University of São Paulo
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Physiological responses of *Bos taurus* and *Bos indicus* cows submitted to hormonal treatments for estrous cycle synchronization

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Dissertation presented to obtain the degree of Master in Science. Area: Animal Science and Pastures

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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DEDICATION

To GOD, because to Him all honor, all glory, forever.

Science can explain the world, but just faith can explain its creator.

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SUMMARY

RESUMO

Respostas fisiológicas de vacas *Bos taurus* e *Bos indicus* submetidas a tratamentos hormonais para sincronização do ciclo estral

Três estudos foram desenvolvidos para avaliar as respostas fisiológicas de fêmeas *Bos taurus* e *Bos indicus* submetidas a estratégias farmacológicas para a sincronização do ciclo estral. No primeiro estudo, o perfil de progesterona (P4) circulante promovido por 10 implantes intravaginais de P4 foi avaliado, bem como o efeito das concentrações circulantes de P4 no desenvolvimento do folículo dominante (FD). Para tanto, vacas holandesas e Nelore não-lactantes sem corpo lúteo (CL) receberam um dos implantes de P4 associado a 2 mg de benzoato de estradiol (BE), para sincronizar a emergência folicular. Os implantes permaneceram por 14 d, enquanto colheitas diárias de sangue e avaliações ultrassonográficas foram realizadas. No geral, todos os implantes foram capazes de aumentar as concentrações circulantes de P4 e, associados ao BE, induzir a emergência de nova onda folicular. Ainda, maiores liberações de P4 foram observadas para implantes com maior área de superfície ou maior carga inicial de P4. Também, maiores concentrações de P4 (principalmente durante os primeiros 3 d) foram associadas um menor diamentro do FD 7 d após a inserção dos implantes, em ambas as raças. No segundo estudo, vacas holandesas não-lactantes foram pré-sincronizadas e, no dia 7 do ciclo estral, designadas a terem ou não um CL funcional. Nesse dia, todas as vacas receberam 100 μg de gonadorelina (GnRH), e simultaneamente, foram designadas a receber ou não um implante de P4 (2 g). Exames ultrassonográficos foram realizados para avaliar a resposta ovulatória ao GnRH e o desenvolvimento subsequente do novo CL, e amostras de sangue foram colhidas para avaliar as concentrações de LH e P4 circulantes. Vacas com CL tiveram maior P4 circulante no momento do GnRH, menor pico de LH e menor ovulação (com CL = 58,1%; sem CL = 95,5%). No entanto, a inserção do implante de P4 não influenciou na liberação de LH nem na ovulação. Independentemente, altas concentrações de P4 no momento do GnRH foram associadas a baixa resposta ovulatória, e o inverso foi observado em relação à amplitude do pico de LH induzido por GnRH. Ainda, o desenvolvimento/manutenção do CL subsequente foi negativamente afetado pela presença do CL e inserção do implante de P4 no momento do GnRH. No terceiro estudo, novilhas púberes e vacas Nelore não-lactantes foram pré-sincronizadas e, no dia 7 do ciclo estral, designadas a receber gonadorelina (100 ou 200 μg) ou buserelina (10 ou 20 μg). No momento dos tratamentos a concentração circulante de P4 não diferiu entre vacas e novilhas (~4 ng/mL). Em novilhas, independente da dose, a buserelina promoveu maior pico de LH e ovulação que a gonadorelina (88,9 *vs*. 16,7%). Ainda, a dupla dose só aumentou a liberação de LH no tratamento com buserelina. Por outro lado, em vacas, embora a dose dupla tenha elevado a liberação de LH para ambos os análogos, apenas a dose dupla de buserelina apresentou resposta ovulatória satisfatória (90,9%), enquanto para os demais tratamentos a ovulação não diferiu (35,7%). Em ambas categorias, maiores picos de LH foram associados à maior ovulação, porém em novilhas a ovulação foi estimulada por picos de menor amplitude comparado às vacas.

Palavras-chave: Progesterona; GnRH; LH; Folículo dominante; Ovulação

ABSTRACT

Physiological responses of *Bos taurus* and *Bos indicus* cows submitted to hormonal treatments for estrous cycle synchronization

Three studies were performed to evaluate the physiological responses of *Bos taurus* and *Bos indicus* females submitted to hormonal strategies for estrous cycle synchronization. In the first study, the circulating progesterone (P4) profile provided by 10 intravaginal P4 implants was evaluated, as well as the effect of circulating P4 concentrations on dominant follicle (DF) development. Then, non-lactating Holstein and Nelore cows without corpus luteum (CL) received one of the P4 implants associated to 2 mg estradiol benzoate (EB), to synchronize follicular emergence. The P4 implants were kept for 14 d. Daily blood samples were taken and ultrasound evaluation was performed. Overall, all implants were able to increase circulating P4 concentrations and induce emergence of a new follicular wave, associated to EB. Moreover, greater P4 concentrations were provided by implants with greater surface area or greater initial P4 load. In addition, higher P4 concentrations (mainly during the first 3 d) were associated with lower DF size at 7 d after implant insertion, for both breeds. In the second study, non-lactating Holstein cows were presynchronized and, on day 7 of the estrous cycle, they were assigned to have or not a functional CL. On this day, all cows received 100 μg gonadorelin (GnRH), and simultaneously, they were designated to receive or not a P4 implant (2 g). Ultrasound evaluations were performed to check the ovulatory response to GnRH and the subsequent CL development, and blood samples were taken to evaluate circulating P4 and LH concentrations. Cows with CL presented higher circulating P4 at the time of GnRH and, consequently, lower LH release and ovulation (with CL = 58.1%; without CL = 95.5%). However, P4 implant insertion did not affect the LH release or ovulation. Moreover, higher P4 concentrations at the time of GnRH were associated with lower ovulatory response, and the opposite effect was observed in relation to the LH peak amplitude induced by GnRH. Additionaly, the development/maintance of subsequent CL was negatively affected by CL presence and P4 implant insertion at the time of GnRH treatment. In the third study, cycling Nelore heifers and non-lactating Nelore cows were presynchronized and, on day 7 of the estrous cycle, they were designated to receive gonadorelin (100 or 200 μg) or buserelin (10 or 20 μg). At the time of treatments, circulating P4 concentrations was similar among heifers and cows (~4 ng/mL). In heifers, regardless of dose, buserelin treatments induced greater LH peak and ovulation than gonadorelin (88.9 *vs.* 16.7%). In addition, the double dose increased LH release only in the buserelin treatment. Otherwise, in cows, although the double dose had increased LH release in both GnRH analogues, only the double dose of buserelin produced high ovulatory response (90.9%), whereas in the other treatments ovulation did not differ (35.7%). In both animal categories, higher LH peak was associated to greater ovulatory response, but in heifers, ovulation was triggered by LH peaks with lower amplitude than cows.

Keywords: Progesterone; GnRH; LH; Dominant follicle, Ovulation

1. INTRODUCTION

From the development of the first fixed-time artificial insemination (FTAI) protocols for cattle [1,2], several research groups have focused their studies on better understand the physiological responses promoted by the exogenous manipulations of reproductive functions. The constant search for the refined synchrony between the hormonal treatments and reproductive events have provided valuable information that contributed to the development of efficient reproductive programs and dramatically improved the fertility of herds [3–5]. It is well established that the efficacy of a synchronization protocol is closely associated to three key-points: emergence of a synchronized follicular wave [6], control of circulating progesterone (P4) concentrations during the follicular development [7,8], and induction of a synchronized ovulation at the end of the protocol [9,10].

In this regard, strategies for estrous cycle synchronization based on the administration of gonadotropin-releasing hormone (GnRH) have been routinely used in beef [11] and dairy [12] cattle. The GnRH treatment at the onset of the protocol aims to induce an ovulation, promoting the emergence of a new follicular wave and originating a corpus luteum (CL), increasing circulating P4 concentrations during the follicular growth. However, some studies have reported unsatisfactory ovulation rates to the GnRH at the beginning of the protocol [13]. In fact, the lack of ovulation in response to the first GnRH treatment of the protocol was associated to an inadequate synchronization and lower circulating P4 concentrations during the follicular growth, affecting the follicle development and, consequently, the fertility of dairy cows [14,15]. Moreover, similar results have been reported in beef cattle submitted to GnRH-based FTAI protocols [16,17]. It is well known that the ovulatory response to a GnRH treatment can be affected by the responsiveness of the dominant follicle (DF) [18,19], stage of the estrous cycle [20,21], and circulating P4 concentrations at the time of treatment [22,23]. In addition, interesting studies have reported the regulatory effects of circulating concentrations of steroid hormones on the GnRH-induced LH release [24,25]. In this sense, as reported, the use of GnRH analogues [26] and increased doses [27] were efficient to increase LH release, suggesting that it could be a potential strategy to improve ovulation in response to GnRH [28]. Nevertheless, a recent study demonstrated that the responsiveness of the pituitary to a GnRH stimulus was differently affected by P4 concentrations in *Bos taurus* and *Bos indicus* cattle [29], supporting the idea that this genetic groups present distinct physiological responses to hormonal manipulations.

Additionally, insertion of an intravaginal P4 implant at the onset of the FTAI protocol has been widely adopted as an strategy to better control circulating P4 concentrations during

follicular growth, and to optimize reproductive outcomes [30,31]. Management of P4 concentrations during the protocol can improve DF development and oocyte quality, by modulating LH pulse frequency [32]. However, it is reported that *Bos taurus* and *Bos indicus* cattle have distinct LH secretion patterns and were differently affected by circulating P4 concentrations [33,34]. Thus, the control of adequate circulating P4 concentrations in these animals is a critical key to improve the synchronization efficacy.

Therefore, the understanding of the physiological responses of cows, as well as, the particularities of *Bos taurus* and *Bos indicus* reproductive function in response to hormonal manipulations is crucial to improve reproductive management, aiming to increase fertility of herds. Thus, the present study performed a sequence of experiments to evaluate the physiological responses of *Bos taurus* an *Bos indicus* cattle submitted to hormonal treatments, specifically, based on P4 manipulation and GnRH administration to induce LH release and ovulation.

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2. PROGESTERONE RELEASE AND FOLLICULAR DEVELOPMENT IN HOLSTEIN AND NELORE COWS RECEIVING INTRAVAGINAL PROGESTERONE IMPLANTS

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ABSTRACT

The aim of this study was to evaluate the progesterone (P4) release profile provided by 10 commercial intravaginal P4 implants, as well as the effect of circulating P4 concentrations produced exclusively by these implants on the development of the dominant follicle (DF) in *Bos taurus* and *Bos indicus* cows. Therefore, non-lactating multiparous Holstein and Nelore cows were submitted to the same experimental design starting with the insertion of a reused P4 implant (2 g) for 7 d, followed by two treatments of cloprostenol (PGF; 0.482 g), 24 h apart, 6 and 7 d after implant insertion. Just before implant removal, a Norgestomet ear implant was inserted and, 2 d later, simultaneously to Norgestomet withdrawal, cows received one of the tested intravaginal implants, and 2 mg of estradiol benzoate (EB) i.m. In Exp.1 (n = 22; three replicates), Holstein cows were randomized to receive: CIDR (1.38 g); PRID-Delta (1.55 g); Prociclar (750 mg); or Repro sync (2 g). In Exp. 2 (n = 33; three replicates), Nelore cows were randomized to receive: CIDR (1.9 g); PRID-Delta (1.55 g); Primer (0.5 g); Prociclar (750 mg); or Sincrogest (1 g). In Exp. 3 (n = 29; four replicates), Holstein cows were randomized to receive: Cue-Mate (1.56 g); DIB 0.5 (0.5 g); DIB (1 g); PRID-Delta (1.55 g); or Sincrogest (1 g). Blood samples were collected before P4 implant insertion, 12 h later and daily over 15 d (1 d after P4 implant removal) and ultrasound examinations were performed to evaluate growth of the DF. Statistical analyses were performed by SAS 9.4 and the results are presented as mean ± SEM. Differences were considered when $P \leq 0.05$. Overall, the circulating P4 profile and mean circulating P4 over 14 d differed among treatments, apparently influenced by implant surface area and initial P4 load. However, no effects were observed on the DF diameter and follicular growth rate from Day 7 to 10 after P4 implant insertion. Moreover, regardless of the treatment, greater circulating P4 concentrations, mainly during the first 3 d of implant insertion, were associated to smaller DF sizes, both in Nelore and Holstein cows. Finally, this study provided a better understanding of the P4 release profile produced by intravaginal P4 implants as well as their effect on circulating P4 concentrations and DF development in *Bos taurus* and *Bos indicus* cows.

Keywords: Circulating P4; Device; Dominant follicle; *Bos taurus*; *Bos indicus*.

2.1 Introduction

Progesterone (P4) plays an important role on regulation of the female reproductive function. Among other events, P4 is responsible for the control of the hypothalamic-pituitary axis, mainly controlling luteinizing hormone (LH) secretion, either by modulating hypothalamic release of gonadotropin-releasing hormone (GnRH) or by regulating pituitary sensitiveness to GnRH stimulation [1]. In cattle, this process is closely related to controlling follicular waves and follicular growth [2]. For this reason, management of circulating P4 concentration is an efficient way to manipulate the estrous cycle and to control follicular development [3,4]. Usually, in synchronization protocols for beef and dairy cattle, it is possible to manipulate circulating P4 through administration of exogenous GnRH and/or prostaglandins, controlling corpus luteum (CL) lifespan [5,6]. Another possibility is to use exogenous sources of P4, such as intravaginal implants, providing a gradual and controlled release of P4.

Intravaginal P4 implants for cattle are commercially available since the 1970's and have been widely used as an efficient tool to optimize reproductive performance of herds [7,8]. Over the last years, several studies reported the use of P4 implants as a strategy to improve synchronization and increase fertility in fixed-time artificial insemination (FTAI) programs, presenting consistent results for dairy [9–11] and beef cattle [12–14]. There is a range of commercial P4 implants, with many approaches for reproductive management. However, it is important to consider physiological aspects of specific animal categories or production systems. For example, it is well known that *Bos taurus* and *Bos indicus* cows have significant physiological differences, including circulating hormone concentrations, follicular development and responsiveness to pharmacological manipulations [15]. Despite having smaller dominant follicle (DF) size and smaller CL, *Bos indicus* have greater circulating P4 concentrations than *Bos taurus* cattle [16]. Moreover, in a comparative study between these genetic groups, Nelore heifers underwent a more pronounced negative effect of high circulating P4 concentrations on LH release than Holstein heifers [17]. Similarly, in a complementary study, Batista et al. [18] confirmed the contrasts between these breeds, indicating an effect of the enzyme complex in the liver and, also, the level of feed intake on P4 metabolism, affecting circulating P4 concentration.

For high-producing dairy cows (*Bos taurus*), it is well established that greater concentrations of P4 during the growth of the DF before AI is associated with better embryo quality [19] and greater pregnancy per AI (P/AI) [20]. Higher circulating P4 reduces LH pulse frequency during DF development, ensuring appropriate growth and avoiding premature oocyte maturation, which results in greater fertility [4]. Therefore, insertion of P4 implants can be an

effective strategy for synchronization protocols of dairy cows in order to provide greater circulating P4 concentrations. In the study by Bisinotto et al. [21], it was demonstrated that supplementing P4 by insertion of two intravaginal implants improved fertility of cows without CL at the beginning of FTAI protocol. In contrast, *Bos indicus* cattle, such as Nelore, present a different LH secretion pattern than *Bos taurus*, in which post-partum Nelore cows have a reduced LH pulse frequency due to a combination of physiological challenges such as negative energy balance and calf presence [22,23]. Thus, for these animals, control of circulating P4 concentration is a critical key to improve follicular growth, aiming to increase fertility.

Studies have shown the effect of circulating P4 concentration in Nelore cows submitted to FTAI protocols, evidencing that P4 concentration was negatively correlated to LH secretion [24] and diameter of the DF [13]. In addition, Meneghetti et al. [25] reported a negative effect of circulating P4 at the time of P4 implant removal (Day 9) on P/AI of Nelore cows submitted to a FTAI protocol. Therefore, in general, protocols that produce higher circulating P4 are desirable for lactating dairy cattle, whereas, for *Bos indicus* cattle, lower circulating P4 throughout the FTAI protocol would be more efficient to produce an adequate size dominant follicle that ovulates at the end of the protocol. Despite that, some studies have shown no increase in fertility when P4 implants with a lower P4 release profile were used in FTAI protocols for *Bos indicus* [13,26]. Hence, it is needed to precisely understand the release pattern of P4 from commercially available implants to optimize their use according to a specific reproductive management demand depending on the animal category and breed.

Thus, the aim of this study was to evaluate the P4 release profile from commercially available implants, as well as to assess growth of the DF associated to the circulating P4 concentration produced exclusively by the implants in non-lactating Holstein and Nelore cows.

2.2 Material and methods

For this study, three experiments were conducted using non-lactating Holstein and Nelore Cows. Experiments 1 and 3 were carried out at the University of São Paulo, Piracicaba, SP, Brazil. Non-lactating Holstein cows were kept in dry lots, fed daily a maintenance diet with haylage (*Cynodon dactylon*) and a corn plus soybean based concentrate supplementation, with free access to water and mineral salt. Experiment 2 was conducted in a commercial beef farm, located in Itatinga, SP, Brazil. Non-lactating Nelore cows were kept on pasture (*Brachiaria brizantha*) with water and mineral salt *ad libitum*. All procedures were approved by the Animal Research Ethics Committee of “Luiz de Queiroz” College of Agriculture (Protocols #2018-22 and #2019-19).

2.2.1 Experiment 1

A total of 22 non-lactating and non-pregnant multiparous Holstein cows (BCS = 3.6 ± 0.1, scale 1 to 5) were enrolled in an experimental design with three replicates (Fig. 1). On Day -9, all cows received a 2 g P4 implant (Repro sync, GlobalGen Vet Science, Jaboticabal, SP, Brazil) that had been previously used for 8 d. Six d later (Day -3), a first PGF injection (0.482 mg cloprostenol sodium, Estron, Agener União, Embu-Guaçu, SP, Brazil) was administered im and, 24 h later (Day -2), a second PGF administration was given simultaneously to P4 implant removal. At that time, cows received a Norgestomet ear implant (CRESTAR, MSD, Cruzeiro, SP, Brazil), which was maintained for 48 h to ensure no residual circulating P4, but preventing ovulation. On Day 0, cows were treated with 2 mg estradiol benzoate (EB, RIC-BE, Agener União, Brazil) and were randomly assigned to receive one of four commercial P4 implants: CIDR (1.38 g, Zoetis, New York, USA); PRID-Delta (1.55 g, Ceva Santé Animale, Libourne, France); Prociclar (750 mg, Ceva Saúde Animal, Brazil); Repro sync (2 g, GlobalGen Vet Science, Brazil). These P4 implants were kept for 14 d. On Day 13, all cows were treated with a PGF injection (0.482 mg) followed by a second dose 24 h later (Day 14), concurrently with P4 implant removal and insertion of another Norgestomet ear implant, to start the next replicate (Day 0) 2 d later. On the second and third replicates, cows were assigned again to receive one of four P4 implants; however, no cow received the same implant twice. Ovarian ultrasound evaluation was performed on Days 0, 7, 8, 9, 10, 12, and 14 to evaluate follicle dynamics, CL presence, and to measure the size of the DF.

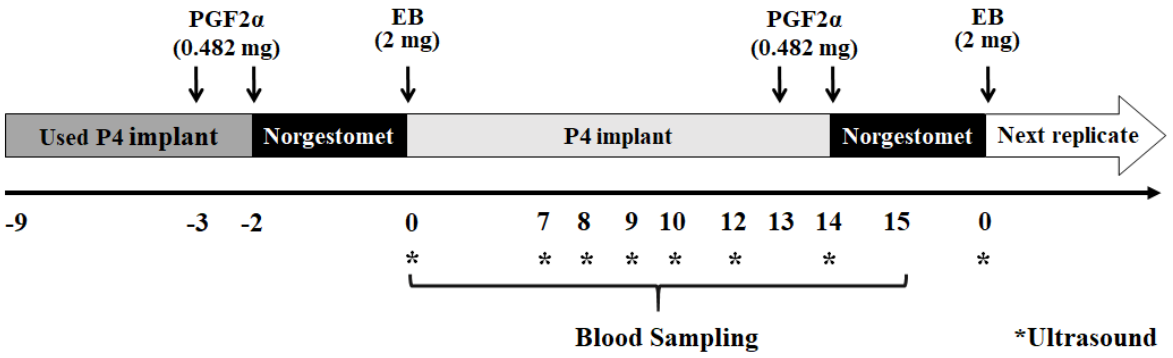

Figure 1. Schematic representation of experimental design for the three experiments. Non-lactating multiparous Holstein or Nelore cows were submitted to a presynchronization protocol starting on Day -9 with insertion of a P4 implant (2 g; previously used for 8 d), followed by two treatments of PGF (0.482 mg) 24 h apart on Day -3 and Day -2. Before implant removal (Day -2), a Norgestomet ear implant was inserted for 2 d. On Day 0, the Norgestomet implant was removed and cows were randomized to receive one of the intravaginal P4 implants to be evaluated, associated to administration of EB (2 mg). The P4 implant remained for 14 d, and on Day 13 and Day 14 cows received two PGF treatments again. On Day 14, the intravaginal implant was removed and a new Norgestomet implant was inserted to start a new replicate.

Three replicates were conducted in Exp. 1 and 2, and four replicates in Exp. 3. Blood samples were collected on Day 0 (before P4 implant insertion), 12 h later and daily until Day 15. (*)Ultrasound evaluations were performed on Days 0, 7, 8, 9, 10, 12 and 14 in Exp. 1 and 2. In Exp. 3, an extra evaluation was performed on Day 5.

2.2.2 Experiment 2

For this experiment, 33 non-lactating and non-pregnant multiparous Nelore cows (BCS = 3.2 ± 0.07) were submitted to the same experimental design described previously (Exp. 1) with three replicates, and they were randomly assigned to receive one of four commercial P4 implants: CIDR (1.9 g, Zoetis, São Paulo, Brazil); Primer (0.5 g, Agener União, Brazil); Prociclar (750 mg, Ceva Saúde Animal, Brazil); Sincrogest (1 g, Ourofino Saúde Animal, Cravinhos, SP, Brazil).

2.2.3 Experiment 3

The third experiment also followed the same experimental design as the others. Thus, 29 non-lactating and non-pregnant multiparous Holstein cows (BCS = 3.2 ± 0.06) were enrolled in four replicates, receiving randomly one of five commercial P4 implants: Cue-Mate (1.56 g, Vetoquinol, Brisbane, Australia); DIB 0.5 (0.5 g, Zoetis, Brazil); DIB (1 g, Zoetis, Brazil); PRID-Delta (1.55 g, Ceva Santé Animale, France); Sincrogest (1 g, Ourofino Saúde Animal, Brazil). In this experiment, one extra ultrasound evaluation was done on Day 5 to more accurately evaluate follicle development.

2.2.4 Blood samples and hormonal assays

For all experiments, blood samples were collected on Day 0, immediately before P4 implant insertion and 12 h later (Day 0.5), and daily from Day 1 to Day 15. Samples were collected by puncture of the jugular vein into 9 mL heparinized evacuated tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) and instantly placed on ice. Tubes were centrifuged at 1,700x*g* for 15 min at 4 °C and plasma was stored at -20 °C.

Progesterone concentrations were determined using a solid-phase RIA kit containing antibody-coated tubes and 125I-labeled P4 (ImmuChem Coated Tube P4 125 RIA Kit, MP Biomedicals, Costa Mesa, CA) validated for bovine plasma as reported [27]. The intra- and inter-assay CVs and the sensitivity were 5.7%, 8.4%, and 0.08 ng/mL, respectively. Each assay contained samples from all treatments within the same experiment.

2.2.5 Statistical analysis

All statistical analyses were performed using the SAS computational software version 9.4 (SAS, Version 9.4 for Windows, SAS Institute Inc., Cary, NC). Data were tested for normality of studentized residuals using the UNIVARIATE procedure, following the Shapiro-Wilk method, and homogeneity of variances were evaluated by Levene test, using Hovtest and Welsh methods. When necessary, data were transformed to logarithm and outliers were removed.

Diameter of the DF and circulating P4 concentrations were analyzed as repeated measures using the MIXED procedure. The model included the fixed effects of treatment, time and interaction between treatment and time, fitting a Kenward-Roger method to calculate the denominator degrees of freedom to approximate the F-tests in mixed models. Replicate was considered as a random effect and cow within treatment was the subject effect. For each model, the appropriate covariance structure was selected, according the smallest AICC value.

