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The effect of circulating progesterone on magnitude of the GnRH-induced LH surge: Are there any differences between *Bos indicus* and *Bos taurus* heifers?

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ABSTRACT

This study evaluated the effects of differing circulating progesterone (P4) levels on the luteinizing hormone (LH) surge profile following treatment with gonadotropin releasing hormone (GnRH) in *B. indicus* (Nelore, n = 13) and *B. taurus* (Holstein, n = 16) heifers. All heifers were synchronized with a hormonal protocol to induce either a Low or High circulating P4 environment at the time of GnRH treatment. Heifers were randomly assigned to a 2 × 2 factorial design composed by two genetic groups (*B. indicus* and *B. taurus*) and two levels of circulating P4 concentrations (Low or High). Blood samples were collected every 30 min from −30 to 210 min and at 270 min after GnRH treatment. As expected, mean P4 concentration was greater for cows in the High than in the Low P4 group (P = 0.0008) and in *Bos indicus* than in *Bos taurus* heifers (P = 0.06). Despite genetic group, the area under the curve of LH release was greater in the Low-P4 than in High-P4 concentration group (P < 0.0001). Interestingly, it appears that High P4 concentrations had a more pronounced effect on LH peak in *B. indicus* than in *B. taurus* heifers, as indicated by the interaction (P = 0.01) between genetic group and P4 levels. In conclusion, circulating P4 concentration have a great impact on the GnRH-induced LH surge of both *B. indicus* and *B. taurus* heifers, but it does not explain the much lower LH peak in *B. indicus* with low circulating P4. Thus, more studies are essential to uncover some of the underlying physiological factors other than circulating P4 that are limiting LH release following a GnRH treatment in *B. indicus* cattle.

1. Introduction

Gonadotropin releasing hormone (GnRH) based synchronization protocols are widely utilized during timed artificial insemination (TAI) both in dairy [1–5] and beef cattle production [6]. Ovsynch-like TAI protocols normally utilize a combination of GnRH and prostaglandin $F_2\alpha$ ($PGF_2\alpha$) to manipulate follicular wave emergence and luteal function allowing for TAI without the need for estrus detection [1]. Thus, GnRH/$PGF_2\alpha$ based programs have been successfully implemented in commercial dairy herds in the last 2 decades [2,3]. However, the use of Ovsynch programs in beef cattle, particularly in *B. indicus* breeds, produce conception results that are normally poor even considering only cycling animals [7], although literature exploring this topic is rather scarce. Thus, the efficacy of a GnRH treatment in inducing an adequate LH surge in *B. indicus* cattle warrants further investigation.

An important factor that has been shown to be closely involved with the amount of LH released following a GnRH treatment is the level of circulating P4 of the cow at the time of GnRH administration [8]. For example, prior studies observed that ovulation to a GnRH treatment in association with synchronization protocols is significantly lower in cows having high circulating P4 concentrations compared to cows with low P4 concentration [4,9,10].

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Altogether, several research groups using beef [11,12] and dairy cows [13] support the hypothesis that the magnitude of GnRH-induced luteinizing hormone (LH) surge in cattle in strongly dependent upon the P4 environment of the animal.

Coincidentally, one of the main physiological differences between *Bos indicus* and *Bos taurus* cattle appears to be related to the greater circulating concentrations of P4 more frequently described for *Bos indicus* breeds [14,15]. For example, Carvalho et al., 2008 observed that *Bos indicus* heifers have consistently greater circulating P4 concentrations than *Bos taurus* heifers. These authors hypothesized that *Bos indicus* heifers are more sensitive to high P4 concentrations, which in turn may be more suppressive on LH pulsatility in zebu breeds [15].

