University of São Paulo
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Hormonal adjustments for fixed-time artificial insemination and physiologic variables influence in the reproductive performance of *Bos indicus* heifers

Juan Pablo Acosta Galindez

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

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DEDICATION

To my family, without a doubt I dedicate this to my parents, my sister and my grandmother, without their support, patience and love would have been impossible, thank you so much.

“The future depends on what you do today”

Mahatma Gandhi

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SUMMARY

RESUMO

Ajustes hormonais na inseminação artificial em tempo fixo e influência de variáveis fisiológicas no desempenho reprodutivo de novilhas *Bos indicus*

O objetivo do presente estudo é avaliar o efeito de diferentes tratamentos hormonais e o entendimento de algumas variáveis fisiológicas na dinâmica ovariana e os resultados reprodutivos de novilhas *Bos indicus* submetidas a protocolos de IATF a base de estradiol (E2)/progesterona (P4). Foi desenvolvido usando novilhas Nelore com uma média de 16,0 ± 0,03 meses de idade. Foram incluídas somente novilhas diagnosticadas como não prenhes 30 dias após uma primeira IA sendo resincronizadas. No Dia -9 todas as novilhas receberam um implante intravaginal de P4 por 7 dias e benzoato de estradiol (BE). No Dia -2, aconteceu a remoção do implante e ao mesmo tempo foi administrado cipionato de estradiol (CE), gonadotropina coriónica equina (eCG) e prostaglandina F2α (PGF). No Dia 0, 48 horas depois da remoção do implante, todas as novilhas foram IA. Além disso, houve dois fatores analisados, um deles foi a administração de PGF (PGF1) ou não (PGF0) ao mesmo tempo da inserção do implante de P4. O outro foi o tratamento com GnRH no momento da IA (G1) ou não (G0), resultando em quatro tratamentos diferentes (PGF0G0, PGF0G1, PGF1G0 e PGF1G1). A expressão de estro até a IA, foi avaliada com o uso do bastão de cera na base da cauda, fazendo as avaliações ultrassonográficas dos ovários nos Dias -9, -2 e 0 e o diagnóstico de prenhez foi executado 30 e 60 dias depois da IA. A P/IA no Dia 60 não teve diferença entre os tratamentos, a PGF no Dia -9 não influenciou na P/IA das novilhas com presença de CL no início do protocolo de IATF. Novilhas com expressão de estro alcançaram uma melhor P/IA assim como houve uma menor perda gestacional para este grupo. A administração de GnRH não gerou diferença dentro do grupo sem estro, assim como não houve influencia deste tratamento hormonal na P/IA geral. Por outra parte, a P/IA tendeu a incrementar somente no grupo das novilhas mais novas que tinham um peso maior, com nenhum efeito dentro das novilhas mais velhas. Além disso, as fêmeas mais leves de maior idade tiveram uma maior probabilidade de prenhez, o que não aconteceu para os grupos de novilhas mais pesadas. Este estudo permite concluir, que os tratamentos propostos não impactaram na fertilidade das novilhas. Melhor desempenho reprodutivo foi atingido pelas novilhas mais velhas, e o maior peso corporal poderia melhorar a P/IA das novilhas mais novas.

Palavras-chave: Novilhas, *Bos indicus*, Fertilidade, IATF, GnRH, Prostaglandina

ABSTRACT

Hormonal adjustments for fixed-time artificial insemination and physiologic variables influence in the reproductive performance of *Bos indicus* heifers

The present study aimed to evaluate the effect of different hormonal treatments and the understanding of some physiological variables on the ovarian dynamics and the reproductive outcomes of *Bos indicus* heifers submitted to a 7d estradiol (E2)/progesterone (P4)-based timed artificial insemination (TAI) protocol. Was performed using Nelore heifers with an average of 16.0 ± 0.03 months of age. Were included just heifers that were non pregnant 30 days after a first AI being resynchronized. On Day -9 all heifers received an intravaginal P4 implant for 7 days and estradiol benzoate (EB). On Day -2, had the implant removed and at the same time was administered estradiol cypionate (EC), equine chorionic gonadotropin (eCG), and prostaglandin F2α (PGF). On Day 0, 48 hours after the implant withdrawn, all heifers were AI. Also, there were two factors analyzed, one of them was the administration of PGF (PGF1) or not (PGF0) at the P4 implant insertion time. The other one was the GnRH treatment at the AI time (G1) or not (G0), resulting in four different treatments (PGF0G0, PGF0G1, PGF1G0 and PGF1G1). The expression of estrus until the TAI, was evaluated with the use of Tail-chalk, the ultrasound of the ovaries was performed on Days -9, -2, 0, and the pregnancy diagnose was executed 30 and 60 days after AI. The P/AI on Day 60 had no difference between the treatments, the PGF on Day -9 had no influence on the P/AI of the heifers with CL at the beginning of the TAI protocol. Heifers with expression of estrus achieved a better P/AI as there was a lower pregnancy loss for this group. The GnRH administration did not generate difference within the group without estrus, as there was no influence of this hormonal treatment on the general P/AI. On the other hand, the P/AI tended to increase just in the youngest heifer's group that had higher body weight, having no effect for the older heifers. Also, the lightest females with an older age had a higher probability of pregnancy, what did not occur for the heavier heifer's groups. This study allows to conclude, that the treatments proposed did not impact on the heifer's fertility. Better reproductive performance was achieved by the older heifers, and the higher body weight might improve the P/AI of youngest heifers.

Keywords: Heifers, *Bos indicus*, Fertility, TAI, GnRH, Prostaglandin

1. INTRODUCTION

The beef cattle production had evolved in different aspects through the last decades in the tropical countries. Two of the main changes experienced in the industry were the development of the timed artificial insemination (TAI) protocols and the genetic improvement programs bringing with them, important advances in productivity for the cow-calf operations in countries like Brazil [1,2].

The improvement of the *Bos indicus* cattle in Brazil has been impressive, many genetic programs with thousands of purebred and commercial cows, allowed the dissemination of material from high genetic merit bulls and females that contributed with the beef commercial herds [3]. Based on the main statement that the best generations are the youngest ones [4], the heifers become one of the most important groups of the herd, because of the genetic load that might be superior compared with the cows, becoming an important challenge for the reproductive programs to achieve the best results as possible in this young female's category, in order to be able of select the best replacement heifers for the herd. In the same way, the herd improvement through the time, will depend on the generation interval, considering that the reduction of the parents average age, will increase the genetic gain, and the productivity of the herd [5]. It is one of the reasons why, the reduction of the first calving age of the females, is an important parameter that should be priority with each new generation, stimulating the reduction of the age at puberty, leading to achieve acceptable fertility outcomes in a young heifer's category in the reproductive programs, keeping in mind that the fertility could be affected by the reproductive status of the heifers at the initiation of the breeding season as described by Moriel *et al.* finding a difference of 32% points on the final pregnancy status at the end of the breeding season between the prepupertal (55.4%) and the cyclic (87.4%) heifers [6].

At the same time, the genetic progress, will be influenced by the bull's genetics introduced in the herd, which implies the need of the most advanced genetic material, which is able of increase the herd's productivity leading to a better calf crop quality as well as the obtention of more efficient replacement heifers. It is the reason why, the artificial insemination had become a relevant biotechnology in the cattle production, mainly supported in the progress that represents for the cow-calf operations [7]. Nonetheless, there are just two alternatives to implement this procedure in the property, one of them is the estrus detection, with different

alternatives in the market to increase the estrus detection rate, being mainly restricted in the beef cattle operations by the low logistic efficiency this method offers and the dependence on the cyclicity of the females introduce in the reproductive programs, a physiologic condition that is determinant for the AI results, especially in *Bos indicus* cattle, that has been described as a genetic group with a low cyclicity incidence at the beginning of the breeding season (Presence of CL at the beginning of the synchronization program in cows: 38.7% and heifers: 14.9%) [8,9]. On the other hand, exist the TAI, a biotechnology that improves the logistic efficiency, allowing the cattle producers to AI a high number of females at the same time, optimizing the labor, and enable the producer to AI cows with postpartum anestrus as well as prepubertal heifers[10,11].

The TAI biotechnology has been supported in years of research in different breeds and production systems, leading to the establishment of two main strategies for the TAI protocols, bringing significant advances in the reproductive management of beef herds [12]. One of those strategies is the Ovsynch protocol and the several modifications widely described in literature [13–16]. On the other hand, an alternative mainly used in South America are the estradiol/progesterone-based TAI protocols, strategy that had different adjustments and alternatives developed in the last years, representing a significant improvement for the fertility results in the breeding herds, opening the possibility of an expressive expansion of the technology application in the field [17–19]. Some of the principal contributions was the implementation of the estradiol cypionate (EC), having no difference on the P/AI compared with the TAI protocols that use the estradiol benzoate (EB) as ovulation inductor (EC: 66.0% vs. EB: 66.7%) leading to a reduction of the managements required in the TAI protocol [20], as well as the use of the equine chorionic gonadotropin (eCG) which improved the reproductive performance, increasing the P/AI of cows (with eCG: 51.7% vs. without eCG: 33.8%) [21] and heifers (with eCG: 50.0% vs. without eCG: 36.8%) [22] submitted to a TAI protocol. Some other important adjustments had been reported in cows and heifers with different results in the literature, one of them is the use of prostaglandin F2α (PGF) at the initiation of the TAI protocol, that was described by Alves *et al.* as a hormonal alternative with a positive influence on the pregnancy per AI (P/AI) on Day 30 after AI, for cows with CL presence at the beginning of the AI protocol (with PGF: 69.1% *vs.* without CL: 62.5%) [23]. However, an anterior study described different results about the use of PGF at the P4 implant insertion, where

there was no influence on the P/AI in Nelore heifers (with PGF: 51.7% *vs.* without CL: 57.7%) [24]. Another important adjustment reported is the use of gonadotropin release hormone (GnRH) analogues at the AI time, which have been described as an important alternative to increase the P/AI, especially for cows that did not express estrus until the AI moment (with GnRH: 33.4% *vs.* without GnRH: 15.0%) [25], nonetheless, there are contradictory studies where the GnRH administration concomitant with the AI had no effect on the P/AI in Nelore heifers without expression of estrus (with GnRH: 46.8% *vs.* without GnRH: 49.3%) [26], different of what was previously described by Sujrus *et al.* who found a tendency of the GnRH treatment to increase the P/AI in heifers (with GnRH: 48.8% *vs.* without GnRH: 42.3%), without interaction with the expression of estrus prior the AI moment [27].