The area under the curve (AUC) of circulating P4 profile was calculated by trapezoid method using the GraphPad Prism software (version 8.4). In addition, mean circulating P4, AUC, P4 peak, DF growth rate and DF diameter on Day 7 and Day 10 were evaluated using the MIXED procedure, with replicate as random effect. For the analyses of DF growth rate and DF diameter on Day 10, the diameter of DF on Day 7 was used as covariate, to adjust the final model.

Finally, linear regression analyses were performed using the REG procedure to identify correlations between the DF diameter and mean circulating P4 concentration. The Tukey adjustment was used to determine differences among treatments and within a time. Significant differences were considered when $P \leq 0.05$ and a tendency was defined when $0.05 < P \leq 0.10$. Data are presented as means ± SEM.

2.3 Results

2.3.1 Experiment 1 (Holstein cows)

For the DF dynamics analysis, 19 observations were removed due to the following events: ovulation between Day 0 and Day 7 (CIDR = 1; PRID = 1; Repro sync = 3), lack of follicular wave emergence (Prociclar = 1; Repro sync = 2), implant loss (PRID = 1; Repro sync = 1), co-dominance (CIDR = 2; PRID = 1; Prociclar = 2; Repro sync = 1) and turn-over of follicular wave (CIDR = 1; PRID = 2). The turn-over of follicular wave was determined when the DF stopped growing and a new follicular wave emergence between Day 7 and Day 10 was detected. Likewise, for circulating P4 evaluation, six observations were not included in the

analysis: five due to ovulation between Day 0 and Day 7 and one due to P4 assay inconsistencies. Data from cows that lost the implant were included until 1 d before implant loss.

Mean circulating P4 and AUC were lower for Prociclar, but similar among the other treatments. Moreover, PRID presented the greatest P4 peak, differing from CIDR and Prociclar, but similar to Repro sync (Table 1). There were effects of treatment, time and interaction treatment*time for circulating P4 concentrations over time ($P < 0.01$, Fig. 2). PRID produced higher circulating P4 in the first 2 d after insertion compared to CIDR and Prociclar, and tended to be higher than Repro sync at those days ($P < 0.10$). In addition, Prociclar presented the lowest P4 release from Day 2 to Day 9 (Fig. 2).

There was no effect of treatment on the DF diameter at any time evaluated, nor on the DF growth rate over time (Table 1). Moreover, no differences were observed when the DF growth rate was analyzed at daily intervals. The pattern of DF growth from Day 7 to Day 10 and daily DF growth rate of Holstein cows is shown in Fig. 3.

Table 1. Circulating progesterone concentrations and dominant follicle dynamics of non-lactating Holstein cows receiving intravaginal P4 implants during 14 days. Experiment 1.

	CIDR (1.38 g)	**PRID** (1.55 g)	**Prociclar** (750 mg)	**Repro sync** (2 g)	***P*-value**
Circulating P4 (n)	11	15	9	12	
Mean concentration (ng/mL)[1]	1.2±0.1[a]	1.4±0.1[a]	0.6±0.1[b]	1.4±0.1[a]	< 0.01
AUC (ng/mL * day)	16.1±1.1[a]	17.5±1.4[a]	8.2±0.9[b]	19.2±1.5[a]	< 0.01
Peak (ng/mL)	2.2±0.2[bc]	2.9±0.2[a]	1.6±0.1[c]	2.4±0.2[ab]	< 0.01
DF (n)	8	11	7	8	
Diameter on Day 7 (mm)	10.7±0.6	10.6±0.5	11.2±0.4	10.5±0.5	0.74
Diameter on Day 10 (mm)	14.6±0.7	14.6±0.6	15.0±0.5	14.3±0.4	0.89
Growth rate (mm/day)[2]	1.3±0.2	1.3±0.1	1.3±0.2	1.3±0.2	0.98

[a,b,c]Values within the same row differ ($P \leq 0.05$).
Values presented as mean ± SEM.
Abbreviations: P4 = progesterone; AUC = Area under the curve; DF = Dominant follicle.
[1]Mean circulating P4 was calculated from Day 0.5 to Day 14.

[2]DF growth rate was calculated from Day 7 to Day 10.

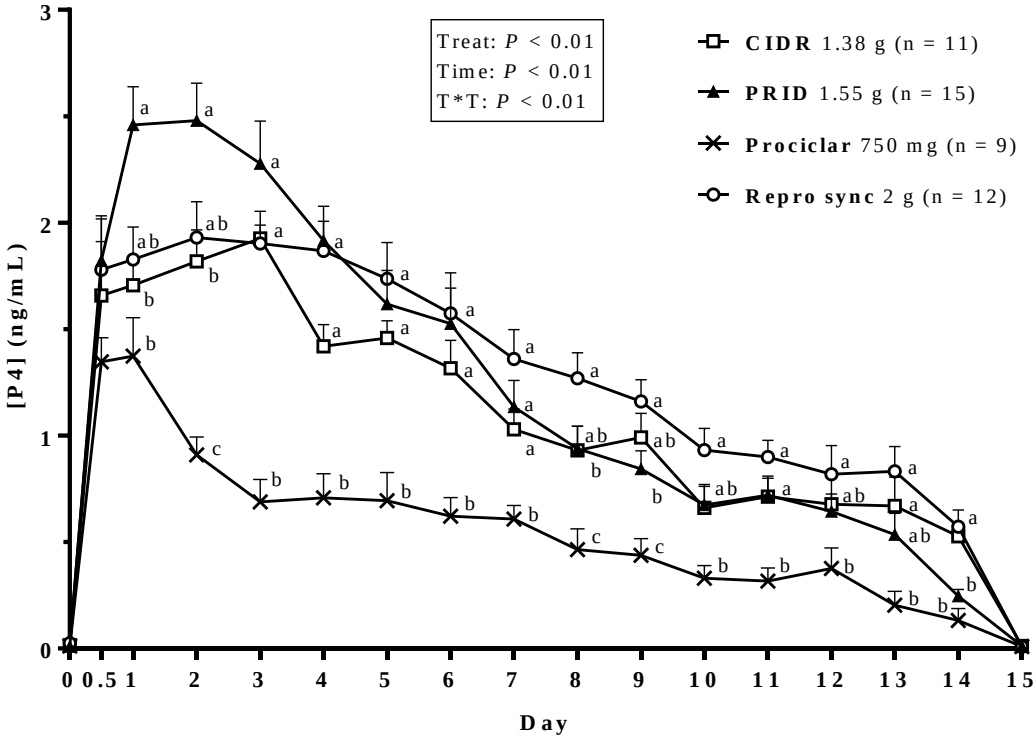

Figure 2. Circulating progesterone (P4) concentrations (mean ± SEM) during 15 d in non-lactating multiparous Holstein cows receiving one of four intravaginal P4 implants: CIDR 1.38 g (n = 11); PRID 1.55 g (n = 15); Prociclar 750 mg (n= 9); Repro sync 2 g (n = 12). The P4 implants were removed on Day 14. Cows that ovulated were excluded from this analysis and for cows that lost P4 implant, circulating P4 concentrations were included just until 1 d before loss. Distinct letters ([a,b,c]) indicate difference among treatments within each time.

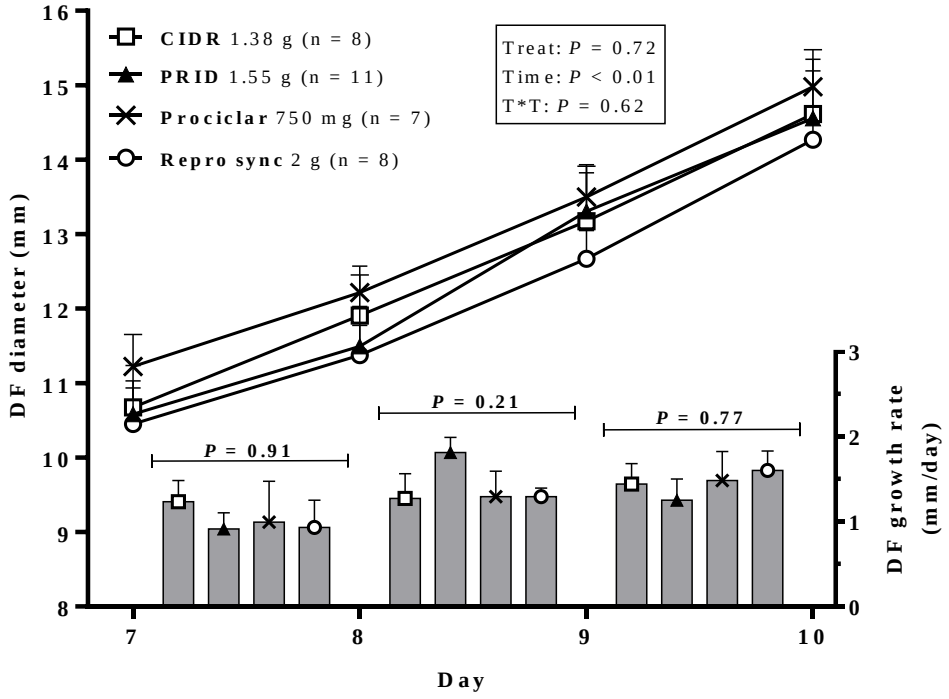

Figure 3. Diameter and daily growth rate (mean ± SEM) of the dominant follicle (DF) from Day 7 to 10 after insertion of an intravaginal P4 implant (CIDR 1.38 g, n = 8; PRID 1.55 g, n = 11; Prociclar 750 mg, n = 7; Repro sync 2 g, n = 8) associated to EB (2 mg) administration in non-lactating multiparous Holstein cows. Continuous lines represent the DF growth profile over time and bars represent the daily growth rate, according to each treatment. Only cows that emerged a new follicular wave were considered. Cows that ovulated, presented follicular codominance, started a new follicular wave between Day 7 and 10 (turn-over), or lost the P4 implant were excluded from this analysis.

2.3.2 Experiment 2 (Nelore cows)

Following Following the same procedure as for Exp. 1, 19 observations were excluded for the DF follicle analysis because of: ovulation between Day 0 and Day 7 (CIDR = 1; Primer = 1; Prociclar = 1; Sincrogest = 1), lack of follicle wave emergence (CIDR = 2; Primer = 1; Prociclar = 1; Sincrogest = 1), implant loss (Prociclar = 1), co-dominance (CIDR = 1) and turn-over of follicular wave (CIDR = 2; Prociclar = 1; Sincrogest = 5). For circulating P4 analyses, four observations were excluded due to ovulation between Day 0 and Day 7 and one because of early implant loss. Moreover, six observations were removed from the analyses because of some inconsistency on P4 assay.

Mean P4 concentration and AUC were affected by treatment ($P < 0.01$, Table 2), where the CIDR had the greatest concentration, the Sincrogest and Prociclar implants presented intermediate values and the Primer implant provided the lowest concentration. In addition, the circulating P4 peak was lower for Primer but similar among others (Table 2). There were effects of treatment, time and interaction treatment*time on P4 release profile from Day 0 to Day 15

($P < 0.01$). Fig. 4 shows the circulating P4 profile for each treatment. The Primer implant provided the lowest circulating P4 concentrations until Day 10, whereas, the CIDR implant had the greatest P4 release. Sincrogest and Prociclar were similar between them and produced intermediate circulating P4 compared to the other treatments (Fig. 4).

No differences were observed for the DF growth rate over time or DF diameter at any specific day (Table 2). However, interestingly, when the DF growth rate was analyzed at daily intervals, a greater growth was observed for the Primer from D7 to D8, differing from the others, except Prociclar. This effect was not observed in the other daily intervals. In addition, there was an effect of treatment ($P < 0.01$) for DF growth profile over time in Nelore cows (Fig. 5), in which cows treated with the Primer implant presented the greatest profile of follicular growth from D7 to D10, whereas no difference was detected among others.

Table 2. Circulating progesterone concentrations and dominant follicle dynamics of non-lactating Nelore cows receiving intravaginal P4 implants during 14 days. Experiment 2.

	CIDR (1.9 g)	**Primer** (0.5 g)	**Prociclar** (750 mg)	**Sincrogest** (1 g)	***P*-value**
Circulating P4 (n)	11	11	10	12	
Mean concentration (ng/mL)[1]	2.6±0.2[a]	1.0±0.1[c]	1.8±0.1[b]	1.8±0.1[b]	< 0.01
AUC (ng/mL * day)	36.6±2.6[a]	13.3±0.9[c]	24.8±1.5[b]	24.9±1.7[b]	< 0.01
Peak (ng/mL)	3.8±0.3[a]	2.2±0.3[b]	3.9±0.6[a]	3.9±0.5[a]	< 0.01
DF (n)	7	10	10	7	
Diameter on Day 7 (mm)	7.9±0.8	8.6±0.7	6.9±0.9	7.7±1.0	0.37
Diameter on Day 10 (mm)	9.9±0.8	10.8±0.8	10.0±0.9	10.3±0.9	0.50
Growth rate (mm/day)[2]	0.7±0.1	0.9±0.1	1.1±0.2	0.9±0.2	0.15

[a,b,c]Values within the same row differ ($P \leq 0.05$).
Values presented as mean ± SEM.
Abbreviations: P4 = progesterone; AUC = Area under the curve; DF = Dominant follicle.
[1]Mean circulating P4 was calculated from Day 0.5 to Day 14.
[2]DF growth rate was calculated from Day 7 to Day 10.

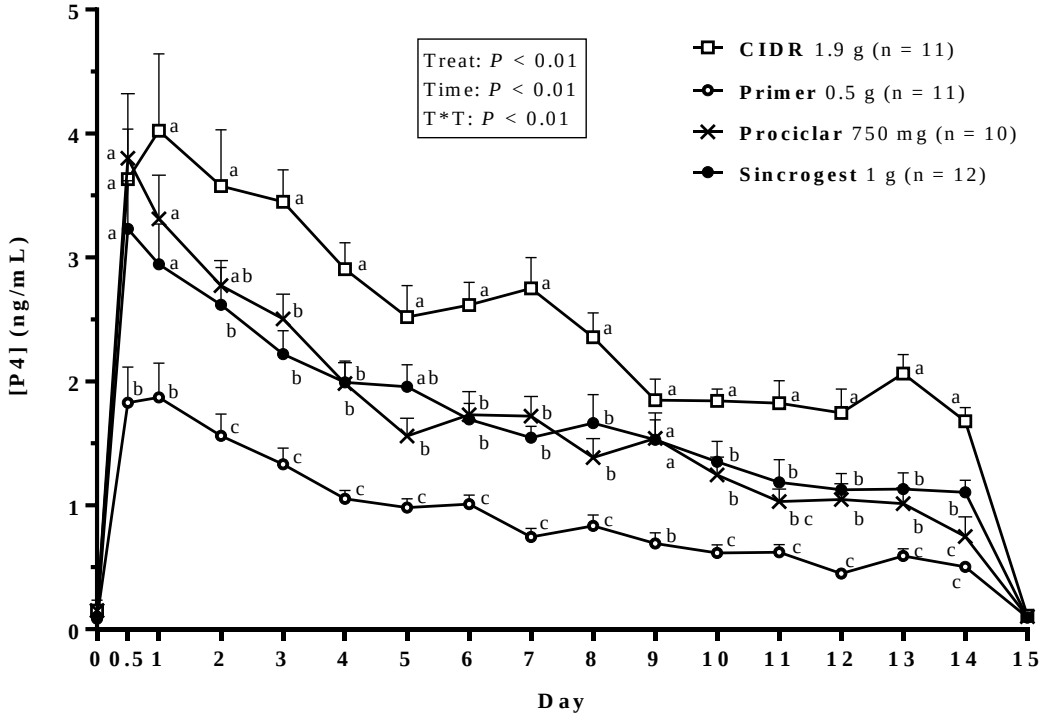

Figure 4. Circulating progesterone (P4) concentrations (mean ± SEM) during 15 d in non-lactating multiparous Nelore cows receiving one of five intravaginal P4 implants: CIDR 1.9 g (n = 11); Primer 0.5 g (n = 11); Prociclar 750 mg (n= 10); Sincrogest 1 g (n = 12). The P4 implants were removed on Day 14. Cows that ovulated were excluded from this analysis and for cows that lost the P4 implant, circulating P4 concentrations were included just until 1 d before loss. Distinct letters (^{a,b,c,d}) indicate difference among treatments within each time.

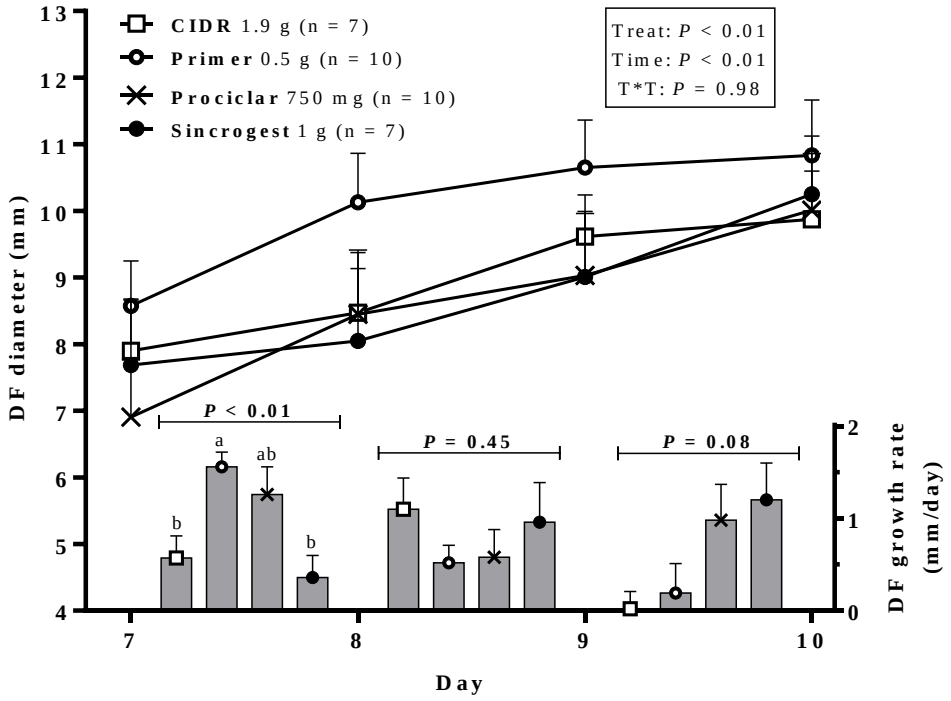

Figure 5. Diameter and daily growth rate (mean ± SEM) of the dominant follicle (DF) from Day 7 to 10 after the insertion of an intravaginal progesterone (P4) implant (CIDR 1.9 g, n = 7; Primer 0.5 g, n = 10; Prociclar 750 mg, n = 10; Sincrogest 1 g, n = 7) associated to EB (2 mg) administration in non-lactating multiparous Nelore cows. Continuous lines represent the DF growth profile over time and bars represent the daily growth rate, according to each treatment. Distinct leters (^{a,b,c}) indicate differences in daily growth rate among treatments. Only cows that emerged a new follicular wave were considered. Cows that ovulated, presented follicular codominance, started a new follicular wave between Day 7 and 10 (turn-over), or lost the P4 implant were excluded from this analysis.

2.3.3 Experiment 3 (Holstein cows)

In the third experiment, a fourth replicate was added to increase the number of observations per treatment. Similar to the other experiments, for the DF dynamics analysis, several data were excluded (n = 34) due to the following circumstances: ovulation between Day 0 and Day 5 (DIB0.5 = 3; DIB = 2; PRID = 2; Sincrogest = 3), lack of follicle wave emergence (Cue-Mate = 2; DIB0.5 = 3; DIB = 6; PRID = 2; Sincrogest = 1), implant loss (PRID = 2), and co-dominance (Cue-Mate = 1; DIB0.5 = 2; DIB = 2; PRID = 2; Sincrogest = 1). No observation was excluded due to follicular turn-over in this experiment. For circulating P4 analyses, data from 10 cows were discarded due to ovulation between Day 0 and Day 5. Moreover, two observations were removed from the analysis because of early implant loss and one due to inconsistency on P4 assay.

Mean circulating P4 concentration, AUC and P4 peak were different between treatments ($P < 0.01$; Table 3). The PRID implant produced the greatest circulating mean P4 and peak, but

not different than what was observed for Sincrogest. Additionally, treatment with Cue-Mate released more P4 than treatment with DIB0.5, but similar to DIB. The P4 peak produced was similar between these last three implants (Table 3). Moreover, the P4 release profile over time was affected by treatment, time and interaction treatment*time ($P < 0.01$; Fig. 6). The PRID implant had the highest peak of P4, about 50% greater than the others on D2. The Cue-Mate, DIB and Sincrogest implants provided similar circulating P4 among each other, and the DIB0.5 presented the lowest P4 release profile, mainly from D4 on (Fig. 6).

There were no differences on DF growth rate over time or DF diameter at any specific day (Table 3). However, an effect of treatment ($P < 0.01$) on DF growth profile over time was observed, although no difference was observed when the DF growth rate was analyzed at daily intervals (Fig. 7). The PRID implant produced lower DF growth profiles than Cue-Mate, DIB0.5, and DIB, which were similar among each other. Treatment with Sincrogest produced an intermediate follicle development, differing from DIB0.5 and tending to be greater than when cows were treated with PRID ($P < 0.10$; Fig. 7).

Table 3. Circulating progesterone concentrations and dominant follicle dynamics of non-lactating Holstein cows receiving intravaginal P4 implants during 14 days. Experiment 3.

	Cue-Mate (1.56 g)	**DIB 0.5** (0.5 g)	**DIB** (1 g)	**PRID** (1.55 g)	**Sincrogest** (1 g)	***P*-value**
Circulating P4 (n)	11	13	15	11	11	
Mean concentration (ng/mL)[1]	1.1±0.1^{b}	0.6±0.1^{c}	1.0±0.1^{bc}	1.5±0.1^{a}	1.2±0.1^{ab}	< 0.01
AUC (ng/mL * day)	15.5±1.2^{b}	8.4±1.1^{c}	13.2±0.9^{bc}	21.4±1.9^{a}	16.4±1.8^{ab}	< 0.01
Peak (ng/mL)	2.0±0.2^{b}	1.8±0.2^{b}	2.2±0.1^{b}	3.1±0.3^{a}	2.2±0.3^{ab}	< 0.01
DF (n)	9	8	7	9	9	
Diameter on Day 7 (mm)	10.2±0.9	11.1±0.7	11.4±0.6	8.8±0.8	10.3±0.3	0.14
Diameter on Day 10 (mm)	14.3±0.7	15.6±0.8	15.9±0.7	13.3±1.1	14.1±0.9	0.12
Growth rate (mm/day)[2]	1.4±0.1	1.4±0.1	1.6±0.2	1.3±0.1	1.3±0.1	0.49

^{a,b,c}Values within the same row differ ($P \leq 0.05$).
Values presented as mean ± SEM.
Abbreviations: P4 = progesterone; AUC = Area under the curve; DF = Dominant follicle.
[1]Mean circulating P4 was calculated from Day 0.5 to Day 14.
[2]DF growth rate was calculated from Day 5 to Day 10.

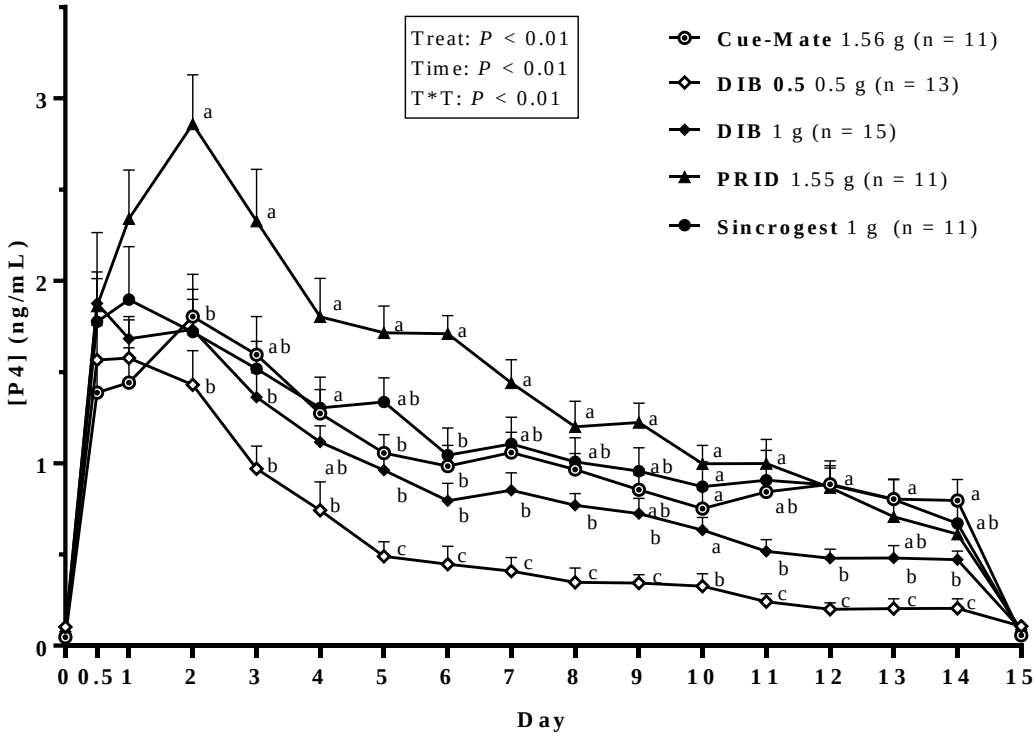

Figure 6. Circulating progesterone (P4) concentrations (mean ± SEM) during 15 d in non-lactating multiparous Holstein cows receiving one of five intravaginal P4 implants: Cue-Mate 1.56 g (n = 11); DIB 0.5 0.5 g (n = 13); DIB 1 g (n = 15); PRID 1.55g (n= 11); Sincrogest 1 g (n = 11). The P4 implants were removed on Day 14. Cows that ovulated were excluded from this analysis and for cows that lost the P4 implant, circulating P4 concentrations were included just until 1 d before loss. Distinct letters (^{a,b,c}) indicate difference among treatments within each time.