Earlier studies in ovariectomized cattle report that *Bos indicus* (Brahman) have a reduced capacity for LH secretion in response to estradiol [16] as well as GnRH treatment [17] compared to *Bos taurus*. However, the comparison of the impact of differing circulating P4 levels on magnitude of the GnRH-induced LH surge in these breeds (Holstein and Nelore) is not well described. Thus, further studies exploring the effect of circulating P4 in *Bos indicus* and *Bos taurus* on the magnitude of the LH surge following GnRH treatment is evidently needed. This information can help explain some of the limiting physiological reasons for inconsistent responses to hormonal treatments across these genetic groups, and serve as basis for future developments in cattle breeding.

Therefore, the objective of the present study was to determine the effects of differing circulating P4 concentration on the magnitude of the GnRH-induced LH release in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) heifers. The hypotheses of this study were twofold: 1) P4 concentration would greatly interfere with the LH surge release induced by a GnRH treatment in both genetic groups and; 2) *Bos indicus* (Nelore) would have a lower response in terms of LH release following the GnRH treatment compared to *Bos taurus* (Holstein) heifers regardless of circulating P4 level.

2. Material and methods

The present study was approved by the Bioethics Commission of the Faculdade de Medicina Veterinária e Zootecnia of the Universidade de São Paulo (FMVZ – USP, protocol number 2565/2012), which complies with the ethical principles in animal research and welfare.

2.1. Animals and management

The current study was conducted at the Sao Paulo University Campus (USP, campus Pirassununga, SP, Brazil) during November and December of 2012. Heifers were selected by an ultrasound examination and only those with a CL present were included in the trial. A total of 29 normally cyclic heifers [13 *Bos indicus* (Nelore; 22–24 mo of age; BCS 3.4 ± 0.1) and 16 *Bos taurus* (Holstein; 18–20 mo of age; BCS 3.2 ± 0.2)] were used. All heifers were kept in the same facility at the School of Veterinary Medicine & Animal Science - Universidade de São Paulo (USP), Pirasununga-SP. Heifers were housed in dry lot pens and fed *ad libitum* a TMR diet based on corn silage, and a corn plus soybean meal based concentrate. The ration was balanced to meet or exceed minimum nutritional requirements according to NRC 2001 guidelines. Heifers were separated by breed and divided in 4 pens of 506 m^2 each (2 pens with Nelore heifers and 2 pens with Holstein heifers), with free access to shade and water, and 55 cm per animal of linear bunk space.

2.2. Experimental design

Heifers were randomly assigned to a 2 × 2 factorial design structure with two genetic groups (*Bos indicus*-Nelore and *Bos taurus*-Holstein) and two circulating P4 concentration levels (High or Low P4) at the time of GnRH treatment.

To create the high and low P4 hormonal milieu all heifers had their ovulation synchronized. All animals were at the same phase of the estrous cycle at GnRH treatment (7 days after ovulation). The hormonal protocol used to synchronize all heifers included the insertion of an intravaginal P4 device (CIDR®, Zoetis, Sao Paulo, SP, Brazil) and an intramuscular injection with 2 mg of estradiol benzoate (EB; Sincrodiol®; Ouro Fino Saude Animal, Cravinhos, SP, Brazil) on Day (−10) of the experimental period (Fig. 1). Eight days later, P4 devices were removed, and all heifers received a treatment with prostaglandin $F_2\alpha$ ($PGF_2\alpha$ 500 μg sodium cloprostenol; Sincrocio®, Ouro Fino Saude Animal, Brazil), estradiol cypionate (ECP 1.0 mg, i.m., Pfizer Animal Health) and equine chorionic gonadotropin (eCG 300 IU, Folligon®, MSD Saude Animal, Brazil; Fig. 1). Then, heifers in the low P4 environment group received additional $PGF_2\alpha$ treatment nine days after P4 device withdrawn (day of ultrasound evaluation to confirm CL presence; D7) and heifers in the high P4 group received placebo (0.9% sodium chloride), as shown in Fig. 1. Further PGF treatment was administered 12 h after the treatment performed on D7 (D7,5) in the low P4 group. Ten days after P4 device removal (D8), heifers received 0.01 mg GnRH, i.m. (buserelin acetate, Sincroforte®, Ouro Fino Saude Animal) for induction of LH surge.