Considering the information described above, it is important to develop strategies that could contribute to the improvement of the reproductive programs in heifers, especially those young females between 12 to 18 months of age that could become pregnant in the first breeding season in their lives, at the same time with the herd cows. Also, there are some possible adjustments in the TAI protocols for heifers, which their benefits are not clear yet, being the main reason of this research, having as objectives of the present study, the understanding of the effects that the PGF administration at the initiation of the TAI protocol, as well as the GnRH administration at AI time, could have in Nelore heifers, and analyze the influence of the variables weight and age of the heifers on their reproductive performance.

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2. HORMONAL ADJUSTMENTS AND PHYSIOLOGIC VARIABLES EFFECTS ON THE REPRODUCTIVE PERFORMANCE OF *Bos indicus* HEIFERS BETWEEN 13 TO 17 MONTHS OF AGE SUBMITTED TO ESTRADIOL/PROGESTERON-BASED TIMED AI PROTOCOLS

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Abstract

The objectives of the present study were to evaluate the ovarian dynamics and the impact of some physiologic variables, on the reproductive performance of *Bos indicus* heifers submitted to a 7d estradiol (E2)/progesterone (P4)-based timed artificial insemination (TAI) protocol using different hormonal treatments. Nelore heifers (n = 784) with an average of 16.0 ± 0,03 months of age, were submitted to resynchronization protocols, being randomly assigned to one of the four treatments: PGF0G0 (no prostaglandin F2α, [PGF] on D-9 and no Lecirelin [GnRH] at AI [n = 197]), PGF0G1 (no PGF on D-9 and GnRH at AI [n = 200]), PGF1G0 (PGF on D-9 and no GnRH at AI [n = 199]) and PGF1G1 (PGF on D-9 and GnRH at AI [n = 188]). On D-9 heifers received an intravaginal P4 implant (0.5 g) for 7 d and estradiol benzoate (EB; 1.5 mg). On D-2, the implant was withdrawn and heifers received cloprostenol (PGF; 0.5 mg), equine chorionic gonadotropin (eCG; 200 UI) and estradiol cypionate (EC; 0.5 mg). On D0 the heifers were AI. Besides the main protocol, received PGF (PGF1) or not (PGF0) on D-9, as well as GnRH (25 µg) at the TAI (G1) or not (G0) on D0. Tail-chalk was used to evaluate the expression of estrus until the TAI, and ultrasound examinations were performed on Days -9, -2, 0, for ovarian dynamics and on 30 and 60 for pregnancy diagnose. Results were different when $P \leq 0.05$, and tendency when $0.05 < P \leq 0.10$. There was no difference on the P/AI on D60 between the treatments (PGF0G0: 36.0; PGF0G1: 35.5; PGF1G0: 35.2 and PGF1G1: 35,6%), as well as there was no effect of the PGF treatment on the P/AI for the heifers with CL on D-9 (PGF0: 37,0 [n = 311] *vs*. PGF1: 38,9% [n = 316]). The expression of estrus increased the P/AI (40,3 [n = 588] *vs*. 21,4% [196]). There was no interaction of the GnRH treatment with the expression of estrus, having no difference within the group without estrus (G0: 22,1 *vs.* G1: 20,8%). Also, the heifer's body weight (BW) tended to increase the P/AI of the youngest females (P = 0.1), but did not influence the oldest heifers (P = 0.5), as well as the heifer's age improved the P/AI of the lightest group (P = 0.03), with no effect for the heaviest heifers (P = 0.69). In conclusion, the PGF and GnRH treatments did not influence the P/AI of the young females studied. Furthermore, the older age of the heifers might contribute for the fertility performance of this young category, as the higher BW could be helpful for the youngest heifers P/AI.

Keywords: TAI, heifer, beef cattle, *Bos indicus*, estrus, pregnancy

2.1. Introduction

Tropical beef cattle industry historically has had to use *Bos indicus* genetic material because of the adaptability and efficiency for different production systems and environments, where the cattle raising activity has place in the tropics, situation that represents different challenges for cattle producers, especially the need for improvement of the reproductive performance in the grass-fed cow-calf operations, which are the main cattle breeding systems in the South America [1,2]. In the last years, different technologies have been developed to face those challenges, as the goal to achieve a younger age at first calving of the *Bos indicus* cattle, considered non-precocious, when compared with the *Bos taurus* cattle [3,4], as well as the need to improve the heifer's reproductive performance [5]. It is thus essential to investigate and understand the main factors and the technologies adjustments that might benefit the cattle producers, leading to reduce the puberty age, age at first calving and increase the calves production through better reproductive parameters. One of those technologies have been the timed artificial insemination (TAI), a relevant advance in the reproductive management that has allowed the commercial cattle producers to increase the use of the artificial insemination on a large scale in countries like Brazil, where there has been an impressive increase in the use of this technology in the last decades, bringing important advantages as the accelerated genetic improvement and the increase in the calves production in the cow-calf operations [6]. It is the main reason why, the principal efforts to achieve the goal of having a younger age of first calving with a good quality calf, have been made through the TAI in the Nelore (*Bos indicus*) heifers [7]. It is the reason why, has been studied which physiologic and hormonal variables could influence the pregnancy results in prepubertal Nelore females [8–10].

Some studies described the impact of the circulating progesterone (P4) concentration on the largest follicle (LF) diameter at the P4 device removal moment, also at the AI time as Dias *et al.* described in post pubertal Nelore heifers when exposed to a TAI protocol with new P4 device with higher serum P4 concentration two days before the device withdrawal (3.0 ± 1.9 ng/mL) or an already twice times used P4 device (2.0 ± 1.2 ng/mL), finding a difference of almost one millimeter on the follicle diameter at the TAI moment (New device: 10.1 ± 2.0 mm *vs.* 11.0 ± 2.1 mm) [11] presenting a positive association of a large follicle diameter with the expression

of estrus and the pregnancy per artificial insemination (P/AI) in Nelore cows, previously presented by Sá Filho *et al.* study where cows with a follicular diameter at the TAI time over 14.4 mm presented a P/AI of 63.3% compared with those cows with the smaller follicles (>7.5 mm) that achieve just a P/AI of 27.5% [12]. The follicle growth and diameter is controlled primarily by the progesterone regulation function over the LH pulse frequency, presenting a negative feedback on the pituitary LH secretion [13,14] resulting in a negative effect on the follicular diameter at the end of the TAI protocol [15] and a reduce expression of estrus, in a high circulating P4 concentration scenario, described before in Holsteins heifers submitted to four different Ovsynch protocols, reveling a 37% points difference in the expression of estrus between the treatment groups of the highest (8.43 ± 0.39 ng/mL) and the lowest (1.21 ± 0.15 ng/mL) P4 concentration at the first PGF treatment of the Ovsynch protocol (45% *vs.* 82%, respectively) [16]. It is the reason why, develop strategies to obtain a low circulating P4 concentration during the TAI protocol would be a relevant alternative to improve the follicular diameter at the end of the TAI and potentially improve the P/AI in Nelore heifers.

There are some researches that have already evaluated different alternatives to achieve a better LF diameter associated with higher fertility, being studied the effect of different P4 concentration intravaginal implants [17], the use of equine chorionic gonadotropin (eCG) [18], or the regression of the present corpus luteum (CL) at the beginning of the TAI protocol, using prostaglandin F2α (PGF), which looks for the reduction of the circulating endogenous progesterone released by the CL, to achieve an increased LH pulse frequency, a greater mean LF diameter and a better reproductive performance, being an alternative previously evaluated by Carvalho *et al*. in two years old *Bos taurus, Bos taurus* x *Bos indicus* and *Bos indicus* heifers (conventional heifers) finding an association of the PGF treatment and a higher follicular diameter at the P4 implant withdrawal (With PGF: 9.5 ± 0.3 mm *vs.* without PGF: 7.9 ± 0.3 mm) influenced by a different circulating P4 concentration for both treatments [19]. Nonetheless, the PGF administration has not been evaluated before in 12 to 18 months of age Nelore heifers (precocious heifers), it is the reason why, one of the objectives of the present study is the evaluation of the CL response, follicle size, expression of estrus, P/AI and pregnancy loss on precocious heifers treated or not with PGF at the TAI protocol initiation.

On the other hand, has been researched the influence on the reproductive performance of the gonadotropin release hormone (GnRH) analogues administration at the AI in cows and heifers, with contradictory results of different studies. In a study of our laboratory, Madureira *et al.* reported the benefits for the P/AI in Nelore cows that did not express estrus until AI, showing an increase of almost 11% points between the cows with no expression of estrus, that receive the GnRH treatment at AI (P/AI = 59.1%) and those that did not have the treatment (P/AI =48.2%), results that did not repeat in the heifers submitted to the same treatments in this study, having no influence of the GnRH administration over the reproductive performance of Nelore heifers [20]. Nonetheless, Surjus *et al.* found a positive association of the P/AI on conventional Nelore heifers and the GnRH administration at the TAI which tended to improve the P/AI (48.8%) *vs.* the non-treated heifers (42.3%), finding no influence on heifers that did not express estrus as was described in cows [21]. However, has not been studied the possible impact of this adjustment in the TAI protocol for precocious Nelore heifers, being our objective to evaluate in that kind of heifers, the influence on the P/IA and the pregnancy loss of the GnRH treatment at AI.