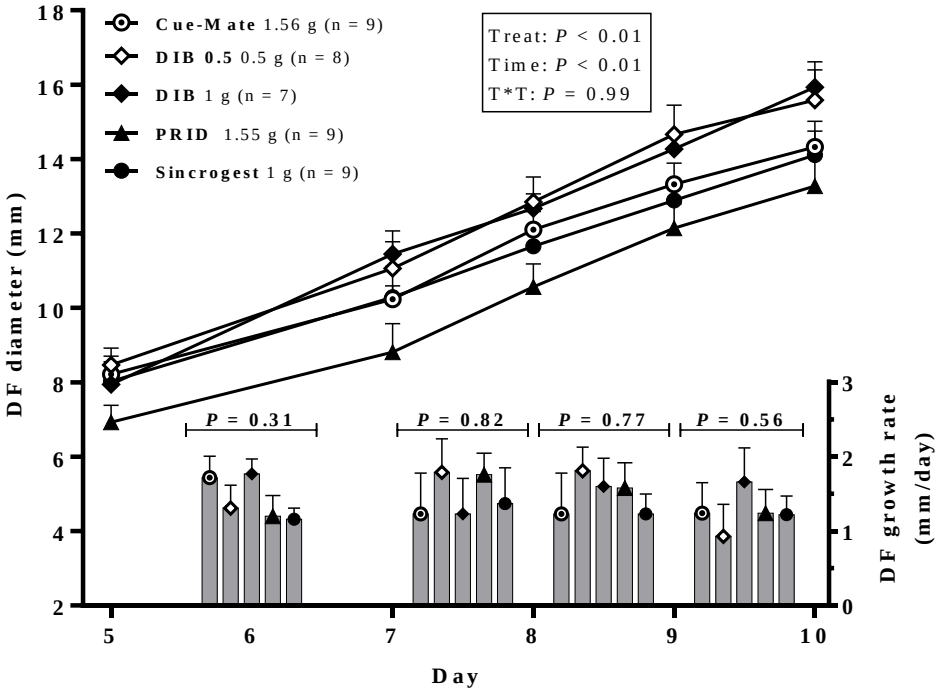

Figure 7. Diameter and daily growth rate (mean ± SEM) of the dominant follicle (DF) from Day 7 to 10 after the insertion of an intravaginal P4 implant (Cue-Mate 1.56 g, n = 9; DIB 0.5 0.5 g, n = 8; DIB 1 g, n = 7; PRID 1.55 g, n = 9; Sincrogest 1 g, n = 9) associated to EB (2 mg) administration in non-lactating multiparous Holstein cows. Continuous lines represent the DF growth profile over time and bars represent the daily growth rate, according to each treatment. Only cows that emerged a new follicular wave were considered. Cows that ovulated, presented follicular codominance, started a new follicular wave between Day 7 and 10 (turn-over), or lost the P4 implant were excluded from this analysis.

2.3.4 Circulating P4 and dominant follicle size

Additional analyses were done to evaluate the relationship between mean circulating P4 concentrations provided by the implants and the DF development within breeds. Therefore, data from Exp. 1 and 3 were combined and the analyses were done according to the genetic group (Holstein or Nelore). Initially, based on the mean time of P4 implant maintenance and the time of AI in synchronization protocols [28,29], the relationship between mean circulating P4 during the first 8 d of implant insertion and DF diameter on D10 was investigated. A significant negative correlation was observed for Holstein cows ($R^2 = 0.10$; $P < 0.01$) and a tendency was detected for Nelore cows ($R^2 = 0.07$; $P = 0.08$). Subsequently, according to the physiological bases of the P4/EB-based protocols [30,31] and the specific stages of follicular wave development [4,32], the period of P4 insertion was divided into three sections: emergence (Day 0.5 to 3), selection (Day 4 to 6) and dominance period (Day 7 to 10). Further analyses were done to study the mean circulating P4 during the emergence and selection periods in relation to the DF diameter on D7, as well as the mean circulating P4 during the dominance period in

relation to DF diameter on D10 or DF growth rate. Then, although no significant effect was observed for the selection or dominance period, a negative correlation between mean circulating P4 during the emergence period and the DF diameter on D7 was observed for Holstein (R^2 = 0.12; $P < 0.01$) and Nelore cows (R^2 = 0.27; $P < 0.01$), as demonstrated in Fig. 8.

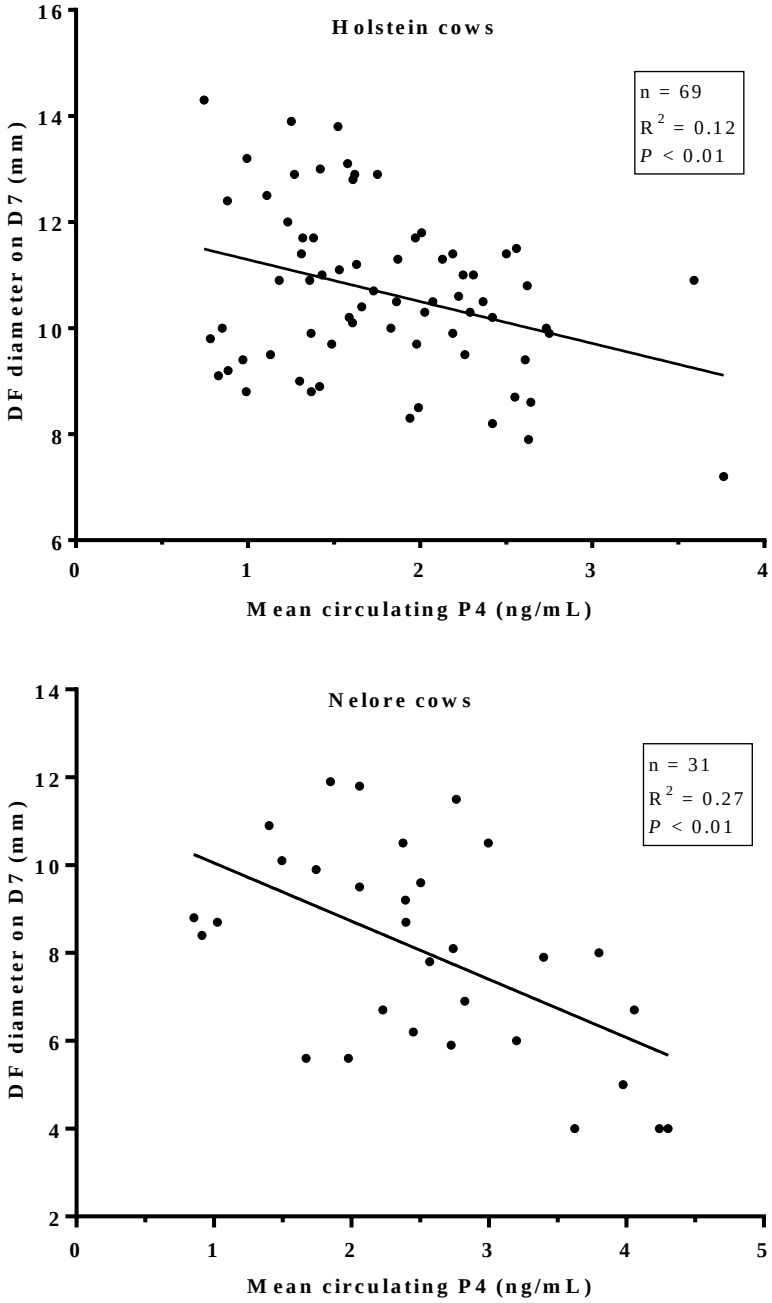

Figure 8. Correlation between dominant follicle (DF) diameter on Day 7 and mean circulating progesterone (P4) concentrations during the first 3 d after intravaginal implant insertion in Holstein (above) and Nelore (below) cows. Data from Exp. 1 and 3 were combined for regression analysis in Holstein cows. The regression lines show linear effects for Holstein (y = 12.1 – 0.8x; R^2 = 0.12; $P < 0.01$) and Nelore (y = 10.8 – 1.1x; R^2 = 0.23; $P < 0.01$).

2.4 Discussion

This study evaluated the circulating P4 profile provided by 10 commercial implants widely used in synchronization protocols for FTAI, as well as the effect of circulating P4 concentrations on the development of the DF in *Bos taurus* and *Bos indicus* cows. Several studies have compared implants with different shapes and materials [33,34] and the same implant shape with distinct P4 loads [35,36]. However, the present study is the first to compare such a large number of implants, evaluating their effectiveness in different breeds.

As previously reported, the efficiency of an implant to release P4 into the bloodstream could be affected by the implant surface area, P4 load and the material of the outer layer [8,36]. In this study, the surface area of implants was not measured. However, PRID is the only tested implant that presents a different shape pattern (triangle-like) while all the others are either Y- or T-shaped. According to van Werven et al. [33], the PRID presents a greater surface area (~ 155 cm^2) than CIDR (~ 120 cm^2). In their study, by comparing circulating P4 concentrations in non-lactating Holstein cows treated with PRID (1.55 g) *vs*. CIDR (1.38 g) for 7 d, it was found greater circulating P4 produced by the PRID during the first 4 d after implant insertion. Similarly, in Exp. 1, circulating P4 concentrations were greater in Holstein cows treated with PRID than CIDR. However, this was observed only between 24 and 48 h after implant insertion and no differences were found for mean circulating P4 or AUC between these implants, considering a 14-d period.

Results in non-lactating Holstein cows obtained from this study supported the idea that surface area affects the capacity of implants to release P4. Considering that PRID has the greatest area, despite having a lower P4 load (1.55 g) than Repro sync (2 g) in Exp. 1, it was able to produce similar circulating P4 concentrations as the other implant. Moreover, in Exp. 3, by comparing implants with similar P4 loads (PRID – 1.55 g *vs*. Cue-Mate – 1.56 g), a greater release profile, mean circulating P4 and AUC was observed for PRID. This could be explained by the fact that the Cue-Mate design was composed by two P4-releasing pods attached to the edge of an inert flexible T-shaped spine [34], yielding a smaller surface area with impregnated P4 in contact with the vaginal mucosa, hence releasing less P4. In addition, findings from the present study suggest that the initial P4 load can affect the P4 release capacity of implants. In their study, Rathbone et al. [36] reported no differences in circulating P4 concentrations when two intravaginal implants (CIDR) with equal surface area, but different P4 loads (1.9 g *vs*. 1.34 g), were compared for a 7-d period in ovariectomized Holstein cows. However, as expected, there was a greater residual P4 load after implant removal for CIDR 1.9 g than 1.34 g [32]. In our study (Exp. 3), the comparison between two equal-shaped implants with distinct P4 loads

(DIB 0.5 [0.5 g] *vs*. DIB [1 g]) resulted in lower circulating P4 concentrations produced by DIB 0.5 from Day 5 to 14. Apparently, despite having produced similar circulating P4 concentrations during the first 4 d, congruently with what has been previously described [36], DIB 0.5 could not maintain an equivalent release compared to the DIB with twice its concentration after this period. Macmillan and Peterson [35], evaluated residual P4 from implants with different initial P4 loads after 15 d of insertion and reported that the proportion of P4 released from the initial load was lower when the initial P4 load was greater. Therefore, possibly due to its lower initial load, DIB 0.5 lost proportionally a greater amount of P4 than DIB during the same insertion period and, from Day 5 on, the residual P4 content in DIB 0.5 was not enough to provide equivalent circulating P4 concentrations as DIB. Additionally, in another study, using the same experimental model as ours, Melo et al. [27] reported ~20% greater circulating P4 provided by CIDR (1.9 g) than Sincrogest (1 g) during an 8-d insertion period in non-lactating Holstein cows, despite of their minor differences in surface area (120 cm^2 *vs*. 129 cm^2, respectively). Moreover, in their study, the observed P4 profile and mean circulating P4 provided by the Sincrogest implant over an 8-d period was similar to what was obtained in Exp. 3, evaluating the same product in non-lactating Holstein cows.

In addition, results obtained in non-lactating Nelore cows also supported the effect of surface area and initial P4 load on the P4 release capacity of implants. In the present study, when CIDR (1.9 g) and Sincrogest (1 g) were compared in Nelore cows (Exp. 2), the circulating P4 provided by the CIDR over 14 d was ~32% greater compared to Sincrogest, although the P4 peak produced did not differ between them, what could be explained by their distinct P4 loads. Moreover, when the Primer implant was evaluated, the circulating P4 profile over 14 d and the P4 peak were lower compared to the other implants, what can be explained by its lower P4 load (0.5 g), but also due to the fact that, in this implant, only the flaps are composed by silicone impregnated with P4, resulting in a smaller surface area.

Furthermore, this study evaluated the effect of circulating P4 concentrations provided by each implant on the growth of the DF, manly from Day 7 to 10 after implant insertion associated to EB administration. Generally, P4/EB-based FTAI protocols have 7 to 9 d of implant P4 exposure, followed by AI 2 d after implant removal [28–30]. Thereby, the major role of an implant in these protocols is to control follicular growth, avoiding early ovulation and providing a DF with adequate size, estradiol production and ovulatory capacity at AI. It is well known that circulating P4 can control LH pulse frequency, directly affecting DF growth. In their study, Cerri et al. [37] reported a greater DF diameter at implant removal and at AI for Holstein cows submitted to low circulating P4 concentrations during the synchronization

protocol. In the present study (Exp. 1), regardless that Prociclar provided lower circulating P4 concentrations, there were no differences in DF dynamics from Day 7 to 10. Otherwise, in Exp. 3, although there were no differences observed for DF growth rate from Day 5 to 10, nor for DF diameter on any specific day, there was an effect of P4 implant on the DF growth profile over time. Implants that produced greater mean circulating P4 were associated with a lower DF growth profile from Day 5 to 10.

It is important to consider that in the experimental model of this study, the cows had no CL during DF growth and the circulating P4 during this period was exclusively from the implant. Thus, despite some implants producing greater circulating P4 than others during DF development, it is still much lower than the circulating P4 provided by a CL during the estrous cycle. For example, while Cerri et al. [38] reported a mean circulating P4 of 0.78 ng/mL produced by insertion of a CIDR implant (1.38 g) for 7 d in lactating Holstein cows without a CL, Sartori et al. [39] reported maximal circulating P4 of 5.6 ng/mL during the estrous cycle of high-producing lactating Holstein cows. A recent study from our laboratory has also observed low circulating P4 peak after CIDR (1.9 g) or Repro synch (2.0 g) insertion in non-cycling high-producing lactating Holstein cows (1.3 and 1.4 ng/mL, respectively; unpublished data). Therefore, regardless of the differences observed among implants, the circulating P4 concentrations produced by them were not enough to dramatically change follicular dynamics in lactating Holstein cows.

Independent of this, it is well established that circulating P4 concentrations during follicular growth prior to AI substantially affect the fertility of lactating dairy cows. There are several reports relating the effect of high circulating P4 on follicular development [37], embryo quality [19] and fertility of dairy cows [20]. Moreover, studies have related the potential improvement of P4 supplementation with intravaginal implants on the reproductive performance of dairy cows submitted to FTAI programs [9,10,21,40].

Additionally, for Nelore cows (Exp. 2), an effect of P4 implant was observed on the DF growth profile from Day 7 to 10. The Primer implant, which produced the lowest circulating P4 concentration, presented a greater DF growth profile over time than the others. However, there were no differences for DF growth rate or DF diameter on any specific day. Likewise, Sales et al. [13] evaluated the effect of distinct circulating P4 concentrations (provided by a new Sincrogest implant – 1 g *vs*. the same implant previously used for 8 and 16 d) on DF growth in anestrous Nelore cows submitted to a P4-based synchronization protocol. There was no effect of treatment on the DF diameter at implant removal on Day 8 of the protocol, nevertheless a larger maximum DF diameter (evaluated from the time of implant removal to ovulation) was

observed for the cows treated with the implant that had been previously used for 16 d. Another similar study comparing the insertion of a new *vs*. previously used for 9, 18, or 27 d CIDR (1.9 g) in anestrous Nelore cows, reported no differences in the diameter of the largest follicle measured at the time of FTAI, despite differences in circulating P4 concentrations among treatments at implant removal on Day 9 of the protocol [25]. In these two studies [13,25], there was no effect of the distinct circulating P4 concentrations provided by the implants on ovulation or P/AI. However, Meneghetti et al. [25], reported a positive correlation between the DF diameter and ovulation or P/AI, which is supported by other studies that observed a relationship between the DF size and expression of estrus, and consequently, positively influencing fertility of cross-bred or Nelore cows [41,42].

Although no direct comparison between genetic groups (*Bos taurus vs*. *Bos indicus*) was done in this study, considerable information has been provided that reinforces differences in circulating P4 concentrations and follicular development between these groups. Our results corroborate data form previous studies that evaluated physiological differences between *Bos taurus* and *Bos indicus* cattle. Carvalho et al. [16] evaluated cycling beef heifers from both genetic groups submitted to a P4-based synchronization protocol with a CIDR (1.9 g) insertion associated or not to a PGF administration on Day 0. Regardless of the PGF treatment, *Bos taurus* heifers presented lower circulating P4 concentrations and a greater follicular growth rate and DF diameter. Moreover, a PGF treatment at the onset of the FTAI protocol decreased circulating P4 and increased follicular growth, regardless of the genetic group. In addition, a recent study has shown similar results comparing Holstein *vs*. Nelore heifers, reporting a potential impact of liver metabolism and feed intake on the endocrine environment and ovarian responses [18]. In this study, besides the effect of breed, there was a substantial effect of the diet (high *vs*. low dry matter intake [DMI]) on DF dynamics and circulating P4 concentrations. Heifers with high DMI had greater DF growth, as well as larger DF diameter on Day 8 and 10 of the synchronization protocol, and circulating P4 was lower than in heifers with low DMI. Moreover, regardless of the diet, Holstein heifers presented greater expression of the main enzymes responsible for P4 metabolism in the liver.

A further analysis on the relationship between circulating P4 concentrations (regardless of the implant used) and follicular dynamics for Holstein and Nelore cows in our study revealed a linear effect of the mean circulating P4 after implant insertion on DF diameter. More specifically, according to this analysis, the greater the mean circulating P4 during the emergence period (from Day 0.5 to 3) the smaller the DF diameter on D7, which was more pronounced for Nelore cows. In fact, in the present study, the emergence of a new follicular

wave was promoted by the administration of EB associated with the P4 implant insertion, promoting a younger and smaller DF on D7 if compared with GnRH-based protocols, due to the moment of the follicular wave emergence [30,31,43]. Moreover, this major effect of circulating P4 concentrations in Nelore cows is supported by findings from two recent studies that reported lower LH pulse frequency [18] and a reduced LH release, mainly under high P4 concentrations, for Nelore compared to Holstein heifers [17]. In our study, the mean circulating P4 during the first 3 d (when circulating P4 reached the highest concentrations) exerted more effect on follicular development than the mean circulating P4 over other and longer periods, and the effect on DF diameter was more pronounced on Day 7 than on Day 10. Therefore, based on the mean time to follicular emergence in P4/EB-based protocols [30,31], and considering that cows had no CL and circulating P4 were provided only by an intravaginal implant, a possible explanation is that the high circulating P4 concentration during the beginning of the protocol promoted a delay in follicular emergence, producing a smaller DF on Day 7. However, this effect did not compromise synchronization of the emergence of follicle wave and follicular growth over time. Moreover, when the DF diameter on Day 10 was evaluated, the effect of mean circulating P4 during the first 3 d was mitigated, probably due to the below physiological circulating P4 concentrations. In fact, recent studies have reported the effect of high circulating P4 during the early follicular development, negatively influencing LH pulse frequency and circulating estradiol concentrations before establishment of follicle dominance [44], as well diminishing the size of the ovulatory follicle [45]. However, these studies were designed to evaluate the effect of circulating P4 concentrations after follicular emergence. The influence of P4 concentrations before emergence, for example during the first 3 d after EB administration at the onset of the protocol is not totally clear. Interestingly, in a study with lactating dairy cows submitted to an EB/P4-based protocol starting either at 3 or 7 d after a confirmed ovulatory treatment with GnRH [46], follicle emergence and deviation was delayed when the protocol started on Day 7 after GnRH, under higher circulating P4 concentrations. Moreover, in their study, there was no differences in the size of ovulatory follicle, consistent with what was observed in the present study. It is also possible, although not properly tested, that greater circulating P4 could delay follicle wave emergence due to a delay in the timing or peak amplitude of the FSH surge after EB treatment. Nevertheless, more specific studies are needed to elucidate the influence of P4 concentrations before emergence of a new follicle wave in EB/P4-based protocols.

In addition, in our experiment, some cows were excluded from the analyses mainly because of ovulation between D0 and first ultrasound evaluation or lack of emergence of a new

follicular wave. For Nelore cows (Exp. 2), ~6% ovulation and ~15% lack of emergence of a new follicle wave was observed, which is similar to what has been reported for Nelore cows submitted to a EB/P4-based protocol (12 and 8%, respectively) [31]. For Holstein cows (Exp. 1 and 3), 11.5% ovulation and 13% lack of emergence was detected, consistent or even lower than what has been reported for lactating dairy cows (17 and 26%, respectively) [46,47]. Moreover, there was an incidence of 14% of follicular codominance in Holstein cows, that could be explained by the subluteal circulating P4 concentrations provided by the experimental design. According to Lopez et al. [48], the development of multiple dominant follicles in lactating Holstein cows was associated with lower circulating P4 concentrations during the initial follicular growth phase and deviation. In their study, cows with codominant follicles had greater circulating FSH and LH prior to follicle deviation (selection of the dominant follicle[s]), that could be due to the lower circulating P4. Therefore, in the present study, probably the subluteal circulating P4 concentrations stimulated greater circulating FSH and LH pulse frequency, which is related to the increase in estradiol and insulin-like growth factor 1 (IGF1) production by the DF [49], and could have contributed to the codominance condition. In this sense, recent studies have reported a greater incidence of double ovulation in lactating dairy cows submitted to low circulating P4 concentrations during the Ovsynch protocol, associated to greater pregnancy loss and, consequently, lower fertility [45,50].

In conclusion, all the intravaginal P4 implants tested in this study were capable of increasing circulating P4 concentrations and synchronizing emergence of a new follicular wave, when associated to EB at the onset of the protocol. Moreover, the implants provided distinct circulating P4 concentrations, especially when the P4 peak after implant insertion was evaluated. Apparently, this effect was primarily influenced by the implant surface area and initial P4 load. In addition, primarily in Nelore cows, greater circulating P4 concentrations during the first 3 d of implant insertion, were associated with smaller DF sizes on Day 7, when submitted to an estradiol/P4-based protocol. Finally, Nelore cows had greater circulating P4 and smaller DF diameter than Holstein cows, even when treated with similar exogenous P4 sources. Results from the present study provide a better understanding of the P4 release pattern of intravaginal P4 implants and the effect of circulating P4 on DF development, in order to optimize their use in reproductive management of *Bos taurus* and *Bos indicus* cattle.