Therefore, heifers were divided into four experimental groups in a 2 × 2 factorial design, as follows: Group 1 - *B. indicus* (Nelore) submitted to a low P4 environment at the time of GnRH treatment (n = 6); Group 2 - *B. indicus* (Nelore) submitted to a high P4 environment at the time of GnRH treatment (n = 5); Group 3 - *B. taurus* (Holstein) submitted to a low P4 environment at the time of GnRH treatment (n = 8); Group 4 - *B. taurus* (Holstein) with high P4 environment at the time of GnRH treatment (n = 5; Fig. 1).

2.2.1. Ultrasound examination

Ovarian transrectal ultrasound examinations were performed using a 7.5 MHz linear array transducer (Mindray DP–200 VET, Shenzhen, China) by the same technician. All heifers were determined to be cyclic defined by the presence of *corpus luteum* (CL) based on two consecutive ultrasound examinations performed 14 days apart before enrolment to the synchronization protocol. Moreover, further ultrasound evaluations took place on the day of P4 device® withdrawal (to evaluate the presence of dominant follicle) and nine days after P4 device removal to confirm ovulation. Ovulation was detected by the disappearance of the dominant follicle and by the presence of a CL structure on the same presumed ovulation site (Fig. 1).

2.3. Blood sampling

Blood samples were collected with vacuum-tubes containing EDTA by jugular venipuncture to determine P4 concentrations at the time of the GnRH treatment in both high and low P4 groups to confirm correct assignment of heifers to their respective circulating P4 level categories (Fig. 1).

For determination of LH concentrations, blood samples were collected at every 30 min for up to 210 min, starting 30 min before GnRH treatment. One last sample was also collected at 270 min after GnRH (Fig. 1). Plasma was stored at - 20 °C until assayed for P4 and LH.

2.4. Ovum pick-up procedure

All visualized follicles in both the high and low P4-heifers and genetic groups (*Bos indicus* and *Bos taurus*) were aspirated on Day 7

