University of São Paulo
“Luiz de Queiroz” College of Agriculture

Reproductive outcomes in progesterone-based fixed-time AI protocols in
Bos indicus

Guilherme Madureira

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

Piracicaba
2019

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Reproductive outcomes in progesterone-based fixed-time AI protocols in
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versão revisada de acordo com a resolução CoPGr 6018 de 2011

Advisor:
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DEDICATION

To my family, especially my parents that supported all my plans, even when I was not sure about my own way. Also to God, being present in my life at moments that I could not expect.

“Try not to become a man of success, but rather try to become a man of value”

Albert Einstein

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RESUMO

Parâmetros reprodutivos em protocolos de IA em tempo fixo à base de progesterona em *Bos indicus*

Com o intuito de aprofundar o conhecimento sobre protocolos hormonais de sincronização da ovulação à base de progesterona (P4) em *Bos indicus*, três estudos foram realizados. O primeiro estudo, composto por três experimentos (dois em vacas Nelore não-lactantes, e um em novilhas Nelore), baseou-se em avaliações ultrassonográficas diárias e colheitas de sangue para P4 circulante em momentos pontuais [início do protocolo (D0) e administração da primeira prostaglandina F2 α (PGF)] de protocolos à base de P4 diferindo em: indutor de emergência de onda folicular; duração do protocolo; e indutor da ovulação ao final do protocolo. A administração de 16,8 μ g de acetato de buserelina (GnRH) ao início do protocolo promoveu maiores taxas de ovulação após o D0 (~55%) e também maior número de corpos lúteos (CL) no momento da PGF. Em alguns momentos, protocolos à base de GnRH estiveram associados a antecipação da emergência de onda folicular (~1,9 d após D0) e/ou maior folículo ovulatório, bem como maior P4 circulante no momento da PGF, e pontualmente em fêmeas que ovularam após a administração deste fármaco. Não obstante, protocolos à base de benzoato de estradiol (BE) promoveram atraso na emergência da onda folicular (~2,4 d após D0), porém ambos (GnRH e BE) estiveram associados a alta taxa de sincronização da emergência de onda folicular entre D0 e D5 do protocolo (~92%). Em contrapartida, protocolos curtos (5 d de implante de P4), apenas com a inserção de implante de P4 no D0, estiveram associados à baixa taxa de emergência de onda folicular (~30%). Entretanto, as três estratégias citadas acima promoveram alta taxa de ovulação ao final do protocolo (~85%), e não diferiram em relação ao horário da ovulação. O segundo estudo, composto por dois experimentos (um em novilhas Nelore e o segundo em vacas Nelore), testou a fertilidade e variáveis fisiológicas de protocolos à base de P4 com duração de 7 d iniciando com BE (convencional) ou com GnRH (16,8 μ g de acetato de buserelina) com adaptações (eCG e PGF no D6, e uma PGF adicional no D7), e com ou sem a administração de GnRH (8,4 μ g) no momento da IATF. Ambos também receberam cipionato de estradiol (CE) no D7. Como esperado, observou-se maior taxa de ovulação (~67%) após o D0 e maior número de CL na PGF em protocolos iniciando com GnRH. A administração de BE ao início do protocolo esteve associada a maior luteólise entre D0 e PGF (~33%). No momento da retirada do implante (D7), protocolos à base de GnRH promoveram maior folículo dominante, e além disso, mais expressão de estro até a IATF. Contudo, apenas em novilhas observou-se maior folículo dominante no momento da IATF para o grupo GnRH. Ambos protocolos (iniciados com GnRH ou BE) obtiveram altas taxas de ovulação após a IATF (~92%). Em novilhas, não se observou efeito de tratamento (BE vs. GnRH no D0, com vs. sem GnRH na IATF) ou interações sobre a prenhez/IA (P/IA). Entretanto, em vacas, protocolos à base de BE obtiveram maior P/IA quando houve a administração de GnRH no momento da IATF (60,2% vs. 69,5%). Além disso, GnRH na IATF também favoreceu a P/IA de vacas que não expressaram estro durante o protocolo (Sem cio: sem GnRH 48,2% vs. com GnRH 59,1%), apesar de não ter sido observado este efeito em

novilhas. O terceiro estudo avaliou a fertilidade e dinâmica ovariana de vacas Nelore submetidas a protocolo à base de P4 com duração de 7 d utilizando 0,5 ou 1,0 mg de CE no momento da retirada do implante (D7). Apesar de 1,0 mg CE produzir menor folículo preovulatório e menor taxa de ovulação após a IA em multíparas, a P/IA foi semelhante para ambas as doses nesta categoria (multíparas: 0,5 mg = 58,4% vs. 1,0 mg = 59,0%). Contudo, primíparas tiveram menor P/IA ao receber 0,5 mg CE (primíparas: 0,5 mg = 30,2% vs. 1,0 mg = 53,6%). Portanto, os estudos permitiram um conhecimento mais refinado em relação a protocolos à base de P4 em *Bos indicus*, especialmente quanto à eficiência de protocolos de 7 d e inclusão de GnRH tanto ao início quanto ao final.

