**University of São Paulo**
**"Luiz de Queiroz" College of Agriculture**

# Hormonal combinations for fixed-time artificial insemination aiming to optimize fertility outcomes of Nelore cattle

**Rodrigo Lemos Olivieri Rodrigues Alves**

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

**Piracicaba**
**2020**

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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# DEDICATION

I would like to dedicate all my efforts to my family, especially my parents and my brother, who have always supported me on my decisions. Also, to God, because of the faith that always kept me moving forward.

“***The important thing is to not stop questioning. Curiosity has its own reason for existing***”

Albert Einstein

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**SUMMARY**

## RESUMO

**Combinações hormonais para inseminação artificial em tempo fixo, com o objetivo de otimizar os resultados de fertilidade em animais da raça Nelore**

Dois estudos foram realizados com o objetivo de avaliar os parâmetros reprodutivos, incluindo dinâmica ovariana e fertilidade, de vacas e novilhas da raça Nelore submetidas a protocolos de inseminação artificial em tempo fixo (IATF), à base de estradiol (E2) e progesterona (P4). O primeiro estudo foi realizado em vacas Nelore lactantes e baseou-se na avaliação de ajustes finos em protocolos de 7 dias de duração do implante de P4. Todas as vacas receberam um implante intravaginal de P4 no Dia 0, associado com a administração de benzoato de E2 (BE), e permaneceram com o implante por 7 dias. Na retirada do implante as vacas receberam gonadotrofina coriônica equina (eCG), cipionato de E2 (CE) e prostaglandina F2α (PGF), e foram inseminadas 48 horas depois. O primeiro ajuste foi feito no início do protocolo (Dia 0) com a administração ou não de PGF; o segundo ajuste foi feito no momento da retirada do implante de P4 (Dia 7) e consistiu na administração de diferentes doses de CE (0,5 ou 1,0 mg). O terceiro ajuste foi a administração ou não de GnRH no momento da IA (Dia 9). Quanto aos resultados de dinâmica ovariana, multíparas apresentaram maior tamanho folicular do que as primíparas, tanto no Dia 7 quanto no momento da IA, além de um maior número de animais com CL no início do protocolo. A prenhez geral do experimento foi elevada (~58%) e não foi afetada pela administração de PGF no Dia 0. A expressão de cio foi maior nas vacas que receberam 1,0 mg de CE, além de uma tendência de maior prenhez por IA (P/IA). Vacas que receberam GnRH na IA apresentaram maior P/IA, especialmente aquelas que não expressaram estro. O segundo estudo avaliou os parâmetros reprodutivos de novilhas Nelore submetidas a protocolos de IATF a base de E2 e P4, com 7 ou 9 dias de duração do implante e PGF administrada em momentos distintos. Uma avaliação ultrassonográfica foi realizada antes do início do experimento, para avaliar ciclicidade das novilhas. Aquelas com CL nessa avaliação, foram aleatorizadas em um dos quatro tratamentos do estudo, um com 7 dias de duração do implante de P4, e PGF nos dias 0 e 7, e os outros com 9 dias de implante, e PGF em momentos distintos (Dia 0 e 9, Dia 7, ou apenas no Dia 9). As novilhas pré-púberes receberam um protocolo de indução de ciclicidade e, depois, foram aleatoriamente distribuídas nos tratamentos, independente da presença de CL. Poucas novilhas possuíam CL na primeira avaliação ultrassonográfica (~17%), entretanto, o protocolo de indução resultou em mais de 80% dos animais com CL no início do protocolo de sincronização. A presença de CL no dia 0, no geral, foi positivamente afetada pelo escore de condição corporal das novilhas, porém não interferiu na expressão de estro e na P/IA. Houve diferença na dinâmica ovariana das novilhas, com menor diâmetro folicular, no momento da IA, nos animais do grupo 7 dias. A expressão de estro foi elevada (~90%) e não influenciou a P/IA, a qual também foi semelhante entre os tratamentos (~53%). Portanto, os estudos permitiram uma avaliação completa de diferentes estratégias em protocolos de IATF, tanto para novilhas quanto para vacas Nelore, possibilitando uma flexibilidade na escolha de protocolos e na elaboração dos calendários dos programas reprodutivos.

Palavras-chave: Expressão de estro, IATF, Fertilidade, Ovulação, PGF, Protocolo

## ABSTRACT

**Hormonal combinations for fixed-time artificial insemination aiming to optimize fertility outcomes of Nelore cattle**

Two studies were performed aiming to evaluate reproductive outcomes, including ovarian dynamics and fertility, of Nelore heifers and cows submitted to estradiol (E2)/progesterone (P4)-based fixed-time artificial insemination (FTAI) protocols. The first study was performed using suckled Nelore cows and evaluated some adjustments in protocols with 7 days of P4 implant duration. Cows received an intravaginal P4 implant on Day 0, associated with E2 benzoate (EB) administration, and the implant remained for 7 days. At the time of P4 implant removal, all cows were treated with equine chorionic gonadotropin (eCG), E2 cypionate (EC) and prostaglandin F2α (PGF), and were inseminated 48 hours later. The first adjustment was made at the beginning of the protocol (Day 0) with or without PGF; the second adjustment was made at the time of P4 implant withdrawal (Day 7) and consisted of treating with either 0.5 or 1.0 mg EC. The third adjustment was administration or not of GnRH at the time of FTAI (Day 9). Regarding ovarian dynamics, multiparous had larger follicules than primiparous cows, on both Day 7 and at FTAI, in addition to a greater number of cows with CL at the initiation of the protocol. Overall, pregnancy per AI (P/AI) was high (~58%) and was not affected by PGF treatment on Day 0. Expression of estrus was higher in cows that received 1.0 mg EC, in addition to a tendency of greater P/AI. P/AI was greater when cows received GnRH at FTAI, particularly in cows not expressing estrus. Thus, GnRH improved P/AI especially in cows with lower expression of estrus, such as primiparous, thinner cows (BCS < 3.0), and cows treated with 0.5 mg of EC on Day 7. The second study evaluated the reproductive outcomes of Nelore heifers submitted to 7 or 9 d E2/P4-based FTAI protocols, with PGF administered at different times. An ultrasound evaluation was performed prior to the start of the experiment, to evaluate cyclicity. Heifers with CL were assigned to one of four experimental treatments, one with 7 days of P4 device, and PGF administered on Days 0 and 7, and the other with 9 days of P4 device, and PGF given at different times (Day 0 and 9, Day 7, or Day 9). Pre-pubertal heifers were submitted to a protocol for induction of cyclicity and, then, were randomly assigned to one of the four treatments, regardless of CL presence. Low number of heifers had CL at the first ultrasound evaluation (~17%), however, protocol for induction of cyclicity resulted in more than 80% of heifers with CL at the initiation of the synchronization protocol. The presence of CL on Day 0, overall, was positively affected by body condition score, although it did not influence expression of estrus and P/AI. There were differences on ovarian dynamics, with lower follicular diameter, at the time of AI, on animals from group 7 d. Expression of estrus was high (~90%) and did not affect P/AI, which also was similar between groups (~53%). Therefore, the studies evaluated strategies using FTAI protocols, either for Nelore heifers or cows, allowing for flexibility in the use of protocols and in schedules for FTAI programs.


Keywords: Expression of estrus, IATF, Fertility, Ovulation, PGF, Protocol

## 1. INTRODUCTION

The main objective of a fixed-time artificial insemination (FTAI) protocol is to mimic the physiology of estrus cycle, which occurs in a follicular wave pattern and consists of the following main phases: follicular wave emergence, follicular deviation, and ovulation [1–3]. In this context, many types of FTAI protocols and adjustments have been tested, aiming to improve fertility outcomes in *Bos indicus* and *Bos taurus* beef cattle.

In *Bos indicus* beef cattle, one of the most common synchronization strategy is based on the combination of estradiol (E2) and progesterone (P4), labelled E2/P4-based FTAI protocols [4,5]. At the beginning of these protocols, a progestogen treatment, such as intravaginal P4 implants, and an E2 ester, mainly estradiol benzoate (EB), are associated in order to synchronize a new follicular wave, after leading to atresia of growing follicles [6,7]. Considering the most common FTAI protocols, at the time of P4 implant withdrawal, prostaglandin F2α (PGF) is administered, to induce luteolysis, together with equine chorionic gonadotropin (eCG), which stimulates the final growth of the pre-ovulatory follicle [8,9]. Then, an ovulation inducer, such as E2 cypionate (EC), E2 benzoate (EB) or gonadotropin-releasing hormone (GnRH) analogues, is administered, in different times [5,10–12].

A number of modifications of these protocols have been made in an attempt to improve ovarian responses and fertility outcomes, in both beef heifers and cows. For example, many studies have reported different protocol lengths, with the intravaginal P4 device remaining in place from 7 to 9 days [13–15]. Also, the induction of early luteolysis, with administration of PGF at the onset of the protocol or two days before P4 implant removal, is another strategy that has been studied, aiming to either increase the length of proestrus or to reduce circulating P4 concentrations during follicle growth [16–19]. Moreover, ovulation inducers, considering type, dose, and time of administration, have been tested. In this context, some studies related different results comparing distinct doses of EC [20,21], as well as GnRH administration at the time of AI [14,22].

Thus, our proposal was to evaluate reproductive outcomes, including ovarian dynamics and fertility, after submitting Nelore cattle to E2/P4-based FTAI protocols, considering hormonal combinations and protocol lengths. Therefore, two studies were performed, and the main objective of the first study was to evaluate reproductive outcomes of Nelore cows submitted to 7d E2/P4-based FTAI protocols with adjustments in terms of hormone administration (PGF on Day 0 and GnRH on Day 9) and/or hormone dose (EC on Day 7). The second study was performed in nulliparous Nelore heifers, aiming to compare reproductive outcomes during 7 or 9 d E2/P4-based FTAI protocols, with PGF administered at different times. Since Nelore cattle is widely utilized in South America, these experiments become relevant and bring interesting and applicable information.

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*Bos taurus*, and *Bos taurus* heifers. Theriogenology 2008;69:167–75. https://doi.org/10.1016/j.theriogenology.2007.08.035.

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# 2. HORMONAL COMBINATIONS AIMING TO OPTMIZE FERTILITY OUTCOMES OF NELORE COWS SUBMITTED TO 7-D FIXED-TIME AI PROTOCOLS

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**ABSTRACT**

The aim was to study reproductive outcomes of Nelore (*Bos indicus*) cows submitted to a 7d estradiol (E2)/progesterone (P4)-based fixed-time artificial insemination (FTAI) protocol, using combinations of hormones or doses. Primiparous (n = 962) and multiparous (n = 1935) cows were submitted to synchronization (n = 2012) and resynchronization (n = 885) protocols, following a 2×2×2 factorial arrangement, as follows: 1) Prostaglandin F2α (PGF) on Day -9 (with = P1; or without = P0); 2) dose of E2 cypionate (EC) on Day -2 (0.5 mg = EC0.5; or 1.0 mg = EC1.0); and 3) GnRH at the time of AI (with = G1; or without = G0). At the initiation of the FTAI protocol (Day -9), all cows received an intravaginal P4 implant (1.0 g), 2.0 mg E2 benzoate and received (P1) or not (P0) 0.5 mg cloprostenol sodium (PGF). On Day -2, the implants were removed, all cows received PGF, 300 IU equine chorionic gonadotropin (eCG) and 0.5 (EC0.5) or 1.0 mg EC (EC1.0). On Day 0, cows were treated with 8.4 µg buserelin acetate (G1) or not (G0), concurrently with FTAI. For estrus evaluation, all cows had the base of their tailhead painted with tail-chalk on Day -2 and were checked at the time of AI. Therefore, on Day -9, all cows were assigned to one of the eight treatments (n): 1) P0-EC0.5-G0 (364), 2) P0-EC0.5-G1 (363), 3) P1-EC0.5-G0 (363), 4) P1-EC0.5-G1 (360), 5) P0-EC-1.0-G0 (360), 6) P0-EC1.0-G1 (363), 7) P1-EC1.0-G0 (361), and 8) P1-EC1.0-G1 (363). Effects were considered significant when $P \leq 0.05$ whereas a tendency was assumed when $0.05 < P \leq 0.10$. Multiparous had larger follicles (mm) than primiparous cows on Days -2 (9.6 ± 0.1 vs. 8.6 ± 0.1) and 0 (12.4 ± 0.1 vs. 11.0 ± 0.1). There was no difference in expression of estrus or P/AI between cows that received or not PGF on D-9. Cows that received 1.0 mg EC expressed more estrus than those treated with 0.5 mg (73.8 [1414] vs. 67.9% [1398]) and had greater P/AI (60.2 [1447] vs. 55.2% [1450]). P/AI was greater in cows with GnRH at FTAI (59.8 [1449] vs. 55.5% [1448]), particularly in cows that did not show estrus (52.7 [393] vs. 38.1% [420]). In conclusion, although PGF treatment on Day -9 did not affect P/AI, 1.0 mg of EC on Day -2 or GnRH at the time of AI improved P/AI, but the association of a higher dose of EC and GnRH treatment at AI did not enhance this effect. Moreover, GnRH improved P/AI especially in *Bos indicus* cows with lower expression of estrus, such as primiparous, thinner cows, and cows treated with 0.5 mg of EC.

Keywords: Artificial insemination, beef cattle, estrus, GnRH, ovulation, synchronization.

## 2.1 Introduction

In South America, *Bos indicus* beef cattle, especially Nelore, are widely utilized due to their greater capacity for adaptation in tropical climates [1–3]. However, *Bos indicus* have a longer postpartum anestrus period than *Bos taurus* beef cattle, and this period is affected by several factors, such as nutrition, suckling and parity number [4–7]. Moreover, poor nutritional conditions in the postpartum period results in lower body condition score (BCS) at the initiation of the breeding season, which can negatively impact cyclicity, ovarian dynamics and fertility outcomes [5,8–10]. In this context, reproductive strategies have been developed to induce earlier resumption of cyclicity and to improve reproductive performance of beef cattle [11], such as fixed-time artificial insemination (FTAI) protocols.

In *Bos indicus* beef cattle, one of the most used FTAI protocol is based on the combination of estradiol (E2) and progesterone (P4), labelled E2/P4-based FTAI protocol [1,12]. These protocols initiate with a progestogen treatment, such as insertion of intravaginal P4 implants, and an E2 ester treatment, mainly E2 benzoate (EB). The combination of E2 and P4 suppresses the circulating gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), leading to atresia of growing follicles and emergence of a new follicular wave [13,14]. At the time of P4 implant removal, prostaglandin F2α (PGF) is administered to induce luteolysis, along with equine chorionic gonadotropin (eCG) that is used to stimulate the final growth of the pre-ovulatory follicle [15,16]. Different ovulation inducers have been used at the end of the E2/P4 FTAI such as E2 cypionate (EC), administered at the time of P4 implant withdrawal, EB, administered 24 h after implant removal, or gonadotropin-releasing hormone (GnRH) analogues, administered either before or at the time of FTAI [12,17–19]. A recent study [20] reported synchronization of a new follicular wave in 92.4% of Nelore cattle, after treatment with EB and an intravaginal P4 device, and ovulation at the end of the protocol was 84.8%, showing good overall synchronization efficiency with this protocol.