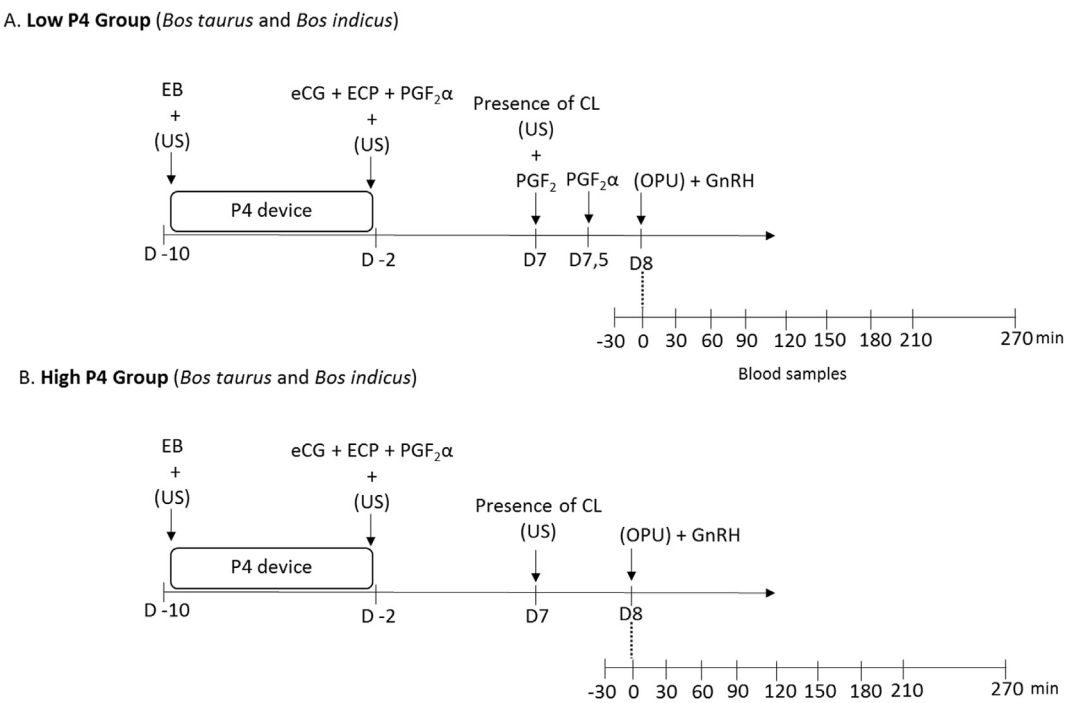

Fig. 1. Schematic representation of experimental procedures. Heifers underwent a protocol to synchronize the time of ovulation, and later to have either a high or low circulating progesterone (P4) level at the time of GnRH treatment (10 μg buserelin acetate). EB – estradiol benzoate, eCG – equine chorionic gonadotropin, ECP – estradiol cypionate, $PGF_2\alpha$ – prostaglandin F2 alpha, CL – *corpus luteum*, OPU – ovum pick up, GnRH – Gonadotropin-releasing hormone.

after ovulation to assure that LH surge occurred in response to the GnRH treatment and were not influenced by endogenous estradiol (Fig. 1).

In preparation for all follicle aspiration procedures, cattle were restrained in a chute and epidural anesthesia was administered using 2% lidocaine hydrochloride (Lidovet®, Bravet, Brazil) to facilitate handling of the ovaries through the rectum. The perineal area was cleaned using water, dried and sprayed with alcohol prior to each session. All follicles ≥2 mm were aspirated using a portable scanner with a 5-MHz convex array transducer (Mindray® DP 2200 vet, China) kept in a plastic vaginal probe with a stainless-steel needle guide (20 G; 0.9 × 50 mm; Terumo Europe NV, Belgium) connected to a vacuum system (V-MAR 5000, Cook Australia, Queensland, Australia). Follicular aspirate contents were recovered via a 1.1-mm inner diameter by a 120-cm length circuit (Watanabe Tecnologia Aplicada, WTA Ltda, Cravinhos, SP, Brazil) connected directly to a 50-mL conical tube containing 15 mL of Dulbecco PBS (DPBS; Nutricell Nutrientes Celulares, Campinas, SP, Brazil) and 5000 IU/mL sodium heparin (Parinex, Hipolabor, Belo Horizonte, MG, Brazil) at 35–37 °C. The vacuum connected to the needle was set at 85–90 mmHg. All retrieval procedures were performed by the same veterinarian.

2.5. P4 and LH assays

Progesterone concentrations were determined in duplicates using a commercial liquid-phase no-extraction RIA kit (HHub, MP Bio P4 DA). To assess the precision of the assay, control samples with a high (27.83 ng/mL) and low (0.53 ng/mL) concentration of P4 were also assayed. Average sensitivity for the P4 assay was 0.005 ng/mL. The average intra-assay CV for the high-control sample was 15%; whereas, the CV was 1.02% for the low-control sample. LH samples were quantified as previously described [18,19]. To assess the precision of the assay, control samples with a high (2.91 ng/mL) and low (0.41 ng/mL) concentration of P4 were concurrently assayed. Average sensitivity for the LH assay was 0.022 ng/mL. The average intra-assay CV for the high concentration control sample was 7.51%; whereas, the CV for the low control was 4.34%.

