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Influence of GnRH analog and dose on LH release and ovulatory response in *Bos indicus* heifers and cows on day seven of the estrous cycle

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ABSTRACT

This study evaluated the influence of GnRH analogs (gonadorelin *vs*. buserelin) and doses (single *vs*. double) on LH release and ovulatory response in *Bos indicus* (Nelore) females on Day 7 of the estrous cycle. Cycling heifers and non-lactating cows were pre-synchronized: Day −10: progesterone (P4) implant insertion plus 2 mg of estradiol benzoate; Day −2: implant removal and 0.53 mg of cloprostenol sodium (PGF); Day 0: 25 μg of lecirelin (GnRH). Over four replicates, heifers (n = 57) and cows (n = 53) that ovulated to the GnRH treatment on Day 0, having a visible corpus luteum (CL) and a dominant follicle (DF) ≥ 8.5 mm, were allocated to receive the following GnRH treatments on Day 7: **G-Single** (100 μg of gonadorelin); **G-Double** (200 μg of gonadorelin); **B-Single** (10 μg of buserelin); and **B-Double** (20 μg of buserelin). At GnRH treatment, a P4 implant was inserted in heifers (0.5 g) and cows (1 g). Ultrasound examinations were done on Days −10, −2, 0, 2, 7, 9, 12, and 14 to evaluate DF diameter, ovulation and presence of CL. Blood samples were collected on Day 7 at 0, 2, and 4 h from GnRH treatment, to evaluate circulating P4 and LH concentrations. On Day 12, the P4 implant was removed, females received two PGF treatments (24 h apart), and 2 d later, 25 μg of GnRH was given to start the next replicate. In both heifers and cows, P4 concentrations were elevated on Day 7, and similar among groups (3.9 and 4.2 ng/mL, respectively). In heifers, buserelin induced greater LH peak (9.5 *vs*. 2.6 ng/mL; $P < 0.01$) and greater ovulation (88.9 [24/27] *vs*. 16.7% [5/30]; $P < 0.01$) than gonadorelin treatments, regardless of the dose. Similarly, in cows, buserelin induced greater LH peak than gonadorelin (9.9 *vs*. 4.9 ng/mL; $P < 0.01$). However, ovulation was only increased in cows from the B-Double group (90.9% [10/11]), whereas in the other groups the ovulatory response was similar (35.7% [15/42]). Regardless of treatment, heifers had similar P4 concentrations ($P = 0.22$), but smaller DF ($P < 0.01$) than cows on Day 7. Only in G-Double group the LH peak was lower ($P = 0.05$) in heifers than in cows, with no difference within other groups. In heifers, but not in cows, the single dose of buserelin resulted in high ovulatory response, equivalent to that produced by the double dose. In conclusion, in *Bos indicus* heifers and cows on Day 7 of the cycle, with elevated P4 concentrations, buserelin induced greater LH release and ovulatory response than gonadorelin treatments. Double doses increased the LH release, however, only resulted in greater ovulation in females treated with buserelin. Finally, although circulating P4 concentrations did not differ between parities, heifers were more likely to ovulate in response to a GnRH-induced LH peak than cows.

1. Introduction

Gonadotropin-releasing hormone (GnRH) is a decapeptide synthesized and secreted by hypothalamic neurons [1], and responsible for the primary regulation of female reproductive functions through the hypothalamic-pituitary-ovarian axis [2]. From its first characterization in mammals [3], native or synthesized GnRH has been used in reproductive management [4,5] as a strategy to stimulate the release of

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gonadotropins (LH and FSH) by the pituitary gland, modulating ovarian responses [6–8]. Treatment with GnRH analogs, therefore, has been widely adopted in reproductive programs for beef cattle [9–11], aiming to induce an LH surge, and thereby ovulation, such as in timed artificial insemination (TAI) protocols. Analogs of GnRH can be used either at the onset of a protocol, to synchronize the emergence of a new follicular wave after ovulation [12,13], or at the end of the protocol, to optimize ovulation and fertility [14,15].

Previous studies, however, have reported unsatisfactory pregnancy per artificial insemination (P/AI) in *Bos indicus* beef cows submitted to GnRH-based TAI protocols [16–18], associated with ovulatory failure and lack of emergence of a new follicular wave at the beginning of the protocol [19,20]. Similar results were reported in *Bos indicus*-influenced beef heifers [21,22]. The ovulatory response to the first GnRH in TAI protocols may be affected by many factors, such as: absence of a dominant follicle (DF) with ovulatory capacity at the time of GnRH treatment [23,24]; stage of the estrous cycle [25,26]; and cyclic status [22]. Moreover, studies have reported that circulating progesterone (P4) concentrations (physiological concentrations during the estrous cycle or exogenously manipulated) had a suppressive effect on the GnRH-induced LH surge, impairing ovulation [26–28].

In order to improve the GnRH-induced LH surge in cows with high circulating P4, some studies have evaluated the administration of various GnRH analogs [29,30] and increased doses [27,31,32]. Since the first characterization of the native GnRH, several analogs have been developed focused on improving the affinity for the GnRH receptor and producing GnRH peptides with greater stability and resistance to degradation, usually through structural modifications in the amino acid chain [33]. Currently, the GnRH analogs commercially available and most frequently used in cattle reproduction are: gonadorelin, buserelin, and lecirelin. Picard-Hagen et al. [29] evaluated the effect of these analogs on the GnRH-induced LH release in Holstein heifers at 6 or 7 d after estrus, and reported lower LH concentrations induced by 100 μg of gonadorelin than 10 μg of buserelin or 25 μg of lecirelin treatments. In a similar study with lactating dairy cows, although there were no differences in the GnRH-induced LH peak, administration of 10 μg of buserelin resulted in greater LH concentrations at 3 and 4 h after treatment compared with 100 μg of gonadorelin [30]. In addition, studies have reported a positive effect of an increased dose of gonadorelin (200 μg) on LH release in beef heifers [27] and lactating dairy cows [32], under high P4 concentrations. Nevertheless, none of these studies was able to detect differences in ovulatory response in relation to the analog or dose administered. Of particular interest, in a study from our research group [12], administration of a higher dose of buserelin (16.8 μg) at the onset of TAI protocols resulted in relatively high ovulatory responses in Nelore heifers (60.3%) and lactating cows (73.6%), and satisfactory P/AI (~58 and ~61%, respectively).