Modifications on these protocols have been made to improve ovarian responses and fertility outcomes. One of the changes is the length of the protocol, with the intravaginal P4 implant remaining in place from 7 to 9 d [9,21,22]. In Brazil, the majority of the E2/P4-based FTAI protocols use P4 implants for 8 or 9 d. However, 7 d E2/P4-based protocols fit very well in the schedules for managing reproduction and previous results from our group have shown similar fertility compared to the conventional protocols with 8 or 9 d of P4 implant [21,23].

Another potential modification is the treatment with PGF prior to P4 device withdrawal, anticipating luteolysis, in order to increase the length of proestrus [24–27]. For example, studies evaluated administration of PGF on the first day of synchronization protocols in cyclic beef cattle and reported either positive effects [25,26], such as greater diameter of preovulatory follicles, more ovulations and greater pregnancy per AI (P/AI), or no effects on those variables [24,27]. Another change in E2/P4-based protocols is altering the dose of EC at the time of P4 implant removal (from 0.5 to 1.0 mg), aiming to increase expression of estrus and fertility [28,29]. In this context, Torres-Júnior et al. [29] described ovulations more synchronized and greater P/AI when 1.0 mg of EC was administered in E2/P4-based FTAI protocols after 8 d of P4 implant in *Bos indicus* cows (55.7% [122/219] vs. 38.6% [85/220] for 1.0 vs. 0.5 mg EC, respectively). In contrast, Bosolasco et al. [30], using an E2/P4-based protocol with 7 d of P4 implant reported greater P/AI in postpartum crossbred Hereford and Angus cows that received 0.5 mg compared to 1.0 mg EC (60.4 [1227/2112] vs. 50.4% [1031/2044].

Another strategy to improve P/AI in beef cattle is treatment with GnRH at the time of AI [21,23]. The aim of this pharmacological strategy is to ensure an LH surge, reducing the occurrence of delayed ovulation, and improving fertility, especially in cows or heifers that had not shown estrus by the time of AI. Madureira et al. [23] and Prata et al. [21] reported positive effects of GnRH treatment at AI on fertility of Nelore cows either detected or not detected in estrus, although the dose of EC administered at P4 implant withdrawal in those experiments was 0.5 mg. Thus, it is likely that treatment with 1.0 mg EC will increase expression of estrus, reducing the necessity for GnRH at the time of AI, which would reduce protocol costs while maintaining fertility.

Thus, the main objective of the present study was to evaluate reproductive outcomes of Nelore cows submitted to 7d E2/P4-based FTAI protocols that had variations on hormonal treatments to: 1) Induce early luteolysis by administering PGF at the initiation of the protocol, 2) Increase circulating E2 and expression of estrus at the end of the protocol by administering a higher dose of EC at P4 device withdrawal to induce final ovulation, and 3) Increase ovulation and, perhaps, improve timing of ovulation by treating with GnRH at the time of AI. Four main hypotheses were tested: 1) Cyclicity, as measured by the presence of CL, and greater BCS at the initiation of the protocol would result in greater expression of estrus and P/AI, 2) Administration of PGF on day 0 of the protocol would increase follicle diameter on

Day 7 (time of P4 implant removal) and at FTAI, expression of estrus, and P/AI in cyclic cows, 3) Increasing dose of EC from 0.5 to 1.0 mg of EC would increase expression of estrus and P/AI of cows, circumventing the need for GnRH at the time of AI, and 4) Administration of GnRH at the time of AI would increase overall P/AI, especially in cows that had not been detected in estrus by the time of AI.

## 2.2 Material and methods

### 2.2.1 Location

This experiment was conducted in Londrina, Paraná, Brazil, at the Experimental Station "Hildegard Georgina Von Pritzelwiltz" (Figueira Farm) and the data were collected at two consecutive breeding seasons. Nelore cows were kept on pastures of *Brachiaria brizantha*, supplemented with mineral salt and had *ad libitum* access to water. The Animal Research Ethics Committee of "Luiz de Queiroz" College of Agriculture of the University of São Paulo (ESALQ/USP) approved all animal procedures (Protocol # 2018-19).

### 2.2.2 Cow management

A total of 2897 suckled Nelore (*Bos indicus*) cows, at 59.4 ± 24.1 d postpartum, were enrolled in this experiment. On the first day (Day -9), BCS was determined using a 1-5 points scale [31] with 0.25 increments (1 = emaciated; 5 = obese). Primiparous (n = 962) and multiparous (n = 1935) cows had an average BCS of 2.9 ± 0.01 and 3.0 ± 0.01, respectively. A total of 2012 cows received the first AI of the breeding season and all non-pregnant cows (n = 885) at pregnancy diagnosis 30 d after FTAI were also randomized, using the same experimental design during the resynchronization protocol.

### 2.2.3 Experimental design

This experiment used a 2×2×2 factorial arrangement, as shown in Fig. 1. The three factors were: 1) treatment with PGF on Day -9 (with PGF = **P1**; or without PGF = **P0**); 2) dose of EC on Day -2 (0.5 EC = **EC0.5**; or 1.0 mg = **EC1.0**); and 3) treatment with GnRH at the time of AI (with GnRH = **G1**; or without GnRH = **G0**). At the initiation of the FTAI protocol (Day -9), all cows received an intravaginal implant containing 1.0 g P4 (Repro neo, GlobalGen vet science, Jaboticabal, Brazil), 2.0 mg EB im (Syncrogen, GlobalGen vet science) and received (**P1**) or not (**P0**) 0.5 mg cloprostenol sodium (PGF) im (Induscio, GlobalGen vet science). Seven d later (Day -2), the implants were removed, all cows were treated with 0.5 mg PGF im, 300 IU equine chorionic gonadotropin im (eCG; eCGen, Globalgen vet science) and 0.5 (**EC0.5**) or 1.0 mg (**EC1.0**) of EC (Cipion, GlobalGen vet science). On Day 0 (48 h after implant removal), cows were treated im (**G1**) or not (**G0**) with 8.4 µg buserelin acetate (GnRH; Maxrelin; GlobalGen vet science), and were inseminated by one of three technicians using 20 x $10^6$ frozen/thawed proven semen (STGenetics, Indaiatuba, Brazil). Therefore, on Day -9, all cows were assigned to one of the eight resulting treatments, according to Fig. 1: 1) **P0-EC0.5-G0** (n = 364); 2) **P0-EC0.5-G1** (n = 363); 3) **P1-EC0.5-G0** (n = 363); 4) **P1-EC0.5-G1** (n = 360); 5) **P0-EC-1.0-G0** (n = 360); 6) **P0-EC1.0-G1** (n = 363); 7) **P1-EC1.0-G0** (n = 361); 8) **P1-EC1.0-G1** (n = 363). For PGF and GnRH administration, "**1**" indicates presence and "**0**" indicates absence of treatments. For EC administration, **0.5** indicates 0.5 mg and **1.0** indicates 1.0 mg. For estrus evaluation, all cows had the base of their tailhead painted with tail-chalk on Day -2, and were checked at the time of AI (Day 0) for the absence of tail-chalk indicating standing estrus.

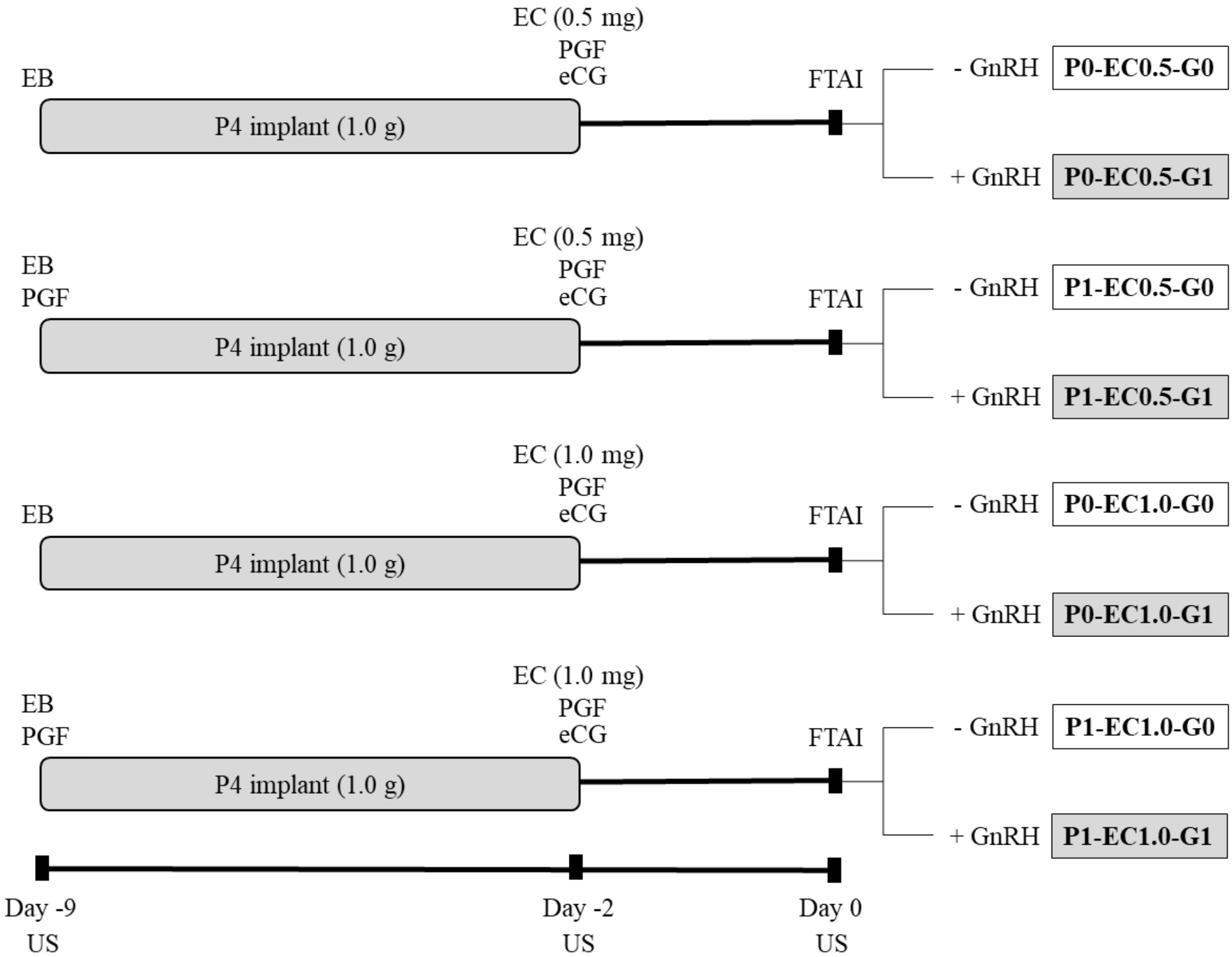


**Fig. 1.** Schematic diagram of the experimental design following a 2×2×2 factorial arrangement. The three factors were: 1) cloprostenol sodium (PGF) on Day -9 (with PGF = **P1**, or without PGF = **P0**); 2) dose of estradiol cypionate (EC) on Day -2 (0.5 EC = **EC0.5**, or

1.0 mg = **EC1.0**); and 3) buserelin acetate (GnRH) at the time of AI (with GnRH = **G1**, or without GnRH = **G0**). On Day -9, cows received an intravaginal implant containing 1.0 g progesterone (P4), 2.0 mg EB im and received (**P1**) or not (**P0**) 0.5 mg PGF im. Seven d later (Day -2), the implants were removed, all cows were treated with 0.5 mg PGF im, 300 IU Ecg im and 0.5 (**EC0.5**) or 1.0 mg (**EC1.0**) of EC. On Day 0 (48 h after implant removal), cows were treated im (**G1**) or not (**G0**) with 8.4 µg GnRH, and were inseminated.

### 2.2.4 Ultrasound examinations

Transrectal ultrasound ovarian examinations in B-mode with a 7.5 MHz linear transducer (DP-2200 VET, Mindray, Shenzhen, China) were performed in all animals on Days -9 and -2 of the synchronization protocol, in order to evaluate for presence or absence of CL. Approximately 20% of both primiparous and multiparous cows were randomly submitted to ultrasound examination of the ovaries to evaluate the diameter (mm) of the largest follicle (LF) on Day -2 (primiparous = 168; multiparous = 370) and at the time of AI (primiparous = 146; multiparous = 422). All measurements were conducted by the same operator. The incidence of ovulation at the end of the protocol (n = 696) was calculated by the presence of CL 7 d after AI (Day 7). The percentage of cows with multiple ovulation was calculated as the proportion of cows with two CL divided by the number of ovulated cows.

Pregnancy diagnosis was conducted by the same operator 30 d after FTAI (Day 30) by means of transrectal ultrasound confirming the presence of an embryo with heart beating and it was calculated as the proportion of cows pregnant divided by the number of cows inseminated. To analyze embryo/fetal loss, ultrasound evaluation was also performed on Day 60 in approximately 50% of cows (n = 1063), and only on the second breeding season.

### 2.2.5 Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC). Analyses of binomial variables (expression of estrus, P/AI, pregnancy losses) were performed using the GLIMMIX procedure fitting a binomial distribution with the Link Logit function. Additionally, the option ddfm = kenwardroger was included in the model statement to adjust the degrees of freedom for variances.

The analysis was performed as a factorial 2x2x2 design, in which the model for P/AI at 30 and 60 d after AI and expression of estrus included effects of treatments (with or without PGF on Day -9, 0.5 or 1.0 mg of EC on Day -2, and with or without GnRH on Day 0) and their interactions. Moreover, the effects of parity (primiparous or multiparous), number of AI (1st AI or resynch), presence of CL on Day 0 (presence or absence), BCS on Day 0 (< 3 or ≥ 3), and expression of estrus (with or without estrus – included only for the P/AI model) were included. The interactions between treatments and the described variables were also evaluated.

The selection of the model that best fitted each variable of interest was performed by finding the model with the lowest value for the Akaike Information Criterion Corrected (AICC) using the stepwise selection procedure that included variables and interactions with $P < 0.20$ from the model. In addition, treatments effects were forced into the final model in all analyses.