3. Data analysis

The analyses of repeated measures with data normalized either for the time of GnRH treatment or normalized for maximum LH amplitude within animal, were both performed using the GLIMMIX procedure of SAS (Version 9.3, SAS Institute Inc., Cary, NC). The final model included the effects of genetic group, P4 circulating level, sampling time, meaningful two- and three-way interactions, and cow, which was treated as a random effect and was the subject for the repeated measures analysis. The area under the curve of the LH surge profile was calculated by the trapezoid method utilizing the SigmaPlot® software (Version 12.5). The comparison between groups of circulating P4 levels at the time of GnRH treatment, maximum circulating LH amplitude levels (LH peak), time to LH peak, and area under the curve were analyzed using the MIXED procedure of SAS. When required, LH records were transformed to attain normality. A probability of $P < 0.05$ was considered to be significant, unless if indicated otherwise.

4. Results

The synchronization protocol utilized successfully induced an ovulation in 84.6% (11/13) of the *Bos indicus* and 92.9% (13/14) of the *Bos taurus* heifers. As proposed in the experimental design, heifers not having a CL 7 days after synchronized ovulation were excluded from the study. In addition, and in accordance to the initially targeted levels of circulating P4, the mean P4 concentration was greater ($P = 0.0008$) in the High-P4 than in the Low-P4 group (Table 1). As expected, *Bos indicus* heifers presented greater P4 concentration than *Bos taurus* heifers ($P = 0.06$).

Table 1
Effects of GnRH treatment on LH-surge release in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) heifers having a Low or High circulating progesterone (P4) concentration at the time of GnRH treatment. All values are expressed as means ± SEM.

Item	*Bos indicus* (Nelore)		*Bos taurus* (Holstein)		*P - value*		
	Low P4 (n = 6)	High P4 (n = 5)	Low P4 (n = 8)	High P4 (n = 5)	Genetic Group	[ ] P4[1]	Genetic Group* [ ] P4
P4 (ng/mL)	0.2 ± 0.02	4.4 ± 0.2	0.1 ± 0.007	2.9 ± 0.4	0.06	0.0008	0.20
LH peak (ng/mL)	8.3 ± 0.8^{cB}	3.3 ± 0.3^{dC}	27.0 ± 1.9^{aA}	6.9 ± 0.4^{bB}	0.0001	<0.0001	0.01
Time to LH peak (min)	125.0 ± 4.9	102.0 ± 3.3	116.3 ± 2.4	96.0 ± 5.0	0.37	0.02	0.54
AUC[2] (units)	1482.5 ± 145.8	612.6 ± 45.7	3862.4 ± 175.2	1188.8 ± 50.5	<0.0001	<0.0001	0.34

[a,b,c] Indicate differences in LH peak (ng/mL) between P4 groups (Low and High P4) in the same genetic group.
a ≠ b $P \leq 0.05$.
c ≠ d $P = 0.07$.
[A,B,C] Indicate differences in LH peak (ng/mL) between genetic groups (*Bos taurus* and *Bos indicus*) in the same P4 environment.
1 P4 concentration.
2 Area under the curve.

The maximum amplitude of LH surge after treatment with GnRH was greater ($P < 0.0001$) in the Low-P4 than in High-P4 concentration group for both *Bos taurus* (27.0 ± 1.9 vs. 6.9 ± 0.4 ng/mL) and for *Bos indicus* heifers (8.3 ± 0.8 vs. 3.3 ± 0.3 ng/mL; Table 1). Regardless of the P4 level at GnRH treatment, the time from GnRH treatment to peak LH was similar ($P = 0.37$) between *Bos indicus* and *Bos taurus* heifers. In contrast, the time to peak LH concentrations occurred later in the Low P4 than in the High P4 concentration in both genetic groups ($P = 0.02$; Table 1). In addition, there were no detectable interactions between P4 level and genetic group on the interval from GnRH treatment to peak LH.

Both in *Bos indicus* and *Bos taurus* heifers the area under the curve (AUC) of LH release was greater in the Low P4 than in the High P4 group ($P < 0.0001$). In addition, regardless of the P4 concentration, the AUC of LH release was greater in *Bos taurus* than in *Bos indicus* heifers ($P < 0.0001$). With no significant interactions between genetic group and circulating P4 levels (Table 1).

