals ovulated 60–72 h after IPD removal.

The CL characteristics such as LTA, CLBF, adjusted CLBF, and plasma progesterone concentration also did not differ ($P > 0.05$) between treatments (Fig. 2).

### 3.2. Experiment 2

There were no significant effects of farm ($P = 0.36$), sire semen used for TAI ($P = 0.26$), BCS (based on VetScore; $P = 0.73$), or calving-TAI interval ($P = 0.49$) on P/AI (Table 3). Proportions of cows detected in estrus at TAI and P/AI according to treatment and animal category are presented in Table 4. The P/AI did not differ among the groups ($P = 0.45$) or between categories ($P = 0.12$). In addition, no treatment × category interaction ($P = 0.5$) on P/AI was observed. Conversely, the P/AI in the group EC-GnRH34 tended to be greater than that in the GnRH34 group ($P = 0.1$). The proportion of cows detected in estrus was greater ($P < 0.0001$) among cows treated with EC (EC-GnRH34 and EC-GnRH48) than among cows in the GnRH34 group. Moreover, the proportion of multiparous cows in estrus was greater ($P < 0.0001$) than for primiparous cows, and no interaction effect of treatment × category ($P = 0.63$) on the proportion of cows in estrus was observed.

The P/AI according to estrus detection is shown in Fig. 3. Considering only cows that did not display estrus in Experiment 2, those treated solely with GnRH 34 h after IPD removal had a greater ($P < 0.05$) P/AI than did cows in the EC-GnRH48 group. In contrast, considering only cows that displayed estrus, those in the GnRH34 group had a lower ($P < 0.05$) P/AI than did cows in the EC-GnRH34 and EC-GnRH48 groups (Fig. 3).

## 4. Discussion

We compared ovulation synchrony and fertility in suckled *B. indicus* beef cows subjected to different treatment protocols that involve use of GnRH along with EC or in the absence of EC. The results of our experiments provided partial evidential support for our hypotheses; although the time of ovulation and ovulation synchrony in cows treated with the GnRH34 protocol were similar

**Table 2**
Ovarian responses in Nelore suckled cows treated with GnRH34 or EC-GnRH34 for ovulation induction in the Experiment 1. All cows were treated with GnRH 34 h after IPD removal. However, only cows from EC-GnRH34 group were treated with 0.6 mg of EC at IPD removal.

| | GnRH34 | EC-GnRH34 | P-value |
|---|---|---|---|
| Time of ovulation (h)[a] (95% confidence intervals) | 61.6 ± 2.7 (55.9–67.4) | 68.0 ± 2.6 (62.5–73.5) | 0.11 |
| Proportion of cows ovulating by 120 h after IPD removal | 84.6% (11/13) | 92.3% (12/13) | 0.53 |
| Diameter of POF[b] (mm) | 13.2 ± 0.69 | 12.8 ± 0.68 | 0.68 |
| Follicle growth rate[c], mm/d | 0.9 ± 0.17 | 1.4 ± 0.16 | 0.04 |

[a] After IPD removal.
[b] POF, preovulatory follicle.
[c] Difference calculated from the maximal diameter of POF minus the diameter of POF on day 9 divided by days.

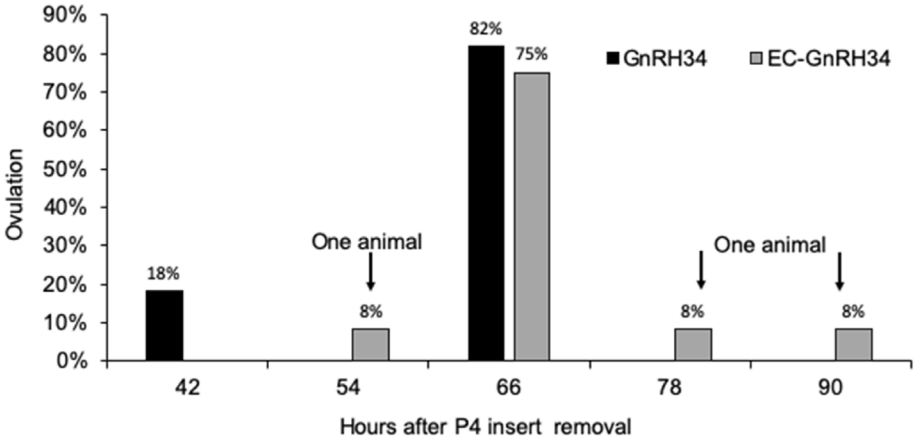


**Fig. 1.** Distribution and percentage of cows ovulating after IPD removal in Nelore suckled cows treated with GnRH34 or EC-GnRH34 for ovulation induction (Experiment 1). All cows were treated with GnRH 34 h after IPD removal. However, only cows from EC-GnRH34 group were treated with 0.6 mg of EC at IPD removal.