Responsiveness to GnRH, under high P4 concentrations, can also be influenced by animal parity. Colazo et al. [26] reported greater LH concentrations in *Bos taurus* beef heifers than in cows, under elevated P4 concentrations, after treatment with 100 μg of gonadorelin. In their study, apparently, cows were more sensitive to the suppressive effect of P4 concentrations than heifers, although the ovulatory response did not differ between heifers and cows. No previous study has evaluated the response to GnRH treatments in *Bos indicus* heifers and cows in the presence of elevated circulating P4 concentrations. As reported, there are several physiological and metabolic differences between *Bos indicus* and *Bos taurus* cattle. For instance, *Bos indicus* have smaller ovulatory follicle and subsequent corpus luteum (CL), and slower follicular growth rate [34] compared with *Bos taurus* cows. However, *Bos indicus* have higher circulating estradiol (E2) and P4 concentrations [34,35], probably due to greater production and lower expression of genes and enzyme pathways associated with steroid metabolism in the liver [36]. Studies also reported a lower LH pulse frequency [36] and a lower GnRH-induced LH peak [37] in *Bos indicus* than in *Bos taurus* heifers, under both low and high P4 concentrations.

Furthermore, studies reported differences in ovarian function and hormonal environment between heifers and cows, in both *Bos taurus* [26,38] and *Bos indicus* [39,40] cattle. In Nelore cattle, although there are few reports comparing ovarian and endocrine function between parities, studies reported lower circulating P4 concentrations [40] and smaller diameter of DF [41] in heifers than in cows.

Therefore, the aim of this study was to evaluate the influence of the GnRH analog (gonadorelin *vs*. buserelin), and the dose (single *vs*. double) on the GnRH-induced LH release in *Bos indicus* heifers and cows, on Day 7 of the estrous cycle, as well as to evaluate the potential impact of these treatments on ovulatory response. Three main hypotheses were proposed: 1) buserelin treatments promote greater LH release and ovulatory response than gonadorelin treatments; 2) regardless of the analog, use of a double dose of GnRH increases LH release and ovulatory response compared with a single dose; and 3) on Day 7 of the estrous cycle, heifers have lower circulating P4 concentrations, but greater GnRH-induced LH release.

2. Materials and methods

2.1. Location, animals and management

This study was conducted from April to June of 2019 in a commercial beef farm located in Itatinga, SP, Brazil. Cycling Nelore heifers (n = 20; body condition score [BCS; 1 to 5 scale] = 3.4 ± 0.1; 26 ± 0.7 mo of age) and non-lactating multiparous Nelore cows (n = 19; BCS = 3.4 ± 0.1; 448.9 ± 54.2 d post-partum; 2 to 5 parities) were enrolled in this study, over four replicates of the experimental design. Non-lactating cows used in this study were designated to be no longer bred, based on productive outcomes, according to the criteria of the farm. However, these cows were kept on the farm for experimental purposes. Prior to the beginning of the experiment, all heifers and cows were examined weekly by ultrasound and confirmed as cyclic based on the presence of CL, in at least 2 consecutive evaluations. All the females were kept on pasture (*Brachiaria brizantha*), with water and mineral salt *ad libitum,* and received a daily supplementation based on corn plus soybean concentrate. The Animal Research Ethics Committee of Luiz de Queiroz College of Agriculture (ESALQ) approved all animal procedures (Protocol CEUA # 2018–18).

2.2. Experimental design

Initially, all the females were submitted to a pre-synchronization protocol, that started on Day −10 with the insertion of a disinfected 1 g intravaginal P4 implant [42], previously used for 8 d (Repro neo, GlobalGen vet science, Jaboticabal, SP, Brazil), with simultaneous administration of 2 mg of E2 benzoate im (EB; Syncrogen, GlobalGen vet science). On Day −2, 0.53 mg of cloprostenol sodium (PGF; Induscio, GlobalGen vet science) was given im, at the same time as P4 implant removal, and 2 d later (Day 0) all females received 25 μg of lecirelin acetate im (GnRH; TecRelin, Agener União, Embu-Guaçu, SP, Brazil) to induce ovulation and synchronize the emergence of a new follicular wave. Over four replicates, only heifers (n = 57) and cows (n = 53) that ovulated in response to the GnRH treatment on Day 0 and had a visible CL and a DF ≥ 8.5 mm on Day 7 (i.e., on Day 7 of the estrous cycle) were enrolled in the experimental treatments (Fig. 1). Immediately after the ultrasound examination on Day 7, synchronized heifers and cows were randomly assigned to a 2 × 2 factorial arrangement, to receive one of two GnRH analogs (gonadorelin *vs*. buserelin) and one of two doses (single *vs*. double). Therefore, females were allocated to one of the following GnRH treatments: **G-Single** (100 μg of gonadorelin); **G-Double** (200 μg of gonadorelin); **B-Single** (10 μg of buserelin); and **B-Double** (20 μg of buserelin). The GnRH analogs evaluated in this study (gonadorelin: Fertagyl, MSD, Cruzeiro, SP, Brazil; and buserelin: Maxrelin, GlobalGen vet science) were kept refrigerated at 4 °C, and the doses were defined according to the manufacturer's instructions. The