Mean LH profile throughout the sampling period were compared by normalizing the data for the time of GnRH treatment or by the maximum LH concentration within each heifer. Both types of normalizations yielded similar results and LH profile was significantly affected by P4 concentration, sampling time, genetic group, genetic group by P4 concentration interaction, P4 concentration by time interaction, and genetic group by time interaction, as shown in Fig. 2.

In *Bos indicus* heifers, GnRH treatment did not increase mean LH beyond 8.3 ng/mL at any time during the sampling period (Table 1; Fig. 2). However, in *Bos taurus* heifers, the mean LH concentration reached up to 27 ng/mL after GnRH treatment (Table 1; Fig. 2). Accordingly, the mean LH concentrations *Bos taurus* heifers were much greater in the Low than in the High P4 concentration from 30 to 270 min after treatment. In contrast, in *Bos indicus* heifers, the mean LH concentrations were also greater in the Low than in the High P4 concentration, but during a narrower window starting only at 90–210 min after GnRH treatment (Fig. 2). In addition, a further analysis of individually distributed AUC of LH release in Fig. 3 shows a greater AUC for individual *Bos taurus* heifers compared to *Bos indicus* when circulating P4 was lower than 1 ng/mL.

5. Discussion

Results from the current trial confirm observations from previous research groups showing that circulating P4 concentration have a major effect on the magnitude of the GnRH-induced LH surge both in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) cattle. Confirming our initial hypothesis, *Bos indicus* heifers had greater circulating P4 than *Bos taurus* heifers and that likely influenced LH release. The greater circulating P4 levels in zebu heifers could be explained by a greater steroidogenic capacity in their CL structures and/or by lower rates of P4 metabolism in the liver of *Bos indicus* animals, as previously proposed [20]. In fact, prior literature suggests that the CL from *Bos indicus* cattle indeed have greater steroidogenic activity [21,22]. In relation to liver metabolism of steroids, it is widely known that increased liver-blood flow [23,24] and the liver enzymes [25–27] are intrinsically involved in P4 metabolism. In addition, several research groups have described that, despite the type of diet - which will drive liver blood flow rate – that *Bos indicus* cows [28] and heifers [15,29] have greater circulating P4 than *Bos taurus* cattle. Moreover, P4 liver metabolism pathways appears to be quite different between *Bos indicus* and *Bos taurus* breeds [29]. In addition, slight differences in body weight and BCS across animals and animal breed are unlikely to have a major influence on progesterone metabolism [30]. Thus, it is logical to speculate that P4 production by the CL and/or liver metabolism might be fairly different in *Bos taurus* and *Bos indicus*, which in turn would have a dramatic effect in circulating P4 levels and supporting the findings in the current trial.

More importantly, one of the main findings in the current study was the consistently lower response to GnRH in *Bos indicus* than in *Bos taurus* heifers, as initially hypothesized. This breed effect following the GnRH treatment was far more evident under low circulating P4 levels. Thus, under low circulating P4, it is quite evident that *Bos indicus* heifers have much lower response in terms of LH peak levels as well as AUC to GnRH treatment.

Similarly to AUC, the maximum amplitude of the LH was significantly greater in *Bos taurus* compared to *Bos indicus* heifers. Moreover, the LH release was considerably greater during the Low P4 than in High milieu in both genetic groups. However, the significant interaction between genetic group by P4 level on LH surge profile indicates that the different cattle breeds responded differently in terms of LH release according to P4 level at GnRH treatment. As expected, the reduction in the GnRH-induced LH surge under a high-P4 environment has been found in the current trial and has also been previously reported in beef heifers and cows [12,31] as well as in dairy cows [8]. For example, Giordano et al., 2012 [13] described marked differences in LH peak profile comparing cows treated with GnRH during proestrus – (Low P4 environment; around 0.2 ng/mL of P4 and 15 ng/mL of LH) and diestrus (High P4 environment; around 3.5 ng/mL of P4 and 3.3 ng/mL). In the same way, recently Armengol – Gelonch et al., 2017 [8] observed during proestrus about 8 ng/mL and 4 ng/mL of LH during diestrus to Holstein cows. In this study, we observed that *Bos taurus* heifers had the maximum amplitude of LH reaching up to 27.0 ng/