Besides the TAI protocols, there have been studied some other variables that could be determinant for the success of the precocious *Bos indicus* heifers breeding programs. The relevance of genetic selection for younger age for first calving is a consensus, necessary to achieve the best results as possible with these young heifers category, being previously studied in *Bos taurus* which have been associated with better reproductive performance of heifers [22], as has been studied the impact of genetic selection for this characteristic in *Bos indicus* heifers [23,24]. Another relevant aspect is the nutritional management, being considered as determinant for the precocious puberty, especially in some special moments of the physical development of the female calf and the young heifer, thought there are contradictory studies. Napomuceno *et al.* described the importance of the post-weaning supplementation to achieve an earlier puberty age in Nelore heifers, associated with a higher body weight gain in that life period, also finding no important effect of the pre-weaning supplementation (creep-feeding) on the puberty age in the same heifers [25]. However, Miszura *et al.* reported the same age at puberty (16-17 months of age) with four different nutritional regimes offered in the post-weaning period for Nelore heifers independent of the diet quality and the average daily weight gain [26]. On the other hand, some studies consider the heifers age and weight as two relevant

factors in the precocious heifer's pregnancy, nevertheless, there are contradictory studies, as was described by finding an association between the heifer's age and the P/AI [27], not being considered as a main factor by Freetly *et al.* who reported an association of the body weight proportion of the adult body weight and the puberty of this young heifer's category [28], . The third objective of the present study was to evaluate the possible whether there is association of the heifer's weight and age at the beginning of the TAI protocol with their reproductive performance or not. Considering the objectives already described above, the hypotheses of the present study were: 1) PGF treatment at the beginning of the TAI protocol would improve the LF diameter at the P4 device withdrawal and at the AI, as well as increase the expression of estrus and P/AI in cyclic Nelore heifers, 2) GnRH administration at the AI moment would increase P/AI without any influence on the pregnancy loss, and 3) Heavier and older Nelore heifers at the TAI protocol initiation, will achieve a better reproductive performance considering P/AI.

2.2. Material and methods

2.2.1. Location

The experiment was carried out in Bataguassu, Mato Grosso do Sul, Brazil, at the Santa Vergínia Farm, where the data was collected during the breeding season in the first trimester of 2021. The Nelore heifers were maintained on *Brachiaria brizantha* pastures, with ad libitum water access and supplemented with proteinic feed. All the animal experimental procedures were approved (Protocol: 2018.5.1235.11.3) by the Animal Research Ethics Committee of "Luiz de Queiroz" College of Agriculture of the University of São Paulo (ESALQ/USP).

2.2.2. Heifers and management

A total of 784 Nelore (*Bos Indicus*) heifers with an average of 16 ± 0.9 months of age were enrolled. The average weight was 323 ± 26.4 Kg and the BCS was 3.28 ± 0.3, which was determined at the AI moment based on a 5-point scale [29] with 0.25 increments (1 = emaciated; 5 = obese). All the heifers were submitted

at their second TAI protocol (resynchronization) during the breeding season, having the beginning of the resynchronization 29 d after the first AI when were submitted to pregnancy diagnosis by ultrasound evaluation, and only non-pregnant heifers were randomized and allocated in one of the four treatments of this experiment.

2.2.3. Experimental design

The experiment was structured as a 2 x 2 factorial arrangement of four treatments as shown in Fig. 1. The two factors were 1) PGF treatment on Day -9 (with PGF = PGF1 or without PGF = PGF0); 2) treatment with GnRH at the time of AI (with GnRH = G1 or without GnRH = G0). All heifers received at the beginning of the TAI protocol (Day -9), an intravaginal P4 insert (0.5 g of progesterone, Repro one, GlobalGen vet science, Jaboticabal, Brazil), EB (2.0 mg of estradiol benzoate, im, Syncrogen, GlobalGen vet science), and some received (**PGF1**) or not (**PGF0**) (0.5 mg of cloprostenol sodium, im, Induscio, GlobalGen vet science). On Day -2 the P4 inserts were removed, was administered 0.5 mg PGF im, eCG (200 IU of equine chorionic gonadotropin, im, eCGen, GlobalGen vet science) and EC (0,5 mg of estradiol cipionate, Cipion, GlobalGen vet science). Also, to evaluate estrus expression, in this day, heifers had their tailhead painted with tail-chalk (Walmur, São Geraldo, Brazil) and were checked at the AI moment for their absence (more than 75% removal), indicating previous estrus expression before AI [30]. Two days later (Day 0), the heifers were treated with a GnRH analog (G1) or not (G0) (25 µg of Lecirelin, im, Tec-relin, Agener União, Embu-Guaçu, Brazil), at the AI moment, having all the heifers inseminated by one of four different technicians, using in this procedure, 20 x 10^6 frozen/thawed proven semen from five bulls (AG Genetics, Ribeirão Preto, Brazil; CRV, Ribeirão Preto, Brazil; Genex, São Carlos, Brazil; Select Sires, Porto Alegre, Brazil). Consequently, there were four different treatments in Fig. 1: 1) PGF0-G0 (n = 199); 2) PGF0-G1 (n = 202); PGF1-G0 (n = 199); PGF1-G1 (n = 191).

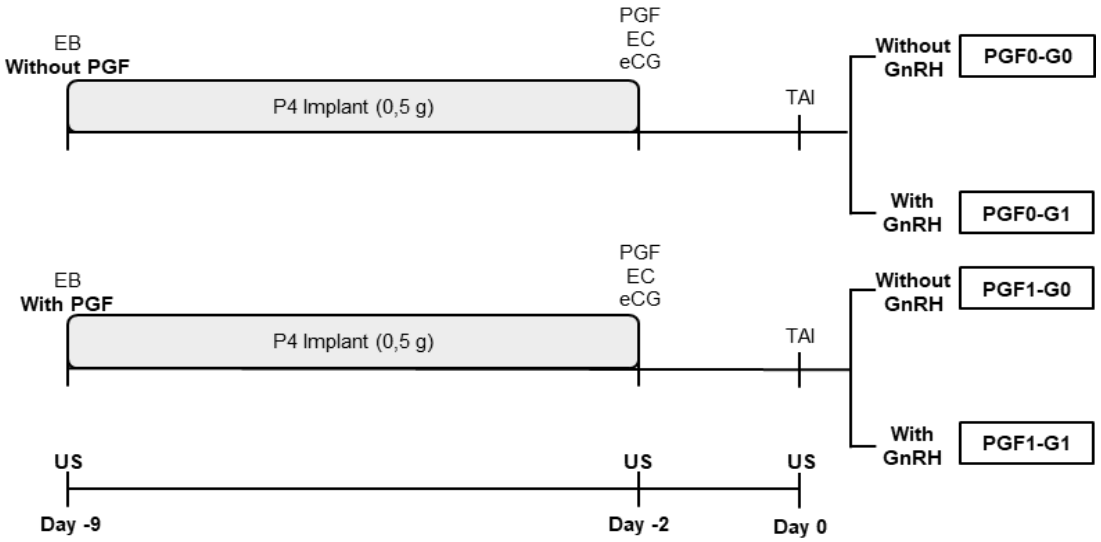

Figure 1. Schematic representation of the experimental design in Bos Indicus beef heifers (Nelore) following a 2 x 2 factorial arrangement which was the administration of cloprostenol sodium (PGF1) or not (PGF0) on Day -9 and the treatment with Lecirelin (G1) or not (G0) on Day 0. On Day -9 the progesterone (P4)-based-fixed-time AI protocol initiated for all heifers with estradiol benzoate (EB; 1.5 mg; im), the intravaginal insertion of a P4 implant (0.5 g) and the administration of PGF (PGF1; 0.530 mg; im) or not (PGF0). On Day -2 the P4 implant was removed and were treated with PGF, estradiol cypionate (CE; 0.5 mg), and equine chorionic gonadotropin (eCG; 200 UI). Two days later the heifers were artificially inseminated and treated with Lecirelin (G1; GnRH; 25 µg) or not (G0). Ultrasound evaluations were performed for CL presence on Day -9 and in part of the heifers on Day -2; for the largest follicle diameter on Day -2 and Day 0 in a subgroup of heifers.

2.2.4. Ultrasound evaluations

Transrectal ovarian evaluations were performed with a B-mode ultrasound using a 7.5 MHz linear transducer (DP-2200 VET, Mindray, Shenzen, China) on Day -9 of the synchronization protocol to determine the presence of CL in all the heifers (n = 784). On Day -2, some heifers were randomly submitted to ultrasound examination of the ovaries in order to evaluate the largest follicle (LF) diameter (mm) (n = 590) and the presence of CL (n = 601). With these two evaluations, was determined the regression of the CL already observed on Day -9, which was considered as the presence of a CL at the beginning of the TAI protocol and later it was not present on Day -2. At the AI moment a subgroup of heifers was submitted to transrectal ultrasound examination to determine the diameter of the largest ovarian follicle (n = 154). All the ultrasound measurements were conducted by two operators.

Between 30 and 31 d after TAI (Day 30) pregnancy diagnosis was performed by 3 different technicians through transrectal ultrasound, confirming the presence of heartbeat of the embryo (n = 784). The P/IA was determined as the quantity of pregnant heifers over the number of inseminated heifers. Also, there was another ultrasound evaluation in the 295 pregnant heifers from Day 30 diagnosis, performed by one operator, to confirm the pregnancy statuses, that was executed 62 days after TAI (Day 60), which was used to analyze the pregnancy loss, considering the number of heifers diagnosed as non-pregnant on Day 60 divided by the total number of pregnant diagnosed heifers on Day 30.