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3. EFFECT OF PROGESTERONE FROM CORPUS LUTEUM, INTRAVAGINAL IMPLANT, OR BOTH ON LH RELEASE, OVULATORY RESPONSE AND SUBSEQUENT CL DEVELOPMENT AFTER GONADORELIN TREATMENT

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ABSTRACT

This study aimed to determine the effect of circulating progesterone (P4) concentrations produced by the corpus luteum (CL) and/or an intravaginal P4 implant (IPI) on the GnRH-induced LH release, ovulatory response and subsequent CL development, after administration of 100 μg gonadorelin acetate (GnRH). Non-lactating multiparous Holstein cows were submitted to a presynchronization protocol: D-17: 2 mg estradiol benzoate and a 2 g reused P4 implant; D-9: 0.5 mg cloprostenol sodium (PGF) and 1 mg estradiol cypionate; D-7: 100 μg GnRH. Only cows that ovulated were enrolled in the experiment (n = 90; BCS = 3.3 ± 0.1; 4 replicates). On D-1.5 cows were randomly assigned to a 2x2 factorial arrangement (presence of CL at GnRH challenge x P4 implant insertion), creating four groups: **CL-IPI**, **CL**, **CL0-IPI** and **CL0**. On D-1.5, CL0 and CL0-IPI groups received 0.5 mg PGF followed by a second dose 24 h later. On D0, all cows were treated with 100 μg GnRH and, simultaneously, cows from IPI groups received a 2 g P4 implant for 14 d. Diameter of the DF, ovulatory response, and subsequent CL volume were assessed by ultrasonography on D-1.5, 0, 2, 7, and 14. For circulating P4, blood samples were collected on D-1.5, 0, 3, 5, 7 and 14 for all groups and, additionally, on D0 at 1, 2, 4 and 6 h, D1 and D2 for IPI groups. For LH release, samples were taken on D0, before GnRH treatment, and at 1, 2, 4 and 6 h later. Moreover, for a subset of cows (n = 36), subsequent CL development was evaluated daily from D5 to D14. Statistical analyses were performed by SAS 9.4, and results are presented as means ± SEM; $P \leq 0.05$). There was a main effect of presence of CL at the time of GnRH challenge on the ovulatory response and GnRH-induced LH peak (with CL = 58.1% and 5.3 ng/mL; without CL = 95.5% and 13.2 ng/mL). In addition, despite producing a rapid increase in circulating P4, there was no effect of P4 implant insertion on ovulatory response and LH release. Regardless of group, ovulatory response was positively correlated to LH peak and negatively correlated to circulating P4 on D0. Moreover, subsequent CL development and function were negatively affected by the presence of CL and by the P4 implant insertion in CL0-IPI group. The results confirm that P4 produced by the CL at GnRH treatment exerts a suppressive effect on ovulatory response of a 7-d old follicle, GnRH-induced LH release and subsequent CL development. Moreover, although the insertion of a P4 implant did not change ovulation and LH release, it negatively influenced CL development/maintenance in cows without CL at the time of GnRH challenge. Thus, it is very likely that P4 supplementation at the time of ovulation induction causes a negative effect on the subsequent CL function and lifespan, producing early luteolysis.

Keywords: Corpus luteum; Progesterone device; Gonadotropin; Ovulation.

3.1 Introduction

Over the last four decades, several studies have developed diverse strategies to manipulate the estrous cycle and induce ovulation in cows by the administration of exogenous reproductive hormones (Knickerbocker et al., 1988; Bo et al., 1995; Sales et al., 2012; Wiltbank and Pursley, 2014). Since then, the gonadotropin-releasing hormone (GnRH) analogues have been widely used as an inducer of ovulation, by the stimulation of the pituitary gland, producing an LH release (Clarke and Cummins, 1982; Peters, 2005; Souza et al., 2009b). From the development of the synchronization protocols for FTAI, such as Ovsynch, GnRH has been used to synchronize follicular wave emergence at the onset of the protocol, after an induced ovulation (Pursley et al., 1995; Haughian et al., 2013). Currently, GnRH-based protocols are intensively applied for reproductive programs in dairy cows (Carvalho et al., 2018; Vasconcelos et al., 2018).

It is well established that the efficacy of synchronization protocols in dairy cows is associated with a suitable emergence of the follicular wave at the beginning of the protocol (Monteiro et al., 2015), promoting an adequate follicular age and length of dominance period, which improves embryo quality and, consequently, fertility of cows (Cerri et al., 2009b; Wiltbank et al., 2011). However, several studies evaluating reproductive strategies based on the Ovsynch protocol, that started at a random day of the estrous cycle of lactating dairy cows, reported around 50% of ovulation in response to de first GnRH of the protocol (Bilby et al., 2013; Bisinotto et al., 2013; Lopes et al., 2013). Moreover, in a recent study from our research group, using a GnRH analogue (gonadorelin diacetate), the ovulatory response to the first GnRH was lower than 35% in lactating Holstein cows (Melo et al., 2016).

In fact, the ovulatory response to a GnRH treatment can be affected by the stage of the estrous cycle (Vasconcelos et al., 1999), type of GnRH product (Souza et al., 2009a; Luchterhand et al., 2019) and circulating P4 concentrations at the time of GnRH administration (Lima et al., 2013; Carvalho et al., 2015). Moreover, interesting studies have reported the effect of circulating concentrations of steroid hormones on the GnRH-induced LH release (Stevenson and Pulley, 2016; Motta et al., 2020). In one of the studies (Giordano et al., 2012a), it has been demonstrated that lactating dairy cows under high circulating P4 concentrations at the time of a treatment with 100 µg of gonadorelin had a 5-fold lower LH peak than cows under low P4 concentrations. Apparently, circulating P4 can modulate the sensitivity of the pituitary gland to a GnRH treatment, with suppression of LH secretion in proportion to circulating P4 (Nett et al., 2002).

Furthermore, besides promoting a synchronized follicular emergence, ovulation to the first GnRH creates a new CL, increasing circulating P4 concentrations during follicular development. For high-producing dairy cows, it is well know that greater P4 concentrations are important for optimal development for the dominant follicle (DF), resulting in better embryo quality (Rivera et al., 2011) and greater P/AI (Wiltbank et al., 2012). For this reason, P4 supplementation by the insertion of an intravaginal implant at the time of the first GnRH treatment has been frequently used to increase circulating P4 concentrations during the synchronization protocol (Chebel et al., 2010; Bisinotto et al., 2015). In this sense, a recent study from our research group (Melo et al., 2018), evaluating the effect of insertion of an implant impregnated with 1.9 g P4 on circulating P4 concentrations in non-lactating Holstein cows, reported an increase of ~1 ng/mL in circulating P4, 2 h after implant insertion. Likewise, a similar increase (~ 0.8 ng/mL) was reported at 15 min after insertion of a similar P4 implant in lactating Holstein cows (Cerri et al., 2009a). The effect of an intravaginal P4 implant insertion at the time of the first GnRH of the protocol on the ovulatory response is still not totally clear. Some studies reported a lesser ovulatory response in cows treated with a P4 implant at the time of GnRH (Galvão et al., 2004; Stevenson et al., 2008), however this effect was not observed in other studies (Bilby et al., 2013; Bisinotto et al., 2013).

There were no previous studies in the literature designed to evaluate the influence of the P4 implant insertion at the time of GnRH treatment on the GnRH-induced LH release and ovulatory response of a presynchronized 7-d old DF. Therefore, the objective of the present study was to determine the effect of circulating P4 concentrations produced by a 7-d CL or a P4 implant insertion, as well as the association of these two treatments on the GnRH-induced LH release, ovulatory response and subsequent CL development, after treatment with a conventional dose of gonadorelin. The specifc hypotheses of this study were: 1) the circulating P4 originating from the CL would suppress the GnRH-induced LH surge and negatively affect the ovulatory response; 2) the insertion of an intravaginal P4 implant at the time of GnRH treatment would promote a rapid increase in circulating P4 concentrations, sufficient to negatively affect the GnRH-induced LH surge, but not enough to compromise the ovulatory response; and 3) the premature increase in circulating P4 by provision of a P4 implant near ovulation would compromise subsequent CL development.

3.2 Material and methods

The current study was conducted from October of 2018 to March of 2019 at the University of São Paulo (USP), Piracicaba, SP, Brazil. All procedures were previously

approved by the Animal Research Ethics Committee of "Luiz de Queiroz" College of Agriculture (ESALQ)/USP (Protocol # 2018-18).

3.2.1 Animals and management

Non-lactating multiparous Holstein cows (n = 36; BCS = 3.3 ± 0.1) were kept in dry lots and were fed daily a maintenance diet based in haylage (*Cynodon dactylon*) and a corn plus soybean based concentrate supplementation, with free access to water and mineral salt.

3.2.2 Experimental design

Previous to the beginning of treatments, all cows were submitted to a presynchronization protocol (D-17) starting with insertion of a 2 g intravaginal P4 implant (Repro sync, GlobalGen Vet Science, Jaboticabal, SP, Brazil) that had been previously used for 7 d, together with 2 mg estradiol benzoate i.m. (Syncrogen, GlobalGen Vet Science). Eight d later, on D-9, P4 implants were removed and all cows received 1 mg estradiol cypionate (Cipion, GlobalGen Vet Science) and 0.53 mg cloprostenol sodium (PGF; Induscio, GlobalGen Vet Science). On D-7, 100 µg of gonadorelin acetate (GnRH; Fertagyl, MSD, Cruzeiro, SP, Brazil) was administered to induce ovulation. For this experiment, 4 replicates (Figure 1) were conducted and only cows that ovulated in response to the GnRH administration on D-7 and had a DF ≥ 10 mm on D0 were submitted to the treatments. Cows that met this criteria (n = 87) were randomly assigned to a 2x2 factorial arrangement composed by presence or not of a 7-d old CL and insertion or not of a P4 implant (2 g) at the time of GnRH challenge on D0. Therefore, 4 groups were created by combination of these factors: **group CL-IPI** (presence of CL and P4 implant insertion); **group CL** (presence of CL and no P4 implant insertion); **group CL0-IPI** (no CL and P4 implant insertion); and **group CL0** (no CL and no P4 implant insertion). Groups designed to have no CL at the time of GnRH challenge received 2 treatments of PGF (0.53 mg) 24 h apart, on D-1.5 and D-0.5, aiming to regress the CL formed after ovulation to GnRH at D-7. Additionally, these cows received a tail-head adhesive patch for estrus detection (BOViFLAG - Bovitime Animal Products LTD, Stellenbosch, South Africa) on D-1.5 at the time of the first PGF treatment, to check estrus behavior between D-1.5 and D0 and, eventually, to exclude cows detected in estrus before the GnRH challenge. On D0, all cows were treated with 100 µg of GnRH (Fertagyl, MSD) and, simultaneously, for groups designated to receive a P4 implant, the cows received a 2 g P4 implant (Repro sync, GlobalGen Vet Science), which remained for 14 d.

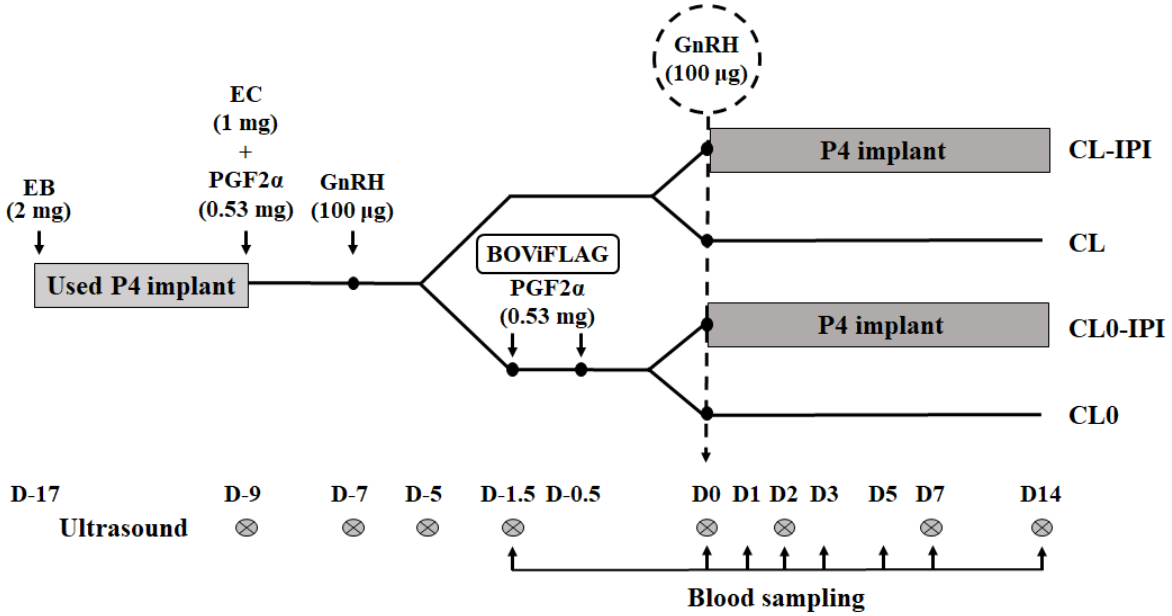

Figure 1. Schematic representation of the experimental design. Non-lactating Holstein cows (n = 90; 4 replicates) were submitted to a presynchronization (D-17: EB + P4 implant; D-9: implant removal + EC + PGF; D-7: GnRH) to synchronize the follicular wave and create a new CL. Only cows that ovulated to the GnRH on D-7 were randomly assigned to the experimental groups, with or without a CL on D0 (CL *vs*. CL0) combined with or without treatment with an intravaginal P4 implant (IPI) on D0. On D-1.5, CL0-IPI and CL0 groups received a PGF administration, followed by a second dose 24 h later to regress the CL, and a tail-head adhesive patch for estrus detection (BOViFLAG). On D0, all cows received the GnRH challenge (100 µg of gonadorelin) and cows from CL-IPI and CL0-IPI groups simultaneously received a P4 implant for 14 d. (⊗) Ultrasound examinations were performed on Days -9, -7, -5, -1.5, 0, 2, 7, and 14. (↑) Blood samples were collected on Days -1.5, 0, 1, 2, 3, 5, 7, and 14 for all cows. On D0, blood samples were collected at 0, 1, 2, 4 and 6 h from the GnRH treatment. Additionally, for a subset of cows (n = 36), ultrasound examinations and blood samplings were performed daily from D5 to 14.

3.2.3 Ultrasound examinations

Transrectal ultrasound evaluations were performed using a 7.5 MHz linear-array transducer (DP-2200 VET, Mindray, Shenzhen, China) and, for all examinations, the ovarian structures (CL and follicles ≥ 5 mm) were mapped. For all these structures, 2 measures were taken, at right angles, to obtain the maximum distances between 2 opposite borders, and the diameter was determined as the mean of these 2 measures. On Days -9, -7 and -5, cows were scanned to evaluate the DF dynamics in response to the presynchronization protocol, and to check the ovulatory response to GnRH treatment at D-7. On D-1.5 and D0 the location and diameters of the DF and CL were evaluated, and 2 d later (D2), the ovulatory response to the GnRH challenge at D0 was accessed. Ovulation was determined by the disappearance of the DF from D0 to D2, and confirmed by the presence of a new CL on D7. The diameter of the subsequent CL and any CL cavity were measured on D7 and D14. Moreover, for a subset of

cows (n = 36), the CL development was evaluated daily from D5 to D14. For the groups designated to keep the CL from the presynchronization (CL-IPI and CL), when cows had ovulation to D0 in the same ovary that the first CL was observed, the CL were distinguished by the presence of a cavity (eventually) or by the position in the ovary, using the map to record the ovarian structures. For the CL analyses, diameter values were used to calculate the volume of CL (cm^3) by the formula for calculation of the volume of a sphere ($V = 4/3\pi r^3$). In CL that had a cavity, the volume of the cavity was also calculated by its diameter value, and the cavity volume was subtracted from the total CL volume.

3.2.4 Blood sampling and hormone assays

Blood samples were collected by puncture of the jugular vein into 9 mL heparinized evacuated tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) and immediately placed on ice. To evaluate circulating P4 concentrations, samples were taken on D-1.5 (before PGF treatment), D0 (before implant insertion), D3, 5, 7, and 14, for all groups. Additionally, for groups that received a P4 implant (CL-IPI and CL0-IPI), blood samples were collected at 1, 2, 4, and 6 h after the P4 insertion on D0, and on D1, 2, and 3. In the subset of cows evaluated for CL development from D5 to 14, samples also were taken daily during this period. Moreover, to assess the LH release in response to the GnRH challenge, blood samples were collected on D0, just before the GnRH treatment, and at 1, 2, 4, and 6 h after, for all groups.

After the collection, tubes were centrifuged at 1,700 x *g* for 15 min at 4°C and plasma was stored at -20°C. Plasma P4 concentrations were determined using a solid-phase radioimmunoassay (RIA) kit containing antibody-coated tubes and 125I-labeled P4 (ImmuChem Coated Tube P4 125 RIA Kit, MP Biomedicals, Costa Mesa, CA) validated for bovine plasma as previously described (Melo et al., 2018). Sensitivity, intra and inter-assay coefficient of variation were 0.02 ng/mL, 5.3% and 8.4%, respectively. The LH concentrations analyses were performed by RIA (Bolt and Rollins, 1983; Bolt et al., 1990), with some modifications (Ginther et al., 1999). Sensitivity, intra- and inter-assay coefficients of variation for LH were 0.04 ng/mL, 7.8 and 12.2%, respectively.

3.2.5 Statistical analyses

The present experiment was designed and analyzed as a 2x2 arrangement. All statistical analyses were performed using the SAS computational software version 9.4 (SAS, Version 9.4 for Windows, SAS Institute Inc., Cary, NC). Data were tested for normality of studentized residuals using the UNIVARIATE procedure, following the Shapiro-Wilk method, and

homogeneity of variances was evaluated by Levene test, using Hovtest and Welsh methods. When necessary, data were transformed to logarithm or square root, and outliers were removed. Nonparametric analysis was performed by the Wilcoxon and Kruskal-Wallis tests, using the NPAR1WAY procedure when data did not normalize even after transformation.

All the continuous data were analyzed by the MIXED procedure and for all the analyses the final model included the effect of presence of CL, P4 implant insertion and interaction between these factors (CL*IPI), fitting a Kenward-Roger method to calculate the denominator degrees of freedom to approximate the F-tests. Also, the replicate was considered as a random effect. For the analyses of CL volume on D7 and 14, the DF diameter on D0 was used as a covariate to adjust the model. Moreover, for circulating P4 concentrations on D3, 5, 7, and 14, the circulating P4 value on D0 was considered as a covariate.

The circulating LH and P4 concentrations, as well as the CL volume over time, were analyzed as repeated measures by the MIXED procedure. The final model included the same fixed effects, the time and the respective interactions with time, except in the analysis of circulating P4 from D0 to D2 for groups that received a P4 implant, in which the group, time and their interaction was considered as fixed effects. Replicate was considered as a random effect and cow within CL*IPI was the subject effect. The Kenward-Rogers method was used for the degrees of freedom calculation and, for each analysis, the appropriate covariance structure was selected, according to the smallest AICC value. Additionally, the area under the curve (AUC) of GnRH-induced LH release was calculated by the trapezoid method using the GraphPad Prism software (version 7.0).

The ovulatory response was evaluated by GLIMMIX procedure, fitting a binary distribution response and considering the presence of CL, P4 implant insertion and their interaction as fixed effects. In addition, the LOGISTIC procedure was used for logistic regression to evaluate the probability of ovulation after the GnRH challenge, as a function of circulating P4 concentrations on D0 and amplitude of LH peak. The final model included the group effect and was selected by backward elimination, testing for linear and quadratic effects.

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 performed to determine differences. Significant differences were declared when $P \leq 0.05$ and a tendency was defined when $0.05 < P \leq 0.10$. Data are presented as means ± SEM.

3.3 Results

Over the 4 replicates, a total of 112 cows were submitted to the presinchronization protocol. The ovulatory response to the GnRH treatment on D-7 was 80.4% (90/112). Moreover, three observations were removed from the analyses because cows had a DF < 10 mm on D0. Therefore, 87 cows were enrolled in the treatments: CL-IPI (n = 21); CL (n = 22); CL0-IPI (n = 22); CL0 (n = 22). There was no difference in BCS between groups (mean BCS = 3.3 ± 0.1; *P* = 0.20).

3.3.1 Ovarian dynamics and ovulatory response

Mean diameters of the DF on D-1.5 and D0 were similar between groups (Table 1). Moreover, no differences were observed in the DF diameter on D0 of cows that ovulated or did not ovulate to the GnRH challenge (12.4 ± 0.34 and 13.0 ± 0.18 mm, respectively; *P* = 0.11). There was a main effect of presence of CL on the ovulatory response (*P* < 0.01), but no effects of P4 implant insertion (*P* = 0.21) or interaction of these factors (*P* = 0.89) were observed (Table 1). Cows with a CL at the time of GnRH treatment had lower ovulatory response than cows without CL (58.1% vs. 95.5%).

Table 1. Dominant follicle diameter before GnRH treatment and effect of CL presence, P4 implant insertion, or both on ovulatory response and subsequent CL development.

	CL		**No CL**		***P*-value**		
IPI	Yes (n = 21)	No (n = 22)	Yes (n = 22)	No (n = 22)	CL	IPI	CL*IPI
DF diameter on D-1.5 (mm)	10.5±0.29	10.8±0.26	10.4±0.37	10.7±0.37	0.44	0.72	0.99
DF diameter on D0 (mm)	12.4±0.27	12.9±0.31	12.8±0.38	13.4±0.29	0.10	0.14	0.93
Ovulation, %	52.4	63.6	90.9	100	< 0.01	0.21	0.89
CL volume on D7 (cm^3)[1]	3.0±0.43	2.5±0.24^{z}	3.7±0.40^{b}	4.9±0.35^{a,y}	< 0.01	0.47	0.05
CL volume on D14 (cm^3)[1]	2.3±0.71	1.4±0.27^{z}	2.9±0.66^{b}	6.5±0.34^{a,y}	< 0.01	0.02	< 0.01

Only cows with a DF ≥ 10 mm on D0 were considered in this analysis (n = 87);
Values presented as mean ± SEM;
Abbreviations: CL = corpus luteum; IPI = intravaginal P4 implant; DF = dominant follicle;
Different letters indicate the interaction (CL*IPI) effect sliced;
[a,b]Effect of P4 implant insertion within groups without CL (*P* < 0.05);
[y,z]Effect of CL presence within groups that did not receive a P4 implant (*P* < 0.05);
[1]Three cows from CL0-IPI group were excluded from the analyses.

3.3.2 Circulating P4 concentrations and LH release

As expected, on D-1.5, circulating P4 concentrations were similar between groups (Table 2). Moreover, PGF treatments on D-1.5 and -0.5 efficiently regressed the CL formed after GnRH treatment on D-7, decreasing circulating P4 concentrations in groups designated to have no CL on D0. Prior to the GnRH challenge, on D0, the mean circulating P4 was greater in groups with CL than groups without CL (2.2 ± 0.18 *vs*. 0.02 ± 0.01 ng/mL; $P < 0.01$). Moreover, in the groups that received a P4 implant on D0, circulating P4 concentrations significantly increased at 1 h after the insertion (Figure 2). In the CL-IPI group, the P4 insert produced a 2-fold increase in circulating P4 concentrations at 1 h after implant insertion, maintaining the mean P4 concentration higher than 5 ng/mL to 48 h. Similarly, in CL0-IPI group, the P4 implant promoted a rapid increase in circulating P4 concentrations, increasing around 1 ng/mL at the first h and doubling at 24 h after P4 insertion.

Table 2. Effect of CL presence, P4 implant insertion, or both on circulating progesterone concentrations at specific days of the study.

	CL		**No CL**		***P*-value**		
IPI	Yes (n = 11)	No (n = 14)	Yes (n = 20)	No (n = 22)	CL	IPI	CL*IPI
D-1.5	1.3±0.21	1.3±0.12	1.1±0.16	1.4±0.20	0.61	0.28	0.77
D0	2.4±0.31	2.1±0.17	0.03±0.01	0.02±0.01	< 0.01	0.58	0.65
D3[1]	5.6±0.30^{a,w}	3.3±0.22^{b,y}	2.1±0.14^{c,x}	0.04±0.01^{d,z}	< 0.01	< 0.01	< 0.01
D5[1]	6.2±0.59^{a,w}	4.2±0.27^{b,y}	2.5±0.12^{c,x}	0.7±0.08^{d,z}	< 0.01	< 0.01	< 0.01
D7[1]	5.9±0.39	5.2±0.46	2.3±0.23	1.9±0.18	< 0.01	0.08	0.95
D14[1]	1.6±0.36^{a}	0.1±0.03^{b,z}	1.1±0.20^{d}	4.4±0.31^{c,y}	< 0.01	0.31	< 0.01

Values presented as mean ± SEM;
Abbreviations: CL = corpus luteum; IPI = intravaginal P4 implant;
Different letters indicate the interaction (CL*IPI) effect sliced;
^{a,b}Effect of P4 implant insertion within groups with CL ($P < 0.05$);
^{c,d}Effect of P4 implant insertion within groups without CL ($P < 0.05$);
^{w,x}Effect of presence of CL within groups that received a P4 implant ($P < 0.05$);
^{y,z}Effect of presence of CL within groups that did not receive a P4 implant ($P < 0.05$);
[1]Only cows that ovulated to the GnRH challenge on D0 were included in analyses.