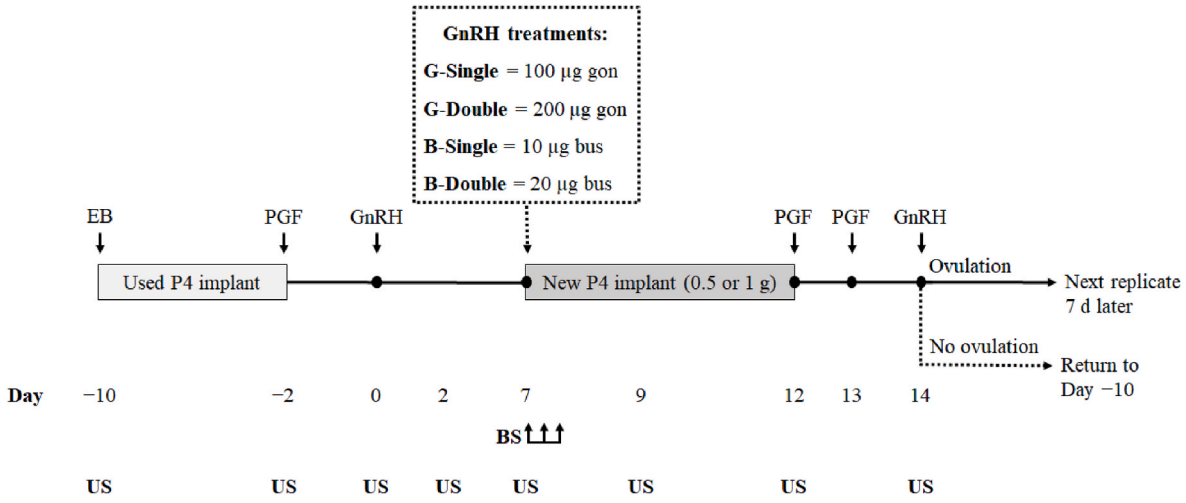

Fig. 1. Schematic representation of the experimental design. Cycling *Bos indicus* (Nelore) heifers and non-lactating cows were submitted to a pre-synchronization protocol starting on Day −10: 2 mg of estradiol benzoate (EB) plus an intravaginal progesterone (P4) implant, previously used for 8 d; Day −2: implant removal and 0.53 mg of cloprostenol sodium (PGF); Day 0: 25 µg of lecirelin (GnRH). Only heifers (n = 57) and cows (n = 53) that ovulated to the GnRH on Day 0 and had a corpus luteum and a dominant follicle ≥ 8.5 mm on Day 7 were assigned to the experimental treatments. On Day 7, heifers and cows were treated with 100 (**G-Single**) or 200 µg (**G-Double**) of gonadorelin, or either 10 (**B-Single**) or 20 µg (**B-Double**) of buserelin, in a 2 × 2 factorial arrangement. Simultaneous with the GnRH treatments on Day 7, heifers received a new 0.5 g P4 implant and cows received a new 1 g P4 implant, which were kept for 5 d. On Days 12 and 13, females received two PGF treatments (24 h apart) and, 2 d later, a new lecirelin treatment was given (correspondingly to Day 0) in order to reassign females to the next replicate. Females that ovulated to this GnRH treatment proceeded directly to the next replicate, whereas females that did not ovulate returned to the pre-synchronization (Day −10). Ultrasound examinations (US) were performed on Days −10, −2, 0, 2, 7, 9, 12, and 14. Blood samples (BS) were collected on Day 7, prior to GnRH treatment, 2 and 4 h later.

single doses were the same as the conventional dose indicated for each analog. In addition, on Day 7, heifers received a new 0.5 g intravaginal P4 implant (Repro one, GlobalGen vet science), and cows received a new 1 g intravaginal P4 implant (Repro neo, GlobalGen vet science). The use of implants with distinct initial P4 loads for heifers and cows was based on strategies commonly used in reproductive management of Nelore heifers and cows submitted to P4-based synchronization protocols [12,14]. The P4 implants were kept until Day 12, and, in order to reassign the females into the next replicate, all of them received two PGF treatments (0.53 mg), 24 h apart, on Days 12 and 13. Then, on Day 14, all females received 25 µg of lecirelin (corresponding to the GnRH treatment on Day 0). The females that ovulated to the GnRH given on Day 14 were assigned to the second replicate 7 d later. Those females that did not ovulate to the GnRH given on Day 14 were resubmitted to the EB + P4 pre-synchronization protocol (Day −10), and reassigned to treatments in the third or fourth replicate of the experiment (Fig. 1). In all replicates, heifers and cows determined as synchronized, based on the criteria established, were designated to their respective treatments on Day 7 by the same technician, always keeping the number of heifers and cows balanced among the four treatments within replicates. However, the same female returning in the next replicate received a different treatment from the previous replicates, to better control the individual effect. In addition, the administration of the experimental treatments on Day 7 was always performed by a different technician, blinded to the treatments.

2.3. Ultrasound evaluations

Ovarian ultrasound evaluations were performed on Days −10, −2, 0, 2, 7, 9, 12, and 14 using a 7.5 MHz linear-array transducer (DP-2200 VET, Mindray, Shenzhen, China) to assess the diameter of the DF, ovulatory response, and presence of CL. During examinations, all visible ovarian structures (CL and follicles ≥ 5 mm) were measured and mapped. For all these structures, two measurements were taken, at right angles, to obtain the maximum distance between two opposite borders, and the diameter was determined as the mean of these two measures. The ovulatory response to the GnRH given on Day 0 was determined by the disappearance of the DF between Days 0 and 2 and confirmed by the presence of a CL in the same ovary on Day 7. Ovulation to GnRH treatments on Day 7 was determined by the disappearance of the DF (from the follicular wave that started after ovulation to GnRH on Day 0) between Days 7 and 9, and confirmed by the presence of an accessory CL in the same ovary on Day 12.

2.4. Blood sampling and hormone assays

Blood samples were taken by puncture of the jugular vein into 9 mL heparinized evacuated tubes (Vaccuete, Greiner Bio-One, Americana, SP, Brazil) to evaluate circulating concentrations of P4 and LH. On Day 7, samples were collected at 0 h (immediately before the GnRH treatments and P4 implant insertion), 2, and 4 h later. After collection, tubes were immediately placed on ice, centrifuged at 1700 × *g* for 15 min at 4 °C, and plasma was stored at −20 °C.

Circulating P4 concentrations were determined using a solid-phase RIA commercial kit containing antibody-coated tubes and 125I-labeled P4 (ImmuChem Coated Tube P4 125 RIA Kit, MP Biomedicals, Costa Mesa, CA), as previously described [43]. All samples were analyzed in a single assay. Standards and quality controls were run in duplicates and experimental samples were run in singlets. Quality control samples were expected to achieve similar P4 concentrations of a cow in mid-luteal phase (~2.0 ng/mL) and were evenly distributed four times over the assay. The average P4 concentration of quality controls was 1.83 ng/mL, sensitivity and intra-assay coefficient of variation were 0.04 ng/mL and 4.5%, respectively. Circulating LH concentrations were evaluated using an in-house RIA previously validated [44,45], with some modifications [46]. Samples were analyzed in two assays, and standards and experimental samples were run in duplicates. Two quality controls were used, the first (Low LH), expected to achieve similar LH concentrations of a cow in mid-luteal phase (< 0.5 ng/mL), and the second (High LH), expected to achieve estrus/peak LH concentrations (~7.0 ng/mL). Low LH quality controls were run in duplicates and were evenly distributed eight times in each assay. High LH quality controls were run in quadruplicates and were evenly distributed 16 times in each assay. The average LH concentration, intra- and inter-assay coefficient of variation for Low LH

quality controls were 0.19 ng/mL, 19.2%, and 7.4%, respectively. The average LH concentration, and intra- and inter-assay coefficient of variation for High LH quality controls were 6.0 ng/mL, 16.9%, and 15.3%, respectively.