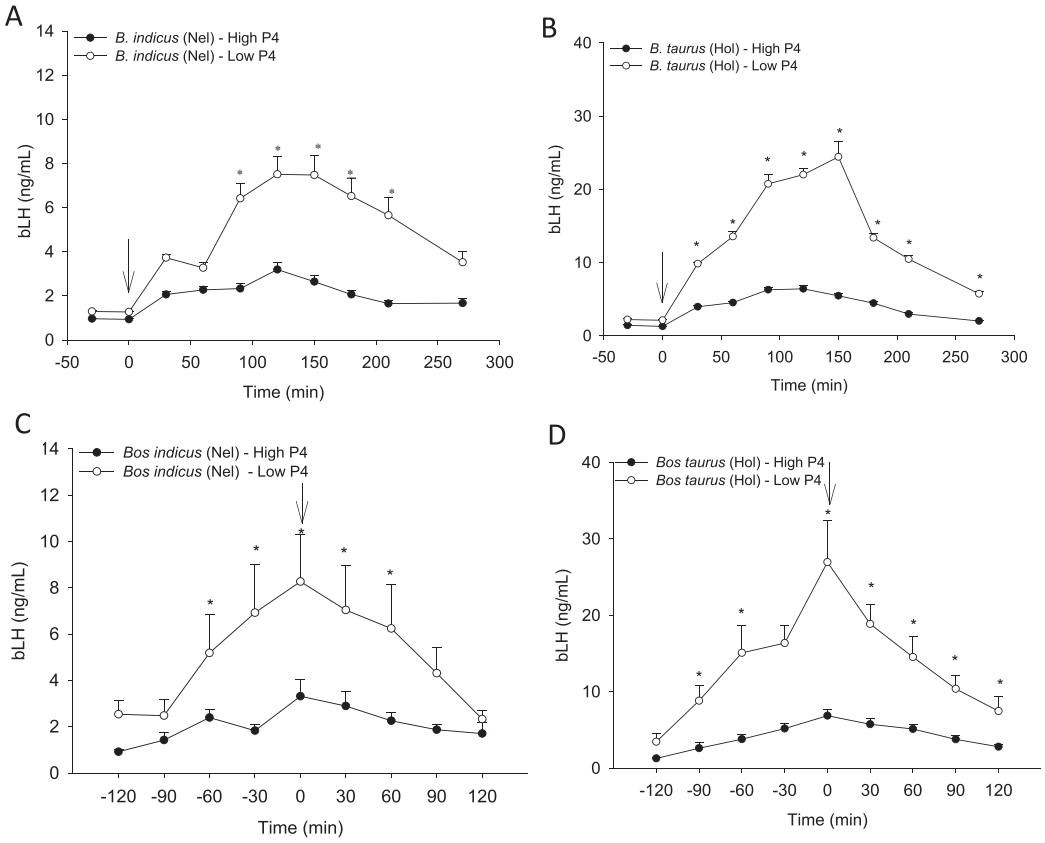

Fig. 2. Mean (±SEM) LH concentrations in *Bos indicus* (Panel A and C) and *Bos taurus* (Panel B and D) heifers having either Low or High circulating progesterone (P4) normalizing for the time of GnRH treatment (Panel A and B) or by maximum LH amplitude (Panel C and D) within each heifer. Asterisks indicate differences in mean LH concentration between Low and High P4 groups. The arrow indicates the time of GnRH treatment (Panel A and B) and the time of LH peak (ng/mL; Panel C and D). LH profile was significantly affected by P4 concentration ($P < 0.0001$), sampling time ($P < 0.0001$), genetic group ($P < 0.0001$), genetic group by P4 concentration interaction ($P < 0.05$), P4 concentration by time interaction ($P < 0.05$), and genetic group by time interaction ($P < 0.05$).