When interactions were declared significant, the SLICE command in the LSMEANS was used to interpret them. 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 $0.10 \geq P > 0.05$. Values are presented as percentage (%; binomial variables). The results of continuous variables are expressed as least squares means ± standard error of the mean (LSM ± SEM), unless otherwise indicated.

The LOGISTIC procedure was used for logistic regression to model the probability of expression of estrus, ovulation and pregnancy on day 31 post AI according to the diameter of the largest follicle on Day -2 and Day 0. Logistic 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 Ovarian dynamics

At the initiation of the protocol (Day -9), regardless of number of AI, less primiparous had CL (13.9% [129/929]) than multiparous cows (30.5% [577/1892]), as shown in Table 1. For all cows, considering the first postpartum AI and the AI after resynchronization, 18.5% (354/1914) and 38.8% (352/907) of the cows had CL on Day -9, respectively. Also, more cows with BCS ≥ 3.0 had CL on Day -9 compared to cows with BCS < 3.0 (33.0 [470/1424] vs. 16.9% [236/1397]; $P < 0.001$). Neither administration of PGF on Day -9 nor cyclicity status influenced LF diameter on Days -2 and 0. In addition, EC dose on Day -2 also did not affect LF diameter on Day 0 ($P = 0.27$). Table 1 also shows that multiparous had greater diameter of the LF than primiparous cows, both on Days -2 (implant removal) and Day 0 (AI). Moreover, BCS ≥ 3.0 was associated with a greater diameter of the LF on Day -2 than BCS < 3.0 (9.5 ± 0.09 [n = 290] vs. 9.0 ± 0.09 mm [n = 248]; $P = 0.008$) but did not differ on Day 0.

**Table 1.** Presence of corpus luteum (CL), largest follicle (LF) diameter on Day -2 and at the time of AI (Day 0) of first AI and resynchronization in primiparous and multiparous Nelore cows submitted to a 7d E2/P4-based FTAI protocol.

| | Parity | | | |
|---|---|---|---|---|
| **Item** | **Primiparous** | **Multiparous** | **Overall** | ***P*-value** |
| **CL on D-9, % (n/n)** | | | | |
| First AI | 7.6 (43/565) | 23.1 (311/1349) | 18.5 (354/1914) | < 0.001 |
| Resynchronization | 23.6 (86/364) | 49.0 (266/543) | 38.8 (352/907) | < 0.001 |
| Overall | 13.9 (129/929) | 30.5 (577/1892) | 25.0 (706/2821) | < 0.001 |
| **LF diameter, mm (n)** | | | | |
| Day -2 | 8.6 ± 0.11 (168) | 9.6 ± 0.07 (370) | 9.3 ± 0.06 (538) | < 0.001 |
| Day 0 (time of AI) | 11.0 ± 0.13 (146) | 12.4 ± 0.1 (422) | 12.1 ± 0.08 (568) | < 0.001 |

After Day -9, 9.1% (209/2293) of the cows ovulated and, therefore, had CL on Day -2 that were not detected on Day -9. Considering only cows with CL on Day -9 and those that did not ovulate after Day -9, the percentage of cows with luteolysis between Days -9 and -2 was greater in cows that received PGF on Day -9 than cows that did not receive (72.0 [183/254] vs. 48.1% [99/206]; $P < 0.001$).

Overall, ovulation after AI was 89.7% (624/696) and was influenced by parity, BCS and presence of CL on Day -9, GnRH treatment on Day 0 and expression of estrus (Table 2). However, percentage of cows that ovulated was not influenced by treatment with PGF on Day -9 or by the dose of EC on Day -2. There was an interaction between BCS on Day -9 and treatment on Day -2 on the percentage of cows that ovulated after AI. More cows with BCS < 3.0 ovulated when 1.0 mg of EC was given than if treated with 0.5 mg of EC (88.1 [140/159] vs. 79.8% [126/158]; *P* = 0.02). Finally, overall multiple ovulation after AI was 8.8% (55/624), and was not influenced by any treatments or variables analyzed.

**Table 2.** Ovulation after AI according to parity, body condition score (BCS) and presence of corpus luteum (CL) on Day -9, dose of estradiol cypionate (EC) on Day -2, treatment with GnRH at AI (Day 0) and expression of estrus in Nelore cows submitted to a 7d E2/P4-based FTAI protocol.

| Item | Ovulation, % (n/n)[1] | *P*-value |
|---|---|---|
| **Parity** | | |
| Primiparous | 81.7 (165/202) | 0.005 |
| Multiparous | 92.9 (459/494) | |
| **BCS on D-9** | | |
| < 3.0 | 83.9 (266/317) | 0.001 |
| ≥ 3.0 | 94.5 (358/379) | |
| **CL on D-9** | | |
| Without CL | 86.9 (419/482) | 0.02 |
| With CL | 95.8 (203/212) | |
| **Treatment on D-2** | | |
| 0.5 mg EC | 88.2 (307/348) | 0.69 |
| 1.0 mg EC | 91.1 (317/348) | |
| **Treatment on D0** | | |
| Without GnRH | 87.2 (299/343) | 0.005 |
| With GnRH | 92.1 (325/353) | |
| **Expression of estrus** | | |
| Without estrus | 77.1 (108/140) | 0.004 |
| With estrus | 92.8 (514/554) | |

Figure 2 shows that the probability of ovulation after AI increased as follicular diameter on Days -2 and 0 increased until it reached a plateau at ~10.5 mm on Day -2 and ~12.5 mm on Day 0 (~100% ovulation). Cows that ovulated after AI had, overall, larger follicles than cows that did not ovulate, on both Day -2 (9.3 ± 0.21 vs 7.6 ± 0.37; $P$ = 0.003) and 0 (11.8 ± 0.18 vs 7.9 ± 0.85; $P$ = 0.003).

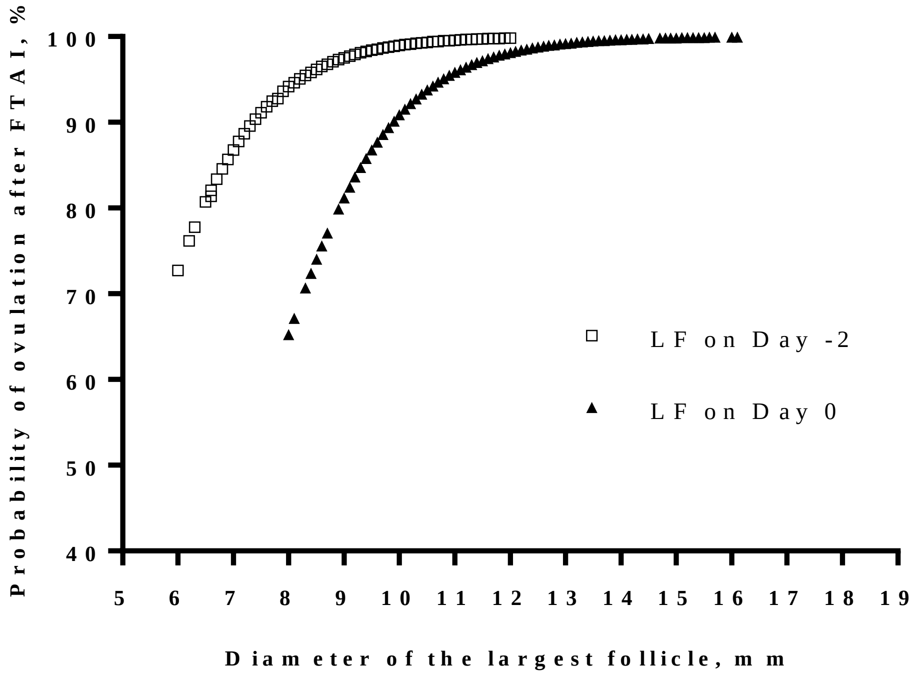


**Fig. 2.** Probability of ovulation after FTAI in Nelore cows according to the diameter of the LF of cows on Day -2 (n = 127) and at the time of AI (n = 127) of a 7d E2/P4-based FTAI protocol.

Probability of ovulation after AI = - 5.8977 + 1.0169 * diameter of the LF on Day -2 ($P$ = 0.03).

Probability of ovulation after AI = - 6.8145 + 0.9172 * diameter of the LF on Day 0 ($P$ = 0.02).

### 2.3.2 Expression of estrus

Overall, estrus was detected in 70.9% (1993/2812) of the cows and multiparous expressed more estrus than primiparous (76.9 [1435/1867] vs. 59.1% [558/945]; $P < 0.001$). As shown in Fig. 3, BCS ≥ 3.0 and presence of CL on Day -9 increased the likelihood of expression of estrus and cows that were treated with 1.0 mg EC had more estrus than cows that received 0.5 mg. Treatment with PGF on Day -9 tended to increase expression of estrus, however, it did not interact ($P$ = 0.28) with presence of CL on Day -9 (with PGF: 83.6 [310/371]; without PGF: 78.6% [243/309]).

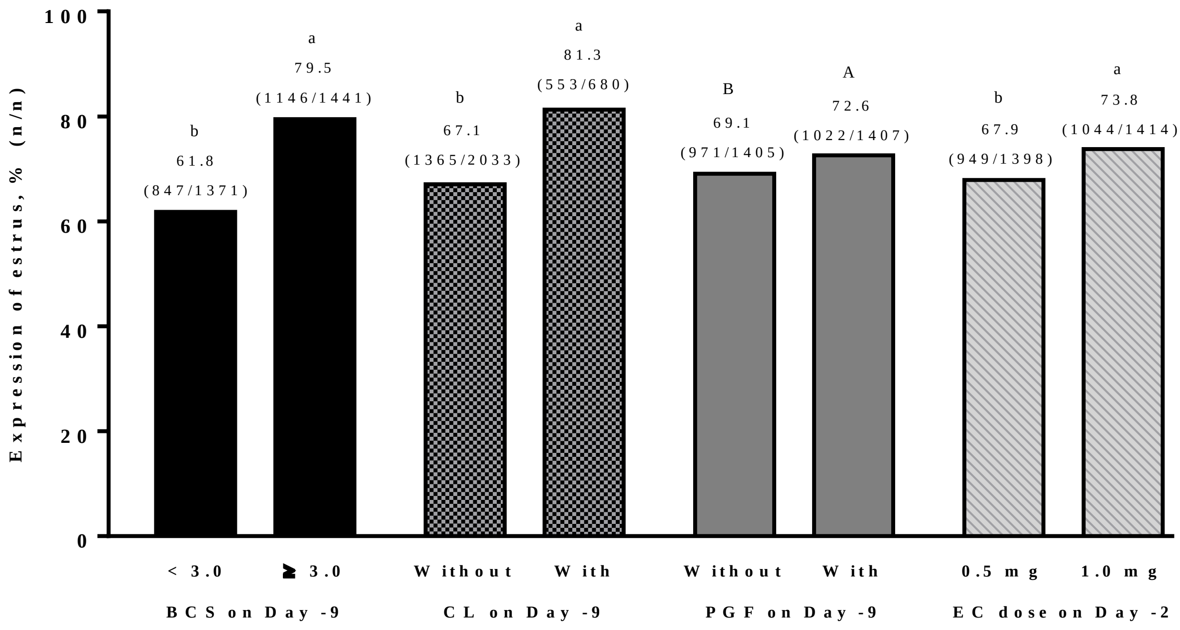


**Fig. 3.** Expression of estrusof Nelore (*Bos indicus*) cows submitted to a 7d E2/P4-based FTAI protocol according to body condition score (BCS) and presence of corpus luteum (CL) on Day -9, PGF treatment on Day -9 and estradiol cypionate (EC) dose at P4 device withdrawal (Day -2)

[a,b]There were effects of BCS ($P$ < 0.001), presence of CL on Day -9 ($P$ < 0.001)and EC dose on Day -2 ($P$ = 0.002). [A,B]There was a tendency for effects of PGF on Day -9 ($P$ = 0.07).

There were interactions for expression of estrus with parity and other factors, such as presence of CL or BCS on Day -9, PGF treatment on Day -9, and dose of EC on Day -2, as shown in Table 3. Presence of CL at the initiation of the protocol increased expression of estrus in both primiparous and multiparous cows, but the effect was much greater in primiparous (32.6%) than in multiparous (12.3%). Also, BCS ≥ 3.0 resulted in greater expression of estrus in multiparous and tended to increase expression of estrus in primiparous cows. There was a tendency for an interaction of EC dose on Day -2 with parity, indicated by the observation that 1.0 mg of EC increased expression of estrus in multiparous ($P$ < 0.001), but not in primiparous cows ($P$ = 0.4).

**Table 3.** Results of expression of estrus (%) of multiparous and primiparous Nelore cows submitted to a 7d E2/P4-based FTAI protocol according to presence of corpus luteum (CL) on Day -9 (with or without), PGF on Day -9, body condition score (BCS) on Day -9 and estradiol cypionate (EC) dose on Day -2.

| **Parity** | **Primiparous** | | **Multiparous** | | ***P*-value** | | |
|---|---|---|---|---|---|---|---|
| **CL on D-9** | **Without** | **With** | **Without** | **With** | **Parity** | **CL** | **Parity*CL** |
| Expression of estrus, % (n/n) | 56.4[b] (436/773) | 74.8[a] (95/127) | 73.7[b] (929/1260) | 82.8[a] (458/553) | < 0.001 | <0.001 | 0.04 |
| **PGF on D-9** | **Without** | **With** | **Without** | **With** | **Parity** | **PGF** | **Parity*PGF** |
| Expression of estrus, % (n/n) | 58.6 (290/495) | 59.6 (268/450) | 74.8 (681/910) | 78.8 (754/957) | < 0.001 | 0.07 | 0.13 |
| **BCS on D-9** | **< 3.0** | **≥ 3.0** | **< 3.0** | **≥ 3.0** | **Parity** | **BCS** | **Parity*BCS** |
| Expression of estrus, % (n/n) | 53.8[Y] (272/506) | 65.2[X] (286/439) | 66.5[y] (575/865) | 85.8[x] (860/1002) | < 0.001 | < 0.001 | < 0.001 |
| **EC dose on D-2** | **0.5 mg** | **1.0 mg** | **0.5 mg** | **1.0 mg** | **Parity** | **EC** | **Parity*EC** |
| Expression of estrus, % (n/n) | 58.4 (289/495) | 59.8 (269/450) | 73.1 (660/903) | 80.4 (775/964) | < 0.001 | 0.002 | 0.08 |

[a,b]Effect of CL on Day -9 within parity ($P < 0.05$).

[x,y]Effect of BCS on Day -9 within parity ($P < 0.05$).

[X,Y]Effect of BCS on Day -9 within parity ($P = 0.10$).