2.2.5. Heifers weight

A subgroup of heifers was weighted at the beginning of the TAI protocol (n = 753) using a cattle squeeze chute scale (Tru-Test, Porto Alegre, Brazil).

2.2.6. 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). The binomial outcome variables analyzed were expression of estrus, presence of CL on Day -9 and on Day -2, CL regression between Day -9 and Day -2, P/IA 30 and 60 days after TAI and pregnancy losses, using the GLIMMIX procedure fitting a binomial distribution with the Link Logit function. The continuous data (follicle diameter on Day -2 and Day 0) was tested for normality of residuals according to Shapiro-Wilk test using the UNIVARIATE procedure, and after that, the continuous outcomes were evaluated using the GLIMMIX procedure fitting a Gaussian distribution. For these analyses, previously stablished cutoff follicle measurements were considered [30].

The model for P/AI and pregnancy loss included the effects of PGF on D-9 (with or without), GnRH on D0 (with or without), CL on D-9 and D-2, expression of estrus. Moreover, body weight and age were included as covariates. The interaction between PGF on D-9 and GnRH on D0 was maintained in all models. Other biologically valuable interactions between treatments and the variables were evaluated, and they are presented and discussed throughout the manuscript. The

final model for each variable of interest was selected by finding the model with the lowest Akaike Information Criterions Corrected (AICC) value, using the forward selection procedure that included effects and interactions with $P < 0.20$.

When an interaction was detected, the SLICE command within the LSMEANS was used to interpret the results. Tukey honest significant difference post hoc test was performed to determine differences. Significant differences were declared when $P \leq 0.05$, whereas tendencies were considered when $0.05 < P \leq 0.10$. Values are presented as percentage (%; binomial variables). The results of continuous outcome variables are expressed as means ± standard error of the mean.

Aiming to evaluate the effects of body weight and age on fertility of the young heifers, probability curves for pregnancy on Day 60 were performed. Moreover, tertiles of body weight and age were stablished to separately evaluate the effect of each physiological measurement within the other. The LOGISTIC procedure was used to model the probability of pregnancy on Day 60 and the regression curves were created using the coefficients provided by the interactive data analysis from SAS and the formula $Y = \exp(\alpha \times X + \beta) / [1 + \exp(\alpha \times X + \beta)]$, where Y = probability of occurrence; exP = exponential; α = slope of the logistic equation; β = intercept of the logistic equation; and X = analyzed variable.

2.3. Results

2.3.1. CL presence (On Day -9 and Day -2)

After a first TAI protocol, 80% (627/784) of the heifers presented CL at the begging of the resynchronization protocol (Day -9). The presence of CL on Day -9 influenced the follicle diameter on Day -2 (with CL: 9.1 mm [480]) when compared with the absence of CL (without CL: 8.3 mm [110]) ($P = 0.0002$), and on the follicle diameter at AI time (with CL: 11.8 mm [125]; without CL: 10.4 [29]) ($P = 0.002$). Nonetheless, even with no impact on estrus expression ($P = 0.17$) the CL presence on Day -9 increased P/IA in heifers 30 ($P = 0.03$) and 60 days after TAI ($P = 0.009$), having a tendency for a reduction in the heifer's pregnancy loss presenting CL, compared with the ones that did not have CL on Day -9 ($P = 0.09$) presented in Table 1.

Table 1. Largest follicle (LF) diameter on Day -2 and Day 0, expression of estrus, pregnancy per AI on Day 30 and Day 60 after AI and pregnancy loss of heifers submitted to estradiol/progesterone-based timed artificial insemination (TAI) protocol based on the presence of CL at the beginning of the TAI protocol (Day -9) and at the time of the progesterone implant withdrawal (Day -2).

Item	CL on Day -9		*P*-value
	Without CL	With CL	
LF diameter Day -2, mm (n)	8,3 (110) [b]	9,1 (480) [a]	0,0002
LF diameter Day 0, mm (n)	10,4 (29) [b]	11,8 (125) [a]	0,002
Expression of estrus, % (n/n)	70,7 (111/157)	76,1 (477/627)	0,17
P/AI on Day 30, % (n/n)	29,3 (46/157) [b]	39,7 (249/627) [a]	0,03
P/AI on Day 60, % (n/n)	26,1 (41/157) [b]	38,0 (238/627) [a]	0,009
Pregnancy loss, % (n/n)	10,9 (5/46) [A]	4,4 (11/249) [B]	0,09
	CL on Day -2		
	Without CL	**With CL**	
LF diameter Day -2, mm (n)	9,3 (377) [a]	8,3 (213) [b]	<0,0001
LF diameter Day 0, mm (n)	11,7 (108) [A]	11,1 (46) [B]	0,07
Expression of estrus, % (n/n)	81,4 (314/386) [a]	67,0 (144/215) [b]	<0,0001
P/IA on Day 30, % (n/n)	38,3 (148/386)	40,5 (87/215)	0,37
P/IA on Day 60, % (n/n)	37,1 (143/386)	36,7 (79/215)	0,80
Pregnancy loss, % (n/n)	3,4 (5/148) [b]	9,2 (8/87) [a]	0,05

[a,b] Effect of CL presence ($P \leq 0.05$).
[A,B] Effect of CL presence ($0.05 < P \leq 0.10$).

On the other hand, presence of CL on Day -2, had the opposite effect on the follicle diameter on Day -2, having difference of a greater follicle with CL absence (with CL: 8.3 mm [213]; without CL: 9.3 mm [377]) (P < 0.0001) and on Day 0 the presence of CL on Day -2 tended to reduce de diameter of the LF (with CL: 11.1 mm [46]; without CL: 11.7 mm [108]) (P = 0.07) (Table 1). The analysis of the effect presented by the presence of a CL in those two moments of the TAI protocol, shows the effect in the follicle diameter on Day -2 when there is CL on Day -9 but there is not on Day -2 compared with the presence of CL in both of the US evaluations (with

CL on Day -9 and without CL on Day -2: 9.6 mm [287] vs. with CL on Day -9 and Day -2: 8.3 mm [193]) (P < 0.0001) different of the impact of the same groups in the follicle diameter on Day 0, where there was no influence of each one of them (with CL on Day -9 and without CL on Day -2: 12.0 mm [85] vs. with CL on Day -9 and Day -2: 11.2 mm [40]) (P = 0.14).

On the other hand, as presented in Table 1, the presence of CL on Day -2 decreased estrus expression at AI moment (*P* < 0.0001). What also happened with the presence of CL on Day -9 and Day -2, compared with the presence of CL on Day -9 but the absence of CL on Day -2 (with CL on Day -9 and without CL on Day -2: 83.1% [245/295] vs. with CL on Day -9 and Day -2: 68% [132/194]) (*P* = 0.004). However, Table 1 shows there was no difference on the P/IA on Day 30 and Day 60 when a CL was detected on Day -2 (*P* = 0.37; *P* = 0.80, respectively), as had no difference, with the presence of CL on Day -9 and detection or not of a CL on Day -2 on P/IA on Day 30 (with CL on Day -9 and without CL on Day -2: 40.7% [120/295] vs. with CL on Day -9 and Day -2: 40.2% [78/194]) (*P* = 0.83) and on Day 60 (with CL on Day -9 and without CL on Day -2: 39.7% [117/295] vs. with CL on Day -9 and Day -2: 36.6% [71/194]) (*P* = 0.75), although the pregnancy loss was reduce (*P* = 0.05) with the absence of CL on Day -2 (Table 1). and tended to be lower even with the presence of a CL at the begging of the TAI protocol and presence or not of a CL on Day -2 (with CL on Day -9 and without CL on Day -2: 2.5% [3/120] vs. with CL on Day -9 and Day -2: 8.9% [7/78]) (*P* = 0.07).

2.3.2. Estrus expression (until the AI time)

The total expression of estrus on the experiment was 75.0% (588/784), finding a difference (*P* < 0.0001) of the heifers that expressed estrus on Day 0, because of the greater follicular diameter on Day -2, when compared with those that did not expressed estrus (with estrus 9.2 mm [450] vs. without estrus 8.1 mm [140]), that also occurred on Day 0 where heifers that expressed estrus presented a greater follicular diameter (11.7 mm [122]) than heifers without expression of estrus (10.6 mm [32]) (*P* = 0.01). Also, as presented in Fig. 2, expression of estrus increased pregnancy per artificial insemination in both of the gestation diagnosis, obtaining a higher P/IA on Day 30 and Day 60 (*P* < 0.0001), as well as there was a positive influence of the estrous, on the pregnancy loss reduction (*P* = 0.04).