2.5. Data handling and statistical analyses

Over the four replicates, 59 heifers and 56 cows ovulated in response to the GnRH treatment given on Day 0, determined by the disappearance of the DF between Day 0 and 2, and confirmed by the presence of CL on Day 7. Since this experiment was designed to evaluate the GnRH-induced LH concentrations and ovulation on Day 7 of the estrous cycle, criteria were established to avoid confounding effects on outcomes. For example, to avoid ovulation failure due to absence of a DF with ovulatory capacity [24], one heifer (replicate 1) and one cow (replicate 3) were not assigned to the experimental GnRH treatments because they had a DF < 8.5 mm on Day 7, even having ovulated to the GnRH given on Day 0. In addition, unexpectedly, one heifer from B-Single group, and two other cows from B-Double group had circulating P4 concentrations < 0.5 ng/mL on Day 7, even having a visible CL. These females were considered as having a non-functional CL [47] at the time of the experimental GnRH treatments and, therefore, were excluded to avoid confounding effects of lower P4 concentrations (inconsistent with what is expected at Day 7 of the estrous cycle [39,48]) on LH concentrations and ovulation. Thus, the final number of heifers included in the analyses was: G-Single = 15; G-Double = 15; B-Single = 13; and B-Double = 14. The final number of cows included in the analyses was: G-Single = 14; G-Double = 14; B-Single = 14; and B-Double = 11. Moreover, exclusively for the analyses of LH concentrations, data from one heifer (B-Double) and two cows (G-Single and B-Double) were considered as outliers and excluded.

This study was designed and analyzed as a 2 × 2 factorial arrangement. All statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows, SAS Institute Inc., Cary, NC). Initially, data from cows and heifers were analyzed separately, and thereafter the effect of parity was studied. For continuous data (diameter of DF, circulating P4 and LH concentrations), the normality of studentized residuals was tested using the UNIVARIATE procedure, following the Shapiro-Wilk method. In addition, homogeneity of variances was evaluated by Levene test, using Hovtest and Welsh methods and, when necessary, outliers were removed. Data on circulating LH concentrations were transformed to logarithm to adjust for normality of residuals. The analyses of DF diameter and P4 and LH concentrations were performed by the MIXED procedure, fitting a Kenward-Roger method to calculate the denominator degrees of freedom to approximate the F-tests. For all these variables, the final model included the effect of GnRH analog, dose, and their interaction (G × D), and the replicate was considered as a random effect. For LH concentration analysis, the P4 concentration at the time of GnRH treatment (0 h) was included in the model as a covariate. In addition, a linear regression analysis was performed by the GLIMMIX procedure, selecting the Solution option, to evaluate the association between the LH peak and circulating P4 concentrations at the time of GnRH treatments.

Circulating LH concentrations over time were analyzed as repeated measures by the MIXED procedure, and the final model included effects of GnRH, dose, time, two-way and three-way interactions, with P4 concentration at 0 h as a covariate and replicate as a random effect. The Kenward-Roger method was also included in this analysis, and the appropriate covariance structure was selected, according to the smallest AICC value. Additionally, the effect of parity on the LH concentrations over time was evaluated within each experimental group, following the same criteria described.

Ovulatory response was analyzed by the GLIMMIX procedure, fitting a binary distribution response and considering the same fixed and random effect as for continuous data, with Kenward-Roger adjustment for the degrees of freedom calculation. The ovulatory response was also evaluated between parities, and the final model included the fixed effects of GnRH analog, dose, parity, and the three-way interaction (G × D × Parity), with replicate as a random effect.

Finally, when interaction effects were observed, the SLICE tool was used to study the effect of each factor within the other, and to evaluate the effect within each time in repeated measure analyses. The Tukey-Kramer post hoc mean separation test was used to determine the differences between means. Significant differences were considered when $P \leq 0.05$ and a tendency was defined when $0.05 < P \leq 0.10$. Continuous data are presented as arithmetic means ± SEM and binomial data are presented as percentage (%, n/n).

3. Results

3.1. Circulating P4 concentrations and LH release

As expected, the experimental design produced elevated circulating P4 concentrations at the time of the GnRH treatment. Mean P4 concentration immediately before the treatments (Day 7), was 3.9 ± 0.1 ng/mL in heifers and 4.2 ± 0.2 ng/mL in cows (Table 1), not differing among experimental groups within each parity (P = 0.28 and 0.46, respectively). In addition, insertion of the intravaginal P4 implant at the time of GnRH treatment rapidly increased circulating P4 concentrations, reaching 7.7 ± 0.3 ng/mL after 2 h and 7.8 ± 0.3 ng/mL after 4 h, in heifers, and 9.5 ± 0.4 ng/mL and 11.3 ± 0.4 ng/mL, respectively, in cows. As expected, circulating P4 concentrations at these timepoints were similar among experimental groups, in both heifers and cows.

The LH release profiles over time according to each experimental group, for heifers and cows, are shown in Fig. 2. In heifers, immediately before the GnRH treatments (0 h), the LH concentrations were similar among groups (0.17 ± 0.01 ng/mL; $P = 0.37$). At 2 h after GnRH treatment, LH concentration was affected by GnRH analog ($P < 0.01$) and dose ($P < 0.01$), but no interaction effect was detected ($P = 0.16$). However, at 4 h after GnRH treatment, an interaction between GnRH analog and dose was observed ($P < 0.01$), in which the LH concentrations were greater in heifers treated with the double dose of buserelin compared with those treated with the single dose ($P < 0.01$), but no difference was observed between gonadorelin doses ($P = 0.72$; Fig. 2). The LH peak concentrations (at 2 h after GnRH treatments) in heifers are presented in Table 1. Treatments with buserelin resulted in greater LH peak compared with gonadorelin (9.5 ± 1.2 *vs.* 2.6 ± 0.4 ng/mL; $P < 0.01$), and regardless of GnRH analog, double doses produced greater LH peaks than single doses (7.5 ± 1.3 *vs.* 4.2 ± 0.8 ng/mL; $P < 0.01$).

In cows, the LH concentrations at 0 h were similar among groups (0.17 ± 0.01 ng/mL; $P = 0.46$). At 2 h after GnRH treatment, the main effects of GnRH analog ($P < 0.01$) and dose ($P < 0.01$) were observed, but there was no interaction effect ($P = 0.26$). However, at 4 h, an interaction effect was observed ($P < 0.01$), similar to what was observed in heifers, in which the double dose resulted in greater LH concentrations only in cows treated with buserelin (Fig. 2). The LH peak concentrations (at 2 h after GnRH treatments) in cows are presented in Table 1. Treatments with buserelin resulted in greater LH peak than treatments with gonadorelin (9.9 ± 1.5 *vs.* 4.9 ± 1.1 ng/mL; $P < 0.01$), as well as treatments with double doses resulted in greater LH peak than single doses (9.2 ± 1.7 *vs.* 5.3 ± 0.9 ng/mL; $P < 0.01$).