Figure 3 represents the probability of expression of estrus based on follicular diameter on Day -2 or Day 0 of the FTAI protocol. As the diameter of the LF increased, expression of estrus also increased. Cows that expressed estrus had, overall, larger follicles than cows that did not express estrus, on Day -2 (9.5 ± 0.07 vs. 8.7 ± 0.12; $P = 0.002$) or Day 0 (12.2 ± 0.09 vs. 11.4 ± 0.17; $P = 0.001$).

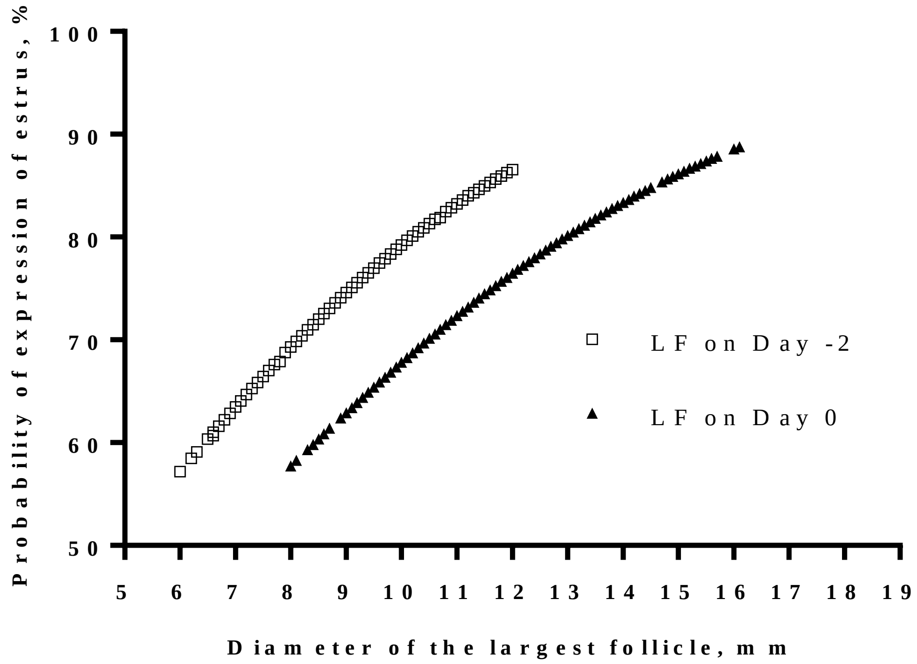


**Fig. 3.** Probability of expression of estrus at the end of fixed-time AI (FTAI) protocols in Nelore (*Bos indicus*) cows according to the diameter (mm) of the largest follicle (LF) on Day -2 (n = 458) and at the time of AI (n = 458) of a 7d E2/P4-based FTAI protocol.

Probability of expression of estrus = - 1.9812 + 0.3326 * diameter of the LF on Day -2 ($P$ < 0.001).

Probability of expression of estrus = - 1.4384 + 0.2273 * diameter of the LF on D0 ($P$ < 0.001).

### 2.3.3 Pregnancy per AI at 30 and 60 d after AI

The overall P/AI on Day 30 was 57.6% (1670/2897) and on Day 60 was 53.2% (566/1063). Overall, pregnancy loss between Day 30 and 60 was 4.9% (29/595) and was not affected by experimental treatments. The P/AI on Day 30 was greater at first FTAI compared to resynchronization protocols (58.9 [1184/2012] vs. 54.9% [486/885]; $P$ = 0.05). The P/AI on Day 30 for each experimental group was: **P0-EC0.5-G0** (50.0 [182/364]), **P0-EC0.5-G1** (57.0 [207/363]), **P1-EC0.5-G0** (52.6 [191/363]), **P1-EC0.5-G1** (60.8 [219/360]), **P0-EC1.0-G0** (59.4 [214/360]), **P0-EC1.0-G1** (60.1 [218/363]), **P1-EC1.0-G0** (59.8 [216/361]), and **P1-EC1.0-G1** (61.4 [223/363]).

The main effects of treatments on P/AI are represented in Fig. 4. The treatment with PGF on Day -9 did not influence P/AI ($P$ = 0.4). However, 1.0 mg of EC increased P/AI (60.2

[871/1447] vs. 55.1% [799/1450]; $P = 0.05$). Moreover, P/AI was greater when cows were treated with GnRH on Day 0 (59.8 [867/1449] vs. 55.5% [803/1448; $P < 0.001$]).

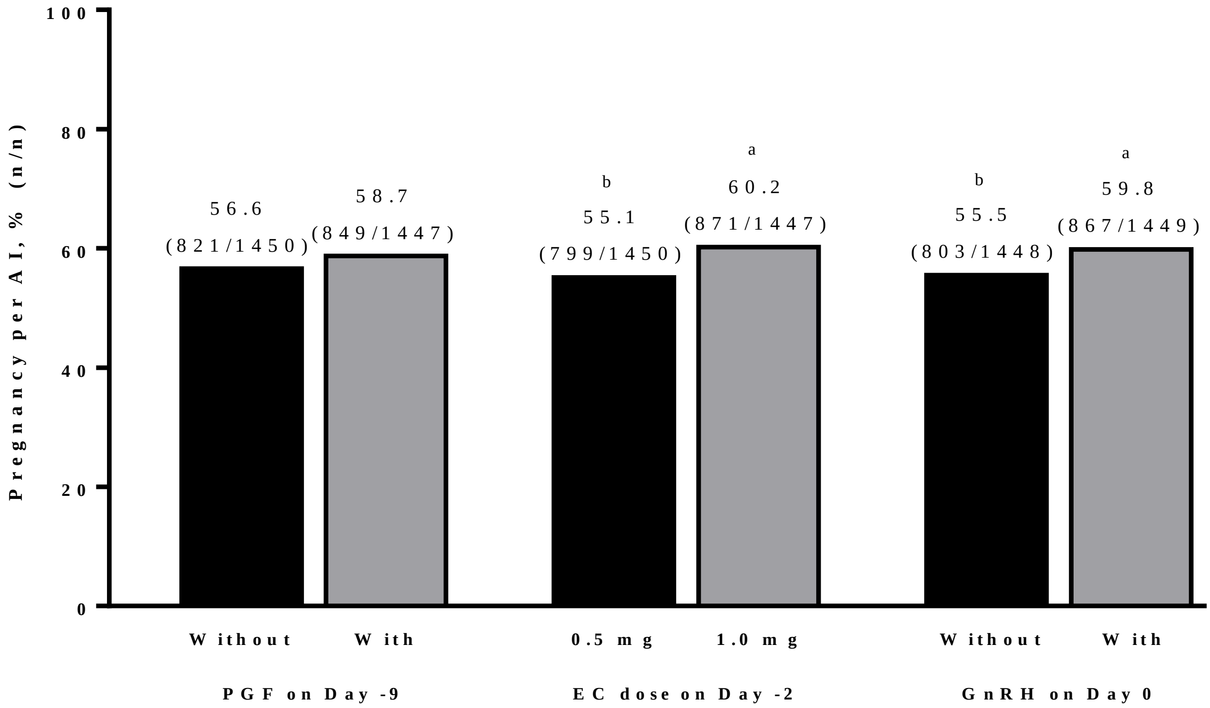


**Fig. 4.** Pregnancy per AI (P/AI) 30 d after AI of Nelore (*Bos indicus*) cows submitted to a 7d E2/P4-based FTAI protocol, with or without PGF on Day -9, using either 0.5 or 1.0 mg of EC at the time of P4 implant removal (Day -2), and with or without GnRH at FTAI. No differences between treatments on Day -9 (with or without PGF; $P = 0.4$).
[a,b]Difference between cows that received 1.0 vs. 0.5 mg of EC on Day -2 ($P = 0.05$), and received or not GnRH at the time of AI ($P < 0.001$).

Cows that expressed estrus had greater fertility than cows not detected in estrus (62.5 [1235/1976] vs. 45.1% [367/813]; $P < 0.001$). Presence of CL on Day -9 was associated with increased P/AI (Fig. 5), although there was no interaction ($P = 0.28$) between treatment with PGF and presence of CL on Day -9 on fertility. Also, PGF tended to increase P/AI of cows with CL on Day -9 (69.1 [259/375] vs. 62.5 [198/317]; $P = 0.07$).

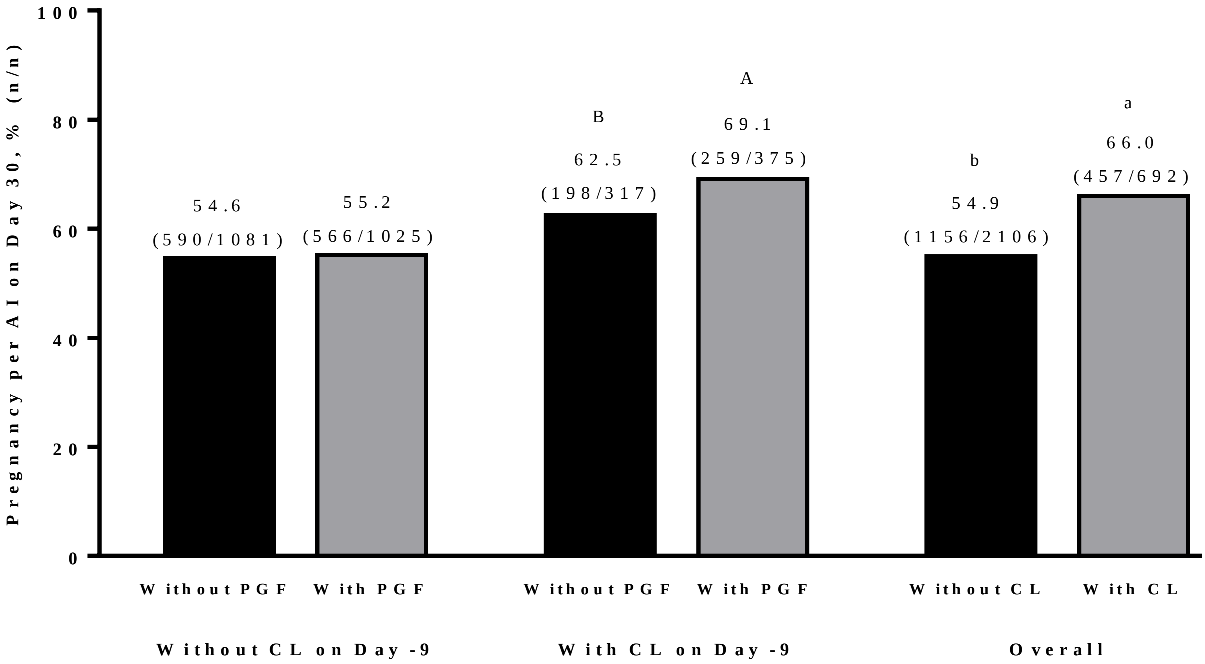


**Fig. 5.** P/AI per AI (P/AI) 30 d after AI of Nelore (*Bos indicus*) cows submitted to a 7d E2/P4-based FTAI protocol according to presence of corpus luteum (CL) on Day -9 of the protocol, receiving or not PGF. [a,b] Effects of presence of CL on Day -9 ($P$ < 0.001). [A,B] Effects of PGF administration in cows with CL on Day -9 ($P$ = 0.07).

There were no interactions of EC dose on Day -2 (0.5 or 1.0 mg) and BCS on Day -9 (< 3.0 or ≥ 3.0) on P/AI ($P$ = 0.79; Fig. 6), however, cows with BCS ≥ 3.0 had greater P/AI than cows with BCS < 3.0 (62.2 [918/1476] vs. 52.9% [752/1421]; $P$ = 0.002). In addition, there was no interaction between parity (primiparous or multiparous) and EC dose (Fig. 6) on P/AI, but multiparous had greater P/AI than primiparous cows (63.5 [1129/1935] vs. 45.8% [441/962]; $P$ < 0.001).

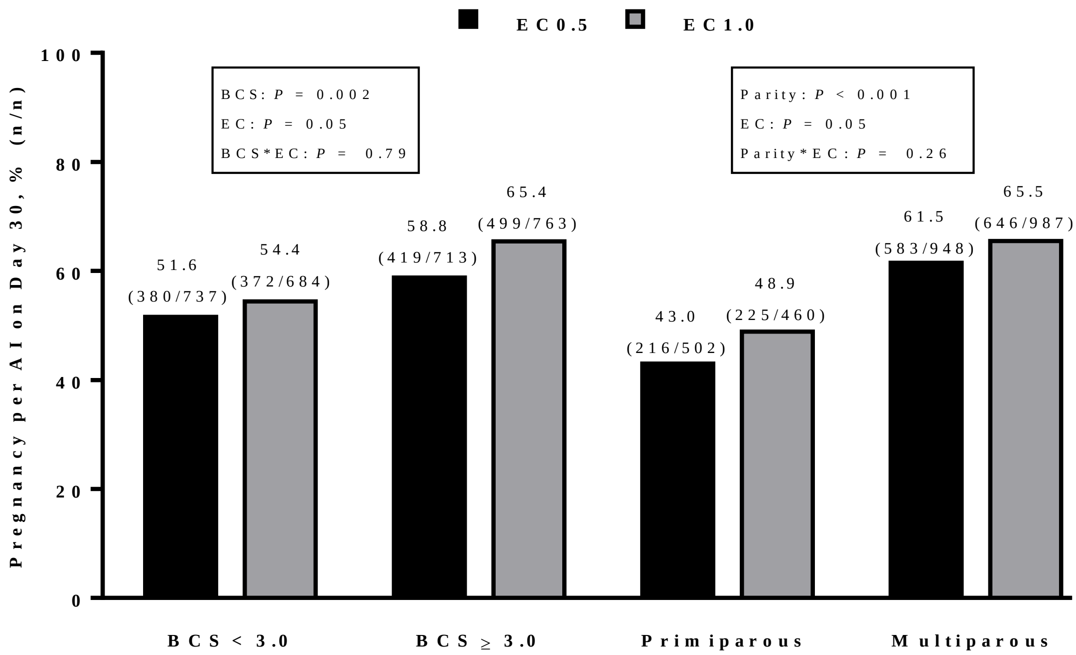


**Fig. 6.** Pregnancy per AI (P/AI) 30 d after AI of Nelore (*Bos indicus*) cows submitted to a 7d E2/P4-based FTAI protocol according to body condition score (BCS) on Day -9, parity (primiparous or multiparous) and dose of estradiol cypionate (EC; 0.5 or 1.0 mg) at the time of P4 implant removal (Day -2).