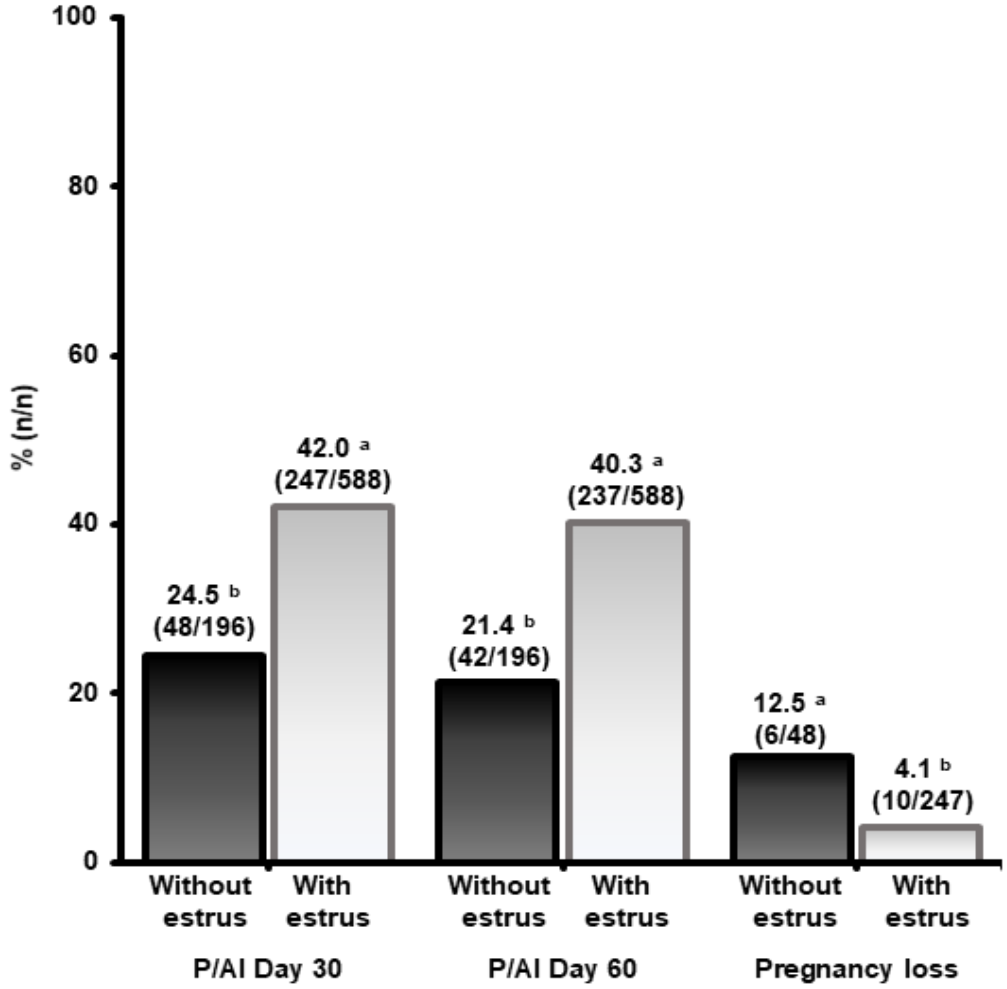

Figure 2. Pregnancy per AI (P/AI) on days 30 (Day 30) and 60 (Day 60) after AI and pregnancy loss between 30 to 60 days of gestation in Nelore heifers submitted to estradiol/progesterone-based timed artificial insemination (TAI) protocol that expressed or did not express estrus until AI moment. [a, b] Difference between heifers that expressed or did not expressed estrus on P/IA on Day 30 and Day 60 (P < 0.0001) and on the pregnancy loss (P = 0.04).

2.3.3. Treatments

The overall proportion of heifers with CL at the beginning of the protocol was 80% (627/784). As expected, the presence of CL on Day -2 and the regression of the CL from Day -9, were influenced, by the prostaglandin treatment (P < 0.0001), having no interaction between the factors PGF on Day -9 and GnRH at AI time (CL on Day -2: P = 0.64; regression of the CL: P = 0.69). In the same way, there was a principal effect of the prostaglandin treatment in the LF diameter on Day -2 (PGF0: 8.6 mm [302]; PGF1: 9.3 [288]) (P = 0.05), not being influenced by anyone of the four treatments (P = 0.84), nonetheless, the follicular diameter on Day 0 did not present

difference by the PGF treatment (PGF0: 11.4 mm [74]; PGF1: 11.6 [80]) (*P* = 0.49), neither for the four treatments (*P* = 0.78) as shown in Table 2.

The expression of estrus was not affected by the experimental treatments or the main effects (*P* = 0.98), as was not found any influence of the main factors or the treatments, on the quantity of pregnant heifers in both of the ultrasonographic diagnoses, over the heifers artificially inseminated (Day 30: *P* = 0.86 Day 60: *P* = 0.86), achieving a general P/IA of 37.6% (295/784) on Day 30 and 35.6% (279/784) on Day 60. On the other hand, the overall pregnancy loss in the experiment was 5.4% (16/295), being affected by the main effect of the GnRH treatment, increasing the pregnancy loss between Day 30 and Day 60 (*P* = 0.02), and having no influence of the experimental treatments (*P* = 0.77).

When analyzed the main effect of the prostaglandin treatment on Day -9, was decided to isolate the results when administered just in heifers with CL presence at the beginning of the TAI protocol, obtaining a reduced LF diameter on Day -2 without the PGF administration than those that received the PGF (with CL - without PGF: 8.7 mm [244]; with CL - with PGF: 9.5 mm [236]) (*P* < 0.0001). Nonetheless, PGF administration did not affect the follicular diameter on Day 0 in this group of heifers (with CL - without PGF: 11.5 mm [59]; with CL - with PGF: 12.0 mm [66]) (*P* = 0.22), even though the expression of estrus was influenced (*P* = 0.004) as shown in Fig. 3, also having no-impact on the P/IA of the PGF treatment on Day 30 (with CL - without PGF: 38.9% [121/311]; with CL - with PGF: 40.5% [128/316]) (*P* = 0.67), also not having influence on the P/IA on Day 60 (*P* = 0.62), as did not affect the pregnancy loss (*P* = 0.82) presented in Fig. 3

Table 2. Presence of corpus luteum (CL) on Day -9 and on Day -2, CL regression between Day -9 and Day -2, largest follicle (LF) diameter on Day -2 and Day 0, expression of estrus until TAI, pregnancy per AI on Day 30 and Day 60, and pregnancy loss between 30 and 60 days of gestation of heifers submitted to estradiol/progesterone-based timed artificial insemination (TAI) protocol for the experimental treatments without prostaglandin (PGF0) or with prostaglandin (PGF1) on Day -9 and without Lecirelin (G0) or with Lecirelin (G1) administration at the time of AI (Day 0).

	Overall	Treatments				*P*-value		
		PGF0G0	PGF0G1	PGF1G0	PGF1G1	PFG	G	PGF x G
CL on Day -2, % (n/n)	35,8 (215/601)	54,7 (82/150)[a]	60,8 (93/153)[a]	11,3 (17/151)[b]	15,7 (23/147)[b]	<0.0001	0.12	0.64
CL Regression, % (n/n)	64,6 (316/489)	40,9 (47/115)[b]	39,2 (51/130)[b]	90,6 (115/127)[a]	88,0 (103/117)[a]	<0.0001	0.50	0.69
LF diameter on Day -2, mm (n)	8,9 (590)	8,5 (149)[b]	8,7 (153)[b]	9,3 (145)[a]	9,3 (143)[a]	0.05	0.54	0.84
LF diameter on Day 0, mm (n)	11,5 (154)	11,3 (39)	11,5 (35)	11,4 (42)	11,8 (38)	0.49	0.49	0.78
Expression of estrus, % (n/n)	75,0 (588/784)	74,1 (146/197)	71,5 (143/200)	77,9 (155/199)	76,6 (144/188)	0.18	0.61	0.98
P/IA on Day 30, % (n/n)	37,6 (295/784)	36,6 (72/197)	39,0 (78/200)	36,2 (72/199)	38,8 (73/188)	0.67	0.43	0.86
P/IA on Day 60, % (n/n)	35,6 (279/784)	36,0 (71/197)	35,5 (71/200)	35,2 (70/199)	35,6 (67/188)	0.57	0.90	0.86
Pregnancy loss, % (n/n)	5,4 (16/295)	1,4 (1/72)[y]	9,0 (7/78)[x]	2,8 (2/72)[y]	8,2 (6/73)[x]	0.54	0.02	0.77

[a,b] Effect of prostaglandin administration on Day -9 within treatments ($P \leq 0.05$).
[x,y] Effect of Lecirelin administration on Day 0 within treatments ($P = 0.02$).

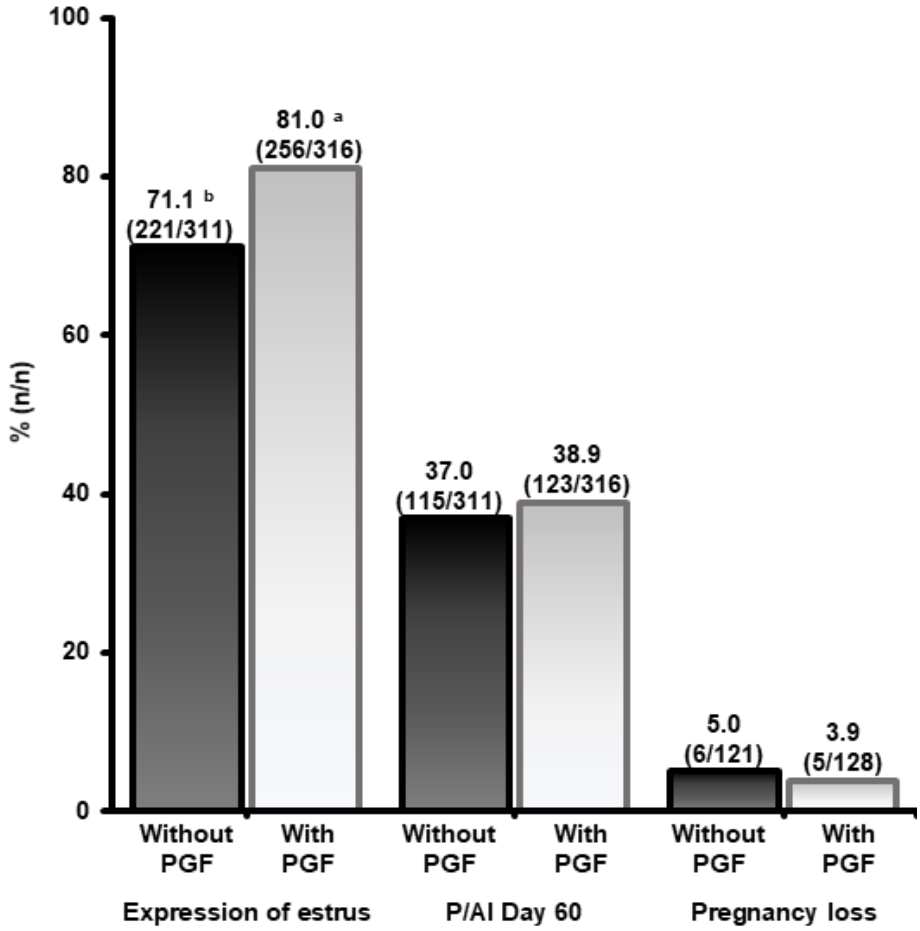

Figure 3. Expression of estrus, pregnancy per AI (P/AI) on day 60 (Day 60) after AI and pregnancy loss between 30 to 60 days of gestation in Nelore heifers **with presence of CL** at the beginning of TAI protocol (Day -9), submitted to estradiol/progesterone-based timed artificial insemination (TAI) protocol, which did not receive the prostaglandin administration (Without PGF) or had prostaglandin administration (With PGF) on Day -9. [a, b] Difference between heifers with CL on Day -9 that did not receive or receive prostaglandin treatment on the expression of estrus until the TAI (P = 0.004).