Regardless of treatment and parity, the magnitude of LH peak was linearly affected ($P < 0.01$) by circulating P4 concentration at the time of GnRH treatment (0 h). Although all heifers and cows had elevated P4 concentration at the time of GnRH treatment, the greater the P4 concentration the lower the magnitude of the LH peak induced by treatments (LH peak = 9.5 – 0.7 × P4 concentration).

3.2. Ovulatory response

Regarding the ovarian dynamics, the pre-synchronization protocol synchronized heifers and cows to be on Day 7 of the estrous cycle on Day

Table 1
Circulating progesterone (P4) concentrations at the time of GnRH treatments and induced LH peak for each GnRH analog and dose, in Nelore heifers and cows.

	Gonadorelin		Buserelin		*P*-value[1]		
	Single	Double	Single	Double	GnRH	Dose	G × D
No. Heifers	15	15	13	14			
Circulating P4 on Day 7, ng/mL	3.8 ± 0.3	4.3 ± 0.2	3.8 ± 0.3	3.7 ± 0.2	0.22	0.49	0.28
LH peak, ng/mL[2]	2.3 ± 0.6	2.9 ± 0.7	6.3 ± 1.3	12.8 ± 1.6	<0.01	<0.01	0.16
No. Cows	14	14	14	11			
Circulating P4 on Day 7, ng/mL	3.8 ± 0.3	4.3 ± 0.5	4.3 ± 0.5	4.5 ± 0.5	0.58	0.66	0.46
LH peak, ng/mL[2]	3.4 ± 0.6	6.3 ± 2.0	7.2 ± 1.6	13.3 ± 2.5	<0.01	<0.01	0.26

Values presented as mean ± SEM.
[1]Evaluated effects: GnRH = GnRH analog administered; Dose = GnRH dose administered; G × D = interaction between GnRH analog and dose.
[2]Data from circulating LH concentrations of one heifer (B-Double) and two cows (G-Single and B-Double) were excluded from this analysis.

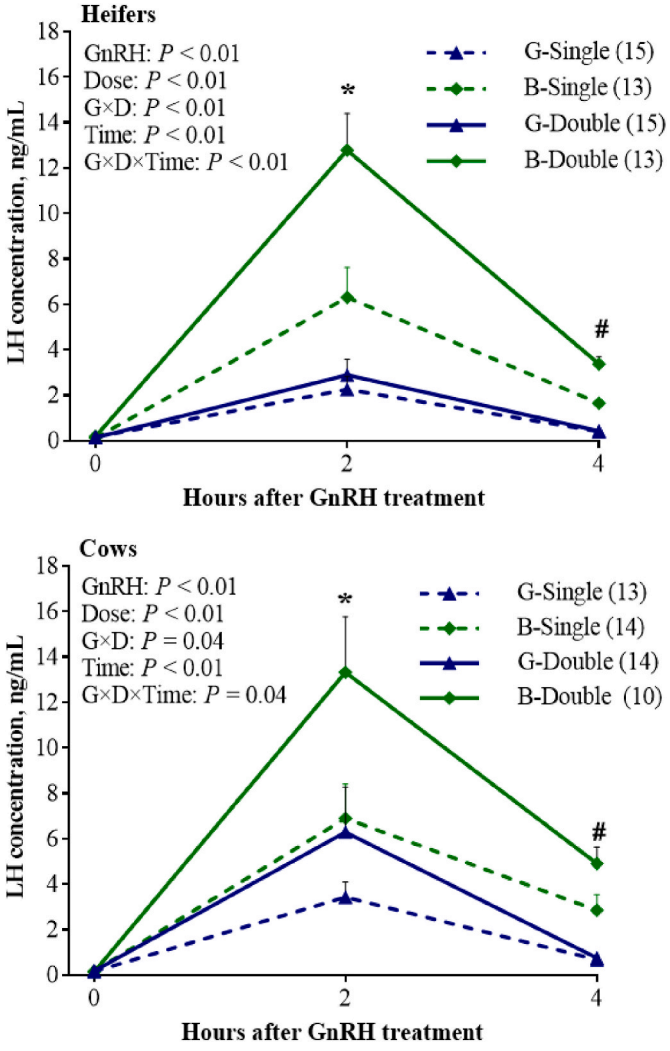

Fig. 2. Circulating LH concentrations (mean ± SEM) in heifers (above) and cows (below), from the GnRH treatment to 4 h later, according to the experimental groups. Females were treated with 100 (**G-Single**) or 200 μg (**G-Double**) of gonadorelin, or either 10 (**B-Single**) or 20 μg (**B-Double**) of buserelin, on Day 7 of the experimental design (corresponding to Day 7 of the estrous cycle). The asterisk indicates the main effects of GnRH analog ($P < 0.01$) and dose ($P < 0.01$) within specific time. Interaction effects (G × D) within specific time are indicated by the number sign ($P < 0.01$), with the effect of dose detected only within buserelin-treated groups ($P < 0.01$).

7 of the experimental design, having a functional CL (as confirmed by the circulating P4 concentrations) and a DF of ovulatory size (≥ 8.5 mm). On Day 7, the mean DF diameter was 10.5 ± 0.1 mm in heifers and 11.2 ± 0.2 mm in cows, and there was no difference among experimental groups within each parity ($P = 0.80$ and 0.38, respectively), as presented in Table 2. In heifers, the ovulatory response was affected by the GnRH analog ($P < 0.01$), but there was no effect of dose ($P = 0.82$) or interaction effect ($P = 0.41$). Heifers that received buserelin treatments, regardless of the dose, had a greater ovulatory response than heifers treated with gonadorelin doses (88.9 [24/27] *vs.* 16.7% [5/30]; $P < 0.01$). Conversely, in cows, besides the main effects of GnRH analog ($P = 0.04$) and dose ($P = 0.03$), an interaction effect ($P = 0.04$) was observed (Table 2). The double dose only resulted in greater ovulation in cows treated with buserelin (90.9% [10/11]), while those cows treated with single buserelin dose or both gonadorelin doses had the same low ovulatory response (35.7% [5/14], in each treatment).