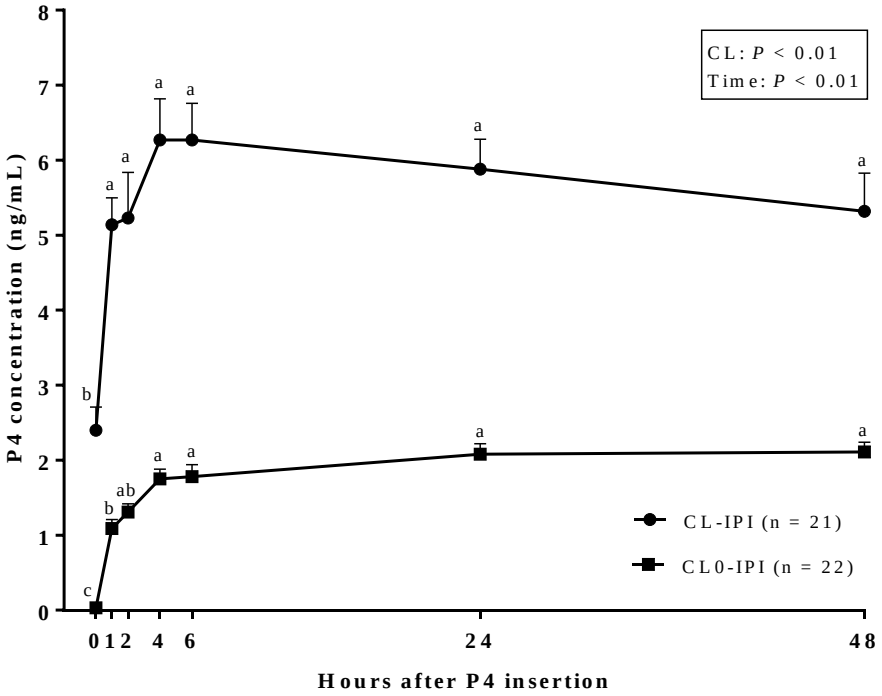

Figure 2. Increase in circulating P4 concentrations (mean ± SEM) provided by the P4 implant insertion from the time of GnRH treatment to 48 h later, in cows with a CL (CL-IPI) or without a CL (CL0-IPI). [a-c]Different letters indicate differences over time within the same group ($P < 0.05$).

Despite the effect on circulating P4 concentrations close to the time of the GnRH - induced LH surge, the insertion of a P4 implant did not affect the LH release profile. Although a tendency was observed for the main effect of P4 insertion on circulating LH concentrations over time ($P = 0.08$; Figure 3), there were no effects on AUC and LH peak ($P = 0.96$ and 0.82, respectively; Table 3). On the other hand, the presence of CL at the time of GnRH treatment negatively affected the LH release over time ($P < 0.01$). Cows without CL had greater circulating LH concentrations during the 6-h period after GnRH than cows with CL. Additionally, the maximum amplitude of LH surge after treatment with GnRH and AUC were greater for cows without CL than cows with CL (13.2 ± 0.68 *vs*. 5.3 ± 0.49 ng/mL and 35.6 ± 1.53 *vs*. 14.2 ± 1.21 ng/mL * hour, respectively; [$P < 0.01$]). The mean time to peak did not differ between groups (Table 3). For the analyses of LH release, data from 3 cows (CL0-IPI: n = 1; CL0: n = 2) were considered outliers by the evaluation of the studentized residual and were excluded.

Table 3. Effect of CL presence, P4 implant insertion, or both on GnRH-induced LH release.

	CL		No CL		*P*-value		
IPI	Yes (n = 21)	No (n = 22)	Yes (n = 21)[1]	No (n = 20)[1]	CL	IPI	CL*IPI
LH peak (ng/mL)	5.3±0.78	5.3±0.62	13.4±0.94	13.0±1.01	< 0.01	0.82	0.83
AUC (ng/mL * hour)	14.0±1.93	14.3±1.54	35.8±2.35	35.3±1.96	< 0.01	0.96	0.84
Time to LH peak (min)	97.1±6.51	106.4±5.49	92.7±6.52	98.2±6.30	0.40	0.11	0.36

Values presented as mean ± SEM;
Abbreviations: CL = corpus luteum; IPI = intravaginal P4 implant; LH = luteinizing hormone; AUC = area under the curve;
[1]Three cows (CL0-IPI: n = 1; CL0: n = 2) were considered outliers and were excluded from the analyses.

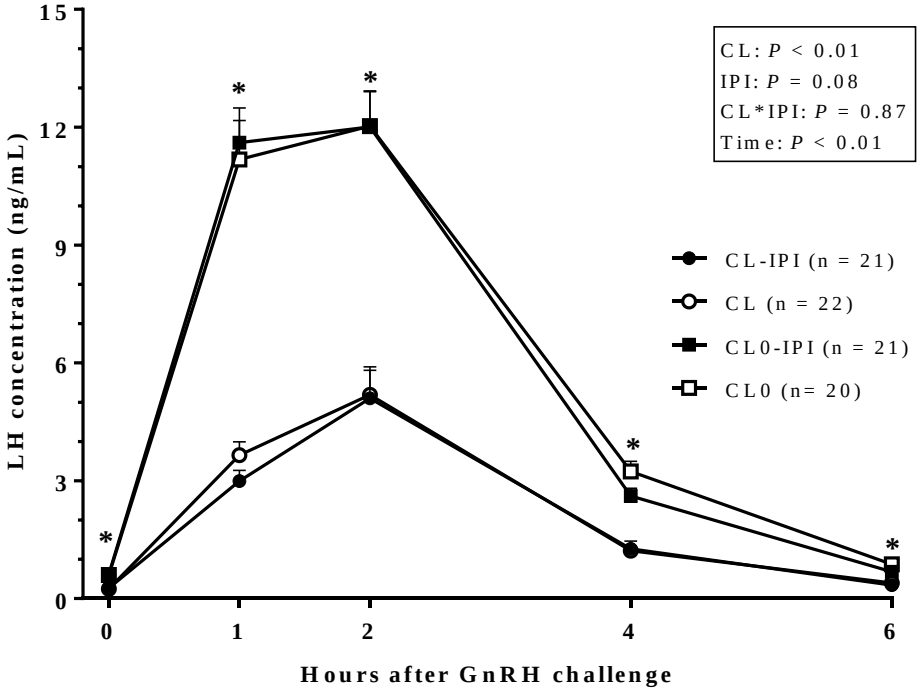

Figure 3. Circulating LH concentrations (mean ± SEM) from the treatment with 100 µg of gonadorelin to 6 h later, on D0, according to the presence of a 7-d old CL, and the insertion of an intravaginal P4 implant (IPI), at the time of GnRH challenge (CL-IPI = with CL and P4 implant; CL = with CL and without P4 implant; CL0-IPI = without CL and with P4 implant; CL0 = without CL and P4 implant). (*) Asterisks indicate the effect of CL presence within each time ($P < 0.05$). Three cows (CL0-IPI: n = 1; CL0: n = 2) were considered outliers and were excluded from the analysis.

Furthermore, regardless of treatments, the mean circulating P4 on D0 differed between cows that ovulated and cows that did not ovulate to the GnRH challenge ($P < 0.01$). Cows that ovulated to the GnRH had lower circulating P4 concentrations on D0 than cows that did not ovulate (0.70 ± 0.12 *vs*. 2.5 ± 0.35 ng/mL). This effect was also observed when only cows from groups with CL were analyzed (1.83 ± 0.15 vs. 2.79 ± 0.33, respectively; $P = 0.01$). Figure 4 illustrates the distribution of cows that ovulated or not to the GnRH challenge, according to their circulating P4 concentrations on D0 and GnRH-induced LH peak. It is possible to observe that most non-ovulated cows had an LH peak < 5 ng/mL. Moreover, the ovulatory response was linearly affected by circulating P4 concentrations on D0 and by the GnRH-induced LH peak. As circulating P4 increased, the probability of ovulation decreased ($P < 0.01$; Figure 5), whereas the increase in amplitude of LH peak increased the ovulation risk ($P < 0.01$; Figure 6).

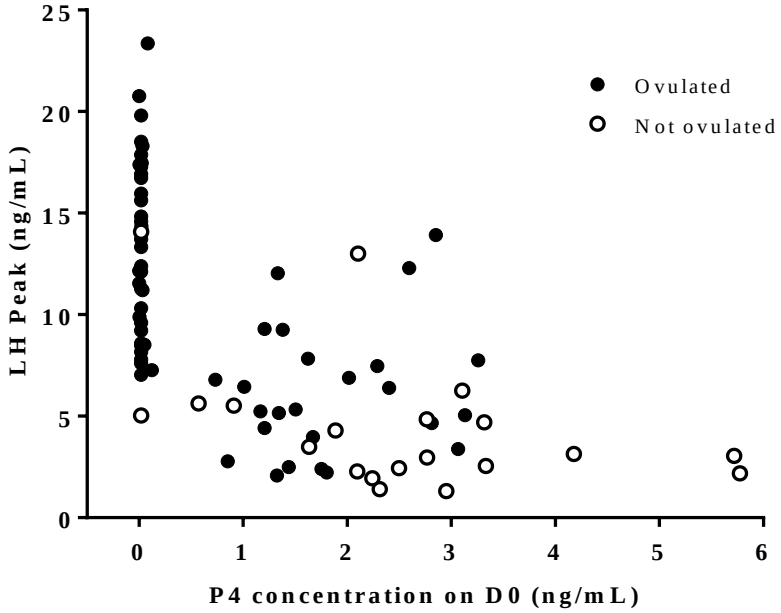

Figure 4. Individual distribution of cows that ovulated (●) or not (○) to treatment with 100 µg of gonadorelin on D0, according to their circulating progesterone (P4) concentration on D0 and amplitude of the GnRH-induced LH peak.

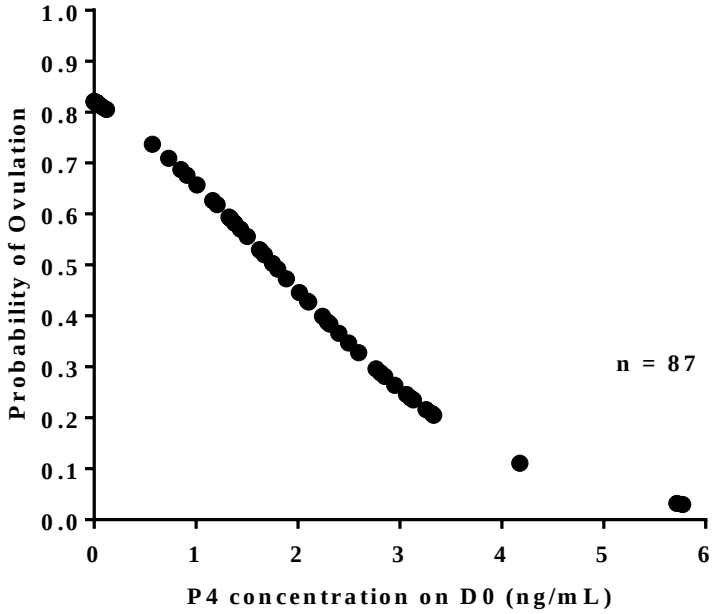

Figure 5. Probability of ovulation after 100µg of gonadorelin administration in relation to circulating progesterone (P4) concentrations on D0. The regression line shows a linear effect (y = 1.52 – 0.86x; $P < 0.01$). Cows with higher P4 concentrations on D0 were less likely to ovulate.

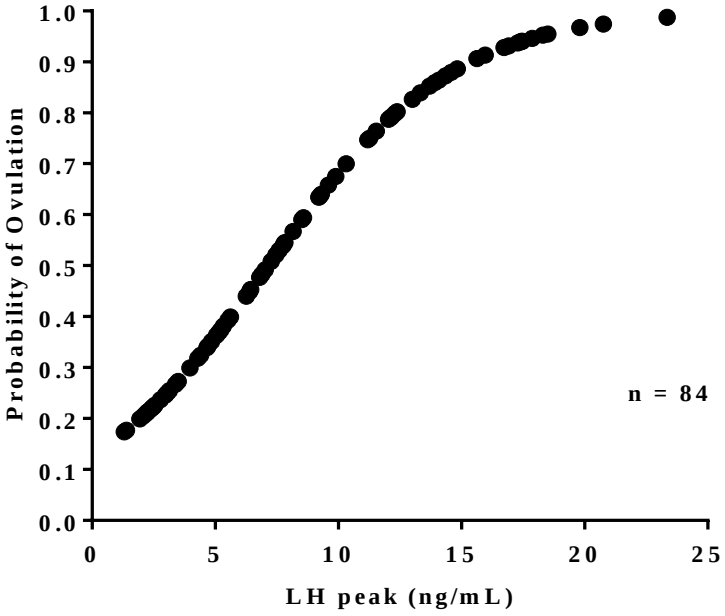

Figure 6. Probability of ovulation after 100µg of gonadorelin administration in relation to the magnitude of GnRH-induced LH peak. The regression line shows a linear effect (y = -1.91 + 0.27x; $P < 0.01$). Cows that had a lower LH peak after the GnRH treatment were less likely to ovulate.

3.3.3 Subsequent CL development

For the analysis of CL volume, 3 cows from the CL0-IPI group were excluded due to the presence of an extremely irregular cavity, precluding an accurate measurement. Mean volume of the CL on D7 and D14 are presented in Table 1. The presence of CL at the GnRH challenge negatively affected the volume of the subsequent CL. Cows with a CL on D0 had a smaller subsequent CL than cows without CL, both on D7 and D14 (2.7 ± 0.23 vs. 4.4 ± 0.29 cm^3 and 1.9 ± 0.38 *vs*. 5.4 ± 0.44 cm^3, respectively; $P < 0.01$). Moreover, an interaction effect of CL presence and P4 implant insertion was observed on D7 ($P = 0.05$) and on D14 ($P < 0.01$). The insertion of a P4 implant on D0 negatively affected the volume of the subsequent CL of cows without a CL at the time of GnRH treatment. In addition, data from 11 cows (CL0-IPI: n = 7; CL: n = 3; CL0: n = 1) were not included in the analysis of volume of CL on D14 because no CL was detected in the ultrasound examination at this day.

The daily evaluations of the subsequent CL development are shown in Figure 7. There was an effect of presence of CL over all periods ($P < 0.01$), in which cows that had a CL on D0 developed a smaller CL after ovulation to GnRH treatment than cows without CL. Moreover, an interaction was observed from D11 to D14 ($P < 0.05$), in which the P4 implant insertion negatively affected the development of the subsequent CL in cows from CL0-IPI group. Apparently, from D10, cows enrolled in CL0 group maintained the CL, whereas in the other groups the mean CL volume decreased consistently.

Circulating P4 concentrations on D3, 5, 7, and 14 for each group are presented in Table 2. For these analyses, only cows that ovulated to the GnRH treatment on D0 were considered. On D3 and 5 after GnRH, there was a clear effect of both factors (presence of CL and P4 insertion) and their interaction on circulating P4 concentrations. Cows with CL had greater circulating P4 than cows without CL ($P < 0.01$) as well as cows that received a P4 implant had greater circulating P4 than cows that did not ($P < 0.01$). Initially, on D3, as can be observed in CL0 group, there was still no detectable increase in circulating P4 concentrations by the subsequent CL, formed from ovulation to the GnRH treatment on D0. On D0, for cows not treated with PGF, the presence of CL (originated from D-7 treatment with GnRH) produced circulating P4 concentration of ~3 ng/mL, whereas the P4 implant insertion provided an increment of ~2 ng/mL. Later, on D5, it was possible to observe an increase in circulating P4 concentrations provided by the subsequent CL. Thereafter, on D7, only the main effect of presence of CL was maintained ($P < 0.01$), whereas a tendency was observed for the P4 insertion effect ($P = 0.08$). On that day, cows from groups with CL still presented a 3-fold greater circulating P4 concentration than cows from groups without CL. However, on D14,

similar to what was observed in CL volume analysis on that day, the presence of CL negatively affected circulating P4 concentrations ($P < 0.01$), and an interaction effect was observed ($P < 0.01$). In the groups without CL, the mean circulating P4 on D14 was substantially lower when cows received a P4 implant. In contrast, in the groups with CL, although both of them had been negatively affected, circulating P4 concentrations were greater in cows that received a P4 implant.

Likewise, when circulating P4 concentrations were evaluated daily from D5 to D14 (Figure 8), there was a main effect of presence of CL until Day 10, when cows from group without CL presented lower circulating P4 concentration than cows from group with CL. Moreover, on D5 and 6, an interaction effect was observed ($P < 0.01$), in which the P4 implant insertion presented a positive effect on circulating P4 concentrations only in cows without CL. From D10 to 14, only the CL0 group maintained a stable circulating P4 profile whereas the other 3 groups presented a decrease in circulating P4 concentrations. In addition, an interaction effect was observed on D13 and 14, when the negative impact of P4 implant insertion on circulating P4 concentrations was detected only in cows from group without CL. Figure 9 presents the pattern of CL development and circulating P4 profile over time, individualized by group.

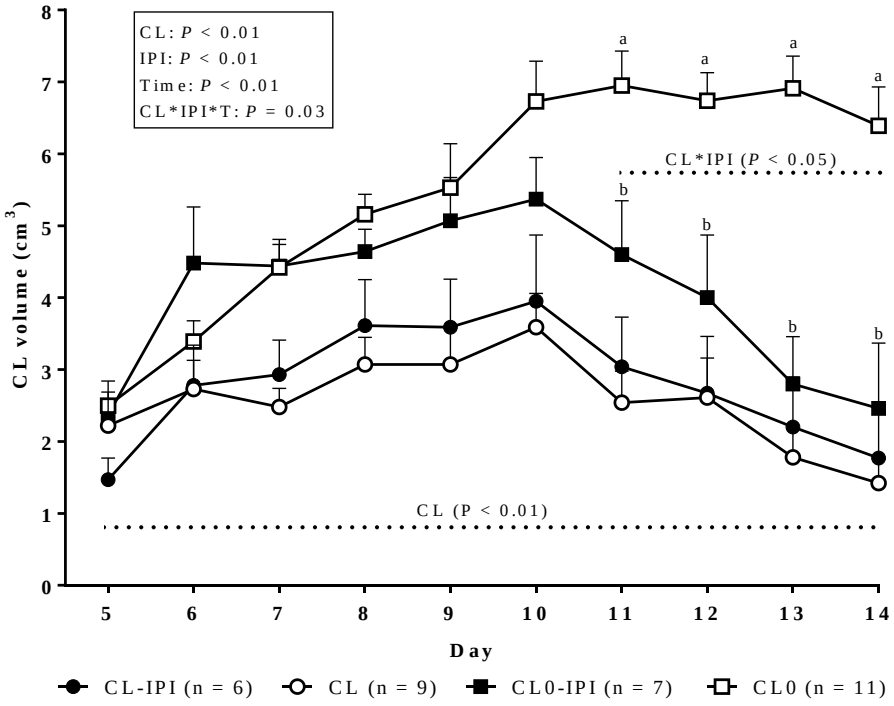

Figure 7. Volume of the subsequent CL (mean ± SEM), originated from the ovulation induced by 100 µg of gonadorelin, from Day 5 to 14, according to the presence of a 7-d old CL, and the insertion of an intravaginal P4 implant (IPI), at the time of GnRH-induced ovulation (CL-IPI =

with CL and P4 implant; CL = with CL and without P4 implant; CL0-IPI = without CL and with P4 implant; CL0 = without CL and P4 implant). Dashed line indicates the period when a significant effect was found. Different letters indicate the interaction (CL*IPI) effect sliced: [a,b]Effect of P4 implant insertion within groups without CL ($P < 0.05$). Three cows from CL0-IPI group were excluded from the analysis.

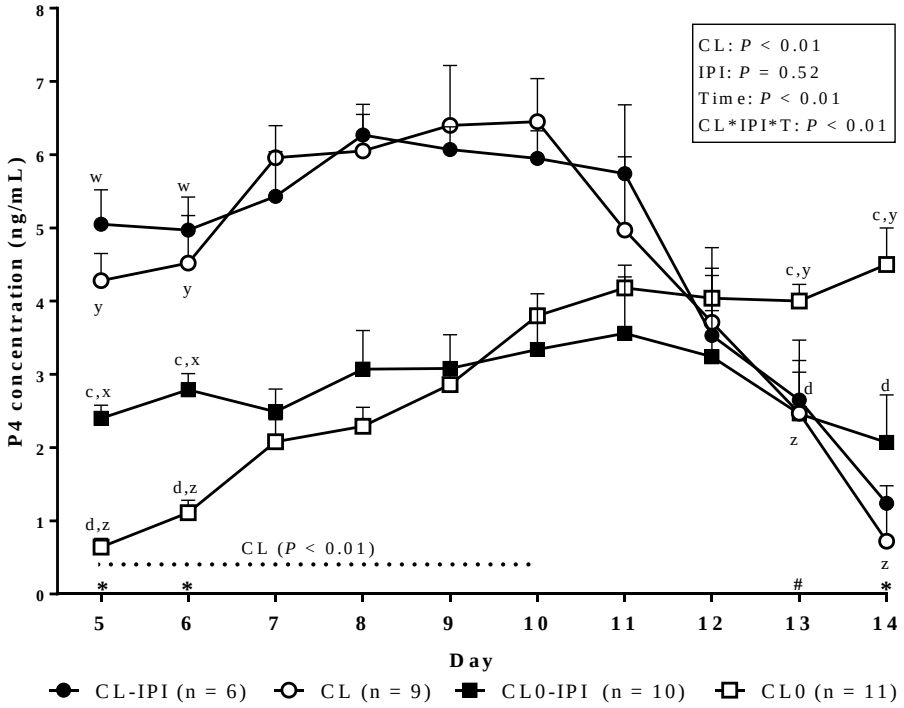

Figure 8. Circulating progesterone (P4) concentrations (mean ± SEM) from Day 5 to 14, according to the presence of a 7-d old CL, and the insertion of an intravaginal P4 implant (IPI), at the time of GnRH-induced ovulation (CL-IPI = with CL and P4 implant; CL = with CL and without P4 implant; CL0-IPI = without CL and with P4 implant; CL0 = without CL and P4 implant). Dashed line indicates the period when the significant effect of CL presence was found. (*) Asterisk indicates interaction (CL*IPI) effect ($P < 0.01$) and (#) number sign indicates a tendency for interaction effect ($P = 10$). Different letters indicate the interaction effect sliced: [a,b]Effect of P4 implant insertion within groups without CL ($P < 0.05$); [w,x]Effect of presence of CL within groups that received a P4 implant ($P < 0.05$); [y,z]Effect of presence of CL within groups that did not receive a P4 implant ($P < 0.05$).

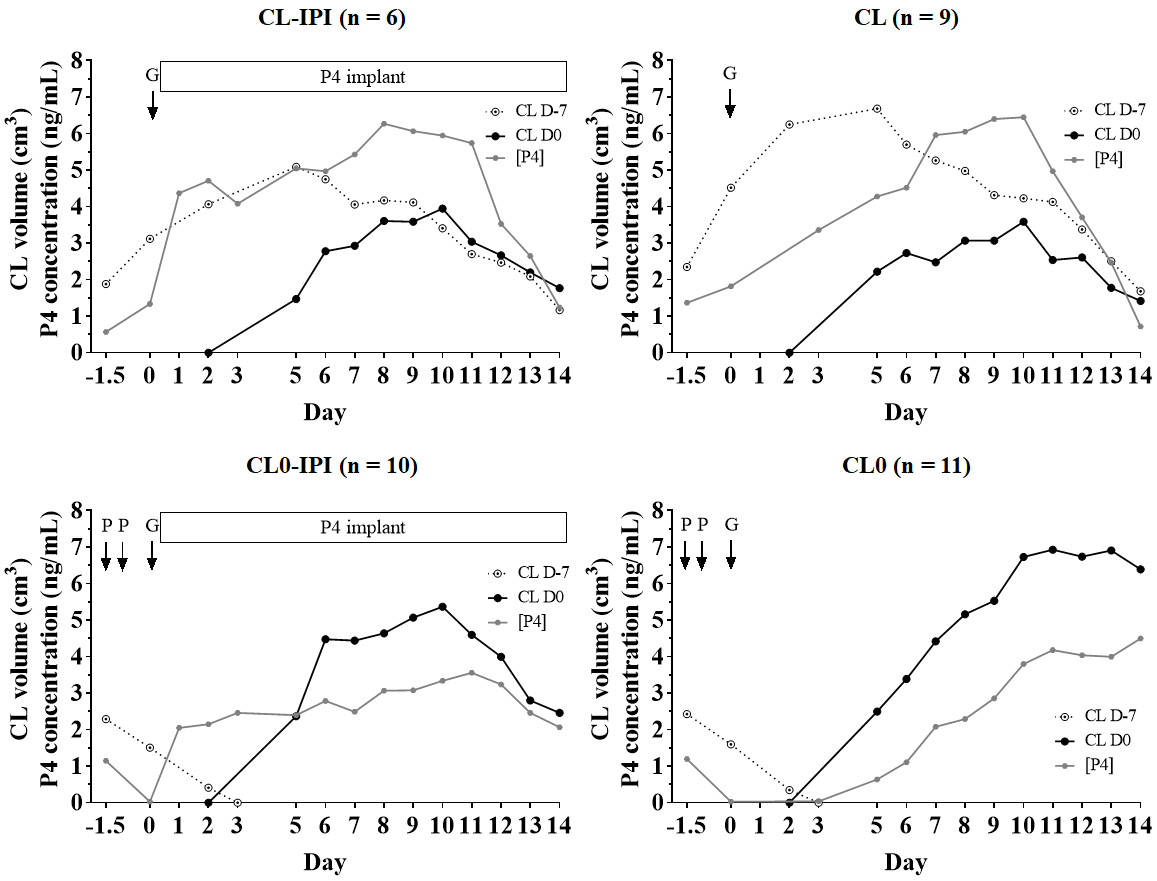

Figure 9. Development patterns of CL from presinchronization (D-7) and CL from GnRH challenge (D0), and circulating progesterone (P4) profile from D-1.5 to 14, individualized by experimental group (CL-IPI = maintenance of CL from D-7 and P4 implant insertion at the time of GnRH challenge; CL = maintenance of CL from D-7 without P4 implant; CL0-IPI = PGF-induced regression of CL from D-7 and P4 implant insertion at the time of GnRH challenge; CL0 = PGF-induced regression of CL from D-7, without P4 implant). (G) Indicates the treatment with 100 µg of gonadorelin; (P) indicates the PGF treatment. The rectangular box indicates the period of intravaginal P4 insertion. (⊙) Open circles with dashed line represent the CL from presynchronization (D-7); (●) dark circles with solid line represent CL from GnRH challenge (D0); (◆) grey rhombuses with solid line represent circulating P4 concentrations. Data from 3 ovulated cows (CL0-IPI group) were not included to calculate the mean volume of CL from D0.