In the same way, the GnRH administration at the AI time, had no impact on the P/AI on Day 30 (Without GnRH: 36.4% [144/396]; with GnRH: 38.9% [151/388]) (P = 0.35) and Day 60 (Without GnRH: 35.6% [141/396]; with GnRH: 35.6% [138/388]) (P = 0.89), even in the absence of expression of estrus, the P/AI on Day 30 was not influence by the GnRH treatment (Without estrus - without GnRH: 24.2% [23/295]; without estrus - with GnRH: 24.8% [25/101]; with estrus - without GnRH: 40.2% [121/301]; with estrus - with GnRH: 43.9% [126/287]) (P = 0.73), and did not affect the P/AI on Day 60 (P = 0.78), as shown in Fig. 4. Also, was not found any interaction between estrus and GnRH treatment for pregnancy loss during the gestation period between 30 and 60 days after AI (Without estrus - without GnRH:

8.7% [2/23]; without estrus - with GnRH: 16.0% [4/25]; with estrus - without GnRH: 0.8% [1/121]; with estrus - with GnRH: 7.1% [9/126]) (*P* = 0.33).

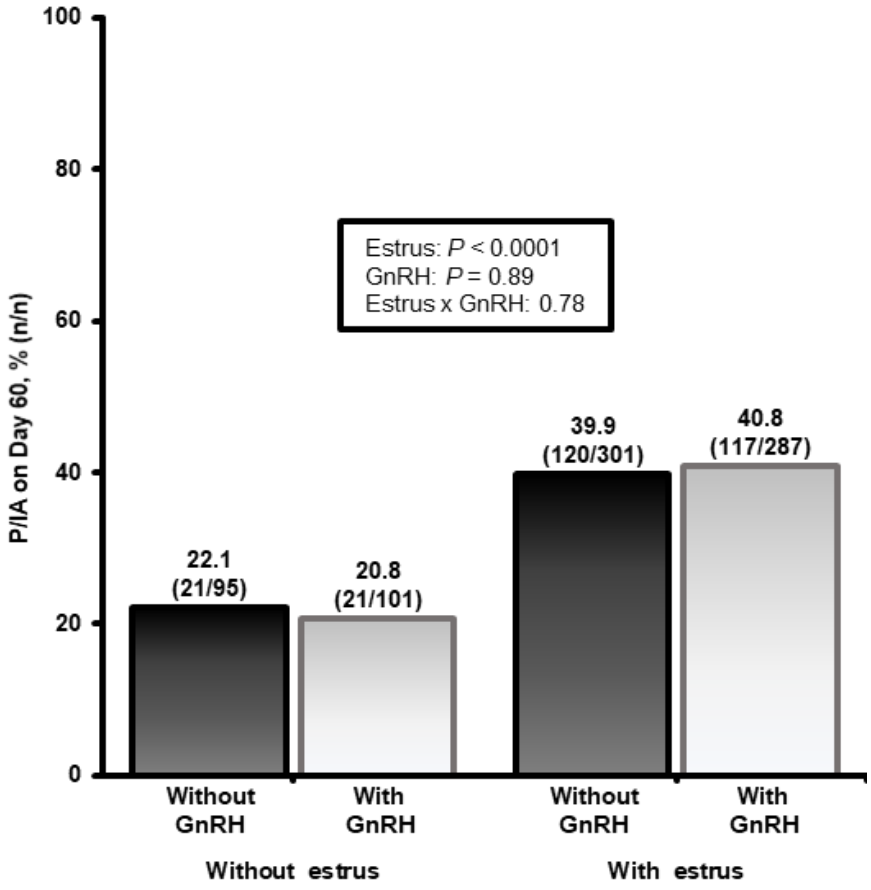

Figure 4. Pregnancy per AI (P/AI) on day 60 (Day 60) after AI in Nelore heifers grouped by their expression of estrus until the TAI (Without estrus; With estrus) and the no administration of GnRH (Without GnRH) or the GnRH treatment at TAI moment (Day 0), heifers submitted to estradiol/progesterone-based timed artificial insemination (TAI) protocol, finding a main positive effect of the expression of estrus for the P/AI on Day 60 of gestation, having no association with the GnRH administration on Day 0.

2.3.4. Weight and age at the beginning of the TAI protocol

The initial approach, showed a difference of a positive relation of the heifer's weight and a better P/AI on Day 60 (*P* = 0.03) finding no association between weight and pregnancy loss (*P* = 0.97). In the same way, there was a higher probability of pregnancy per AI on Day 60 in older heifers when analyzed the isolated age effect for the fertility efficiency (*P* = 0.003), nonetheless, had no impact of this variable in the

pregnancy loss between 30 and 60 days of gestation (*P* = 0.84). On the other hand, through the combined analysis of these two variables, as shown in the Fig. 5A, there was a tendency within the youngest category, to achieve a better pregnancy per AI of the heavier heifers (< 15.5 months: *P* = 0.1 [average weight: 307.4 Kg]), unlike of the two older age categories, with no impact of the heifer's weight on the reproductive performance (15.5 to 16.2 months: *P* = 0.14 [average weight: 318.5 Kg] and > 16.2 months: *P* = 0.50 [average weight: 334.5 Kg]). At the same time, there was no influence of the age within the two heavier categories, exhibited on Fig. 5B, except for expected higher P/AI of the older heifers on the lightest category (< 309 Kg: *P* = 0.03 [average age: 15.6 months]; 309 to 333 Kg: *P* = 0.11 [average age: 16.1 months]; > 333 Kg: *P* = 0.69 [average age: 16.5 months]).

2.4. Discussion

The advances and expansion of the TAI industry in countries like Brazil has been extraordinary, where there has been a representative increase in the use of this technology of more than 24% from 2020 to 2021, leading to achieve a 93,3% of the total inseminated females in Brazil, through the TAI technology, which in 2021 corresponded to approximately 26 million TAI procedures [31–34]. The search for production increase in commercial herds and the need to accelerate the genetic improvement, had promoted the interest to achieve a younger pregnancy age in the *Bos indicus* heifers around the country. In the same way, academy and industry had been developing new technologies to accomplish better reproductive results in precocious *Bos indicus* heifer's category. The aim of this study was to evaluate the effects of different hormonal adjustments in TAI protocols in physiologic response and reproductive performance of 13 to 17 months old Nelore heifers besides of the impact of some heifer's characteristics that could be relevant in the success of the young heifers breeding programs.