There were interactions for P/AI with GnRH treatment on Day 0 and other factors, such as BCS on Day -9, parity and expression of estrus, as shown in Table 4. Treatment with GnRH on Day 0 increased P/AI in cows with BCS < 3.0 ($P$ < 0.001), but there was no difference in cows with BCS ≥ 3.0 ($P$ = 0.11). Also, GnRH treatment on Day 0 increased P/AI in primiparous ($P$ = 0.003) but did not change P/AI in multiparous cows. Moreover, cows that did not express estrus had greater P/AI when GnRH was given on Day 0 ($P$ < 0.001), but no effect of GnRH was observed in cows that expressed estrus ($P$ = 0.46).

Table 4 also shows a tendency for an interaction on P/AI on Day 30 between dose of EC on Day -2 and GnRH on Day 0 ($P$ = 0.10). Thus, GnRH on Day 0 increased P/AI in cows that received 0.5 mg EC on Day -2 ($P$ < 0.001), but did not influence P/AI in cows receiving 1.0 mg EC.

**Table 4.** Pregnancy per AI (P/AI) on Day 30 of Nelore (*Bos indicus*) cows submitted to a 7d E2/P4-based FTAI protocol according to BCS on Day -9, parity, EC dose on Day -2, expression of estrus, and GnRH treatment at the time of AI (Day 0).

| **Variable** | **GnRH treatment[1]** | | ***P-value**** | | |
|---|---|---|---|---|---|
| | **Without** | **With** | **T** | **V** | **T*V** |
| BCS on D0 | | | | | |
| < 3.0 | 48.6[b] (339/698) | 57.1[a] (413/723) | 0.001 | 0.002 | 0.05 |
| ≥ 3.0 | 61.9 (464/750) | 62.5 (454/726) | | | |
| Parity | | | | | |
| Primiparous | 41.9[b] (208/497) | 50.1[a] (233/465) | 0.001 | < 0.001 | 0.05 |
| Multiparous | 62.6 (595/951) | 64.4 (634/984) | | | |
| EC dose on D7 | | | | | |
| 0.5 mg | 51.3[b] (373/727) | 58.9[a] (426/723) | 0.001 | 0.05 | 0.1 |
| 1.0 mg | 59.6 (430/721) | 60.7 (441/726) | | | |
| Expression of estrus | | | | | |
| Without | 38.1[b] (160/420) | 52.7[a] (207/393) | 0.001 | < 0.001 | 0.003 |
| With | 62.5 (607/972) | 62.6 (628/1004) | | | |

[1] Treatment was GnRH at the time of AI (with or without).

[*] T = treatment effect; V = variable effect; T × V = treatment × variable interaction.

Figure 7 presents the probability of P/AI on Day 30 based on diameter of the ovulatory follicle on Days -2 and 0. Thus, as the diameter of the largest follicle increased on Day -2 and on Day 0, P/AI also increased. Pregnant cows on Day 30 had, overall, larger follicles on both Days -2 (9.5 ± 0.08 vs 8.8 ± 0.11; $P$ = 0.002) and 0 (12.2 ± 0.02 vs 11.7 ± 0.16; $P$ = 0.004) compared to non-pregnant cows.

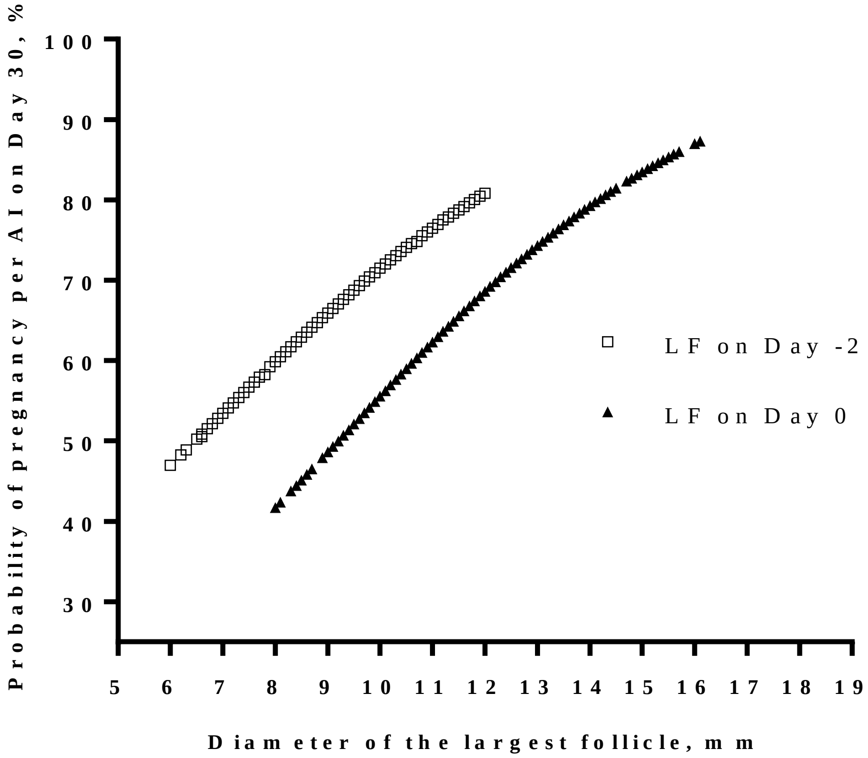


**Fig. 7.** Probability of pregnancy per AI (P/AI) in Nelore cows according to the diameter of the LF of cows on Day -2 (n = 458) and at the time of AI (n = 458) of a 7d E2/P4-based FTAI protocol.

Probability of P/AI = - 2.3579 + 0.3151 * diameter of the LF on Day -2 ($P < 0.01$).

Probability of P/AI = -11.1991+ 1.8441 * (diameter of the LF on Day 0)$^2$ ($P < 0.01$).

## 2.4 Discussion

Synchronization protocols based on the combination of E2 esters and P4 implants are widely used in dairy and beef cattle, and modifications in these protocols are currently being evaluated to optimize synchronization and fertility. The main objective of the present study was to evaluate the reproductive outcomes of *Bos indicus* (Nelore) cows due to logical modifications in a 7d E2/P4-based FTAI protocol (PGF on Day -9, dose of EC on Day -2, and GnRH on Day 0). Thus, using an expressive number of cows for fertility and ovarian dynamics parameters, this experiment provides clear information on the value of these hormonal manipulations and describes the interactions of these protocol adjustments with important physiologic factors, such as parity, BCS, expression of estrus, cyclicity status, and ovulation after AI. Moreover, a comparison of the eight experimental groups in the present study shows that P/AI can be improved over 10% points comparing the conventional treatment protocol (no PGF on Day -9, 0.5 mg EC on Day -2 and no GnRH at AI) with the optimized group (PGF on Day -9, 1.0 mg EC on Day -2, and GnRH at AI). Thus, the present results, based on results of almost 3,000 FTAI, can be used to optimize fertility outcomes in commercial operations with *Bos indicus* beef cattle.

The first hypothesis was supported since cows with greater BCS and with presence of CL at the initiation of the protocol expressed more estrus and had greater P/AI. It is well known that resumption of cyclicity is affected by many factors including nutritional status, suckling, parity, change in body weight after calving, and BCS, and that these factors can impact reproductive performance [32–37]. In the postpartum period, cyclicity resumption is regulated by the frequency and amplitude of LH pulses, in order to increase follicular E2 production sufficiently to produce a GnRH and LH surge and first postpartum ovulation. The timing of first postpartum ovulation is dependent on several factors including body condition at calving and loss/recovery of body condition after calving [33,36,38,39]. Similar to our results, Ayres et al. [4] reported a lower percentage of cows with CL at the initiation of a synchronization protocol (42 d postpartum) when the BCS was < 3.0, compared with cows with BCS > 3.0 (33.8 vs 43.4%). In the present study, more cows with greater BCS had CL on Day -9, and after the FTAI protocol this was associated with a greater expression of estrus and P/AI, confirming previous reports that described earlier resumption of cyclicity and better reproductive performance when beef cows gained weight or lost less BCS in the postpartum period and had greater BCS at the initiation of the protocol [5,35,36,40–42]. Thus, greater BCS on the first day of the protocol reflects better nutritional management during the pre and postpartum periods and this was associated with improved reproductive outcomes.

Normally, primiparous beef cows have longer postpartum anestrus periods and lower fertility than multiparous cows [5,43]. In the present study, a greater percentage of multiparous cows had a CL at the beginning of the protocol compared to primiparous cows, consistent with the results of Dimmick et al. [6], that described a longer interval between parturition and first ovulation in primiparous than multiparous cows (112 vs. 46 d). Guedon et al. [7] also reported that the first postpartum ovulation occurred earlier in multiparous than primiparous beef cows (7.7 vs. 9.9 wk). Therefore, the greater percentage of multiparous cows with CL on the first day of the FTAI protocols in our study are consistent with previous results with *Bos indicus* cattle.

In our study, the diameter of the LF at P4 device removal and at AI influenced expression of estrus, ovulation and P/AI, as observed previously [5,8]. Several negative impacts on reproductive outcomes may occur by reducing the size of the ovulatory follicle, such as lower circulating E2 during the proestrous period [44] leading to an inappropriate environment in the uterus and oviduct [45–47], decreased expression of estrus [8], lower ovulation efficiency [5], development of smaller CL with lower P4 secretion [48–51], and lower P/AI [9,51]. In a manipulative study, Vasconcelos et al. [48] generated smaller ovulatory follicles in Holstein cows and observed decreased circulating E2 prior to ovulation, decreased CL volume and circulating P4 concentrations in the following diestrus, and decreased fertility. In the present study, multiparous had larger follicles than primiparous cows, and cows with greater BCS also had larger follicles, likely resulting in greater E2 secretion, that resulted in the greater expression of estrus and ovulation after AI. These results are consistent with previous findings, which described greater probability of expression of estrus, ovulation and P/AI as follicle diameter increases [5,8,9]. Thus, although some studies did not find parity effects on P/AI [8,22], in the present study, the parity differences on fertility may be explained by the parity differences in cyclicity status at the initiation of the protocol and the subsequent differences in diameter of the ovulatory follicle and expression of estrus.

One of the advantages of using FTAI protocols based on P4 releasing implants combined with E2 esters is that these protocols can induce cyclicity in postpartum anovular cows [1]. The presence of the P4 device, providing sub-luteal concentration of P4, leads to an increased LH pulse frequency, stimulating follicular growth in anestrous animals [11,52,53], and potentially allowing for ovulation by the end of the protocol [42]. In the current study, the percentage of cows with CL was greater at the initiation of the resynchronization protocol than at initiation of the first AI protocol. Nevertheless, the observation that most of the cows did not have a CL at the initiation of the resynchronization protocol, indicates that cows returned to an anovular condition even after being exposed to a FTAI protocol and ovulating. Thus, the resynchronization protocol can be particularly important during a complete reproductive management program for beef cattle, in order to have the majority of cows pregnant early in the breeding season.

Ovulation at the end of the protocol is obviously a critical determinant of fertility in FTAI programs [12]. All protocols in the present study generated high percentages of ovulation after AI, consistent with previous studies that reported final ovulation ranging from 80 to 94% [5,8,9]. However, ovulation was greater in cows treated with GnRH at the time of AI than cows not treated with GnRH and this was likely the underlying reason that GnRH improved fertility in the study. In addition, many of the other factors that improved P/AI also increased percentage of cows ovulating to the protocol such as CL on Day -9, BCS at Day -9, and expression of estrus during the protocol. One other interesting observation is the multiple ovulation incidence with 8.8% found in this study, similar to the 8.0% multiple ovulation observed in a previous study [23] using Nelore cows submitted to a 7d E2/P4-based FTAI protocol. In addition, in the present study, the overall pregnancy loss was 4.9%, which is similar to what has been previously reported [4].

High P4 concentration suppresses LH pulse frequency and negatively impact follicular growth, ovulation efficiency, and fertility in beef cattle [25,26,53,54–56]. Carvalho et al. [26] described lower circulating P4 concentrations in cycling beef heifers receiving PGF on Day -9 of an E2/P4-based FTAI protocol, resulting in greater diameter of the dominant follicle and higher ovulation incidence (78.8 vs. 54.0%). Therefore, our second hypothesis was based on the idea that the administration of PGF at the initiation of the protocol would induce luteolysis in cyclic cows, resulting in greater follicular diameter and better fertility outcomes. However, even with greater CL regression in cows that had CL on Day -9 and received PGF, this hypothesis was not supported, since there was no effect of PGF on Day -9 on expression of estrus and P/AI. The possible explanation is that a high percentage of cows did not have CL at the initiation of the protocol (>70%), so we could not detect statistical effect of treatment with PGF on Day -9. Moreover, as the administration of PGF on the first day of the protocol did not increase the size of the ovulatory follicle, both expression of estrus and P/AI were not improved. Similar results were described by Sá Filho et al. [27], that did not report differences in follicle diameter, ovulation incidence and P/AI between cycling Nelore heifers receiving or not PGF on the first day of an E2/P4-based FTAI protocol. Moreover, Surjus et al. [24] described no effect on expression of estrus and P/AI of Nelore heifers treated or not with PGF on the first day of a 7d E2/P4-based FTAI protocol. However, in disagreement with our findings, Dadarwal et al. [25] reported larger follicles at the time of AI and greater P/AI in beef cattle receiving PGF at the initiation of the synchronization protocol.

In FTAI protocols, different ovulation inducers can be used with similar results for synchronization of ovulation, expression of estrus and P/AI, and some adjustments in terms of type of inducer and doses have been tested [12,17,18,29,57]. Torres-Júnior et al. [29] compared three inducers of ovulation (0.5 or 1.0 mg of EC, administered at the time of P4 implant removal, and EB, administered 24 h later) in Nelore cows submitted to an 8d E2/P4-based FTAI protocol, and did not report differences in follicle diameter (24 h after P4 implant removal) or percentage of cows ovulating to the protocols. However, 1.0 mg of EC resulted in shorter interval to ovulation than 0.5 mg (71.1 ± 3.6 vs. 78.0 ± 3.5 h), in addition to more synchronized ovulations and greater P/AI. Martins et al. [58] compared the administration or not of 1.0 mg of EC at the time of P4 implant withdrawal, during an 8d FTAI protocol in Nelore cows, and described that both primiparous and multiparous cows had greater expression of estrus when treated with 1.0 mg of EC. Moreover, Sá Filho et al. [28] reported greater expression of estrus and P/AI in suckled anestrus Nelore cows comparing 1.0 mg of EC vs. no EC at the time of P4 implant removal. In contrast, as previously mentioned, Bosolasco et al. [30], using an 7d E2/P4-based FTAI protocol, reported greater P/AI in postpartum multiparous crossbred Hereford and Angus cows when 0.5 mg of EC was administered compared to 1.0 mg of EC. Moreover, in the same study, the authors reported a shorter interval to ovulation for 1.0 compared to 0.5 mg EC (58.7 ± 2.7 vs. 66.7 ± 2.5 h) and an interaction between dose of EC and insemination time on P/AI. When 1.0 mg of EC was administered, P/AI was greater if FTAI was performed 46-50 h after P4 implant removal compared to 52-56 h (54.0 [564/1045] vs. 46.7% [467/999]).