3.4 Discussion

The current study evaluated the effect of circulating P4 concentrations produced by a CL, as well as the impact of an intravaginal P4 implant insertion at the time of a treatment with a conventional dose of gonadorelin (100 µg), on the GnRH-induced LH release, ovulatory response and subsequent CL development in non-lactating Holstein cows. The experimental design aimed to determine the circulating P4 effects on these responses in a synchronized 7-d old DF, simulating the physiologic conditions provided by presynchronization strategies at the first GnRH of the synchronization protocol (Bello et al., 2006; Souza et al., 2008). It is reported

that steroidal hormones can regulate the sensitivity of the pituitary gland (Nett et al., 2002) and expression of GnRH receptors in the pituitary changes according the stage of the estrous cycle (Rispoli and Nett, 2005). For LH secretion, this regulation seems to be influenced by both ovarian steroids (Stevenson and Pulley, 2016; Motta et al., 2020). In this sense, a recent study from our research group reported that the GnRH-induced LH release was equivalently affected bby circulating estradiol (E2) and P4 concentrations, being stimulated by E2 and suppressed by P4 (Motta et al., 2020). In the present study, circulating P4 was clearly different between groups and although circulating E2 concentration was not measured in this study, it may have been different, however, the dominant follicle at the moment of GnRH treatment was of a similar age and size among treatments.

Our first hypothesis that the circulating P4 produced by a 7-d old CL would suppress the GnRH-induced LH surge and negatively affect the ovulatory response after the GnRH treatment was confirmed. Initially, the proposed experimental design was efficient in producing two distinct P4 environments in groups with and without CL at the time of GnRH. The mean circulating P4 in groups with CL on D0 was consistent with what has been reported in Holstein cows on day 7 of the estrous cycle (Sartori et al., 2004). In the absence of a functional CL, the mean circulating P4 was very low, providing a greater GnRH-induced LH release over all observed times. Moreover, regardless that all groups had low circulating LH concentrations (< 1 ng/mL) before the GnRH challenge, the groups without CL had around 2-fold greater LH concentrations at this time, differing from groups with CL. This fact can be explained by the dramatic decrease in circulating P4 concentrations from D-1.5 to D0, due to the PGF treatments in CL0 and CL0-IPI groups. Probably, the decrease in circulating P4 during this short period stimulated the endogenous LH secretion in response to the increased GnRH pulse frequency, as previously described (Clarke and Pompolo, 2005). However, this period under low circulating P4 before the GnRH challenge was not able to trigger an endogenous pre-ovulatory GnRH peak, and no cow had the estrus detection patch activated prior to the GnRH challenge. In addition, the circulating LH maximum amplitude was substantially suppressed (2.5-fold lower) by the circulating P4 concentrations from the CL. Several studies using distinct experimental models have reported the suppressive effect of high circulating P4 concentrations on the GnRH-induced LH peak (Giordano et al., 2012a; Lima et al., 2013; Stevenson and Pulley, 2016; Motta et al., 2020). However, curiously, the absolute LH values observed in these studies are highly variable, probably due to differences in the LH assays. Furthermore, as expected, the ovulatory response was affected by the presence of a functional CL on D0, consistent with previous studies (Giordano et al., 2012b; Stevenson and Pulley, 2016).

The second hypothesis proposed by this study was that the insertion of a P4 implant at the time of GnRH treatment would promote a rapid increase in circulating P4 concentrations, sufficient to negatively affect the GnRH-induced LH surge, but not the ovulatory response. This hypothesis was refuted by our results. Although the intravaginal P4 increased the circulating P4 concentrations by 1 h after P4 implant insertion, as previously described (Cerri et al., 2009a), there was no effect on GnRH-induced LH release. The LH release profile over time and maximum LH amplitude were very similar between cows that received or did not receive the P4 implant, indicating that the increase in circulating P4 close to the time of the GnRH-induced LH release was not able to modulate the pituitary sensitiveness to GnRH. Moreover, in our study, the ovulatory response was not affected by the implant insertion at the time of GnRH, unlike what has been reported in previous studies (Galvão et al., 2004; Stevenson et al., 2008). Interestingly, in a similar study, Lima et al. (2013) evaluated the GnRH-induced LH release and ovulatory response of Holstein heifers with or without CL and receiving a P4 implant 24 h before the treatment with 100 μg of gonadorelin. In the group without CL, luteolysis was induced by 2 PGF administrations, at the time of P4 implant insertion and 12 h later. The ovulatory response in heifers without CL was low (~48%), although it was greater than in heifers with CL (~19%). These results suggest that even in the absence of a functional CL at the time of GnRH treatment, previous exposure to circulating P4 provided by the P4 insertion for, at least, 24 h was sufficient to suppress the ovulatory response. However, despite presenting a similar suppressive effect of high P4 concentrations on LH release, the magnitude of the absolute values for LH concentrations are somewhat puzzling in this study. Additionally, another interesting aspect of these findings (Lima et al., 2013) is that, due to the P4 implant insertion at the time of the first treatment with PGF, probably there was no dramatic decrease in circulating P4 concentrations, even after the induced luteolysis. In our study, after the PGF treatments on Days -1.5 and -0.5, circulating P4 dramatically decreased to D0, and apparently, this period under low P4 concentrations was sufficient to reestablish the pituitary sensitivity to GnRH, as demonstrated by the LH release profile and ovulatory response observed in groups without CL. In this sense, a very elegant study reported the effect of the magnitude of change in circulating P4 concentrations on the amplitude of LH pulses (Fike et al., 2004). It seems that the more acute the change in P4 concentrations, the longer is the time required for reestablishing of physiological pre-ovulatory LH pulse amplitude. More studies are necessary to better understand the effect of changes in circulating P4, as well as the period of exposure to distinct P4 concentrations on the responsiveness of the pituitary to an exogenous GnRH stimulus.

Furthermore, regardless of CL presence or P4 insertion, results from this study support the understanding about the regulatory effect of circulating P4 on LH release by the pituitary, directly affecting ovulation. Analysis of individually distributed LH peak according to circulating P4 at the time of GnRH challenge clearly demonstrated the suppressed LH release under high P4 concentrations, despite variability among cows. For instance, all cows with P4 ≤ 0.5 ng/mL had a GnRH-induced LH peak > 5 ng/mL, of which 70% were above 10 ng/mL, whereas only 10% (4/39) of cows with P4 ≥ 1 ng/mL presented LH peak > 10 ng/mL. Moreover, almost all cows that did not ovulate had an LH peak < 5 ng/mL. These results are consistent with what has been previously reported (Giordano et al., 2012a). Additionally, the results obtained from this study generated a logistic model to predict the probability of ovulation after treatment with 100 μg of gonadorelin, as a function of circulating P4 at GnRH treatment and of the GnRH-induced LH peak. Then, considering the average circulating concentration of the LH peak observed for cows with and without CL in this study, it was possible to estimate that the probability of ovulation to GnRH in the presence of a functional CL was around 30%, whereas in the absence of CL, the probability increased to around 80%, relatively close to what has been reported in lactating cows (Giordano et al., 2012b) and heifers (Lima et al., 2011). However, in these studies, not all cows/heifers had an adequately synchronized DF, what could explain the lower ovulatory response, especially in cows without CL at the time of GnRH treatment.

The detailed mechanism by which P4 modulates the sensitiveness of the pituitary to the GnRH stimulus remains unclear. It is well known that the GnRH receptor (GnRHR) is a G protein-coupled receptor (GPCR) expressed on the membrane of the pituitary gonadotroph cells (Hapgood et al., 2005), and the binding of these receptors by GnRH triggers intracellular signaling pathways to induce synthesis and secretion of gonadotropins: LH and FSH (Rispoli and Nett, 2005). Moreover, the response of gonadotropes to GnRH is directly correlated to the number of GnRHRs on the cell surface (Wise et al., 1984). The regulation of GnRHR gene expression, and thereby the number of receptors is associated to multiple factors, including gonadal steroids, inhibin and activin and GnRH itself (Rispoli and Nett, 2005). Studies have reported the down-regulation effect of P4 on GnRHR expression and amounts of mRNA encoding for this receptor in cultured ovine pituitary cells (Laws et al., 1990; Wu et al., 1994). However, it seems the inhibitory effect of P4 on the GnRHRs expression did nor occur by a direct action on the pituitary gland. Apparently, P4 decreases the pituitary responsiveness to GnRH by regulating the expression of GnRHRs by controlling the GnRH pulse frequency in the hypothalamus. In fact, circulating P4 can regulate the GnRH pulse generator via intermediary steroid-sensitive neurons, reducing the pulse frequency secreted (Clarke and

Pompolo, 2005). Moreover, the GnRH pulse frequency has been indicated as an effective regulator of GnRHR expression on the pituitary cells (Rispoli and Nett, 2005; Kanasaki et al., 2013). In this sense, in a very elegant study, anestrous ewes (under suppression by the photoperiod) had ovulation induced by a GnRH administration and, thereafter, received a PGF treatment to induce luteolysis. Interestingly, the induction of luteolysis did not affect the GnRHR and its mRNA expression. Considering the inhibitory effect of the photoperiod on the GnRH pulse generator in these ewes, the findings of the study (Turzillo et al., 1995) suggest that the isolated event of decrease in circulating P4 concentration was not sufficient to up-regulate the GnRHR expression. In contrast, in the same study, when GnRH pulses were administered hourly for 12 h, there was an increase in both expression of mRNA and its encoded GnRHR, even in ewes with a functional CL. These results indicate that, probably, the suppressive effect of circulating P4 concentrations on the pituitary responsiveness occurs indirectly, mediated by the hypothalamic GnRH pulse frequency. Nevertheless, further studies are required to elucidate these mechanisms and possible implications in cows.

The present study also evaluated the subsequent CL development, after the ovulation induced by exogenous GnRH treatment, according to each P4 environment provided by the experimental groups. As expected, cows with a CL from the presynchronization at the time of the induced ovulation developed a smaller subsequent CL than cows without a CL originated from the presynchronization, despite having ovulated a DF with similar size after GnRH treatment on D0. In cows, during a normal estrous cycle, ovulation and subsequent CL development are preceded by proestrus, characterized by a dramatic decrease in circulating P4 concentration and increase in circulating E2 (Wiltbank et al., 2014). In our model, cows designated to have a CL at the time of GnRH did not have a proestrous period before the induction of ovulation, hence, for these cows the GnRH treatment on D0, acted as an inducer of accessory CL, on day 7 of the estrous cycle (Souza et al., 2009a). In their review, Niswender et al. (Niswender et al., 2000) described that the CL development in ewes was mediated by LH and growth hormone (GH), and that LH induces expression of vascular endothelial growth factors (VEGF), which probably is associated with the proliferation of luteal endothelial cells. In addition, in cattle, suppression of LH pulse frequency during the luteal development negatively affected the normal function of the CL (Peters et al., 1994). These results suggest that, in our study, the high circulating P4 concentrations produced by the CL could have affected the LH pulse frequency, impairing CL development, similar to what has been reported in ewes (Letelier et al., 2011; Christensen et al., 2012). Unfortunately, the model used in the present study did not allow us to distinguish the individual concentration of P4 produced by

each CL and the P4 implant, however, by analyzing the circulating P4 concentrations observed after ovulation in our results, it is possible to infer the functionality of the subsequent CL. On Day 3 and 5 after ovulation, it seems that the P4 implant was contributing with ~2 ng/mL on circulating P4 concentrations (Table 2). However, from Day 5 to 10 (Figure 8), despite the presence of the P4 implant, the circulating P4 concentrations did not differ between groups CL-IPI and CL. During this period, it is expected that the P4 implant would still be responsible for 1.5 ng/mL of circulating P4 on average (data from chapter 1 – Exp. 1), suggesting that the P4 insertion could have suppressed even more the subsequent CL function, probably by regulating the LH pulse frequency during early CL development. Likewise, the same effect can be observed in the groups without CL at the time of GnRH treatment, in which the P4 insertion clearly affected the CL volume on D7 (when all cows were considered; Table 1), and by the same logic, excluding the expected P4 concentration provided by the implant, apparently the subsequent CL function was impaired by the P4 insertion. Similar results were previously reported by interesting studies in beef (Parr et al., 2017) and dairy (Burke et al., 1994) heifers.

Furthermore, another interesting finding from this study was that in the 3 groups (with exception of the CL0) from Day 10 after the GnRH challenge, a progressive decrease in the subsequent CL volume was observed, simultaneously to a reduction in circulating P4 concentrations, evidencing the occurrence of luteolysis. Not surprisingly, Day 10 corresponded to the Day 17 of the estrous cycle in cows from CL-IPI and CL groups, considering the ovulation to the GnRH on D-7 of the study. Based on the day of natural luteolysis reported in Holstein cows (Sartori et al., 2004), the reasonable explanation for the findings from these groups is that, due to the physiological moment of the cycle, there was the activation of the luteolytic process, promoting the regression of both CL and decreasing circulating P4 concentrations. Additionally, the evaluation of the development of the CL originated from presynchronization, demonstrated an apparent greater growth profile of this CL until D5 in the CL than CL-IPI group, reinforcing the idea that the P4 insertion can compromise CL development. Moreover, regardless of this fact, it seems that the CL originated from the presynchronization treatment started to gradually reduce in volume from D5 (corresponding to Day 12 of the cycle), although no decrease in circulating P4 was observed. Finally, 2 feasible explanations were considered for the regression of the subsequent CL in the CL0-IPI group. The first is that the short cycle had been induced by the supplementation of P4 at the early stages of CL development, consistent with what has been reported (Burke et al., 1994; Parr et al., 2017). As an alternative explanation, is possible that the lack of an adequate proestrus after the induced ovulation had promoted a short cycle, as described previously (Peters and Pursley,

2003; Rantala et al., 2009). The first explanation seems to be more reasonable, since cows from the CL0 group were submitted to the same proestrous period and did not have short cycle or early luteolysis.

In summary, the circulating P4 concentrations produced by a 7-day age CL at the GnRH treatment negatively affected the GnRH-induced LH release, ovulatory response and subsequent CL development in non-lactating Holstein cows. In addition, the insertion of a P4 implant simultaneously to the GnRH administration did not affect the GnRH-induced LH release and ovulatory response, but compromised the subsequent CL development and function, inducing short cycles in cows without CL at the GnRH. Finally, the results from this study confirmed that greater circulating P4 concentrations at the time of the GnRH treatment decrease the chance of ovulation, whereas greater magnitudes of the GnRH-induced LH peak increase the ovulatory risk.

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4. INFLUENCE OF ANALOGUE AND DOSE OF GnRH ON LH RELEASE AND OVULATORY RESPONSE IN *Bos indicus* HEIFERS AND COWS WITH HIGH CIRCULATING PROGESTERONE

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ABSTRACT

This study aimed to evaluate the influence of GnRH analogues (gonadorelin [GON] *vs*. buserelin [BUS]), as well as GnRH dose (single *vs*. double) on the LH release and ovulatory response in Nelore females with high circulating progesterone (P4) concentrations. Cycling heifers (n = 59) and non-lactating cows (n = 56) were previously enrolled in a presynchronization protocol (D-17: P4 implant and 2 mg estradiol benzoate; D-9: implant removal and 0.53 mg cloprostenol sodium [PGF]; D-7: 25 μg lecirelin [GnRH]). Females that ovulated to GnRH at D-7 were randomly assigned to receive one of the following treatments on D0: 100 μg GON (**G**); 200 μg GON (**2G**); 10 μg BUS (**B**); or 20 μg BUS (**2B**). At GnRH treatment, a P4 implant was inserted in heifers (0.5 g) and cows (1 g). Ultrasound examinations were performed on Days -9, -7, -5, 0, 2, 5, and 7 to evaluate the ovulatory response to presynchronization, the diameter of the dominant follicle (DF) and ovulation to the GnRH treatments. Moreover, blood samples were taken on Day 0 at 0, 2, and 4 h after GnRH, to evaluate circulating P4 and LH concentrations. Five d after the treatments, the P4 implant was removed, females received two PGF treatments, 24 h apart, and 2 d later, 25 μg lecirelin was given to reassign the females into the next replicate. Statistical analyses were performed by SAS 9.4 and results are presented as mean ± SEM ($P \leq 0.05$). In heifers, regardless of the dose, BUS induced greater LH release than GON. Additionally, the double dose increased the LH release only in heifers treated with BUS. Likewise, the ovulatory response was greater in heifers treated with BUS than GON (88.9 [24/27] *vs*. 16.7% [5/30]), but there was no effect of dose or interaction. In cows, the GnRH-induced LH peak was greater for BUS than GON. Moreover, the double dose induced a higher LH peak than the single dose, regardless of the GnRH analogue. However, no interaction effect on LH peak was observed in cows. Otherwise, the ovulatory response was affected by an interaction effect, in which the double dose increased ovulation only in cows treated with BUS (single = 35.7% [5/14]; double = 90.9% [10/11]), whereas for GON treatments, there was no effect of dose (single = 35.7% [5/14]; double = 35.7% [5/14]). Moreover, cows presented a higher LH peak than heifers only with the 2G treatment, and the ovulatory response was greater in heifers than cows only with the B

treatment. In conclusion, regardless of the category, BUS treatment produced greater LH release and ovulatory response, under high circulating P4. In addition, the double dose increased the LH release for both analogues in cows, but only for BUS treatment in heifers, and improved the ovulation only in cows treated with BUS. Finally, regardless of treatment, the amplitude of GnRH-induced LH peak positively affected the ovulatory response. However, apparently, heifers were more sensitive to this effect.

Keywords: Gonadorelin; Buserelin; Ovulation; Nelore; Beef cattle.

4.1 Introduction

Gonadotrophin-releasing hormone (GnRH) is a decapeptide synthetized and secreted by hypothalamic neurons [1], and responsible for the primary regulation of female reproductive function through the hypothalamic-pituitary-ovarian axis [2]. From its first characterization in mammals [3], native or synthesized GnRH has been used as a reproductive strategy [4,5], specifically to stimulate the LH and FSH release by the pituitary gland modulating ovarian responses [6–8]. In this context, administration of exogenous GnRH has been routinely adopted in reproductive programs for beef cattle [9,10], aiming to stimulate an LH-peak release and induce ovulation, such as at fixed-time artificial insemination (FTAI) protocols, either at the onset of the protocol, to synchronize a new follicular wave emergence [11,12], or to optimize the ovulatory response at the end of the protocol [13,14].

However, especially in *Bos indicus* cattle, previous studies have reported unsatisfactory results using GnRH-based protocols for FTAI [15–17], apparently associated with failure in ovulation and lack of a new follicular wave emergence at the beginning of the protocol [18]. Similar results were reported in crossbred beef heifers [19,20]. In fact, the ovulatory response to the first GnRH of the protocol can be affected by factors such as the absence of a responsive DF at the time of GnRH treatment [21,22], the stage of the estrous cycle [23,24] and cyclic status [20]. Moreover, interesting studies have reported the suppressive effect of circulating P4 concentrations on the GnRH-induced LH surge, compromising ovulation [24,25].

In order to improve the response to GnRH in females with high circulating P4, some studies have evaluated the administration of different GnRH analogues [26,27] and increased doses [25,28,29]. As reported, since the characterization of the amino acid sequence that encode the native GnRH molecule, several different analogues have been developed aiming to produce agonists with higher affinity for the GnRH receptor and greater stability or resistance to degradation, usually through structural modifications in positions 6 and 10 of the amino acid chain [30]. Currently, the GnRH analogues commercially available and most frequently used in cattle reproduction are: gonadorelin, buserelin and lecirelin. In this sense, Picard-Hagen et

al. [26] evaluated the effect of these analogues on the GnRH-induced LH release of Holstein heifers on day 7 of the estrous cycle, and reported lower LH release induced by gonadorelin (100 μg) than buserelin (10 μg) and lecirelin (25 μg) treatments. In a similar study with lactating dairy cows, although no differences had been observed in GnRH-induced LH peak, the buserelin treatment promoted higher circulating LH concentrations at 3 and 4 h after challenge [27]. On the other hand, studies have reported a positive effect of an increased dose of gonadorelin (200 μg) on LH release in beef heifers [25] and lactating dairy cows [29], under high circulating P4 concentrations. However, none of these mentioned studies reported differences in ovulatory response in relation to the analogue or dose administrated. Interestingly, in a recent study from our research group, administration of an increased dose of buserelin (16.8 μg) at the onset of the FTAI protocol resulted in relatively high ovulatory responses in Nelore heifers (60.3%) and lactating cows (73.6%), and satisfactory P/AI (~58 and ~61%, respectively) [11].

Furthermore, an elegant study with *Bos taurus* beef cattle, comparing heifers and lactating cows under high circulating P4, demonstrated a greater LH release in heifers than cows after treatment with gonadorelin (100 μg) [24]. Additionally, in their study, it seemed that cows were more sensitive to the suppressive effect of circulating P4 concentrations than heifers. However, ovulatory response did not differ among heifers and cows [24]. Until now, no previous study had evaluated the response to GnRH treatments in *Bos indicus* heifers and cows submitted to high circulating P4 concentrations. It is well known that *Bos taurus* and *Bos indicus* cattle presented several physiological differences [31], including circulating P4 concentrations [32] and the sensitiveness of the pituitary gland to a GnRH stimulus [33]. In addition, Figueiredo et al. [34] reported lower circulating P4 concentrations during a normal estrous cycle in *Bos indicus* heifers compared to cows.

Therefore, the aim of the present study was to evaluate the influence of the GnRH analogue (gonadorelin *vs*. buserelin), as well as the dose (single *vs*. double) on the GnRH-induced LH release and ovulatory response in *Bos indicus* heifers and cows. Then, three hypotheses were created: 1) buserelin treatments would promote greater LH release and ovulatory response than gonadorelin treatments; 2) regardless of the analogue, the double dose would promote greater LH release and ovulatory response than the single dose; 3) Heifers would present lower circulating P4 concentrations and greater LH release, but similar ovulatory response in relation to cows.

4.2 Material and methods

The present study was conducted from April to June of 2019 at the facilities of Moro do Brumado Farm, a commercial beef farm located in Itatinga, SP, Brazil. The Animal Research Ethics Committee of "Luiz de Queiroz" College of Agriculture (ESALQ) previously approved all animal procedures (Protocol CEUA # 2018-18).

4.2.1 Animals and management

Cycling Nelore heifers (n = 20; BCS = 3.4 ± 0.03; 26 ± 0.7 mo of age) and non-lactating multiparous Nelore cows (n = 19; BCS = 3.4 ± 0.04) were used in this study, over four replicates of experimental design. The females were kept on pasture (*Brachiaria brizantha*), receiving a daily supplementation based on corn plus soybean concentrate, with water and mineral salt *ad libitum*.