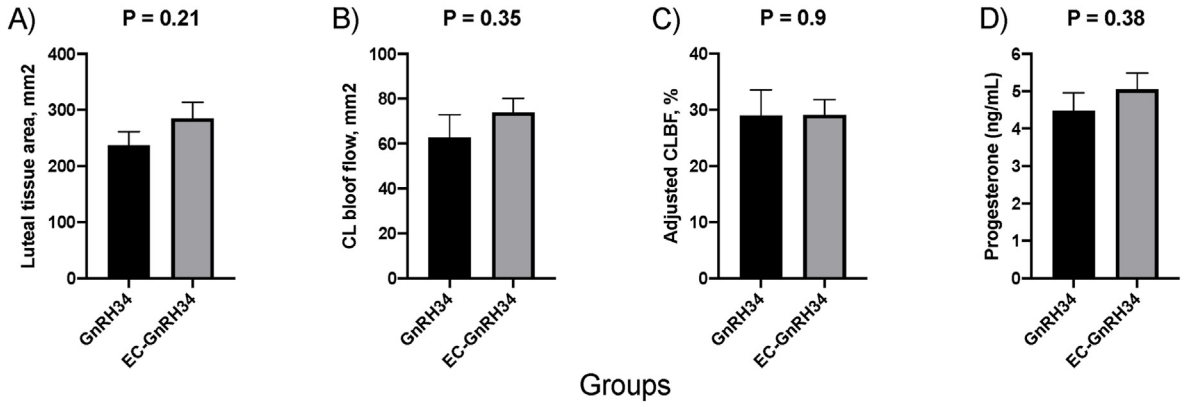


**Fig. 2.** Features of the corpus luteum (CL) and plasma progesterone concentration in Nelore suckled cows treated with GnRH34 or EC-GnRH34 for ovulation induction (Experiment 1). All cows were treated with GnRH 34 h after IPD removal. However, only cows from EC-GnRH34 group were treated with 0.6 mg of EC at IPD removal. Data are mean ± SEM. (A) Luteal tissue area (LTA); (B) amount of blood flow to the CL (CLBF); (C) CLBF:LTA ratio (adjusted CLBF in %), and (D) plasma progesterone concentration.

to those in cows treated with EC-GnRH34, P/AI was generally greater for cows treated with EC-GnRH34 than for those treated with GnRH34, and was similar to those treated with EC-GnRH48.

Although no differences were observed in POF diameter between the groups in Experiment 1, follicle growth rate was higher among cows treated with EC than in cows that were not given EC. Previous study has shown that when injected at the end of the E2/P4-based protocol, EC promotes ovulation by inducing an LH peak in *B. indicus* [19] and *B. taurus* cows [20]. Estrogens has been implicated in synergizing action with FSH for granulosa cell differentiation including CYP19A1 (aromatase) and LHCGR (LH receptor) upregulation [21,22]. This suggests that the higher follicle growth rate observed in cows treated with EC might be due to increased LH receptor abundance, which is maximal in granulosa cells from preovulatory follicles immediately before ovulation induction [23].

In the E2/P4-based protocols, in which ovulation was induced with 0.5 mg or 1 mg EC at the time of IPD removal, an increase in serum estradiol concentration was observed 24 h after treatment [24]. Application of EC increases circulating E2 in the preovulatory period, and such an increase, along with additional E2 released from the follicle, may be required to trigger estrus, and has also been associated with postovulatory uterine function [24–27]. These changes appear to play critical roles in the programming of uterine function during early pregnancy. For instance, serum E2 concentrations at the time of GnRH treatment is considered to be among

**Table 3**
Pregnancy per artificial insemination (AI) according to farm, sire semen used for timed AI, body condition score (BCS) evaluated by the Vetscore's device, and calving-TAI interval in the Experiment 2.

| | | Pregnancy per AI, % | P-value |
|---|---|---|---|
| Farm | 1 | 61.6 (446/724) | 0.36 |
| | 2 | 58.4 (150/257) | |
| Sire semen | 1 | 55.6 (75/135) | 0.26 |
| | 2 | 68.1 (64/94) | |
| | 3 | 59.5 (179/301) | |
| | 4 | 66.9 (109/163) | |
| | 5 | 62.9 (56/89) | |
| | 6 | 56.8 (113/199) | |
| BCS by Vetscore[a] | Red | 61.9 (65/105) | 0.73 |
| | Green | 60.8 (509/837) | |
| | Yellow | 56.4 (22/39) | |
| Calving-TAI interval | Early | 59.3 (182/307) | 0.49 |
| | Middle | 59.7 (221/370) | |
| | Late | 63.5 (193/304) | |

[a] Devices used to classify the BCS of cows subjected to TAI protocols into red (low), green (adequate), and yellow (excessive).

the most important factors determining pregnancy outcomes after embryo transfer in recipient cows [28]. Increased P/AI in cows treated with EC-GnRH34 indicates that EC injection, as performed in Experiment 2, may contribute to greater pregnancy rates in TAI beef cows. Therefore, although GnRH34 provided optimal ovulation synchronization (with ~85% of the cows ovulating between 60 and 72 h), our results suggest that EC injection may be necessary to achieve satisfactory fertility outcomes in GnRH34 cows.