Expression of estrus prior to AI is associated with greater P/AI on both primiparous and multiparous beef cows [5,22,23]. The E2 concentration required to generate estrus behavior is related to the ovulatory capacity, since it induces a GnRH and LH surge, essential for ovulation. In this context, our third hypothesis was that treating cows with 1.0 mg of EC and inseminating 48 h after P4 implant withdrawal would increase expression of estrus and P/AI, reducing the requirement for GnRH at the time of AI. In fact, 1.0 mg EC increased expression of estrus and P/AI on Day 30, with similar results on fertility compared to cows that received GnRH, supporting our hypothesis. However, a higher dose of EC did not alter neither the diameter of the LF at the time of AI nor the ovulation frequency, in accordance with Torres-Júnior et al. [29]. Thus, in the present study, 1.0 mg of EC may have promoted a more synchronized ovulation, due to a possible greater stimulation of the pre-ovulatory LH surge. In addition, the greater pharmacological E2 dose probably produced adequate uterus and oviducts hormonal milieu during proestrus, providing a better support for the sperm and improving the fertilization process and embryo development [28,45,59,60].

Our fourth hypothesis was supported because treatment with GnRH simultaneously to FTAI increased overall P/AI. Moreover, since GnRH increased P/AI of cows that had not expressed estrus by the time of AI, this treatment improved fertility of groups of cows with lower expression of estrus, such as primiparous, thinner cows, and cows treated with 0.5 instead of 1.0 mg of EC on Day -2. These results could be justified by an increased ovulation frequency, since GnRH increased the percentage of cows ovulating after AI (without GnRH: 87.2 [307/348] vs. with GnRH: 92.1% [325/353]). In addition, although not evaluated in the present study, it is very likely that the GnRH treatment provided a better synchronization of ovulation, preventing an occurrence of a delayed LH surge, optimizing the time of FTAI in relation to ovulation[61–63]. Similarly, two recent studies from our laboratory reported improved fertility outcomes when GnRH treatment was included at the time of AI. Madureira et al. [23], after submitting Nelore cows to 7d FTAI protocols, reported that treatment with GnRH at FTAI improved P/AI of cows that did not express estrus by the time of AI (59.1 vs. 48.2%). In addition, two other experiments [21] compared protocol lengths in Nelore cows (7, 8, or 9 d of P4 implant) and treatment or not with GnRH at FTAI. Protocol length did not influence P/AI, but GnRH treatment had a positive effect on fertility in both experiments. In experiment 1, GnRH tended to increase overall P/AI of cows that did not express estrus, although in experiment 2 this tendency was found on cows that expressed estrus, in disagreement with our findings, since we did not find differences in P/AI between cows that expressed estrus, receiving or not GnRH.

In conclusion, larger follicles at the time of P4 device removal and at AI reflected in greater expression of estrus, ovulation frequency and P/AI. The use of PGF at the initiation of FTAI protocols has a potential benefit on fertility outcomes considering cyclic cows and further studies are necessary to explore this subject. Administration of 1.0 of EC improved expression of estrus, reducing the need for GnRH treatment at FTAI. Finally, GnRH at the time of AI improved P/AI especially in cows with lower expression of estrus, such as primiparous, thinner cows (BCS < 3.0), and cows treated with 0.5 mg of EC.

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## 3. FERTILITY OF NELORE HEIFERS SUBMITTED TO 7 OR 9-D FIXED-TIME AI PROTOCOLS WITH prostaglandin F2α ADMINISTERED AT DIFFERENT TIMES

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### ABSTRACT

The aim of this study was to compare reproductive outcomes of Nelore heifers (n = 831) submitted to a 7 or 9 d E2/P4-based FTAI protocols, with PGF administered at different times, considering synchronization (n = 533) and resynchronization (n = 298) protocols. Prior to the onset of the breeding season (BS), heifers with corpus luteum (CL; n = 89) on the first ultrasound (US) evaluation were assigned to the treatments and those without CL received a protocol for induction of cyclicity [Day -35: insertion of a 7 d used intravaginal progesterone (P4) implant (0.5 g); Day -23: P4 withdrawal and 0.5 mg estradiol (E2) cypionate (EC)]. Twelve (for 9 d protocols) or 14 (for 7 d protocol) d later, all heifers, regardless of CL presence, received a P4 device (0.5 g) and 1.5 mg E2 benzoate (EB). On the day of implant removal (7 or 9 d later), 0.5 mg PGF, 0.5 mg EC and 200 IU equine chorionic gonadotropin (eCG) were administered. For estrus evaluation, all heifers had the base of their tailhead painted with tail-chalk at the time of P4 removal and were checked 48 h later, at FTAI. Therefore, on Day -11 heifers were assigned to one of four treatments (n): The groups 9dP4-PGd-2 (n = 216) and 9dP4-PGd-4 (198) received PGF on Days -2 or -4, respectively, whereas the groups 9dP4-PGd-11&-2 (215) and 7dP4-PGd-9&-2 (202) were treated with PGF on Days -11 and -2 or -9 and -2, respectively. Effects were considered significant when $P \leq 0.05$ whereas a tendency was assumed when $0.05 < P \leq 0.10$. The percentage of cyclic heifers at the beginning of the BS was 16.7% (533) and from those without CL submitted to the protocol for cyclicity induction, 84.2% (444) had CL on Day 0. Heifers with body condition score (BCS) ≥ 3.0 had greater percentage of CL than those with BCS < 3.0 [84.5 (666) vs. 74.5% (161)]. Group 9dP4-PGd-11&-2 had greater CL regression (94.4% [107]), between Days 0 and 9, than groups 9dP4-PGd-4 and 9dP4-PGd-2 but did not differ from 7dP4-PGd-9&-2. The diameter of largest follicle (LF) at the time of P4 removal did not differ among treatments, but the presence of CL at this time had a negative impact on follicle size (9.6 ± 0.4 vs. 10.4 ± 0.2 mm). The LF diameter at AI was smaller in group 7dP4-PGd-9&-2 (12.1 ± 0.3 mm) compared to 9dP4-PGd-4 and 9dP4-PGd-11&-2 (13.2 ± 0.3 mm) but did not differ from 9dP4-PGd9 (12.9 ± 0.4 mm). Overall expression of estrus (91% [831]) did not affect pregnancy per AI (P/AI; 52.5% [831]) and both variables were similar among groups. Cyclic heifers at the beginning of the BS had greater P/AI than heifers with induced cyclicity with or without CL on Day 0 (64.0 [89] vs. 51.3 [374] vs. 47.0% [66], respectively). In conclusion, despite differences in ovarian dynamics, Nelore heifers submitted to either 7 or 9 d E2/P4-based FTAI protocols, with PGF administrated in different moments, had similar expression of estrus and P/AI.

Keywords: Beef cattle, cyclicity, fertility, ovulation, prostaglandin.

**3.1 Introduction**

Beef cattle herds are mainly composed by *Bos indicus* and *Bos taurus* cattle, and both have physiological particularities that affect reproductive performance [1,2]. Due to high adaptability [3,4], particularly in tropical climates, *Bos indicus* cattle, such as Nelore, have been widely explored in beef farms, so that many strategies have been tested aiming to optimize production outcomes of those animals. In this context, hormonal treatments, including fixed-time AI (FTAI) programs, are generally associated with genetic improvement and economic gains [5,6]. Moreover, optimizing the reproductive efficiency of heifers is particularly important, as this category usually has the most developed genetic of the herd.

The time of the onset of puberty depends on nutritional and genetic factors [7,8] and occurs when the estradiol (E2) receptors in the hypothalamus decrease, reducing the negative feedback on GnRH-induced LH secretion and allowing for ovulation [9–11]. Several studies have tested strategies to induce cyclicity in beef heifers using E2/progesterone (P4)-based protocols, and described efficient results [12,13]. Also, similar protocols are widely used to synchronize follicular wave emergence and ovulation, allowing for FTAI at the end [14–16]. The physiology behind these protocols are based on the induction of a new follicular wave, after an association between E2 (mainly E2 benzoate [EB]) and P4 (mostly intravaginal implants), which occurs ~2.2 d after this association in Nelore heifers [16]. At the end of these protocols, the growing dominant follicle receives an ovulation inducer followed by FTAI is performed.

Adjustments have been tested on E2/P4-based FTAI protocols in Nelore heifers, considering protocol length, types of hormones and doses. In this type of protocol, the duration of P4 implant usually varies between 7 and 9 d and PGF is administered either at the time of P4 implant withdrawal or earlier, at the onset of protocol or between P4 implant placement and removal [1,15,17–20]. Prata et al. [18] reported similar P/AI in Nelore heifers treated with 7 or 8 d E2/P4-based FTAI protocols (49.1 [182/371] vs. 45.6% [169/371]), receiving PGF at the initiation of the protocols. Also, Carvalho et al. [1] described positive effects on the diameter of the largest follicles when PGF was administered at the initiation of an 8 d E2/P4-based protocol in cyclic beef heifers. However, some studies reported an absence of effect on P/AI of earlier PGF administration, either on Day 0 of 7 or 8 d E2/P4-based protocols [19,21], or on Day 7 of 9 d E2/P4-based protocol [17]. Additionally, Martins et al. [22] reported greater fertility in postpubertal Nelore heifers when the P4 implant remained for 9 d, instead of 7 (45.3 [173/382] vs. 37.6% [128/341]).

Nevertheless, none of these studies directly compared 7 d protocol with PGF administered at the same time of P4 implant placement to 9 d protocols, with PGF administered on the three most common times (Days 7, 9 and both 0 and 9). In addition, previous results from our laboratory reported relatively high fertility in heifers treated with the 7 d E2/P4-based FTAI protocols with [18] or without [20] PGF at the initiation of the protocols. Therefore, the main objective of this study was to directly compare reproductive variables of nulliparous Nelore heifers during 7 or 9 d E2/P4-based FTAI protocols, with PGF administered at different times. Our hypotheses were: 1. Presence of CL at the initiation of protocol would increase expression of estrus and P/AI, regardless of treatment; 2. The 7 d protocol would induce smaller follicles at the time of P4 implant removal and at FTAI, resulting in lower expression of estrus compared to 9 d protocols; 3. Within 9 d protocols, earlier PGF administration would increase follicle diameter, expression of estrus and P/AI; 4. Despite differences in expression of estrus, P/AI would be similar among the 9 d groups with earlier administration of PGF and 7 d group.

## 3.2 Material and methods

### 3.2.1 Location

This experiment was conducted in Londrina, Paraná, Brazil, at the Experimental Station “Hildegard Georgina Von Pritzelwiltz” (Figueira Farm) and the data were collected from the 2018/2019 and 2019/2020 (November to April) breeding seasons. Nelore nulliparous heifers (*Bos indicus*) were kept on pastures of *Brachiaria brizantha*, supplemented with mineral salt and had *ad libitum* access to water. The Animal Research Ethics Committee of “Luiz de Queiroz” College of Agriculture of the University of São Paulo (ESALQ/USP) approved all animal procedures (Protocol CEUA # 2018-19).

### 3.2.2 Animal management

A total of 831 Nelore (*Bos indicus*) nulliparous heifers, 26.0 ± 2.0 mo old and with body weight of 307.1 ± 22.6 kg, were enrolled in this study. On the first day of the synchronization protocol (Day -11), body condition score (BCS) was measured, considering a scale of 1-5 points [23], using 0.25 increments (1 = emaciated; 5 = obese). Pubertal heifers (with corpus luteum [CL] prior to the initiation of the protocol) had overall BCS of 3.27 ± 0.02 and heifers that received a protocol for induction of cyclicity had overall BCS of 3.19 ± 0.01. A total of 533 heifers received the first AI and all the non-pregnant heifers (n = 298) were submitted randomly to a resynchronization protocol, using the same experimental design.

### 3.2.3 Experimental design

At initiation, all heifers with more than 265.0 kg were evaluated by ultrasound for the presence of a corpus luteum (CL). Heifers with CL were assigned to one of four experimental treatments described below, and heifers without a CL were submitted to a protocol for induction of cyclicity, as shown in Fig. 1 [D-35: insertion of a 7-d used intravaginal P4 implant containing 0.5 g (Repro one, GlobalGen Vet Science, Jaboticabal, Brazil); D-23: P4 implant withdrawal and administration of 0.5 mg E2 cypionate im (EC; Cipion, GlobaGen Vet Science)]. After 12 d (Day -11), all heifers, regardless of CL presence, were randomly assigned to one of the four treatments (Fig. 2). Heifers received on the first day of each treatment a new P4 implant containing 0.5 g (Repro one, GlobalGen Vet Science) and 1.5 mg of EB im (Syncrogen, GlobalGen Vet Science). At the time of P4 implant removal (Day -2), heifers were treated im with 0.5 mg cloprostenol sodium (PGF; Induscio, GlobalGen Vet Science), 0.5 mg EC and 200 IU equine chorionic gonadotropin (eCG; ECGen, GlobalGen Vet Science). For estrus evaluation, all heifers had the base of their tailhead painted with tail-chalk at the time of P4 implant removal, and were checked at the time of AI based on the disappearance of the tail-chalk signaling for positive activity of standing estrus. All heifers were inseminated 48 h later (Day 0) by one of three technicians using 20 x $10^6$ frozen/thawed proven semen (STGenetics, Indaiatuba, Brazil). The groups **9dP4-PGd-2** (n = 216) and **9dP4-PGd-4** (n = 198) received 0.5 mg PGF im on Days -2 or -4, respectively, whereas the groups **9dP4-PGd-11&-2** (n = 215) and **7dP4-PGd-9&-2** (n = 202) were treated with PGF on Days -11 and -2 or -9 and -2, respectively.

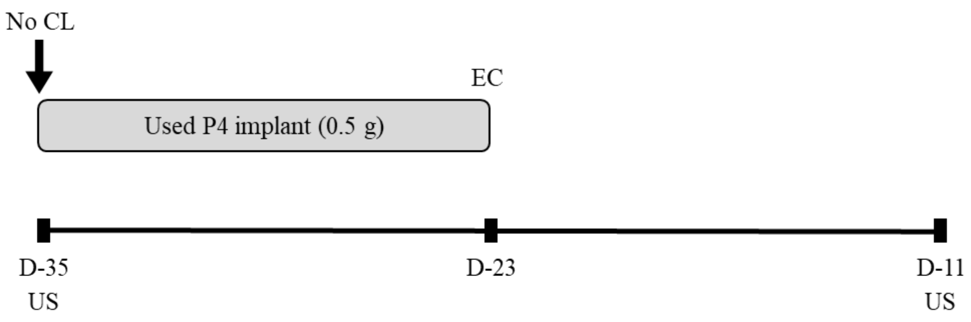


**Fig. 1.** Schematic diagram of the protocol used for induction of cyclicity. On Day -35, all heifers were submitted an ultrasonographic evaluation, and heifers without CL received a disinfected intravaginal P4 implant [24] previously used for 7 d with 0.5 g. Twelve d later (Day -23), the day of P4 implant withdrawal, 0.5 mg of EC was administered im. After 12 d (Day -11), all heifers, regardless of CL presence, heifers were randomly assigned to one of the four experimental groups.