4.2.2 Experimental design

All females were submitted to a presynchronization protocol that started on Day -17 with the insertion of a disinfected 1 g intravaginal P4 implant [35], previously used for 8 d (Repro neo, GlobalGen Vet Science, Jaboticabal, SP, Brazil), associated to 2 mg EB i.m. (Syncrogen, GlobalGen Vet Science). After 8 d, on Day -9, 0.5 mg of cloprostenol sodium (PGF; Induscio, GlobalGen Vet Science) was given simultaneously to P4 implant removal, and 2 d later (D-7) all females received 25 µg of lecirelin acetate (GnRH, TecRelin, Agener União, Embu-Guaçu, SP, Brazil) to induce ovulation and synchronize the emergence of a new follicular wave. Only cows and heifers that ovulated in response to this GnRH administration were submitted to the treatments. Then, synchronized heifers (n = 59) and cows (n = 56) were randomly assigned into a 2x2 factorial design experiment (Fig. 1), composed by the GnRH analogue (gonadorelin *vs*. buserelin) and dose (single *vs*. double). On Day 0, females were treated with GnRH according to the following groups: **G** (100 µg of gonadorelin); **2G** (200 µg of gonadorelin); **B** (10 µg of buserelin); and **2B** (20 µg of buserelin). The GnRH analogues evaluated in this study (gonadorelin; Fertagyl, MSD, Cruzeiro, SP, Brazil; and buserelin; Maxrelin, GlobalGen Vet Science) were kept refrigerated at 4ºC, and the doses were defined according to the manufacturer's instructions, being the single dose equal to the conventionally indicated for each analogue. In addition, simultaneously to the GnRH treatment on D0, cows received a 1 g intravaginal P4 implant (Repro neo, GlobalGen Vet Science) and heifers received a 0.5 g intravaginal P4 implant (Repro one, GlobalGen Vet Science). In order to reassign the animals into the next replicate, the P4 implant was removed 5 d after the insertion and cows

received two treatments of PGF (0.5 mg), 24 h apart. Then, 7 d after the GnRH treatment, all females received 25 µg lecirelin again. The females that ovulated to this GnRH administration were submitted to another experimental group 7 d later. Those who eventually have not ovulated were reassigned to the presynchronization protocol (D-17), returning on the third or fourth replicate (Figure 1).

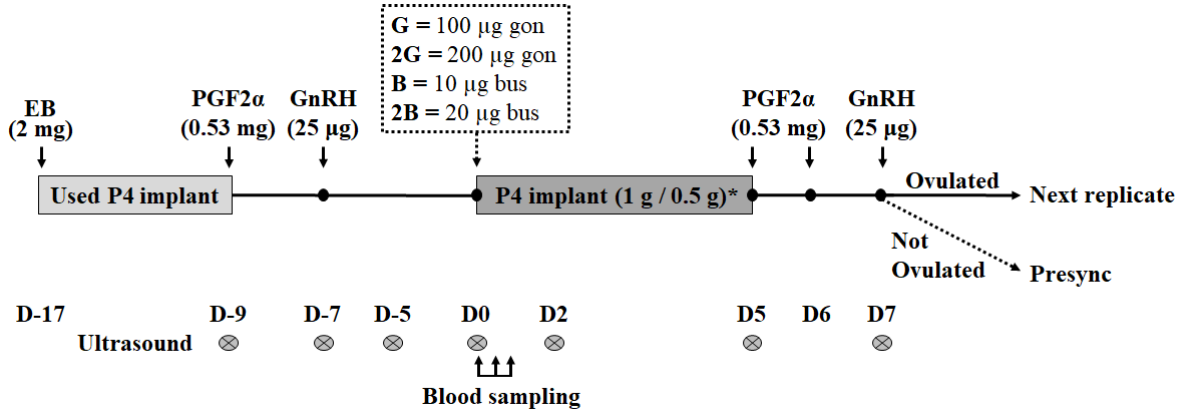


Figure 1. Schematic representation of the experimental design. Cycling heifers (n = 59) and non-lactating cows (n = 56) were submitted to a presynchronization protocol (Day -17: EB + P4 implant; Day -9: implant removal + PGF; Day -7: GnRH) to synchronize ovulation and create a new CL. Only cows that ovulated to the GnRH on Day -7 were randomly assigned into a 2x2 factorial arrangement. On Day 0, according to each experimental group, females received either 100 or 200 µg gonadorelin (G; 2G), or either 10 or 20 µg buserelin (B; 2B). Simultaneously, all females received a new intravaginal progesterone (P4) implant. (*) Cows received a 1 g P4 implant and heifers received a 0.5 g P4 implant. Five d later, the P4 implant was removed, females received two PGF treatments (24 h apart) and, after 2 d, a new GnRH injection was given to reassign females to the next replicate. Females ovulated to this GnRH followed directly to the next replicate, whereas females that did not ovulate returned to the presynchronization. (⊗) Ultrasound examinations were performed on Days -9, -7, -5, 0, 2, 5, and 7. (↑) Blood samples were collected on D0, at 0, 2, and 4 h.

4.2.3 Ultrasound evaluations

Ovarian ultrasound evaluations were performed using a 7.5 MHz linear-array transducer (DP-2200 VET, Mindray, Shenzhen, China) to access the diameter of the dominant follicle (DF) and check the ovulatory response. During examinations, all visible ovarian structures (CL and follicles ≥ 5 mm) were measured and mapped. Cows and heifers had their ovaries scanned on Days -9, -7, and -5 to evaluate the response to the presynchronization protocol. On Day 0, ovulation to presynchronization was confirmed by the presence of a CL, and the diameter of the DF was measured. Ovulation to GnRH treatment on Day 0 was determined by the disappearance of the DF between Day 0 and Day 2 and confirmed by the presence of a new CL on Day 5. Moreover, on Day 7 and Day 9, ultrasound examinations were done to check the

ovulatory response to the new GnRH administration on Day 7 and to determine the reassignment of animals to the next replicate or the return to the presynchronization protocol.

4.2.4 Blood sampling and hormonal assays

Blood samples were taken by puncture of the jugular vein into 9 mL heparinized evacuated tubes (Vacuette, Greiner Bio-One, Americana, SP, Brazil) to evaluate circulating concentrations of P4 and LH. On D0 of each replicate, samples were collected at 0 h (immediately prior to the GnRH treatment and P4 implant insertion), also 2 and 4 h later. After collection, tubes were instantly placed on ice, posteriorly centrifuged at 1,700 x *g* for 15 min at 4ºC and plasma was stored at -20ºC.

Plasma P4 concentrations were determined using a solid-phase RIA kit containing antibody-coated tubes and 125I-labeled P4 (ImmuChem Coated Tube P4 125 RIA Kit, MP Biomedicals, Costa Mesa, CA) validated for bovine plasma as previously described [35]. Sensitivity, intra and inter-assay coefficient of variation were 0.02 ng/mL, 4.2% and 6.7%, respectively. The LH concentrations analyses were performed by RIA [36,37], with some modifications [38]. Sensitivity, intra- and inter-assay coefficients of variation for LH were 0.03 ng/mL, 8.2 and 13.1%, respectively.

4.2.5 Statistical analyses

The present experiment was designed and analyzed as a 2x2 factorial. 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 separately analyzed, and thereafter the effect of category was studied. For continuous data, the normality of studentized residuals were tested using the UNIVARIATE procedure, following the Shapiro-Wilk method, and homogeneity of variances was evaluated by Levene test, using Hovtest and Welsh methods. When necessary, data were transformed to logarithm and outliers were removed. The analyses of DF diameter, P4 concentration on Day 0, LH peak, and area under the curve (AUC) for LH 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 of these variables, the final model included the effect of GnRH analogue, dose, and their interaction (G*D), and the replicate was considered as a random effect. The AUC for GnRH-induced LH release was calculated by trapezoid method using the GraphPad Prism software (version 7.0).

Ovulatory response was analyzed by logistic regression using the GLIMMIX procedure, fitting a binary distribution response and considering the same fixed and random effect as for continuous data, with Kenward-Rogers adjustment for the degrees of freedom calculation. The ovulatory response was also evaluated between animal categories, and the final model included the fixed effects of GnRH analogue, dose, category and the three-way interaction (G*D*Cat), with replicate as a random effect. Additionally, the LOGISTIC procedure was used to determine the probability of ovulation for cows and heifers after the GnRH treatment, as a function of the amplitude of LH peak.

Data from circulating LH and P4 concentrations were analyzed as repeated measures by MIXED procedure, and the final model included effects of GnRH, dose, time, two-way and three-way interactions, with replicate as a random effect. For P4 repeated measure analysis, the initial P4 concentration was used as a covariate to adjust the final model. For these analyses, the Kenward-Rogers method was also included, and the appropriate covariance structure was selected, according to the smallest AICC value. Moreover, the effect of animal category on the GnRH-induced LH release over time was evaluated within each experimental group. Additionally, to determine the effect of circulating P4 concentrations on the GnRH-induced LH peak, a cutoff was established according to the median of data, creating two distinct classes for circulating P4 on Day 0. The analysis was performed by MIXED procedure, and the final model included the fixed effect of GnRH, dose, P4 class and the three-way interaction (G*D*P4).

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. Significant differences were considered when $P \leq 0.05$ and a tendency was defined when $0.05 < P \leq 0.10$. Data are presented as means ± SEM.

4.3 Results

In order to minimize the effect of confounding factors on the results obtained by this study, only cows and heifers that were synchronized by the presynchronization protocol as shown by the presence of a DF with ovulatory capacity [22] and a functional CL [39] at the administration of treatments were included in the analyses. Therefore, one heifer and one cow from group 2B were excluded because of the absence of a DF ≥ 8.5 mm on Day 0, and one heifer from group B, two other cows from group 2B were excluded due to circulating P4 concentrations ≤ 1 ng/mL at the time of GnRH treatment. Moreover, data from one heifer (2B)

and two cows (G and 2B) were not included in analyses of circulating LH concentrations, due to inconsistencies in the LH assay.

4.3.1 Effect of GnRH analogue and dose

For these analyses, the results were evaluated separately for cows and heifers. On Day 0, the DF diameter was similar between experimental groups for cows (11.3±0.17 mm; P = 0.38) and heifers (10.5±0.14 mm; P = 0.80), as presented in Table 1. In heifers, the ovulatory response was affected by the GnRH analogue ($P < 0.01$), but there was no effect of dose (P = 0.82) or interaction of these factors (P = 0.41). Heifers that received buserelin treatments, regardless of the dose, presented greater ovulatory response than heifers treated with gonadorelin (16.7 [5/30] *vs*. 88.9% [24/27]). For cows, the same effect of GnRH analogue was observed ($P < 0.04$), however, there was also an effect of dose (P = 0.03) and an interaction effect was observed (P = 0.04). The double dose increased ovulation only when cows were treated with buserelin (Table 1).

Table 1. Dominant follicle diameter and ovulatory response in heifers and cows treated with single or double doses of GnRH analogues.

	Gonadorelin		**Buserelin**		***P*-value**		
	Single	Double	Single	Double	GnRH	Dose	G*D
Heifers (n)	15	15	13	14			
DF diameter on Day 0 (mm)	10.3±0.33	10.7±0.28	10.4±0.27	10.7±0.26	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
Cows (n)	14	14	14	11			
DF diameter on Day 0 (mm)	11.2±0.33	11.5±0.38	10.8±0.27	11.6±0.42	0.53	0.11	0.38
Ovulation, % (n/n)	35.7 (5/14)	35.7^{z} (5/14)	35.7^{b} (5/14)	$90.9^{a,y}$ (10/11)	0.04	0.03	0.04

Only heifers (n = 57) and cows (n = 53) with a DF ≥ 8.5 mm and circulating progesterone concentrations > 1ng/mL on D0 was considered in this analysis;
Values presented as mean ± SEM;
Abbreviations: DF = dominant follicle;
Different letters indicate the interaction (G*D) effect sliced;
$^{a\text{-}b}$Effect of dose within Buserelin group ($P < 0.05$);
$^{y\text{-}z}$Effect of GnRH analogue within double dose group ($P < 0.05$).

In addition, the experimental design produced high circulating P4 concentrations at the time of GnRH treatment, in all experimental groups in cows (4.2 ± 0.23 ng/mL; $P = 0.28$) and heifers (3.9 ± 0.14 ng/mL; $P = 0.46$). Nevertheless, the LH release was influenced by the GnRH analogue and dose (Table 2). In heifers, the LH peak and the AUC for circulating LH concentrations over 4 h after GnRH treatments were affected by GnRH analogue, dose and their interaction ($P < 0.01$). Despite the dose, buserelin produced a greater LH peak and AUC than gonadorelin. Moreover, the administration of the double dose increased the LH release only when heifers were treated with buserelin ($P < 0.01$), whereas no effect was observed when doubling the gonadorelin dose ($P = 0.92$). Similar effects were observed at 4 h after GnRH treatment (Fig 2A). For cows, the GnRH-induced LH peak and AUC were also affected by GnRH analogue ($P < 0.01$) and dose ($P < 0.01$), but no interaction effect was observed ($P = 0.23$; Table 2). Treatments with buserelin produced a greater LH peak and AUC than treatments with gonadorelin (9.6 ± 1.47 *vs*. 4.9 ± 1.09 ng/mL and 22.9 ± 3.43 *vs*. 10.9 ± 2.43 ng/mL*hour, respectively). Likewise, regardless of the GnRH analogue used, the double dose produced a greater LH peak and AUC than single dose (9.2±1.68 *vs*. 5.2±0.89 ng/mL and 21.3±3.82 *vs*. 12.3±2.19 ng/mL*hour, respectively). In addition, when the GnRH-induced LH release was evaluated over time, an interaction effect was observed at 4 h after GnRH treatment ($P < 0.01$), when circulating LH concentrations were higher in cows that received a double dose of buserelin compared to cows treated with the single dose ($P < 0.01$), but it was similar between gonadorelin doses ($P = 0.72$; Fig. 2B).

Table 2. Circulating progesterone concentrations at the GnRH treatments and the effect of GnRH analogues and doses on the GnRH-induced LH release, in heifers and cows.

	Gonadorelin		**Buserelin**		***P*-value**		
	Single	Double	Single	Double	GnRH	Dose	G*D
Heifers (n)	15	15	13	14			
Circulating P4 on Day 0 (ng/mL)	3.8±0.32	4.3±0.20	3.8±0.30	3.7±0.25	0.22	0.49	0.28
LH peak (ng/mL)	2.4±0.61^{w}	2.3±0.41^{z}	5.1±0.45^{b,x}	10.9±1.08^{a,y}	< 0.01	< 0.01	< 0.01
AUC LH (ng/mL * hour)	5.4±1.30^{w}	5.18±0.88^{z}	11.7±0.95^{b,x}	23.3±2.11^{a,y}	< 0.01	< 0.01	< 0.01
Cows (n)	14	14	14	11			
Circulating P4 on Day 0 (ng/mL)	3.8±0.33	4.3±0.50	4.3±0.52	4.5±0.49	0.58	0.66	0.46
LH peak (ng/mL)[1]	3.4±0.65	6.3±1.99	6.9±1.51	13.3±2.46	< 0.01	< 0.01	0.23
AUC LH (ng/mL * hour)[1]	7.6±1.49	13.9±4.40	16.6±3.69	31.7±5.49	< 0.01	< 0.01	0.23

Values presented as mean ± SEM;
Abbreviations: CL = corpus luteum; LH = luteinizing hormone; P4 = progesterone; AUC = area under the curve;
Different letters indicate the interaction (G*D) effect sliced;
$^{a-b}$Effect of dose within Buserelin group ($P < 0.05$);
$^{w-x}$ Effect of GnRH analogue within single dose group ($P < 0.05$);
$^{y-z}$Effect of GnRH analogue within double dose group ($P < 0.05$);
[1]Data from circulating LH concentrations of 1 heifer (2B) and 2 cows (G and 2B) were not included in the analysis.

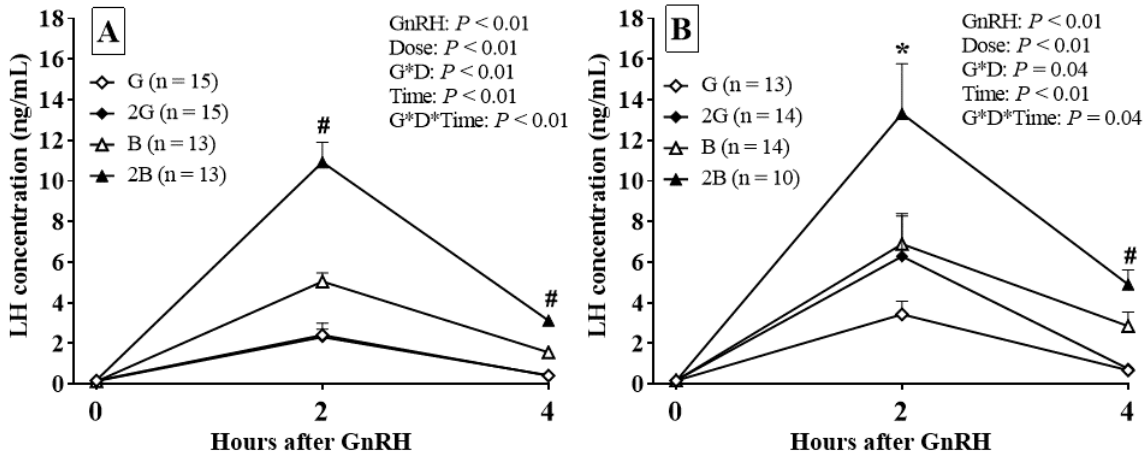


Figure 2. Circulating LH concentrations (mean ± SEM) in (A) heifers and (B) cows, from the GnRH treatment to 4 h later, according to each experimental group. (*) Asterisk indicates the main effects of GnRH analogue and dose (P < 0.01) and (#) number sign indicates the interaction effect (G*D; P < 0.01), in which the effect of dose differs only within buserelin groups (P < 0.01).

4.3.2 Effect of circulating progesterone concentrations

As expected, insertion of an intravaginal P4 implant at the time of GnRH treatment increased circulating P4 concentrations after 2 h, in heifers and cows. Because there were no differences among treatments for both categories (P = 0.27 and 0.32, respectively), data of circulating P4 concentrations over time were combined and are presented by category in Fig. 3. Moreover, circulating P4 concentrations at the time of GnRH treatment did not differ between heifers that ovulated or not (3.7 ± 0.19 *vs*. 4.1 ± 0.19 ng/mL; P = 0.48). The same was observed for cows (4.0 ± 0.33 *vs*. 4.4 ± 0.31, respectively; P = 0.48). Additionally, the circulating P4 profile from 0 to 4 h after P4 implant insertion was similar between heifers (P = 0.75), and cows (P = 0.68) that ovulated or did not ovulate. Figure 4 presents the individual distribution of heifers and cows according to circulating P4 concentrations at the time of GnRH treatment and LH peak.

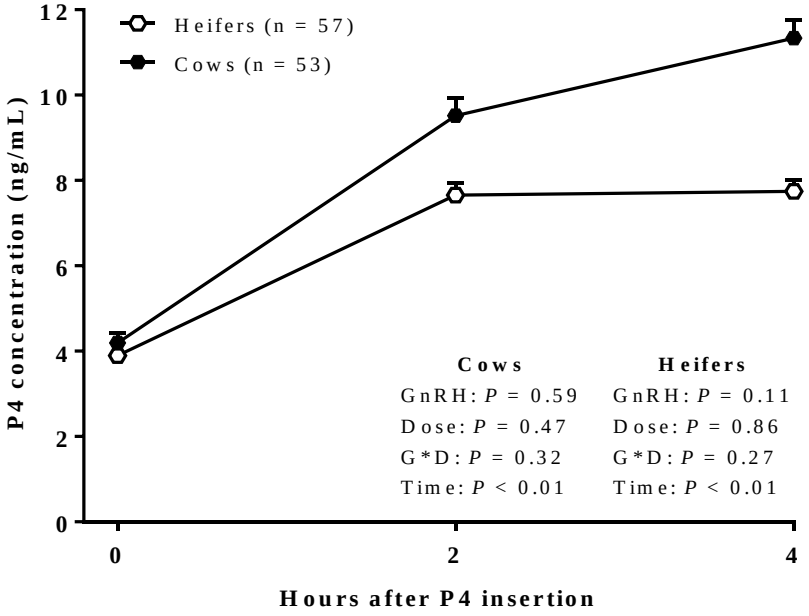
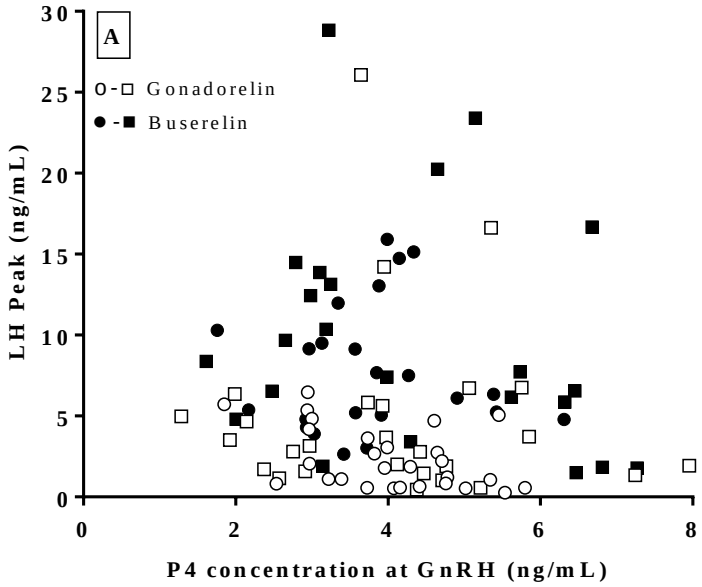


Figure. 3. Circulating progesterone concentrations (mean ± SEM) produced by the intravaginal P4 implant from the insertion to 4 h later, in heifers and cows. Heifers received a 0.5 g P4 implant and cows received a 1 g P4 implant. Circulating P4 concentrations at 2 h were greater than at 0 h, in both categories ($P < 0.01$). At 0 h, P4 concentrations did not differ among categories ($P = 0.22$), but were greater in cows than heifers at 2 and 4 h ($P < 0.01$).



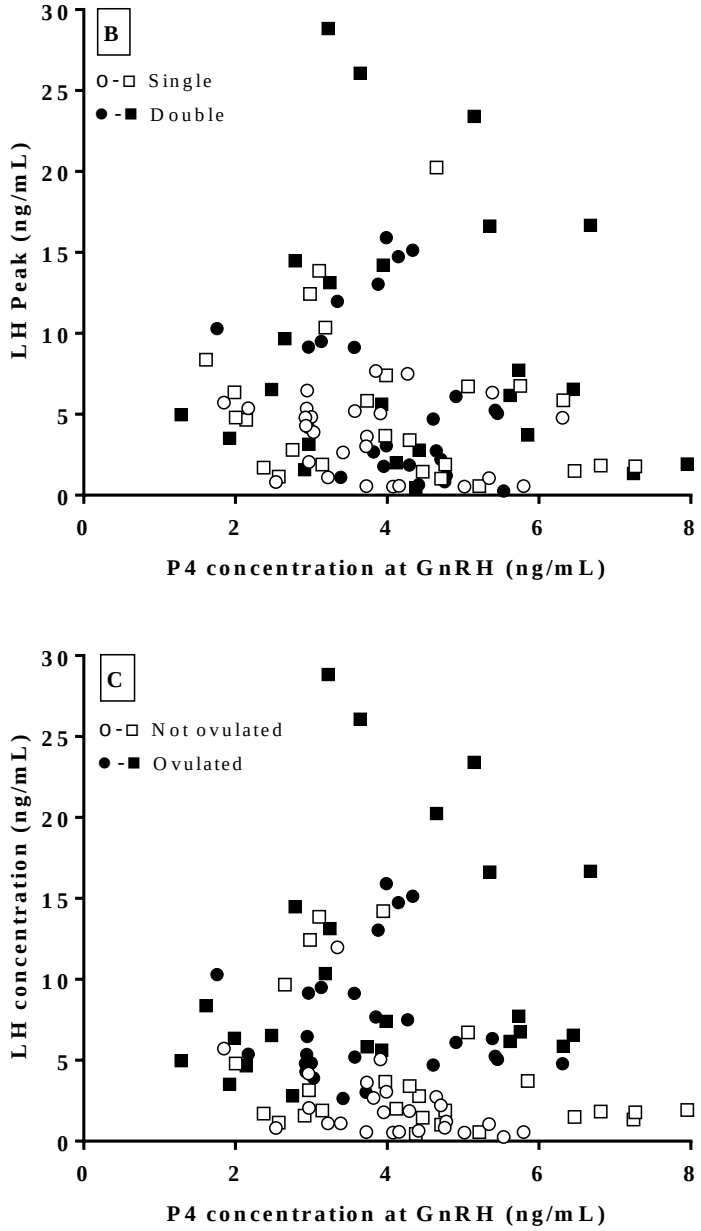


Figure 4. Individual distribution of heifers (represented by circles) and cows (represented by squares) according to their circulating progesterone (P4) concentration at the time of GnRH treatments and amplitude of the LH peak. Open and dark symbols represent respectively: gonadorelin and buserelin treatments (panel A); single and double GnRH doses (panel B); and not ovulated and ovulated females (panel C).