The individual distribution of GnRH-induced LH peaks and ovulation status of heifers and cows, according to each GnRH analog within single or double doses, is shown in Fig. 3. Regardless of treatment and within both parities, approximately 80% of females that did not ovulate in response to the GnRH treatment had an LH peak ≤ 5 ng/mL. In addition, when treated with gonadorelin, regardless of dose and within both parities, approximately 75% of females had an LH peak ≤ 5 ng/mL. Furthermore, when females were evaluated according to their ovulation status, regardless of treatment, there was no difference in circulating P4 concentrations at the time of GnRH treatment between females that ovulated or not, for both heifers (3.7 ± 0.2 *vs.* 4.1 ± 0.2 ng/mL; $P = 0.66$) and cows (4.0 ± 0.3 *vs.* 4.4 ± 0.3 ng/mL; $P = 0.37$). Likewise, circulating P4 concentrations at 2 and 4 h after GnRH treatment did not differ between females that ovulated or not. However, the mean LH peak induced by GnRH was ~2.7-fold greater in females that ovulated compared with those that did not ovulate, for both heifers (8.4 ± 1.1 *vs.* 3.2 ± 0.9 ng/mL; $P = 0.01$) and cows (10.8 ± 1.5 *vs.* 3.2 ± 0.8 ng/mL; $P = 0.01$).

3.3. *Effect of parity*

The characteristics of evaluated responses between heifers and cows were also explored in this study. Different than our expectation, the circulating P4 concentrations at the time of GnRH treatment did not differ between heifers and cows (3.9 ± 0.1 *vs.* 4.2 ± 0.2; $P = 0.22$). However, the diameter of the DF on Day 7 was greater in cows than in heifers (11.2 ± 0.2 *vs.* 10.5 ± 0.1 mm; $P < 0.01$). In addition, when GnRH-induced LH peaks were compared within each experimental group, there was no difference between heifers and cows, except in group G-Double, in which cows had a greater LH peak compared with heifers (6.3 ± 2.0 *vs.* 2.9 ± 0.7 ng/mL; $P = 0.05$). The individual distribution of GnRH-induced LH peaks and ovulation status of heifers *vs.* cows within each experimental group is shown in Fig. 4. Regarding ovulatory response, although no main effect of parity was observed ($P = 0.70$), there was an interaction effect between experimental group and parity ($P = 0.02$), in which the ovulatory response was lower in cows than heifers from group B-Single (35.7 [5/14] *vs.* 84.6% [11/13]; $P < 0.01$), whereas no difference was observed within the other

Table 2
Dominant follicle (DF) diameter and ovulatory response of Nelore heifers and cows treated with single or double doses of GnRH analogs on Day 7 of the estrous cycle.

	Gonadorelin		Buserelin		*P*-value[1]		
	Single	Double	Single	Double	GnRH	Dose	G × D
No. Heifers	15	15	13	14			
DF diameter on Day 7, mm	10.3 ± 0.3	10.7 ± 0.3	10.4 ± 0.3	10.7 ± 0.3	0.81	0.24	0.80
Ovulation, % (n/n)	20.0 (3/15)	13.3 (2/15)	84.6 (11/13)	92.9 (13/14)	<0.01	0.82	0.41
No. Cows	14	14	14	11			
DF diameter on Day 7, mm	11.1 ± 0.3	11.6 ± 0.4	10.6 ± 0.3	11.7 ± 0.4	0.55	0.15	0.47
Ovulation, % (n/n)	35.7^{a,w} (5/14)	35.7^{d,w} (5/14)	35.7^{a,z} (5/14)	90.9^{c,y} (10/11)	0.04	0.03	0.04

Values presented as mean ± SEM or as percentage.
[1]Evaluated effects: GnRH = GnRH analog administered; Dose = GnRH dose administered; G × D = interaction between GnRH analog and dose.
Different letters indicate the interaction (G × D) effect sliced.
[a-b]Effect of GnRH analog within Single dose group ($P \leq 0.05$).
[c-d]Effect of GnRH analog within Double dose group ($P \leq 0.05$).
[w-x]Effect of Dose within Gonadorelin group ($P \leq 0.05$).
[y-z]Effect of Dose within Buserelin group ($P \leq 0.05$).

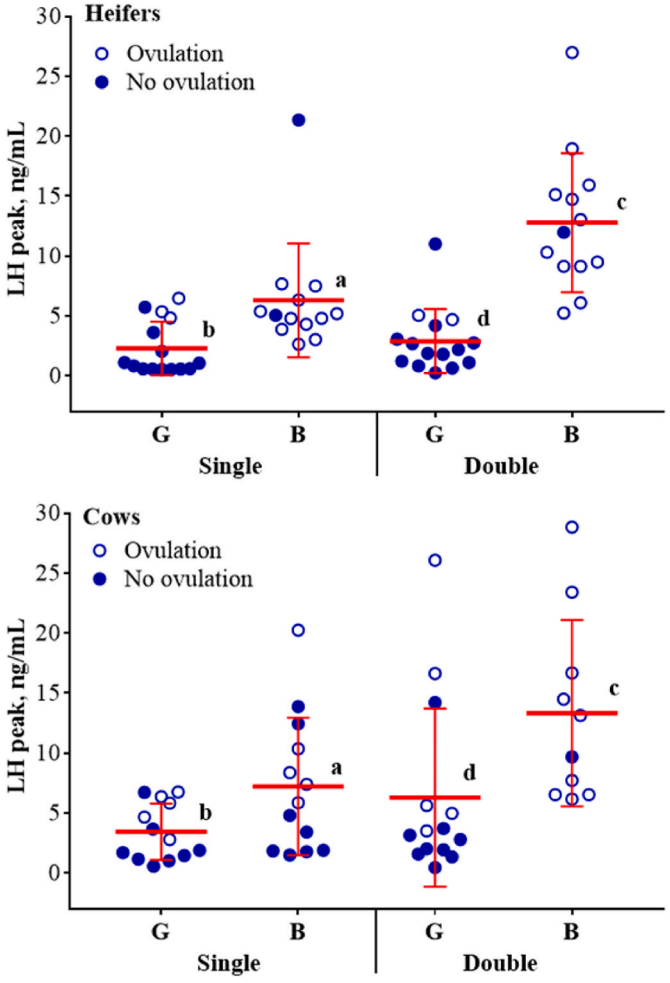

Fig. 3. Individual distribution of LH peak of heifers (above) and cows (below) that ovulated (blue open circles) or not (blue closed circles) in response to each GnRH treatment. Females were treated with 100 (**G-Single**) or 200 μg (**G-Double**) of gonadorelin, or either 10 (**B-Single**) or 20 μg (**B-Double**) of buserelin, on Day 7 of the experimental design (corresponding to Day 7 of the estrous cycle). Red lines indicate the mean ± SEM. [a-b]Mean LH peak induced by the Single dose of each GnRH analogs differ ($P \leq 0.05$). [c-d]Mean LH peak induced by the Double dose of each GnRH analogs differ ($P \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