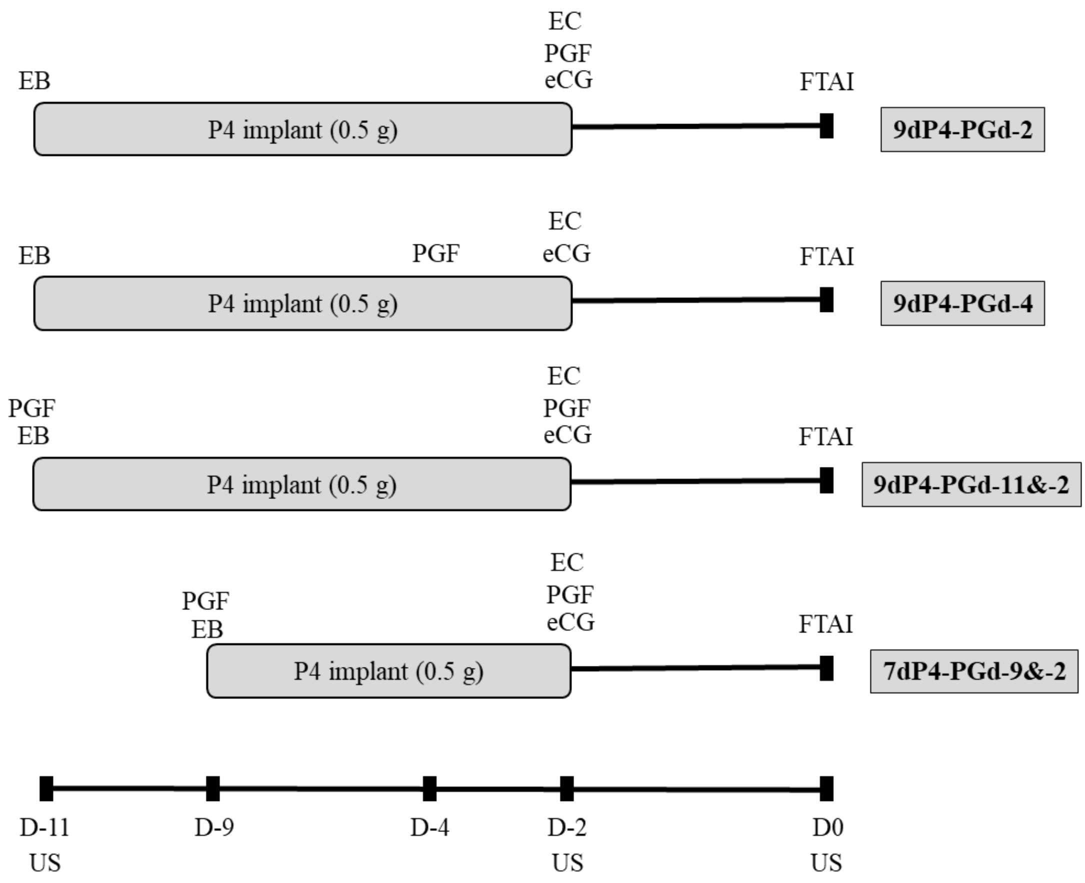


**Fig. 2.** Schematic diagram of the experimental design. All heifers, regardless of treatment, received on the first day of each treatment a new P4 implant and 1.5 mg EB im. On Day 9, the animals were administered with PGF, EC, eCG and had the base of their tailhead painted with tail-chalk for estrus detection. All heifers were inseminated 48 hours later (Day 11). The

groups **9dP4-PGd-2** (n = 216) and **9dP4-PGd-4** (n = 198) received 0.5 mg PGF im on Days -2 or -4, respectively, whereas the groups **9dP4-PGd-11&-2** (n = 215) and **7dP4-PGd-9&-2** (n = 202) were treated with PGF on Days -11 and -2 or -9 and -2, respectively.

### 3.2.4 Ultrasound examinations

Transrectal ultrasound ovarian examinations in B-mode with a 7.5 Mhz linear transducer (DP-2200 VET, Mindray, Shenzhen, China) were performed in all heifers prior to the initiation of the breeding season, and on Days -11 and -2 of the synchronization protocol, in order to evaluate for presence or absence of CL.

During the synchronization protocol, approximately 20% of the heifers were randomly submitted to ultrasound examination of the ovaries to evaluate the diameter of the largest follicle (LF) at the time of P4 implant removal (n = 96) and at AI (n = 146). All measurements were conducted by the same operator. Ovulation at the end of the protocol was calculated by the presence of a CL 7 d after FTAI on 204 heifers. Multiple ovulation was calculated as the proportion of heifers with two or more CL divided by the number of ovulated heifers.

Pregnancy diagnosis (pregnancy per AI; P/AI) was conducted by the same operator 30-35 d after FTAI by means of transrectal ultrasound confirmation of embryo heart beating and it was calculated as the proportion of heifers pregnant divided by the number of heifers inseminated.

### 3.2.5 Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC). Analyses of binomial variables (expression of estrus and P/AI) were performed using the GLIMMIX procedure fitting a binomial distribution with the Link Logit function. Additionally, the option ddfm = kenwardroger was included in the model statement to adjust the degrees of freedom for variances.

The analysis was performed following a completely randomized design, in which the model for P/AI 30 d after AI and expression of estrus included effects of treatments. Due to the number of heifers used and the high BCS, for analyses, we were able to assign the BCS into two categories (BCS $< 3.0$ or BCS $\geq 3.0$). Thus, the effects of reproductive status (induced or cycling), number of AI (first AI or resynchronization), presence of CL on Day -11 (presence or absence), BCS on Day -11 ($< 3$ and $\geq 3$), and expression of estrus (with and without estrus – included only for the P/AI model) were included. Interactions between treatments and the described variables were evaluated.

The selection of the model that best fitted each variable of interest was performed by finding the model with the lowest value for the Akaike Information Criterion Corrected (AICC) using the stepwise selection procedure that included variables and interactions with $P < 0.20$ from the model. In addition, treatment effect was forced into the final model in all analysis.

When interactions were declared significant, the SLICE command in the LSMEANS was used to interpret them. 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 $0.10 \geq P > 0.05$. Values are presented as percentage (%; binomial variables). The results of continuous variables are expressed as least squares means ± standard error of the mean (LSM ± SEM), unless otherwise indicated.

**3.3 Results**

At the initiation of the BS, 16.7% (89/533) of heifers were considered cycling (presence of CL at the first ultrasound evaluation). After the protocol for induction of cyclicity, 84.2% (374/444) of the induced heifers had CL on Day -11 (day 0 of the FTAI protocol). Overall, considering the first AI and the AI after resynchronization, 82.7% (687/831) of the heifers had CL on Day -11 of the protocol, and was higher on first AI than on resynchronization (87.6 [467/533] vs. 73.8% [220/298]; $P < 0.001$). Also, overall, more heifers with BCS ≥ 3.0 had CL on Day -11 (synch and resynch) than heifers with BCS < 3.0 (84.6 [566/669] vs. 74.7% [121/162]; $P = 0.008$).

Ovarian dynamics results evaluated during the protocol are represented in Table 1. A lower percentage of heifers of group **9dP4-PGd-11&-2** had CL at P4 implant removal, in comparison with group **9dP4-PGd-2**. However, no differences were found between the other groups. The diameter of the largest follicle (LF) at the time of P4 implant removal (Day -2) was not different between treatments. Moreover, presence of CL on Day -11 was associated to a greater diameter of the LF on Day -2 compared to absence of CL on Day -11 (10.4 ± 0.2 [n = 77] vs. 9.6 ± 0.4 mm [n = 18]; $P = 0.03$). However, presence of CL on Day -2 reflected in smaller follicles on this day, compared with heifers without CL (9.4 ± 0.4 [n = 21] vs. 10.5 ± 0.2 mm [n = 74]; $P = 0.003$). The diameter of the LF at FTAI was smaller in group **7dP4-PGd-9&-2** than in groups **9dP4-PGd-4** and **9dP4-PGd-11&-2** but did not differ from group **9dP4-PGd-2**. Also, diameter of the LF in group **9dP4-PGd-2** did not differ from the other groups. The diameter of the LF at the time of AI was not influenced by the presence of CL neither on Day -11 nor on Day -2 ($P > 0.05$). Moreover, luteolysis between Days -11 and -2 was higher on group **9dP4-PGd-11&-2** than group **9dP4-PGd-4** or **9dP4-PGd-2**, which were not different from each other. Also, luteolysis on group **7dP4-PGd-9&-2** was greater than group **9dP4-PGd-2** but did not differ from the other groups.

Table 1 also shows that, overall, estrus was detected in 91.0% (756/831) of the heifers and was not different between groups. Heifers that received the cyclicity induction protocol expressed more estrus than pubertal heifers (94.1 [418/444] vs. 80.9% [72/89]; $P < 0.001$). Also, presence of CL on Day -11, regardless of protocol for induction of cyclicity, increased the likelihood of expression of estrus (with CL: 91.4 [628/687]; without CL: 88.9% [128/144]; $P = 0.03$).

**Table 1.** Presence of corpus luteum (CL) on Day -11 and at the time of P4 implant removal (Day -2), CL regression after Day -11, measurements of the largest follicle (LF) diameter on Days -11 and -2, and expression of estrus and ovulation of Nelore heifers submitted to a 7 or 9-d E2/P4-based FTAI protocols with PGF administered at different times.

| | **Experimental Groups** | | | | |
|---|---|---|---|---|---|
| **Item** | **9dP4-PGd-2** | **9dP4-PGd-4** | **9dP4-PGd-11&-2** | **7dP4-PGd-9&-2** | ***P*** |
| CL on Day -11, % (n/n) | 76.9 (166/216) | 84.3 (167/198) | 83.3 (179/215) | 86.6 (175/202) | 0.1 |
| CL on Day -2, % (n/n) | $33.8^{a}$ (47/139) | $28.6^{ab}$ (36/126) | $11.5^{b}$ (15/131) | $20.2^{ab}$ (26/129) | 0.01 |
| CL regression between Days -11 and -2, % (n/n) | $68.0^{c}$ (70/103) | $77.6^{bc}$ (83/107) | $94.4^{a}$ (101/107) | $87.3^{ab}$ (96/110) | < 0.001 |
| LF diameter on Day -2, mm (n) | 9.8 ± 0.4 (24) | 10.4 ± 0.3 (21) | 10.8 ± 0.3 (21) | 10.2 ± 0.2 (30) | 0.37 |
| LF diameter on Day 0, mm (n) | $12.9^{ab}$ ± 0.4 (34) | $13.2^{a}$ ± 0.3 (38) | $13.2^{a}$ ± 0.3 (41) | $12.1^{b}$ ± 0.3 (33) | 0.03 |
| Expression of estrus, % (n/n) | 89.8 (194/216) | 96.0 (190/198) | 88.8 (191/215) | 89.6 (181/202) | 0.28 |
| Ovulation after AI, % (n/n) | 96.2 (50/52) | 97.9 (47/48) | 96.2 (51/53) | 94.1 (48/51) | 0.75 |

Overall, ovulation frequency after FTAI was 96.1% (196/204) and was not different among groups ($P = 0.75$) neither was affected by expression of estrus ($P = 0.74$). However, heifers that received the cyclicity induction protocol tended to ovulate more after AI than pubertal heifers (98.3 [113/115] vs. 93.3% [83/89]; *P* = 0.08). Finally, overall multiple ovulation after AI was 2.0% (4/196) and was not influenced by or interacted with any other factor.

A total of 436 out of 831 (52.5%) heifers were diagnosed pregnant 30 d after FTAI, as shown in Fig. 3. However, P/AI was not different neither among experimental groups (*P* = 0.89) nor between first AI and resynchronization AI (52.9 [282/533] vs. 51.5% [154/298]; *P* = 0.74).

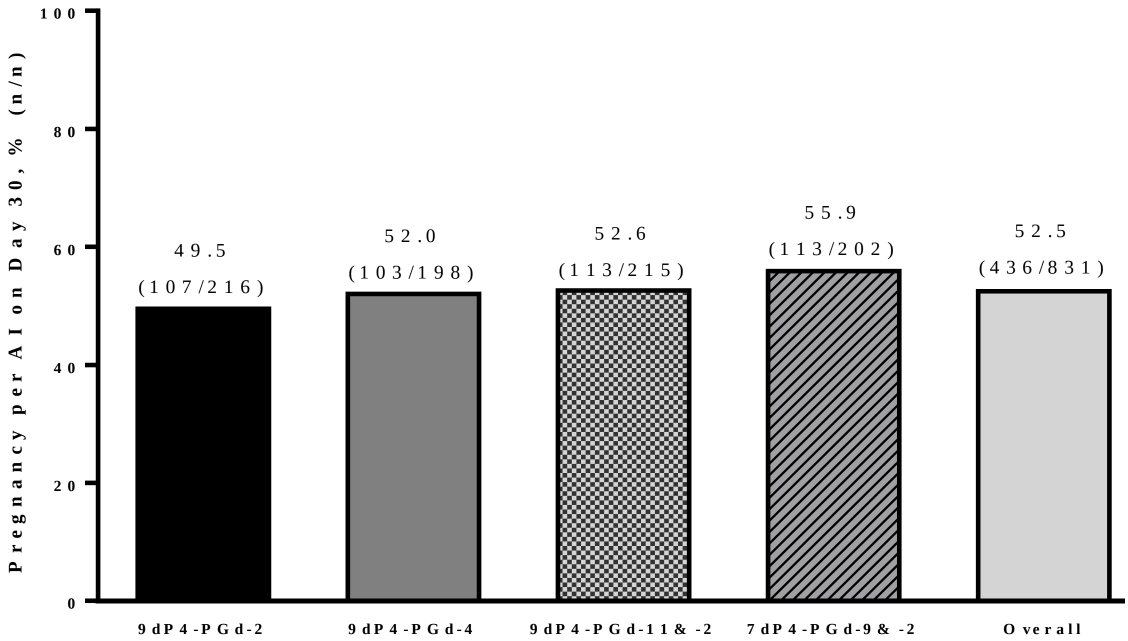


**Fig. 3.** P/AI (%, n/n) 30 d after FTAI of Nelore (*Bos indicus*) heifers submitted to 7 or 9-d E2/P4-based FTAI protocols, with PGF administered at different times. 9 d groups had the P4 implant for 9 d, although 7 d group had P4 for 7 d. The groups **9dP4-PGd-2** (n = 216) and **9dP4-PGd-4** (n = 198) received 0.5 mg PGF im on Days -2 or -4, respectively, whereas the groups **9dP4-PGd-11&-2** (n = 215) and **7dP4-PGd-9&-2** (n = 202) were treated with PGF on Days -11 and -2 or -9 and -2, respectively (*P* = 0.89).