Furthermore, an additional analysis was performed to evaluate the influence of circulating P4 concentrations at the time of GnRH treatments on LH peak and ovulatory response. For that, heifers and cows were divided into two classes according to their circulating P4 concentrations at GnRH: lower P4 (≤ 4 ng/mL) or higher P4 (> 4 ng/mL). In heifers there was a tendency to P4 class affecting the amplitude of GnRH-induced LH peak (P = 0.07). Moreover, also an interaction was observed between experimental group and P4 class (P =

0.04). Heifers treated with the single dose of gonadorelin presented lower LH peaks under higher than lower P4 concentrations ($P < 0.01$), whereas no differences were observed for the other treatments (Fig. 5). Otherwise, in cows, although the same tendency to P4 class main effect was observed ($P = 0.07$), there was no interaction between experimental group and P4 class ($P = 0.21$; Fig 6). Moreover, when ovulatory response was evaluated, there was no effect of P4 class within heifers ($P = 0.24$) or cows ($P = 0.22$). However, when data from all females were combined and analyzed, regardless of treatment and category effects, a tendency was observed for P4 class affecting ovulatory response, in which females with lower P4 concentrations had greater ovulatory responses than those with higher P4 concentrations (56.5 [35/62] vs. 39.6% [19/48]; $P = 0.08$).

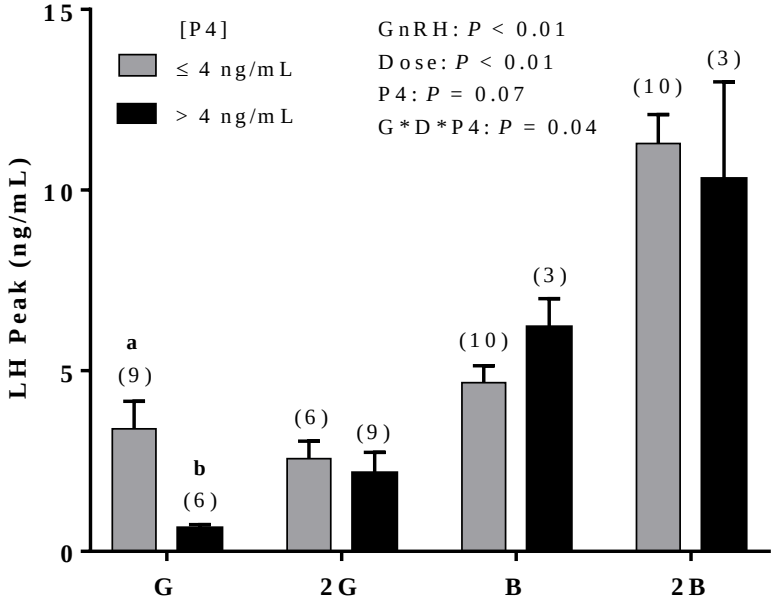


Figure 5. Amplitude of the GnRH-induced LH peak (mean ± SEM) produced by each GnRH treatment, in heifers with lower (≤ 4 ng/mL) or higher (> 4ng/mL) circulating progesterone concentrations at the time of GnRH. Distinct letters ($^{a\text{-}b}$) indicate differences within experimental treatment ($P < 0.01$).

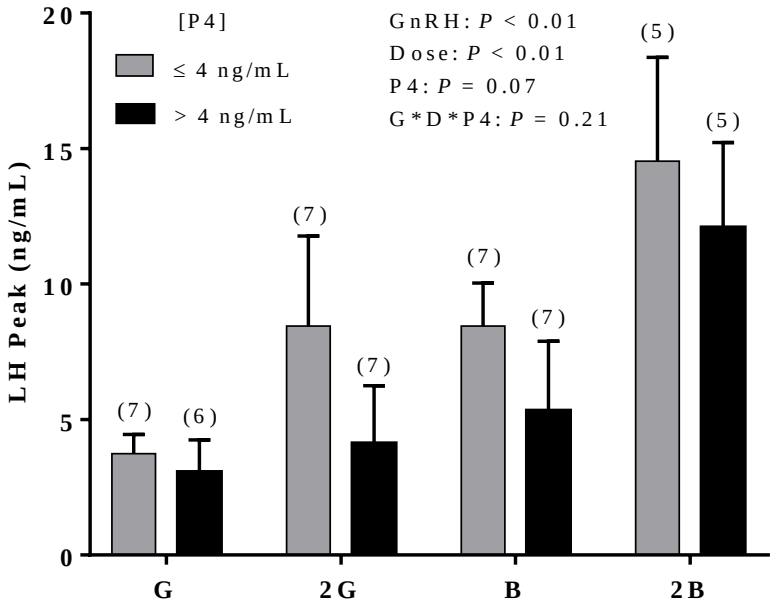


Figure 6. Amplitude of the GnRH-induced LH peak (mean ± SEM) produced by each GnRH treatment, in cows with lower (≤ 4 ng/mL) or higher (> 4ng/mL) circulating progesterone concentrations at the time of GnRH.

4.3.3 Effect of animal category

The effect of animal category was also evaluated in this study. As mentioned before, the DF diameter on Day 0 was greater for cows than heifers ($P < 0.01$; Table 1), but no difference was observed for circulating P4 concentrations at the time of GnRH treatment ($P = 0.22$; Table 2). Moreover, at 2 and 4 h after the P4 implant insertion, circulating P4 concentrations was greater for cows than heifers ($P < 0.01$; Fig. 3). Additionally, when the GnRH-induced LH release was evaluated within each experimental group, there was an effect of category only in females treated with double dose of gonadorelin (Fig. 7), in which cows presented greater circulating LH concentrations at 2 h after treatment than heifers ($P = 0.05$), whereas in the other experimental groups the GnRH-induced LH release did not differ between cows and heifers ($P > 0.10$).

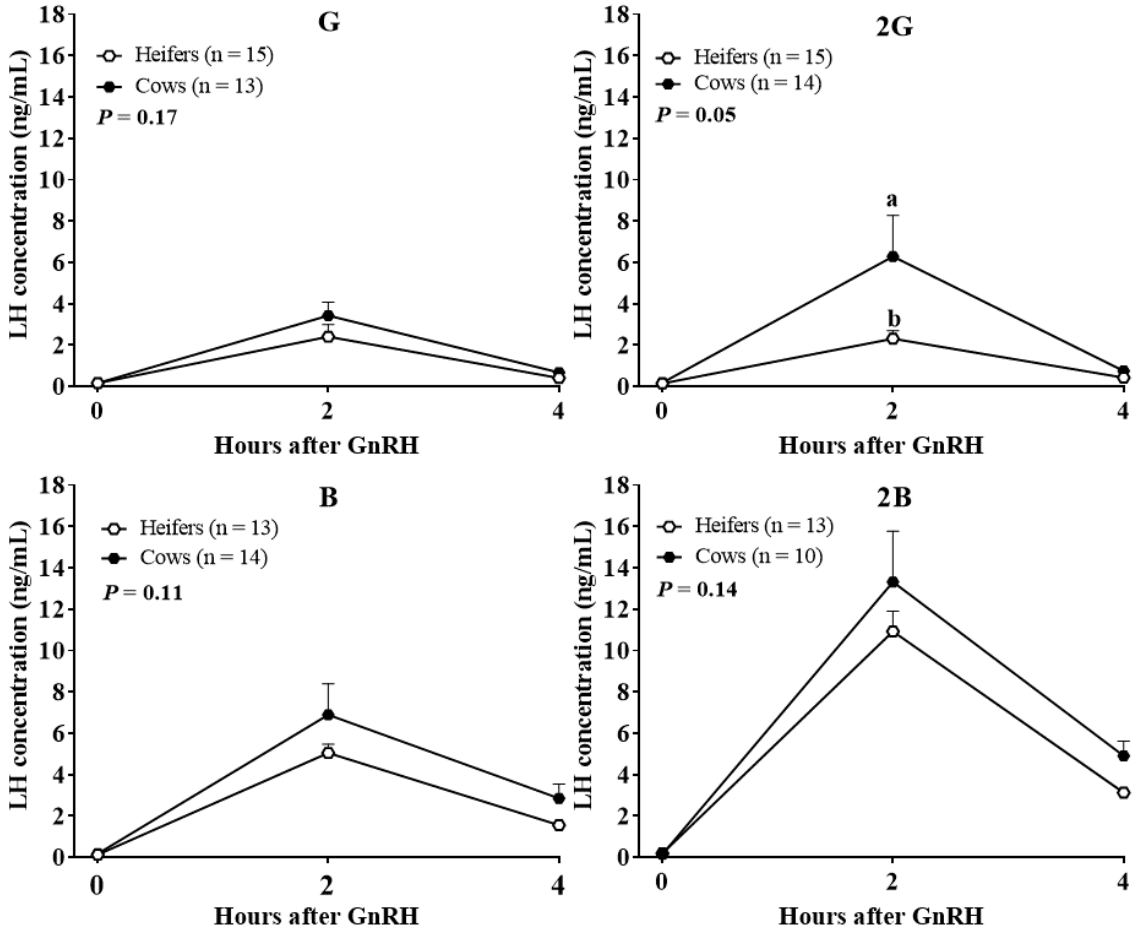


Figure 7. Circulating LH concentrations (mean ± SEM) in heifers and cows, from the time of GnRH treatment to 4 h later, individualized by each experimental group. Indicated *P*-value represents the effect of animal category on the LH release. Distinct letters ($^{a\text{-}b}$) indicate differences between heifers and cows within a time ($P < 0.01$).

A further analysis was done to estimate the probability of ovulation as a function of the LH peak amplitude in heifers and cows. Curves demonstrated in Fig. 8 represent positive linear effects for both categories ($P < 0.01$). Regardless of treatment, the higher the GnRH-induced LH peak the greater was the ovulatory risk. Nonetheless, it seems that this effect was more pronounced in heifers than cows. Finally, although no main effect of category was observed on ovulatory response ($P = 0.70$), there was an interaction between experimental group and category ($P = 0.02$), in which the ovulatory response was lower for cows than heifers receiving a single dose of buserelin ($P < 0.01$), but no difference was observed within the other treatments ($P > 0.15$; Table 1).

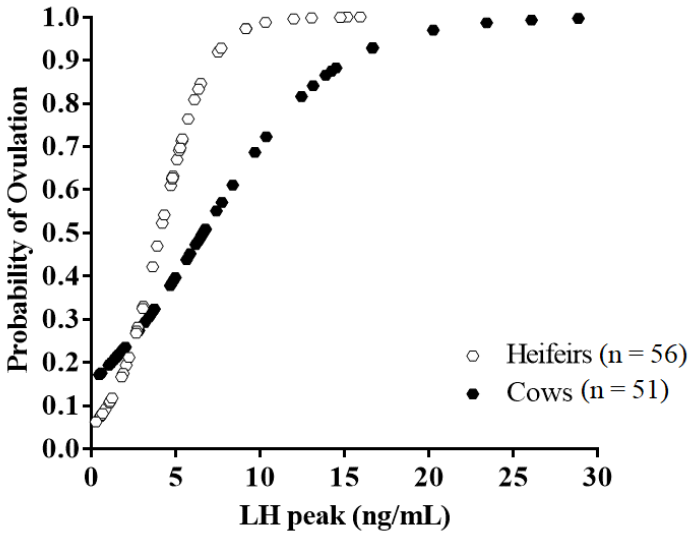


Figure 8. Probability of ovulation after GnRH, regardless of analogue or dose, in relation to the magnitude of GnRH-induced LH peak. The regression lines show linear effects for heifers ($y = -2.88 + 0.71x$; $P < 0.01$) and cows ($y = -1.69 + 0.26x$; $P < 0.01$). Females that presented a greater LH peak after GnRH were more likely to ovulate.

4.4 Discussion

The present study compared the GnRH-induced LH release and ovulatory response produced by two GnRH analogues and their respective doses, in *Bos indicus* heifers and cows under high circulating P4 concentrations. To ensure the accuracy of the responses, all females were previously synchronized to be on Day 7 of the estrous cycle at the time of GnRH treatments. The diameter of the DF before GnRH was consistent with what has been previously reported in Nelore cattle [34], and with an adequate size to respond to an ovulatory stimulus [22,40]. Moreover, circulating P4 concentrations observed on Day 7 of the estrous cycle in this study were similar to what has been reported in Nelore cattle [32,41].

The first hypothesis that the 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 produced higher circulating LH concentrations than gonadorelin. As reported, buserelin differs from gonadorelin (native GnRH) by substitution of glycine for a D-serine at position 6 and the replacement of the carboxyl-terminal glycinamide by ethylamide at position 10, producing a nonapeptide supposed to have greater affinity to the GnRH receptor and greater stability [30]. In fact, previous studies in cattle reported that buserelin was around 50-fold more potent to stimulate the pituitary gland than gonadorelin [42,43]. Moreover, similar to our results, a previous study in Holstein heifers reported lower LH release and lower LH peak induced by 100 μg gonadorelin compared to 10 μg buserelin, on

Day 7 of the estrous cycle. Nevertheless, in lactating dairy cows, the same treatments produced similar LH peaks on Day 7 of the estrous cycle, but buserelin had higher circulating LH concentrations for a longer time [27]. Interestingly, despite the differences on the LH surge, these studies did not find differences in ovulation between the GnRH analogues. In the present study, administration of 100 μg gonadorelin stimulated only 20% of ovulation in heifers, whereas 84.6% of the heifers ovulated to 10 μg buserelin. Conversely, in Holstein heifers, the study by Picard-Hagen et al. [26] reported 72.7 and 100% ovulation, respectively. These results suggest that the ovulatory response in *Bos indicus* heifers are more suppressed by high circulating P4 concentrations than in *Bos taurus* heifers, reinforcing the findings about the more pronounced suppressive effect of P4 on the LH release in Nelore than Holstein heifers [33]. In addition, in the present study, results from cows demonstrated that the single dose of buserelin (10 μg) was not enough to increase ovulatory response compared to 100 μg gonadorelin, despite producing a greater LH release. In lactating Holstein cows, same treatments also presented similar ovulatory responses [27], but apparently superior than what was observed in Nelore cows in our study (66.7 *vs*. 35.7%, respectively).

Furthermore, the second hypothesis created suggested that, regardless of the GnRH analogue, administration of double dose would increase LH release and ovulatory response. This hypothesis was partially supported. In heifers and cows treated with buserelin, the double dose produced around 2-fold greater LH peak. However, although by increasing the gonadorelin dose from 100 to 200 μg improved LH release in cows (around 2-fold increase), surprisingly, it did not influence the LH release in heifers. Previous studies evaluating doses of gonadorelin reported greater LH release when the double dose was administrated, even under high circulating P4, in *Bos taurus* heifers [25] and lactating cows [29]. However, there was no published study that evaluated the LH release induced by distinct GnRH analogues and doses in *Bos indicus* cattle. Thus, although it was not totally clear, a possible explanation for this lack of difference on LH peak produced by both gonadorelin doses may be that, in heifers, the maximum LH amplitude have occurred earlier, before 2 h after gonadorelin administration, as reported in *Bos taurus* heifers [26,44]. In this case, the double dose of gonadorelin could have promoted a greater LH peak than the single dose, but it could not be detected by this study due to the interval of blood collections. Moreover, the ovulation results did not confirme entirely what was hypothesized. Clearly, the double dose of gonadorelin was not enough to increase the ovulatory response in both heifers and cows. In fact, previous studies reported low ovulatory response in *Bos taurus* heifers and cows after treatment with 100 μg of gonadorelin [20,23]. Similarly, in a recent study with *Bos indicus*-influenced cows the ovulatory response after 100

μg gonadorelin was only 14.3% [45]. Additionally, according to their study, the LH release was greater when the gonadorelin treatment was given on day 3 of the estrous cycle compared to days 7 and 10. Nonetheless, results from the present study indicate no ovulation improvement by administering the double dose, contrary to what was reported in lactating dairy cows [46]. Otherwise, by increasing 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 unsatisfactory ovulation [18]. However, in a recent study from our research group [11], the use of an increased dose of buserelin (16.8 μg) at random stages of the cycle in Nelore cows resulted in high incidence of ovulation (73.6%), corroborating the results of this study. Conversely, the single dose of buserelin was sufficient to induce ovulation in a high percentage of heifers, equivalent to the ovulation percentage induced by the double dose. These findings suggest that, even under high circulating P4 concentrations, treatment with 10 μg buserelin was efficient to promote an adequate ovulatory response in Nelore heifers. Supporting this idea, the study by Chenaut et al. [43] demonstrated that treatment with 10 μg buserelin resulted in greater LH release than 100 or 250 μg gonadorelin, in Holstein heifers. Nevertheless, although the results from the present study have provided a better understanding about the potential of both analogues and respective doses on the ovulatory response in *Bos indicus* cattle, studies with a greater number of animals are necessary to consolidate this information.

In addition, in the present study, both heifers and cows were under high circulating P4 concentrations, provided by a 7-d old CL, at the time of GnRH treatments. Moreover, an intravaginal P4 implant was inserted simultaneously to the GnRH administration, simulating what occurs at the beginning of a GnRH-based FTAI protocol [12,47]. For this reason specific P4 implants were used in each category, as routinely used in Nelore cattle [11]. As expected, the insertion of a P4 implant stimulated a rapid increase in circulating P4, reaching 2-fold greater concentrations at 2 h after the insertion, similar to what was reported in Holstein cows [35]. However, according to a recent study from our research group (unpublished), the insertion of a P4 implant at the time of GnRH treatment was not able to suppress the GnRH-induced LH release or affect ovulation in non-lactating Holstein cows. Even so, it is well established that circulating P4 can modulate the responsiveness of the pituitary gland to a GnRH stimulus [48], and previous studies have reported the suppressive effect of high circulating P4 concentrations on GnRH-induced LH release after gonadorelin treatment in heifers [25] and cows [24], directly affecting the ovulatory response. Moreover, similar suppressive effect on LH release was demonstrated when buserelin was given under high circulating P4 concentrations [27,33]. The

present study did not detect a direct effect of circulating P4 concentrations on ovulation after GnRH treatments. Nevertheless, the analyses of the individualized distribution of females according to their circulating P4 at GnRH and LH peak observed provided some interesting information. When the GnRH analogues were contrasted, it was possible to observe that, regardless of the P4 concentration, no heifer and almost no cow treated with gonadorelin presented an LH peak greater than 7 ng/mL. Moreover, the contrast between ovulated and non-ovulated females demonstrated that only 24.1% (7/29) ovulated heifers and 12% (3/25) ovulated cows have had an LH peak lower than 5 ng/mL. Additionally, although the present study was not designed to provide distinct circulating P4 concentrations at the time of GnRH treatments, cows and heifers were classified into two groups, according to their circulating P4 at the time of GnRH, aiming to investigate the influence of P4 concentrations on the LH release. Interestingly, in both categories, females with lower circulating P4 ($\leq$ 4 ng/mL) tended to present a greater LH peak after GnRH treatment than females with higher circulating P4 (> 4 ng/mL), supporting the idea that the greater is circulating P4 concentration, the greater is the suppressive effect on LH release by the pituitary gland [24,25]. Moreover, this effect was more pronounced in heifers treated with the single dose of gonadorelin. In this sense, previous studies have reported that circulating P4 regulates the pituitary sensitiveness to GnRH by down regulating the GnRH receptors expression on the gonadotropic cells surface [49,50]. Therefore, a possible explanation for this finding is that, under higher P4 concentrations, the expression of GnRH receptors was more suppressed and, associated to that, probably the conventional dose of gonadorelin provided a low availability of agonist molecules with known lower potency [43] resulting in a very low stimulation of the pituitary. Curiously, this effect was not observed in cows or with other treatments in heifers.

Finally, the present study also compared the responses between heifers and cows. The third hypothesis suggested was that heifers would present lower circulating P4 concentrations, greater LH release and similar ovulatory response than cows. This hypothesis was not supported. First, diverging to what has been previously reported in Nelore females [34], the mean circulating P4 on day 7 of the estrous cycle did not differ between heifers and cows, probably due to the similarity of diet and metabolic status among them. Some interesting studies have reported the influence of these factors on circulating P4 concentrations both in *Bos taurus* and *Bos indicus* cattle [51,52]. However, in cows the diameter of the 7-day old DF was greater than in heifers, which could be explained by a probable greater incidence of three follicular waves in heifers than in cows, previously reported in Nelore cattle [34]. In addition, the GnRH-induced LH release did not differ among categories when females were treated with both doses

of buserelin or a single dose of gonadorelin, but unexpectedly, was greater in cows than heifers treated with double dose of gonadorelin. Conversely, Colazo et al. [24] reported that the LH release induced by 100 μg gonadorelin was greater in *Bos taurus* heifers than in cows, suggesting a more pronounced suppressive effect of high circulating P4 in cows. Two main possibilities were hypothesized to explain the results from the present study. First, as mentioned before, it is possible that, in heifers, the GnRH-induced LH peak had occurred earlier than the second blood collection, at 2 h after the GnRH treatment, underestimating the LH release results in this category, mainly when gonadorelin was administrated. It could explain the lack of the expected difference in LH release after the treatment with 100 μg gonadorelin, as well as the unexpected greater LH release in cows with the double dose. Alternatively, these results could be suggesting a more intense down-regulation of the GnRH receptors on the pituitary, mediated by high circulating P4 concentrations, in Nelore heifers than in cows. In fact, *Bos indicus* heifers presented much lower LH release induced by 100 μg gonadorelin than *Bos taurus* heifers, even under low P4 concentrations [33]. However, unfortunately, there is very little information about these physiological responses in *Bos indicus* cattle. More intensive studies are needed to better understand the effect of circulating P4 concentrations on the pituitary gland of *Bos indicus* cattle. On the other hand, the results from this study demonstrated that the single dose of buserelin was able to induce a greater ovulatory response in heifers than in cows. Besides that, in heifers, the ovulation rate promoted by the single dose was equivalent to that promoted by the double dose. Moreover, a further analysis indicated that a lower LH peak was enough to trigger ovulation in heifers compared to cows, suggesting that heifers are more sensitive to an LH ovulatory stimulus. It is well established that LH binds its receptor on granulosa cells resulting in ovulation of the DF [53]. In *Bos indicus* cattle, increased expression of mRNA encoding LH receptor was detected in granulosa cells of follicles > 7 mm [40], and an elevated ovulatory response was reposted in DF > 8.5 mm [22]. In this regard, results provided by this study demonstrated that a 7-d old DF in heifers, although smaller, was potentially more sensitive to the LH stimulus than in cows. Considering the predominance of the three follicular wave pattern in heifers and the shorter duration of its first wave compared to two-wave cycles [34] it is possible that, at day 7, the DF in heifers was more close to its maximum development and, consequently, responsiveness to LH than in cows.

In summary, buserelin treatments induced greater LH releases and ovulatory responses than gonadorelin treatments in *Bos indicus* heifers and cows, under high circulating P4. In addition, the double dose of GnRH increased LH release for both analogues in cows, but only for buserelin in heifers. The ovulatory response was increased by the double dose of buserelin

in cows, whereas in heifers, the single dose of buserelin was enough to induce ovulation in a high number of animals. Moreover, circulating P4 concentrations did not differ among heifers and cows, and the GnRH-induced LH release was similar between them in all treatments, except when doubling the dose of gonadorelin. Finally, regardless of treatment, the amplitude of GnRH-induced LH peak positively affected ovulation. However, apparently, heifers were more sensitive to this effect.

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5. FINAL CONSIDERATIONS

Results from the present study confirmed the suppressive effect of circulating P4 concentrations on the pituitary responsiveness to a GnRH stimulus in *Bos taurus* cows, *Bos indicus* cows and *Bos indicus* heifers. Moreover, for both genetic groups, greater ovulatory response to a GnRH treatment was associated to higher GnRH-induced LH peaks, and specifically in *Bos indicus*, heifers seem to be more sensitive to this effect than cows. Additionally, findings from this study demonstrated that the insertion of an intravaginal P4 implant at the time of GnRH treatment was not able to impair LH release or ovulation, but compromised the development of the subsequent CL, anticipating luteolysis. Finally, this study provided valuable information about the potential of P4 release from commercially available intravaginal P4 implants, as well as the efficacy of treatments with distinct GnRH analogues and doses, which can be applied to better adjust the synchronization protocols for FTAI, according to specific reproductive management demands depending on the hormonal status, animal category, or breed.