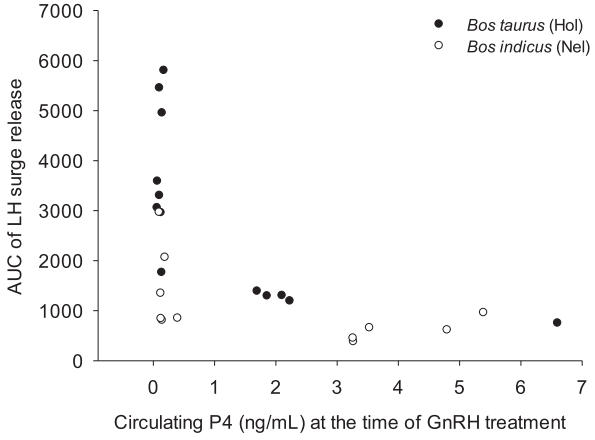

Fig. 3. Distribution of area under the curve (AUC, ng/ml × time) of LH surge release in *Bos taurus* (dark circles) and *Bos indicus* (open circles) according to circulating progesterone concentration in ng/ml at the time of GnRH treatment.

mL in Low P4 and 6.9 ng/mL in High P4 milieu. These differences across studies could be explained likely by the type of LH assay used and/or possibly the female category in the experimental design (cow vs. heifer).

Thus, the relatively low response in terms of LH release following the GnRH treatment in *Bos indicus* is quite intriguing. It might perhaps simply indicate that a greater GnRH dose ought to be applied in *Bos indicus* cattle to achieve a more adequate LH release. Studies in cattle have described a wide range of results using different doses of GnRH and reporting either enhanced or similar LH release in response to increasing doses of GnRH [12,31,32]. Later reports showed though a consistent increase in GnRH dose been associated to greater magnitude of LH surges regardless of circulating P4 environment in Holstein cows [13]. Altogether, an intriguing opportunity to try to overcome this low response to GnRH, particularly in *Bos indicus* cattle, could then be to increase the dose of GnRH. Alternatively, the low response of LH release to GnRH treatment in *Bos indicus* may suggest that the blood-brain barrier in these cattle breeds is less permeable to the GnRH molecule when given intramuscularly and further investigation addressing whether GnRH given directly into the spinal cord fluid [33] could bring important insights on this plausible question. Additionally, LH storage can perhaps be depleted sooner in *Bos indicus* cattle following a gonadotropin release stimulus. Yet, it is possible that even in the low P4 group that circulating levels of P4 already had reached an inhibitory threshold to GnRH-induced LH secretion for the *Bos indicus* heifers [13]. All these hypotheses need to be further explored by future studies.

Despite genetic group, the interval from GnRH treatment to maximum amplitude of LH was greater in heifers exposed to low P4 compared to heifers in the high P4 group. It is hard to draw final conclusions around this unexpected finding with the current experimental design. However, it is possible that lower P4 allowed

gonadotroph cells to continue to release LH for a longer period of time, achieving maximum levels at a later time in relation to GnRH treatment. Future studies utilizing a greater number of experimental units and more frequent blood sampling, ideally with controlled-exogenous P4 supplementation are needed to conclusively confirm this hypothesis.

In conclusion, circulating P4 concentration at GnRH treatment had a dramatic effect on the GnRH-induced LH release in both genetic groups. However, the maximum LH surge amplitude and AUC is considerably lower in *Bos indicus* than *Bos taurus* heifers. This information indicates that the pituitary of *Bos indicus* heifers is less responsive in terms of LH release following a GnRH treatment if compared to the pituitary of *Bos taurus* heifers.

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