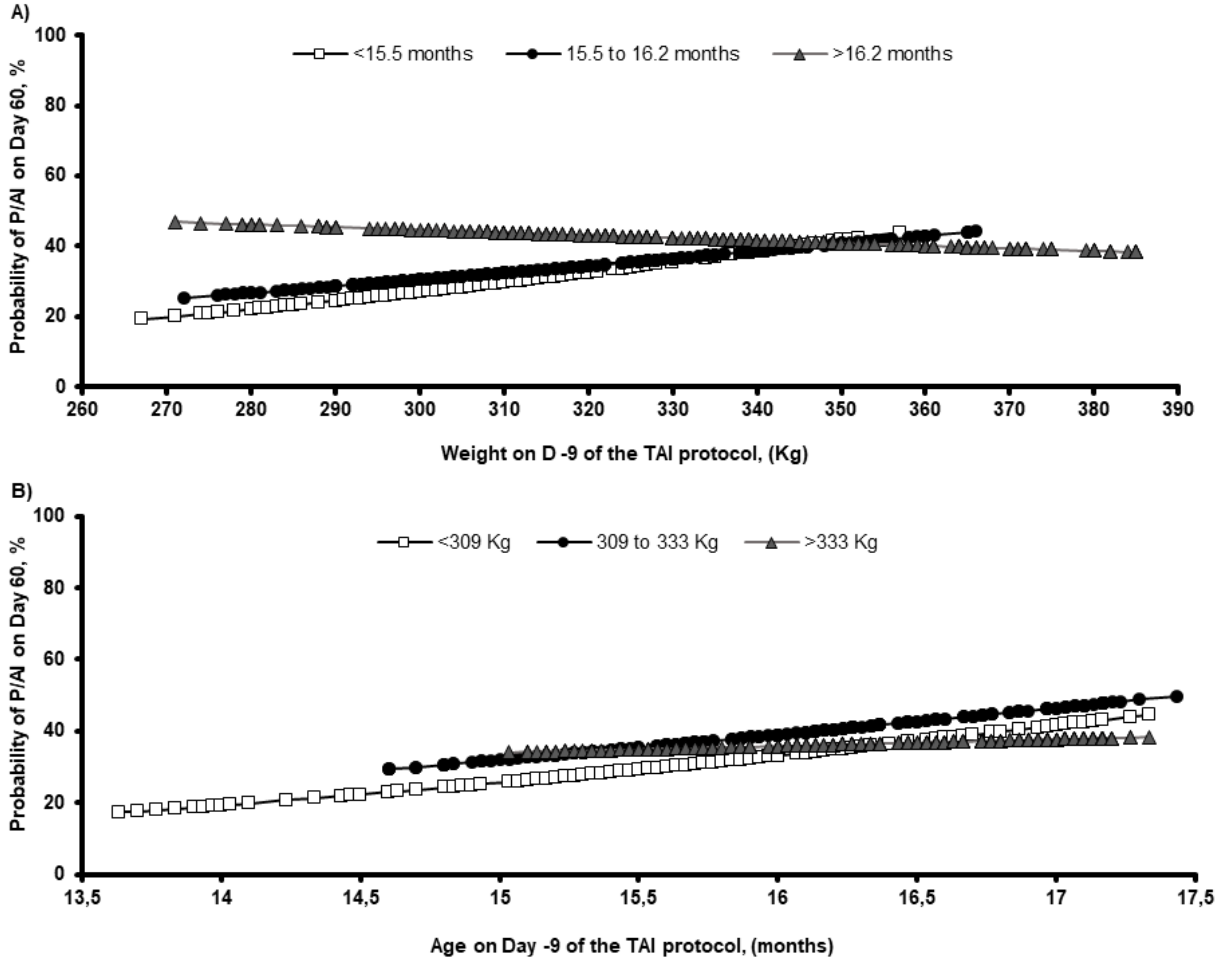

Figure 5. Panel A: Probability of pregnancy per AI (P/AI) on Day 60 after TAI, for three different categories of heifers, grouped by their age on Day -9:
Square: Heifers under 15.5 months of age (n = 180,). P/IA on Day 60 = -4.96 + 0.01 * heifer's weight on D -9 (*P* = 0.10).
Circle: Heifers between 15.5 to 16.2 months of age (n = 263). P/IA on Day 60 = -3.52 + 0.009 * heifer's weight on D -9 (*P* = 0.14).
Triangle: Heifers over 16.2 months of age (n= 283). P/IA on Day 60 = 0.71 + -0.003 * heifer's weight on D -9 (*P* = 0.50).
Panel B: Probability of pregnancy per AI (P/AI) on Day 60 after TAI, for three different categories of heifers, grouped by their weight on Day -9:
Square: Heifers under 309 Kg (n = 241). P/IA on Day 60 = -6.52 + 0.37 * heifer's age on D -9 (*P* = 0.03).
Circle: Heifers between 309 to 333 Kg (n = 240). P/IA on Day 60 = -5.35 + 0.31 * heifer's age on D -9 (*P* = 0.11).
Triangle: Heifers over 333 Kg (n= 256). P/IA on Day 60 = -1.88 + 0.08 * heifer's age on D -9 (*P* = 0.69).

There are studies establishing the well none benefits of the presence of CL before any hormonal treatment (considered as pubertal), one of those benefits reported is the higher expression of estrus of pubertal heifers when compared with those that did not have CL presence (considered as prepubertal) (Pubertal: 68.1%

vs. prepubertal: 39.5%) leading to a better P/AI in heifers (Pubertal: 46.6% *vs.* prepubertal: 30.0%) [35], results partially supported for this study, having a higher LF diameter on D -2 and on D0 within the heifers group with CL on Day -9, having no influence on the expression of estrus as described above, nonetheless there was a relevant difference of 11.9% points for P/IA on Day 60 for heifers with CL presence at the P4 implant insertion, considering the CL presence could be considered as a puberty indicator of heifers, indicating a better reproductive status and functionality of the hypothalamus – pituitary – gonadal axis [22,36], also heifers with CL on Day -9, tended to present a reduced pregnancy loss, possibly due to the better embryo quality of the cycling heifers [37] or a previous exposition to high levels of endogenous or pharmacologic estradiol concentration, derived from the first TAI protocol that the heifers received, influencing the uterine tissues, having a possible positive impact on the uterine environment [38].

One the other hand, the CL presence on the P4 implant withdrawal, was undesirable, affecting the LF diameter at that protocol time, with a reduction of 1 mm of diameter for heifers with CL, that also tended to have a lower dominant follicle at the AI time, mainly explained by the progesterone secretion from de CL, different from an anterior study that evaluated de influence of exogenous circulating P4 in Nelore cows, which found no association of higher circulating P4 levels and the follicle diameter on the P4 device withdrawal or at the AI time [39]. Nonetheless Dias *et al.* reported the benefits of low circulating P4 concentration on the follicle diameter at TAI in Nelore heifers, in agreement with the present study considering the higher P4 levels in those females with CL presence [40], primarily explained by the P4 negative influence over the GnRH and LH secretion[41], leading to a lower LF diameter at the P4 implant withdrawal between the treatments with highest and the lowest circulating P4 concentration described by Claro Júnior *et al.* (High P4: 9.45 ± 0.24 mm vs. low P4: 11.42 ± 0.16 mm) influencing the expression of estrus as well (High P4: 19.5% vs. low P4: 39.3%) [8], also confirmed in the present study where there was a difference of 14% points between the higher expression of estrus of the heifers without CL no Day -2 compared with the ones that had a present CL in that moment of the TAI protocol. Even though the P/IA was not influenced by the CL presence at the P4 implant removal, there was a lower pregnancy loss in those heifers without the CL presence, what could be explained mainly by a better uterine environment of a cycling heifer. When analyzed the influence of having or not CL on

Day -2 within the heifer's group with CL presence on Day -9, the absence of CL on Day -2 was especially important to improve the LF diameter in the P4 device withdrawal moment, leading to a greater expression of estrus, also tending to reduce the pregnancy loss, reinforcing the possible beneficial effects of the CL regression during the P4 implant permanence due to the positive effect on the follicular diameter and the better uterine environment because of the higher expression of estrus [42].

In the same way, the study presents the relevant contribution of estrus to the improvement of the reproductive performance in this young heifer's category, representing an important pregnancy gain of 18.6% points of P/IA on Day 60 for the heifers that expressed estrus until the TAI, in agreement with the previously reported in cows [43] and heifers, as was described by Nuñez *et al.* finding a positive effect for the heifers that expressed estrus (With estrus: 63.9%; without estrus: 45.9%) [44], reinforcing the benefits of the estrus in the pregnancy results of the TAI programs, contributing no just for the conception but also in the pregnancy loss reduction as described by Bó *et al.* who reported a high difference on the pregnancy loss in TETF programs between the recipients that expressed estrus and those without expression of estrus between 30 and 60 days of gestation (With estrus: 5,6%; without estrus 66,7%) [45,46] what also is supported by the present study, finding a pregnancy loss increase of 8% points for the heifers that did not express estrus, compared with those that expressed estrus.

The four experimental treatments did not present any difference for the variables studied in our research, having no interaction between both factors previously established in the experimental design. However, the first hypothesis of this research was the positive effect of the prostaglandin administration at the initiation of the TAI protocol in some evaluated physiologic variables, including the CL regression, follicular size at different times, expression of estrus and P/IA. As expected, the CL regression in the present study was significantly different in the grouped treated, achieving more than 2 times of CL regression than the heifers non treated, reflecting as well in a lower of CL presence on Day -2. Likewise, a reduce CL presence on Day -2, contributed to the higher LF diameter at progesterone implant withdrawal, found in an anterior study with *Bos taurus* heifers, where reported a positive association of the PGF treatment at the TAI protocol initiation, on the LF size at the P4 implant removal (With PGF: 12.2 ± 0.5 mm *vs.* without PGF: 9.6 ± 0.5 mm) [47] like found in the present study, where treated heifers presented a higher follicular

diameter on Day -2 (difference of 0.7 mm) partially accepting the first hypothesis of the study, nonetheless, there was no influence of the PGF on Day -9 over the LF diameter at the TAI moment, differing from the previously reported by Carvalho *et al.* who described the influence of the PGF treatment at the beginning of the protocol with the maximum diameter of the ovulatory follicle, which tended to be higher in the treated heifers (With PGF: 12.6 ± 0.4 mm *vs.* without PGF: 11.7 ± 0.5 mm) [19] as was initially hypothesized in the present study, expecting that the greater follicle from Day -2 would maintain the size difference until the AI time, however, the main difference in this study compared with the Carvalho *et al.* research, is the implementation of the eCG administration at the P4 implant withdrawal, treatment that offers an exogen LH support at the end of the TAI protocol, hormonal strategy previously reported as favorable, which tended to increase the diameter of the ovulatory follicle (With eCG: 14.5 ± 0.4 mm *vs.* without eCG: 13.1 ± 0.7 mm) and the ovulation rate to the TAI protocol [48], as could be happening in the present study, that is why the authors believe that the eCG administration could be helping to reduce the LF diameter difference at the TAI time, that was already found on Day -2, and reducing the effect that the PGF did present on the follicular diameter, mainly explained by the eCG treatment, which contributes to counteract the negative influence of the high circulating P4 concentration at the intravaginal device removal on the follicular diameter at the TAI [49].

In the same way, have been reported the positive effect of the PGF administration at the initiation of the TAI protocol, which tended to increase the expression of estrus in cows (With PGF: 72.6% *vs.* without PGF: 69.1%) [30] and Holstein heifers (With PGF: 82% *vs.* without PGF: 45%). However, even with the increase of CL regression between Day -9 and Day -2 in the present study, those heifers with the PGF administration did not express more estrus until the TAI compared with the non-treated heifers for the overall result, on the other hand, when analyzed the PGF influence within the heifers with CL presence on Day -9, there was an increase on the expression of estrus of 10% points as expected, because of the higher CL regression, the positive effect of having no CL on Day -2 on the follicle size, producing a LF with a higher diameter and steroidogenic capacity, leading to an increased expression of estrus, in accordance of the described by Martins *et al.* where the heifers with the smallest follicles had lower expression of estrus compared

with the ones with the biggest follicles (Small follicle at AI: 12.4% vs. big follicle 46.7%) [15].