Table 3 describes P/AI 30 d after AI according to cyclicity status, BCS and presence of CL on Day -11 of the protocols, and expression of estrus. There was an effect of cyclicity (*P* = 0.05), with greater P/AI in pubertal heifers prior to the induction protocol than heifers with or without a CL after the induction protocol, although no difference was found between the last two categories (*P* = 0.6). Moreover, P/AI was not affected by BCS on Day -11, presence of CL on Day -11, or expression of estrus.

**Table 2.** P/AI (%, n/n) 30 d after AI of Nelore (*Bos indicus*) heifers submitted to 7 or 9-d E2/P4-based FTAI protocols with PGF administered at different times.

| **Item** | **Condition** | **P/AI, % (n/n)** | ***P-value*** |
|---|---|---|---|
| **Cyclicity Status** | **Pubertal** | 64.0[a] (57/89) | 0.05 |
| | **Induced with CL** | 51.3[b] (194/378) | |
| | **Induced without CL** | 47.0[b] (31/66) | |
| **BCS on Day 0** | **< 3.0** | 50.6 (82/162) | 0.58 |
| | **≥ 3.0** | 52.9 (354/669) | |
| **CL on Day 0** | **Without** | 46.5 (67/144) | 0.58 |
| | **With** | 53.7 (367/683) | |
| **Expression of estrus** | **Without** | 52.0 (39/75) | 0.69 |
| | **With** | 52.5 (397/756) | |

### 3.4 Discussion

*Bos indicus* cattle are widely explored in the world due to their greater adaptability to heat stress, parasites and food shortage than *Bos taurus*, particularly in tropical climates [3,4,25]. However, zebu cattle (e.g. Nelore) present a longer prepubertal period and advanced age of first calving than *Bos taurus*, which can result in economic losses to the producers [7,9,26]. In this context, many reproductive programs and strategies using FTAI protocols have been tested aiming to reduce the prepubertal period and improve reproductive outcomes in Nelore heifers. Therefore, this study becomes relevant since it directly compared reproductive outcomes of nulliparous heifers submitted to E2/P4-based FTAI protocols with different lengths (7 or 9 d of P4 implant) and PGF administered at different times.

Overall P/AI greater than 52% (52.5% [436/831]) was notable and consistent with previous reports of our group [20]. Puberty in beef heifers is achieved when the number of E2 receptors decreases in the hypothalamus, reducing the negative feedback of E2 on GnRH-induced LH secretion, allowing GnRH release, LH surge and ovulation [9–11,26]. Nutritional and genetic factors can explain the low percentage (16.7% [89/533]) of heifers with CL at the first day of ultrasound examination, according to previous reports [7,8,20]. To support this argument, in the present study, overall, higher BCS (≥3.0) resulted in greater percentage of

CL at the initiation of the protocol, indicating a positive effect of nutrition on cyclicity. Moreover, in agreement with other studies [12,13,20], after the 12 d induction protocol, more than 85.0% (374/440) of the previously supposed prepubertal (without CL) heifers had a CL at the initiation of synchronization protocol, indicating a high efficiency of this strategy to induce cyclicity. Additionally, at the beginning of synchronization protocol for first FTAI, the percentage of heifers with CL was composed by pubertal heifers and heifers that ovulated at the end of induction protocol, and it could explain the greater percentage of CL at this time than at the start of resynchronization.

Our first hypothesis was based on previous reports that described greater follicle size and better reproductive performance of postpubertal than prepubertal heifers [19,27–32]. Also, presence of CL is indicative of reproductive activity and suggests development of the reproductive tract, resulting in greater fertility [28]. Thus, this hypothesis was partially supported since heifers that had CL at the initiation of the protocols, considering first FTAI and resynchronization, regardless of treatment, presented greater diameter of the LF on Day -2 and higher expression of estrus than those without CL (91.4 [624/683] vs. 88.9% [128/144]), but no differences on P/AI were detected. In disagreement with our data, Locke et al. [29] described greater P/AI in *Bos indicus* influenced pubertal than pre-pubertal beef heifers, submitted to different reproductive strategies, although no differences on expression of estrus was detected. Oosthuizen et al. [30] also reported greater P/AI in cycling than non-cycling *Bos indicus* influenced beef heifers submitted to a 5-d-CO-synch + CIDR protocol or to a natural service (control group). The low number of heifers not expressing estrus in our study (9.0% [75/831]) can explain the absence of effect of this condition in some variables, such as ovulation after AI and P/AI, differing from previous studies that reported better reproductive performance in beef heifers that expressed estrus [20,33].

In the present study, considering first FTAI, even with lower expression of estrus, P/AI was higher in pubertal heifers (64.0%) than in heifers that received the protocol for cyclicity induction, with (51.3%) or without (47.0%) CL at the initiation of the synchronization protocols. In contrast with this result, Madureira et al. [20] reported greater P/AI in pubertal Nelore heifers (62.0% [90/145]) and heifers with CL after an induction protocol (56.8% [367/646]) than heifers without CL after this protocol (46.2% [84/182]). Since these heifers received the same induction protocol as ours, proposed by Rodrigues et al. [12,13], and were synchronized with 7 d E2/P4-based FTAI protocols, this difference was not expected. One possible explanation is the fact that pubertal heifers achieved this condition naturally and may have developed a more “mature” reproductive tracts compared to inducted heifers, resulting in greater P/AI [28,34]. Moreover, the low number of inducted heifers not having CL at the initiation of the synchronization protocols (n = 66) can explain the absence of effect of this condition on P/AI. Also, in the same study [20], they reported 2.4% (2/84) of multiple ovulation after AI, in agreement with our data (2.0% [4/196]).

Different protocol lengths, types of hormones and administration at distinct times alter ovarian dynamics and follicular growth pattern in beef cattle [16,35–38]. The second hypothesis of this study was that the shorter protocol (group 7dP4-PGd-9&-2) would produce smaller follicles at the time of P4 implant removal and at AI, resulting in a lower percentage of heifers expressing estrus. Recently, Madureira et al. [16] reported that the follicle wave emergence in nulliparous Nelore heifers occurred 2.2 ± 0.2 d after an association between 1.5 mg of EB and P4 implant (0.5g), in a 7 d FTAI protocol. Also, Burke et al. [39] described higher proportion of prepubertal and cycling beef heifers emerging a new follicle wave 3 d after administration of 1 mg of EB, in association with a P4 implant. Consequently, considering different protocol lengths and follicle wave emergence occurring between days 2 and 3, longer protocols (9 d) should result in larger follicles, which probably would produce more E2 [40], leading to a greater expression of estrus [41]. Our hypothesis was partially supported since there was difference in LF diameter only at the time of AI, in which follicles of heifers treated with the 7 d protocol were smaller than follicles of heifers treated with 9 d protocols, with PGF administered earlier (Day -11 or -4). PGF administration at the initiation of the 7 d protocol may have led to a decrease in circulating P4 concentration [1,42,43], since it induced the same percentage of CL regression as 9dP4-PGd-4 and 9dP4-PGd-11&-2 groups, and greater luteolysis than the 9dP4-PGd-2. Thus, probably, the anticipated PGF treatment allowed for higher LH pulse frequencies during the development of the dominant follicle [44,45] and greater follicle growth rate [19], allowing to achieve the same size as the follicles of heifers from group 9dP4-PGd-2. The lack of difference on expression of estrus is consistent with previous reports [18] and can also be explained by the earlier PGF administration in three of four groups, in addition to the physiological condition of the heifers, with almost 83% presenting CL at the initiation of the protocol. Moreover, at the time of P4 implant removal, all heifers were treated with 0.5 mg of EC, which could also be associated to the high expression of estrus in all groups [46].

Many studies have tested different protocol lengths, which commonly vary between 7 and 9 d of P4 implant duration [16,18,22] in Nelore cattle, in addition to distinct strategies related to the time of PGF administration, which is usually done either at the same time or before the P4 implant removal [1,15,17]. Dias et al. [17] elaborated two experiments to compare the P4 concentrations and time of PGF administration on fertility of postpubertal Nelore heifers, during a 9 d FTAI protocol. In the first experiment, lower circulating P4 on Day 7, larger follicles at the time of AI and greater P/AI were described in heifers that received a previously twice used disinfected P4 device (1.9 g). In the second experiment, heifers received the same treatments as the first one, but PGF was given on Day 7 or on Day 9 of the 9 d FTAI protocol (equivalent to Day -4 or -2 of our study), in addition to administration or not of 300 IU of eCG at the time of P4 implant removal. No difference in P/AI was detected between the time of PGF administration, but eCG improved fertility of heifers that received a first use P4 device and heifers administered with PGF on Day 9. Additionally, Peres et al. [15] submitted nonlactating Nelore cows to a 9 d E2/P4-based FTAI protocol, and reported lower circulating P4, greater follicle size at AI, higher ovulation frequency, and greater P/AI (52.0 [182/350] vs. 36.4% [128/352]) when PGF was administered on Day 7 instead of Day 9.

The positive effects of PGF administration at the beginning of FTAI protocols have also been reported. Carvalho et al. [1] evaluated the ovarian dynamics of cyclic beef heifers submitted to a 8 d E2/P4-based protocol with or without PGF at the time of P4 implant insertion and treatment with EB (initiation of the protocol) and described lower P4 concentrations, between Days 3 and 8 of the protocol, and larger follicles, between Days 6 and 10, on heifers treated with PGF, due to several reasons, including prevention of turnover of the dominant follicle that emerged after synchronization. Núñez-Olivera [19] evaluated the effect of the time of PGF administration (Day 0 and Day 7 or only on Day 7 of a FTAI protocol), in a 7 d E2/P4-based FTAI protocol, on ovarian dynamics and fertility of cyclic *Bos taurus* beef heifers. No differences were detected on fertility (with PGF: 64.3 [872/1356]; without PGF: 66.5% [887/1334]), although PGF administration on both Days 0 and 7 resulted in larger follicle size at ovulation. Also, heifers that received PGF at both times and were inseminated 54 h after P4 implant removal, instead of 48 h, presented lower fertility. In our study, considering that a large portion of heifers that received the induction protocol described by Rodrigues et al. [12,13], and ovulated after that protocol would likely be on day 7-10 of the estrus cycle, a time of high efficiency of PGF to induce luteolysis [47,48]. Then, although treatment with PGF on Day 0 resulted in greater CL regression (94.4% [101/107]), considering 9 d protocols, our third hypothesis was not supported since no differences were detected in follicle diameter, expression of estrus and P/AI, consistent with Núñez-Olivera [19]. In agreement with our data, Sá Filho et al. [21] reported no differences in follicle size, ovulation frequency and P/AI between cyclic Nelore heifers receiving or not PGF at the onset of a 8d

E2/P4-based FTAI protocol. This lack of effect can be explained by the fact that all heifers received intravaginal implants with low P4 concentrations (0.5 g), in addition to eCG administration at the time of P4 implant withdrawal. Additionally, heifers that ovulated at the end of the induction protocol and were assigned to group 9dP4-PGd-2 probably underwent spontaneous luteolysis before P4 device withdrawal, leading to a greater follicle development, due to low P4 concentrations, and similar P/AI compared to the other treatments [13].

As discussed in this section, the administration of PGF an 8 d E2/P4-based FTAI protocol resulted in positive effects on ovarian dynamics of beef heifers, when compared with heifers that were not administered with PGF at this time [1]. Moreover, PGF administration at the onset of a 7 d FTAI protocol was reported not to have had effect on fertility of beef heifers [19] and there is a study that detected greater fertility in 9 d protocols than the 7 d E2/P4-based FTAI protocol in post-pubertal Nelore heifers [22]. However, Prata et al. [18] reported similar P/AI in Nelore heifers submitted to either a 7 or 8 d E2/P4-based FTAI protocols (49.1 [182/371] vs. 45.6% [169/371]), all heifers receiving PGF at the onset of the protocols, and with or without GnRH at the time of AI. Madureira et al. [20] also reported high overall fertility when Nelore heifers were synchronized with 7 d E2/P4-based FTAI protocols (55.6% [541/973]). Nevertheless, none of these studies directly compared the 7 d protocol with PGF treatment at the time of P4 implant insertion with 9 d protocols, with different times of PGF administration. Then, our fourth hypothesis was that, despite differences in expression of estrus, P/AI would be similar among groups with earlier administration of PGF. Thus, this hypothesis was supported since P/AI of group 7dP4-PG-9&-2 was similar to the other groups. The PGF administration on Day -9 of the 7dP4-PGd-9&-2 protocol may have caused luteolysis in most heifers, reducing P4 concentrations and hence decreased the potential negative effect of smaller follicles on fertility in shorter protocols [22]. Also, in our study, all heifers received FTAI 48 h after P4 implant withdrawal and this time was reported to be adequate with greater P/AI in protocols with 7 d of P4 implant [19].

In conclusion, despite earlier administration of PGF in 7 or 9 d E2/P4-based FTAI protocols altered ovarian dynamics, there were no detectable effects of these types of protocols on expression of estrus and fertility. Moreover, this is the first study that directly compared 7 d E2/P4-based FTAI protocol with PGF administered at the onset with the three main types of 9 d protocols and reported similar reproductive outcomes, allowing for flexibility in schedules for reproductive programs in Nelore heifers.

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## 4. FINAL CONSIDERATIONS

The present study was designed to evaluate reproductive outcomes of *Bos indicus* cattle, submitted to E2/P4-based FTAI protocols, considering hormonal combinations and protocol lengths. The first experiment was based on previous reports from our group that described the physiology behind the 7 d E2/P4-based protocol, in addition to fertility outcomes, comparing with other protocols. Our results, based on results of scientific trials of almost 3,000 FTAI, showed good overall P/AI (~58%) on this type of protocol and described interesting adjustments and combinations that can be done, resulting in better reproductive performance. In the second experiment, new strategies were performed aiming to improve fertility outcomes in Nelore heifers and described similar results among treatments. Finally, these results, using more than 3500 Nelore cattle (2897 cows and 831 heifers) allow for flexibility in the schedule of FTAI programs and can be used to optimize fertility outcomes in commercial operations with *Bos indicus* beef cattle.