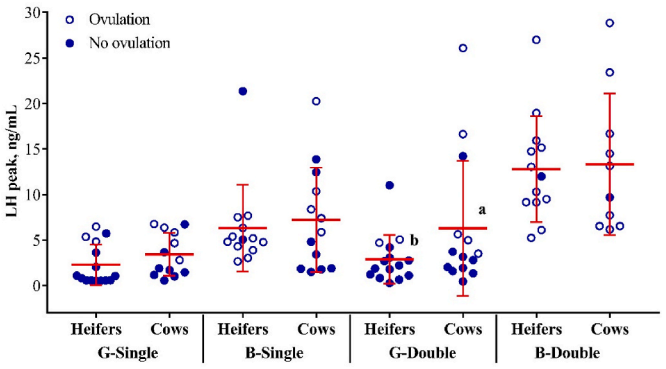

Fig. 4. Individual distribution of LH peak of heifers and cows that ovulated (blue open circles) or not (blue closed circles) in response to each GnRH treatment, analyzed by the effect of the parity within each treatment. Females were treated with 100 (**G-Single**) or 200 μg (**G-Double**) of gonadorelin, or either 10 (**B-Single**) or 20 μg (**B-Double**) of buserelin, on Day 7 of the experimental design (corresponding to Day 7 of the estrous cycle). Red lines indicate the mean ± SEM. [a-b]Mean LH peaks differ between heifers and cows within specific experimental group ($P \leq 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

experimental groups (Table 2).

4. Discussion

This study demonstrated the influence of GnRH analogs and doses on LH release in *Bos indicus* heifers and cows under elevated P4 concentrations, on Day 7 of the estrous cycle. Although the sample size used in this study may be a limitation to determine differences in ovulation, our results provide an interesting insight about the potential impact of these treatments on ovulatory response, which may be supported by further studies with a greater number of females. In addition, to ensure greater reliability of the results, only females with a DF with adequate size and ovulatory capacity (≥ 8.5 mm) [24,49], as well as under elevated circulating P4 concentrations (> 1 ng/mL) at the time of GnRH treatments (Day 7) were included in this study. The mean diameters of the DF in heifers and cows [39,40] as well as the circulating P4 concentrations on Day 7 [39,48] were consistent with what was reported in Nelore cattle on Day 7 of the estrous cycle.

The first hypothesis, that buserelin treatments would induce greater LH release and ovulatory response than gonadorelin was fully supported by the findings. For both heifers and cows, treatment with buserelin resulted in greater circulating LH concentrations than gonadorelin, regardless of the dose administered. As reported, the buserelin molecule

differs from gonadorelin (structurally analogous to the native GnRH) due to the substitution of glycine for a D-serine at position 6 and the replacement of the carboxyl-terminal glycinamide at position 10 of the peptide chain by an ethylamide, resulting in a nonapeptide with greater stability and higher affinity for the GnRH receptor [33]. Consistent with these ideas, previous studies in cattle reported that buserelin was ~50-fold more potent in stimulating LH secretion from the pituitary gland than gonadorelin [50,51]. Chenault et al. [51] reported that a treatment with 10 μg of buserelin resulted in greater LH release than 100 or even 250 μg of gonadorelin, in Holstein heifers. Moreover, coinciding with our results, a previous study with Holstein heifers on Day 6 or 7 after estrus reported greater LH concentrations and LH peak after 10 μg of buserelin compared with 100 μg of gonadorelin [29]. However, in lactating dairy cows 7 d after a synchronized ovulation, these treatments resulted in a similar LH peak, although buserelin induced greater circulating LH concentrations at 3 and 4 h after treatment [30].

Surprisingly, despite the differences in LH surge, none of these studies reported differences in ovulation between the GnRH analogs. In our study, treatment with 100 μg of gonadorelin induced ovulation in only 20% (3/15) of heifers, whereas 84.6% (11/13) ovulated to 10 μg of buserelin. In contrast, in Holstein heifers on Day 6 or 7 of the estrous cycle, the study by Picard-Hagen et al. [29] reported 72.7 (8/11) and 100% (12/12) ovulation, when comparing gonadorelin and buserelin, respectively. In their study, for both analogs the LH peak concentrations reported were approximately 3-fold greater than what was observed in Nelore heifers in our study. These findings corroborate a previous study reporting a more pronounced suppressive effect of P4 concentrations on GnRH-induced LH release in Nelore than in Holstein heifers [37]. Hence, in *Bos indicus*, the P4 effect may be further impairing ovulatory response, especially when treated with less potent GnRH analogs, because the induced LH peak may not be enough to trigger ovulation. Nevertheless, in our study, although 10 μg of buserelin resulted in greater LH peak concentration than 100 μg of gonadorelin in cows, it was not enough to increase ovulatory response. In lactating dairy cows receiving the same treatments, Armengol-Gelonch et al. [30] also reported no difference in ovulatory response. However, regardless of treatment, the mean ovulatory response reported in their study was superior to what was observed in Nelore cows in our study (66.7 *vs.* 37.5%; respectively).

The second hypothesis suggested that the administration of a double dose of GnRH would increase LH release and ovulatory response, regardless of the analog. This hypothesis was partially supported. Treatment of heifers and cows with a double dose of buserelin resulted in a ~2-fold greater LH peak. Similarly, doubling the gonadorelin dose resulted in a 2-fold increase in the LH peak in cows. Nevertheless, surprisingly, doubling the gonadorelin dose did not increase LH release in heifers. No difference was detected on the LH peak in heifers treated with gonadorelin, and both doses resulted in a low LH peak. Previous studies evaluating the same doses of gonadorelin reported greater LH release when the double dose was administered, even under high circulating P4, in *Bos taurus* heifers [27] and lactating cows [32]. Nevertheless, no published study evaluated the LH release induced by distinct GnRH doses in *Bos indicus* cattle. In this regard, although unexpected, a possible explanation for the lack of difference observed in LH peak when doubling the gonadorelin dose is that, particularly in heifers, the maximum LH concentration may have occurred earlier, before the blood collection at 2 h after the GnRH treatment. Previous studies with *Bos taurus* heifers under elevated P4 concentrations treated with gonadorelin reported the maximum LH concentration at 1 h after treatment, or even earlier [29,52]. Therefore, it is possible that the double dose of gonadorelin produced a greater LH peak than the single dose in heifers, but our experimental design was not able to detect it due to the timing of blood collections.