Intramuscular injection of GnRH induces ovulation within 26–30 h [8,29–31]. Previous studies have shown that cows treated with EC-GnRH34 ovulated at 66.0 ± 0.0 h, whereas cows treated only with EC, as an ovulation inducer, ovulated ~11 h later [6]. In the present study, cows subjected to the GnRH34 and EC-GnRH34 protocols ovulated at 61.6 ± 2.7 h and 68 ± 2.6 h after IPD removal, respectively. Therefore, for these groups, we estimated that the mean intervals from TAI to ovulation were approximately 14 h and 20 h, respectively. The timing of AI in relation to ovulation is important for attaining a successful pregnancy rate [32]. Previously, we found that fertility was improved when synchrony between the time of ovulation and TAI was increased [33]. A second reproductive characteristic closely associated with fertility in cows subjected to E2/P4-based TAI programs is the capacity of cows to display estrus before TAI. Previous work has shown that cows that have undergone TAI in estrus were more likely to become pregnant than were cows that did not display estrus [11,34,35], inspiring researchers to explore the strategic use of GnRH in cows that do not enter into estrus during TAI [14,36,37]. Programs of TAI, in which cows that were not in estrus until 48 h after IPD removal received GnRH and were inseminated 8 h later have generated intriguing results [9], suggesting that TAI performed 8 h [9] or 14 h [6] after GnRH administration are nearer to the time of ovulation. Thus, cows that did not display estrus and were conventionally treated with GnRH concurrently with AI would have a longer interval between TAI and ovulation, consequently resulting in ovulation more likely to occur in the absence or reduced number of viable sperm cells in the female oviducts [38]. Moreover, it was also reported that treatment with EC at the time of IPD removal may induce estrus independent of the presence of a preovulatory follicle in the ovary – that is, pharmacological estrus with no endogenous E2 production [39]. In light of the fact that some EC-treated cows in the EC-GnRH48 group displayed pharmacological estrus and that the TAI-ovulation interval of these cows may not be optimal for fertility,

**Table 4**
Estrus expression rate at timed AI, and pregnancy per AI according to treatment (GnRH34, EC-GnRH34, and EC-GnRH48), and to animal category (primiparous and multiparous). Cows from GnRH34 group were not treated with EC at IPD removal and were treated with GnRH 34h later. Only cows from EC-GnRH34 and EC-GnRH48 groups were treated with EC at IPD removal; however, EC-GnRH34 cows were treated with GnRH 34h later. In EC-GnRH48 group, only cows that did not display estrus received GnRH at TAI.

| | Group | | | Category | | P-value | | |
|---|---|---|---|---|---|---|---|---|
| | GnRH34 | EC-GnRH34 | EC-GnRH48 | Primiparous | Multiparous | Group | Category | Group*Category |
| Estrus expression rate, % (n/total) | 45.6$^{A}$ (147/322) | 69.0$^{B}$ (231/335) | 64.8$^{B}$ (210/324) | 37.8 (81/214) | 66.1 (507/767) | <0.0001 | <0.0001 | 0.63 |
| Pregnancy per AI, % (n/total) | 58.0$^{a}$ (187/322) | 64.2$^{b}$ (215/335) | 59.9$^{ab}$ (194/324) | 54.7 (117/214) | 62.4 (479/767) | 0.45 | 0.12 | 0.5 |

$^{AB}$Different letters indicate difference between treatments ($P < 0.05$).
$^{ab}$Different letters indicate tendency between treatment ($P \leq 0.1$).