Nonetheless, the PGF administration at the beginning of the TAI protocol, had no impact on the general P/IA or within the heifers with CL presence on Day -9 even with a higher expression of estrus, differing of the positive effect of this hormonal strategy already reported in *Bos taurus* heifers and cows in an anterior study which presented a higher proportion of pregnant females in the group treated with PGF at the TAI protocol initiation [40], as well as Nuñez *et al.* found a tendency in *Bos taurus* 14 months old heifers, of better a pregnancy per AI results in the group that received a half dose PGF treatment at the beginning of the TAI protocol (54.7%) compared with the heifers with no additional PGF treatment during the TAI protocol (48.5%), nonetheless, the same study found no association of that treatment in two years old heifers [47]. In the same way, the results of the present study do not differ from those results in the older heifers, having no difference on the P/AI of the heifers treated or not with PGF on Day -9, partially rejecting the study's first hypothesis, confirming the information described in another study, where there was no difference in two years old Nelore heifers, between those treated or not with PGF on the TAI protocol initiation (With PGF: 51.7% *vs.* without PGF: 57.7%) [50], especially explained by the support provided by the eCG treatment, which increase the probability of pregnancy of beef heifers [49], strategy that allowed the increase in fertility of the TAI programs in heifers, independent of the genetic group, either with *Bos taurus* (With eCG: 57.1% *vs.* without eCG: 53.1%) [51] or *Bos indicus* (With eCG: 50.0% *vs.* without eCG: 36.8%) [18].

Contradictory results have been reported in some studies about the impact of the GnRH administration at AI time on the fertility performance of two years old heifers, obtaining a positive association of the use of this hormonal strategy in the P/AI, with a tendency for the GnRH treatment to improve the reproductive performance of Nelore heifers (With GnRH: 48.8% *vs.* without GnRH: 42.3%) [21]. On the other hand, Prata *et al.* described the no effect of the GnRH treatment at the AI time of the 2 years old heifer's category (With GnRH: 47.3% *vs.* without GnRH: 47.2%) [5], as was not different for the P/AI in this research, rejecting the second hypothesis of the present study, having no difference between the two experimental groups (With or without GnRH at the TAI), as well as there was no influence even for those heifers without expression of estrus, different from the reported benefits in

reproductive performance of cows with no expression of estrus prior the TAI (Without estrus - without GnRH: 15.0% *vs.* without estrus - with GnRH: 33.4%) [52]. However, despite of the non-relevant results for the P/AI, the GnRH administration increase the pregnancy loss between 30 and 60 days of gestation, results that could be supported on the GnRH effect on the induction of ovulation of a small follicle, leading to a reduction on the steroidogenic capacity of those follicles submitted to the GnRH treatment, which decreases the influence of the endogenous estradiol on the uterine environment which have been described as positive for the pregnancy maintenance in dairy cattle [53], probably explaining the negative effect of the GnRH treatment described above on the pregnancy loss, different of what was reported by our laboratory, describing in an anterior study the no association of the GnRH treatment at the AI moment with the pregnancy loss in Nelore cows [54].

Some other important findings of the present study, were the effect of the weight and age of these young heifers on their probability of pregnancy. Considering the influence of the weight, was found a tendency for the heavier heifers to increase the probability of pregnancy, as was already described in another study that determined the parameter weight as the principal criterion to decide the moment to breed the beef heifers [28]. Nonetheless, if consider just the heifer's weight, the older heifers and the ones with a better genetic potential for weight gain, are going to be primarily considered to be part of the breeding programs, however, some lighter heifers that already achieve puberty or are able to become pregnant with the TAI protocols, are not going to be part of the reproduction programs, excluding them as potential replacement females just because of their lighter weight with the same or older age of their heavier contemporaries, as well as would be exclude from the breeding season the older heifers that did not achieve a specific weight but could be potentially capable to become a replacement female for the herd. Based on previous studies that described the no impact over puberty of the genetic merit for weight gain [55] and the apparently no influence of the heifers age on their reproductive performance [56], was decided to analyzed in the present study the potential influence of the heifer's weight, finding an initial association with the heifers P/AI, nonetheless, when evaluated the weight's effect within 3 different age categories, unexpectedly there was no association between the heifer's weight and the fertility response of the two older categories. On the other hand, was found a positive relation for the heavier heifers, that tended to achieve a higher P/AI than those

females with a low body weight at the beginning of the TAI protocol, within the youngest category, what suggest that a better body weight could improve the reproductive performance of the heifers born from the middle to the end of the calving season, as was previously described by Nepomuceno *et al.* who found a positive association between the pregnancy per AI and better nutritional management with a higher body weight of 18 months old heifers (10% points higher of P/AI) [25], on the other side, taking into account that was offered the same diet for all heifers, and the reported benefits of the early birth during the breeding season for the calf performance [57], the authors believe that the higher weight and good performance of the heavier females in the youngest heifers category, could be consider an indicator of good health from the birth of the female-calf, until the beginning of the breeding season, giving an idea of the real productive and reproductive potential of the heifers that were born at the end of the calving season, a hypothesis that would be necessary to study, looking for the understanding of the possible impact on the productive and reproductive performance, of possible health issues from the heifers birth until the beginning of the breeding season. On the other hand, with the results obtained in the present study, it is possible to infer that the higher weight in older heifers would not offer any benefits for the reproductive performance of those females, which might indicate that the strategy used in the precocious heifers of high energy diets to achieve a better body weight, could not influence the pregnancy results at the end of the breeding season, in agreement with an anterior study which found no difference in the total pregnancy percent for *Bos taurus* heifers with low or high gain diets in a 47 days breeding period in a natural mating system [58].

At the same time, was researched the influence of the heifer's age on their probability of pregnancy, where there was a positive association suggesting the older heifers would achieve a better reproductive performance. Nevertheless, despite of the benefits for fertility of the heifer's age, was not found any influence of this variable for the two categories with the heavier heifers. Nonetheless, there was a clear difference of pregnancy improvement for the older heifers within the lightest weight category, explained primarily by the reduced time period to achieve puberty and a better reproductive tract development for the youngest heifers, reported in a study which found a difference of 19 days of age between the highest and the lowest reproductive tract score (RTS: 5 and 1, respectively), as well as the better P/AI

results of those groups of *Bos taurus* heifers with just one artificial insemination (RTS 5: 80% vs RTS 1: 31%) [59], also considering the influence of the moment of birth during the calving season, on the calf performance and the female development until the heifer first s as Funston *et al.* found in a yearling *Bos taurus* heifers, describing a better proportion of cycling females at the beginning of the breeding, and a higher pregnancy rate of those heifers born in the first to two periods of the calving season, when compared with the youngest ones [60].

Based on the described information above, it is possible to infer that the main contribution of the heifer's weight is to improve the reproductive performance of the young precocious Nelore females born at the end of the calving season, being careful about the real contribution of a high weight gain for the youngest heifers, and the better moment to offer a high quality diet, keeping in mind that the nutritional strategies not necessarily ensured a better fertility [61] and might be necessary to understand the factors that make some young heifers to be lighter in comparison with the heavier contemporaries, as well as must be analyzed the cost/benefit relationship of the better nutritional management of that specific group of young lighter females. Also, the present study shows the importance of the heifer's birth at the beginning of the calving season, to achieve the best reproductive performance of the 12 to 18 months of age category, indicating as well, that there are some other factors involved in the better reproductive results of this young heifers, like the nutritional management offered through the female growth, and as already know, the relevance of the genetic selection, with the precocious heifer's identification and the use of evaluated genetic material, with high potential to reduce the first calving age, variables that were previously described, finding an important difference of pubertal heifers proportion at the 18 months of age, highlighting the ideal scenario of good nutrition and specialized genetics, achieving a 62% of pubertal heifers, when compared with the other groups that did not present puberty until the 18 months of age [62].

In conclusion, the adjustments proposed for the TAI protocol in this research article did not bring benefits for the reproductive performance of 13 to 17 months old Nelore heifers, considering factors as the presence of CL at the initiation of the TAI protocol and expression of estrus until the AI, two determinant variables to achieve the best P/AI results and reduce pregnancy loss in this precious heifers breeding programs, where the prostaglandin treatment on Day -9 could bring, somehow, a

better expression of estrus increasing the CL regression during the P4 implant permanence. Also, there was found partially no impact of the heifer's weight on the precocious pregnancy of *Bos indicus* young females, except of the youngest heifer's group, which apparently would achieve a higher P/AI with a better body weight. Furthermore, the reproductive results of the precocious heifers were mainly affected by the birth moment of the female-calf, increasing the probability of pregnancy when the heifer was born at the beginning during the calving season, having a relevant contribution from the correct nutritional management and the genetics as well, to achieve the best reproductive performance.

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3. FINAL CONSIDERATIONS

The present study provided important information about the use of PGF and GnRH as complementary strategies for the TAI protocols in Nelore heifers, finding clear evidence about the irrelevant impact from those two hormonal adjustments in the reproductive performance of the young females included in this research. In the same way, there was a general positive influence of the older age on the fertility outcomes of the heifers, as well as should be consider the body weight as an important parameter in the reproductive programs of the heifers born at the end of the calving season.

Finally, the time of the Master's process was challenging but rewarding. The knowledge acquired on the bovine reproductive physiology was the base for an important evolution on my development as professional in this area. The development of studies, the participation and the contribution with science, brought experiences that were essential for the critical thinking and lead to a continuous learning, bringing great opportunities for my growth process as a veterinarian with a deep conviction that this might contribute to the advance of the cattle industry where ever my work could have place.