Regarding ovulation, the results did not confirm entirely what was hypothesized. In heifers, the ovulatory response to the double dose of gonadorelin was not increased compared with the single dose, and both groups had very low ovulatory response. Therefore, even if the double dose of gonadorelin did produce a greater LH peak before the blood collection at 2 h, it was not enough to increase the ovulatory response in heifers. Moreover, despite increasing the LH peak in cows, the double dose of gonadorelin did not increase ovulation. Indeed, several studies have reported low ovulatory response after 100 μg of gonadorelin treatment in *Bos taurus* heifers and cows [25,53] and in *Bos indicus*-influenced cows [22,54]. Thus, our results differed from what has been reported in lactating Holstein cows [55], as doubling the gonadorelin dose did not result in more ovulation in Nelore heifers or cows.

Conversely, in support of the second hypothesis, doubling the dose of buserelin positively affected ovulation in cows. As reported in Nelore cows, a single dose of buserelin (8 μg) administered at random stages of the estrous cycle resulted in low percentage of ovulation [19]. However, in the study by Madureira et al. [12], treatment with an increased dose of buserelin (16.8 μg) at random stages of the cycle in Nelore cows resulted in high ovulatory response (73.6%), consistent with the results of the present study. Conversely, in our study, a different effect was observed in heifers treated with buserelin. The single dose was sufficient to induce high ovulatory response, equivalent to that induced by the double dose. These findings suggest that, even under elevated P4 concentrations, treatment with 10 μg of buserelin produced a high ovulatory response in Nelore heifers, but not in cows. Nonetheless, although the results have provided a better understanding about the potential of these two GnRH analogs and respective doses on the ovulatory response in *Bos indicus* cattle, studies with a greater number of females are necessary to confirm these findings.

This experimental study was designed so that both heifers and cows were under elevated circulating P4 concentrations, from a 7-d old CL, at the time of GnRH treatments. Moreover, an intravaginal P4 implant was inserted simultaneously with the GnRH administration, similar to what routinely occurs at the beginning of GnRH-based TAI protocols for beef cattle [12,13]. The specific P4 implants that were used for heifers and cows are similar to what is commonly used in Nelore cattle [12,56]. As expected, the P4 implant produced a rapid increase in circulating P4. In a recent study, the insertion of a P4 implant at the time of GnRH treatment did not alter the GnRH-induced LH release or ovulation [28]. Nevertheless, it is well established that elevated P4 concentrations decrease LH release in response to a gonadorelin treatment in heifers [27] and cows [26], negatively affecting the ovulatory response [28]. Moreover, similar suppressive effects were reported when buserelin was given to heifers [37] and cows [30] under elevated P4 concentrations. Consistent with these previous results, in this study a negative relationship was observed between higher P4 concentration at the time of GnRH treatments and the induced LH peak, regardless of treatment. In addition to the suppressive effect of P4, and of particular interest, circulating E2 concentrations may also have influenced the LH release and even the ovulatory response in this study, since lower E2 concentrations were associated with reduced GnRH-induced LH release [57]. Although circulating E2 concentrations were not measured in this study, all females were synchronized to have a 7-d old DF at the GnRH treatments and were expected to have similar E2 concentrations. However, a previous study with Nelore heifers reported smaller and less steroidogenic DF under higher (4.52 ng/mL) than lower (0.48 ng/mL) circulating P4 concentrations [58].

Furthermore, this study also compared the responses between heifers and cows. The third hypothesis, that heifers would have lower circulating P4 concentration, but greater GnRH-induced LH release than cows on Day 7 of the estrous cycle, was not supported. The mean circulating P4 on Day 7 did not differ between heifers and cows, differing from the study by Figueiredo et al. [40], which reported lower P4 concentrations in Nelore heifers than in cows. A possible explanation for these distinct findings may be the nutritional and metabolic status of females. In our study, non-lactating cows and heifers had similar BCS and were under the same nutritional conditions, while in the study by Figueiredo et al. [40] heifers and cows received different diets. In this regard, studies reported that greater feed intake was associated with greater

metabolism of steroid hormones in the liver, resulting in lower circulating P4 concentrations [36,48]. However, as expected, the diameter of the DF on Day 7 was greater in cows than in heifers, consistent with what was previously reported [26,41]. Regarding the GnRH-induced LH release, there was no difference between parities within experimental groups, except in G-Double treatment, in which cows had a greater LH peak than heifers (6.3 ± 2.0 *vs.* 2.9 ± 0.7 ng/mL). However, this effect could be due to an earlier occurrence of the LH peak in heifers, as mentioned before. Conversely, Colazo et al. [26] reported greater LH release in response to a 100 μg of gonadorelin treatment in *Bos taurus* heifers than in cows under elevated P4 concentrations.

Finally, our results indicated that a single dose of buserelin was able to induce a high ovulatory response in heifers (84.6% [11/13]) under elevated P4 concentrations, but not in cows (35.7% [5/14]), despite having similar LH peak concentrations (6.3 ± 1.3 *vs.* 7.2 ± 1.6 ng/mL; respectively). This finding suggests that a lower LH peak may be needed to trigger ovulation in heifers compared with cows, implying a possible effect of parity at the follicular level impacting ovulatory response. In *Bos indicus* cattle, increased expression of mRNA encoding LH receptor was detected in granulosa cells of follicles >7 mm [49], and satisfactory ovulatory response to GnRH treatment was reported with DF ≥ 8.5 mm [24]. Therefore, findings from our study suggest that a 7-d old DF in Nelore heifers, although smaller, may be more responsive to an LH ovulatory peak than in cows. However, further studies with a larger number of females are needed to confirm the influence of parity on ovulatory response after a GnRH-induced LH peak.

In summary, buserelin treatment induced greater LH release and ovulatory response than gonadorelin treatment in *Bos indicus* heifers and cows under elevated circulating P4, on Day 7 of the estrous cycle. In addition, doubling the dose of both GnRH analogs increased the LH release, however, only resulted in greater ovulatory response in females treated with buserelin. Finally, despite inducing similar LH peak concentrations, the single dose of buserelin was enough to induce high ovulatory response in heifers, but not in cows, suggesting that heifers were more likely to ovulate in response to a GnRH-induced LH peak than cows.

CRediT authorship contribution statement

Lucas O. e Silva: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Project administration. **Jessica C. L. Motta:** Methodology, Investigation, Writing – review & editing. **Abraham L. Oliva:** Investigation. **Guilherme Madureira:** Data curation, Writing – review & editing. **Rodrigo L.O.R. Alves:** Methodology, Writing – review & editing. **Natália P. Folchini:** Investigation, Writing – review & editing. **Taynara J.B. da Silva:** Investigation. **Mateus A. da Silva:** Investigation. **Carlos E.C. Consentini:** Investigation. **Milo C. Wiltbank:** Conceptualization, Methodology, Writing – review & editing. **Roberto Sartori:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision.

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