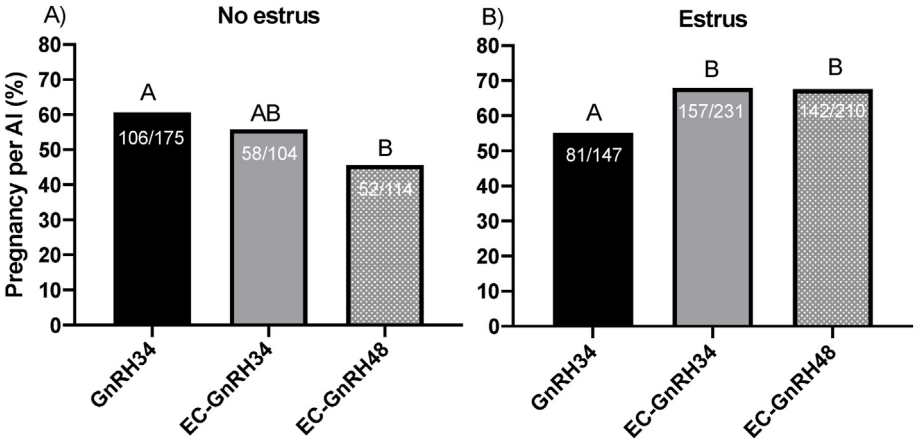


**Fig. 3.** Pregnancy per AI in suckled Nelore beef cows according to treatment (GnRH34, EC-GnRH34, and EC-GnRH48), and estrus detection at TAI (Experiment 2). Cows from GnRH34 group were not treated with EC at IPD removal and were treated with GnRH 34h later. Only cows from EC-GnRH34 and EC-GnRH48 groups were treated with EC at IPD removal; however, EC-GnRH34 group was treated with GnRH 34h later. Cows from EC-GnRH48 received GnRH at TAI only in cows that did not display estrus. Different letters indicate the effect between treatments ($P < 0.05$). The number of pregnant cows and the total number of cows per treatment are represented on each bar.

we were optimistic that P/AI would be greater among EC-GnRH34-treated cows than it would be in cows in the EC-GnRH48 protocol. However, although P/AI was 7.2% higher in cows treated with EC-GnRH34 than those treated with EC-GnRH48, fertility did not significantly differ between the two groups.

Notably, the GnRH34 protocol achieved the highest P/AI in cows that did not exhibit estrus. It has been shown that cows in estrus ovulate earlier than those that did not show estrus at TAI [20,40], and cows with longer AI-ovulation intervals had a lower likelihood of pregnancy [33]. As such, we inferred that the greater rate of P/AI in cows that did not display estrus at the time of TAI was most likely due to the shortened AI-ovulation interval in cows treated with GnRH 34 h after IPD removal, disregarding estrus status, as was observed in the GnRH34 and EC-GnRH34 groups. However, it is important to note that the GnRH34 group had a greater proportion of cows that did not display estrus.

To evaluate the effects of GnRH34 and EC-GnRH34 on CL function in Experiment 1, we examined luteal tissue area (LTA), blood flow to the CL (CLBF), the CLBF:LTA ratio, and plasma P4 concentration, but none of these variables differed among the experimental treatments. Likewise, Barbosa et al. [6] also did not observe differences in CL function between cows treated with EC-GnRH34 and cows treated solely with EC at the time of IPD removal. We therefore concluded that the enhanced P/AI we observed following EC-GnRH34 treatment was not associated with increased ovulation synchrony or improved luteal function. However, the effect EC has on the endometrium might account for this result, as reported previously [24].

## 5. Conclusion

Although ovulation synchronization did not differ between the groups, cows treated with the EC-GnRH34 protocol tended to have improved P/AI relative to cows treated solely with GnRH34. Moreover, no differences in P/AI were observed between cows in the EC-GnRH34 and EC-GnRH48 groups, which represent the conventional protocols used in South American beef production operations. As such, considering the extent of direct handling required to treat cows with GnRH 34 h after P4 removal, we suggest that administration of GnRH at TAI for cows that fail to show signs of estrus up to 48 h after IPD removal is the most cost-effective and efficient approach to TAI, as this strategy results in a P/AI similar to that observed in cows treated with the EC-GnRH34 protocol.

## Declaration of competing interest

The authors declare no conflicts of interest that could be perceived as prejudicing the impartiality of the reported research.

## CRediT authorship contribution statement

**Samira A. Silva:** Data curation, Writing – original draft, Perform the experiment, writing the MS. **Rafael G. Mondadori:** Data curation, Formal analysis, Writing – original draft, Formal analysis, writing the MS. **Gabrielly S. Noleto:** Perform the experiment. **Ingrid P. Barbosa:** Perform the experiment. **Reuel L. Gonçalves:** Formal analysis, writing the MS. **Bernardo G. Gasperin:** Data curation, Formal analysis, Writing – original draft, Formal analysis, writing the MS. **Monique T. Rovani:** Data curation, Formal analysis, Writing – original draft, Formal analysis, writing the MS. **Eanes F. Paz:** Perform the experiment. **Leonardo S. Gomes:** Perform the experiment. **Luiz F.M. Pfeifer:** Formal analysis, Writing – original draft, Perform the experiment, Formal analysis, Data curation, writing the MS.

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