Palavras-chave: Expressão de cio; Fertilidade; GnRH; IATF; Nelore

ABSTRACT

Reproductive outcomes in progesterone-based fixed-time AI protocols in *Bos indicus*

To improve the knowledge on progesterone (P4)-based hormonal protocols for synchronization of ovulation in *Bos indicus*, three studies were performed. The first study, consisting of three experiments (two in non-lactating cows, and one in Nelore heifers), were based on daily ultrasound evaluation and blood sampling for circulating P4 at specific times [onset of the protocol (D0) and first prostaglandin F2 α (PGF) administration] of P4-based protocols differing in: inducer of the follicle wave emergence; protocol lengths; and ovulation inducer at the end of the protocol. Administration of 16.8 μ g of buserelin acetate (GnRH) at the beginning of the protocol was associated with better ovulation rate after D0 (~55%), and higher number of corpus luteum (CL) at PGF. In addition, sometimes GnRH-based protocols were associated to early emergence of the follicle wave (~1.9 d after D0) and/or bigger ovulatory follicle, as well as greater circulating P4 at the time of PGF, especially in females that ovulated after GnRH administration. Nevertheless, protocols based on estradiol benzoate (EB) promoted delayed follicular wave emergence (~2.4 d after D0), but both (GnRH and EB) were associated to satisfactory synchronization rate of follicular wave emergence between D0 and D5 (~92%). In contrast, short protocols (5 d of P4 implant) only with insertion of a P4 implant on D0 were associated to low follicle wave emergence between D0 and D5 (~30%). However, the three strategies mentioned above promoted high ovulation rate at the end of the protocol (~85%), and did not differ in relation to the time of ovulation. The second study, consisting of two experiments (one in Nelore heifers, and the second in Nelore cows), evaluated fertility and some physiological variables of P4-based 7 d protocols beginning with EB (conventional protocol) or GnRH (16.8 μ g of buserelin acetate) with adaptations (eCG and PGF on D6, and an additional PGF on D7), and with or without GnRH (8.4 μ g) administration at the time of AI. As expected, higher ovulation rate after D0 (~67%) and more CL at PGF were observed in protocols starting with GnRH. Administration of EB at the beginning of the protocol was associated to greater luteolysis rate between D0 and PGF (~33%). At the time of implant withdrawal (D7), GnRH-based protocols induced bigger dominant follicles, and more estrus expression by the time of AI. However, only in heifers it was observed a bigger dominant follicle at the time of AI for GnRH group. Both (GnRH and EB) obtained satisfactory ovulation rate after AI (~92%). In heifers, no treatment effect was observed (EB vs. GnRH on D0, with vs. without GnRH at the time of AI) or interactions on pregnancy/AI (P/AI). However, in cows EB-based protocols obtained great P/AI when GnRH was administered at the time of AI (60.2% vs. 69.5%). In addition, this administration also increased P/AI of cows that did not express estrus during the protocol (no estrus: no GnRH = 48.2% vs. GnRH = 59.1%), although this effect was not observed in heifers. The third study evaluated ovarian dynamics and fertility of Nelore cows submitted to a P4-based 7 d FTAI protocol using 0.5 or 1.0 mg of estradiol cypionate (EC) at the time of implant removal (D7). Although 1.0 mg EC induced a smaller preovulatory follicle and lower ovulation rate after AI in multiparous, the P/AI was similar for

both doses in this parity (multiparous: 0.5 mg = 58.4% vs. 1.0 mg = 59.0%). However, primiparous had lower P/AI when receiving 0.5 mg EC (primiparous: 0.5 mg = 30.2% vs. 1.0 mg = 53.6%). Therefore, both studies allowed a more refined knowledge regarding P4-based protocols in *Bos indicus*, especially on the efficiency of 7 d protocols and the inclusion of GnRH at the start or in the end of this type of synchronization protocol.

Keywords: Estrus expression; Fertility; GnRH; FTAI; Nelore

1. INTRODUCTION

The knowledge of the estrous cycle in cattle, involving circulating hormones and ovarian dynamics, made possible the control of follicles and corpus luteum (CL) development and regression in synchronization programs for fixed-time AI (FTAI) in large scale, without estrus observation.

Therefore, it is well known that follicle development in the ovaries during estrous cycles occurs in a wave-like pattern, and the start of each follicle wave is preceded by a peak in blood concentrations of FSH [1-3]. This event, called follicle wave emergence, is represented by simultaneous growth of several small follicles (~4 mm of diameter), and after 2 or 3 days, the dominant follicle of the current follicle wave assumes a different growth rate compared to their largest subordinate (deviation phase), acquiring LH receptors (LHr) on granulosa cell, and LH dependence for final development [4-6]. Under low circulating progesterone (P4), an estradiol (E2) peak promoted by the ovulatory follicle is responsible for the GnRH peak by the hypothalamus, and subsequent LH/FSH peaks by the pituitary, causing ovulation and emergence of a new follicle wave. On the other hand, high circulating P4 suppresses circulating LH pulse frequency and the growth of the dominant follicle, and consequently its E2 and inhibin secretion, promoting an FSH peak and follicle wave emergence [7]. Thus, two ways for synchrony of the follicle wave have been usually applied. The first, based on GnRH administration, acts by inducing ovulation of an adequate and growing dominant follicle [8-12]. Also, P4/E2-based protocols can be used to synchronize the follicle wave emergence by promoting atresia of the current growing dominant follicle [13]. In addition, P4-based strategies have been studied and are showing that extremely high circulating P4 (~50 ng/mL) can promote atresia of the dominant follicle and induce emergence of a new follicle wave [14].

Despite not widely used in *Bos indicus*, GnRH can be an important tool for synchronization strategies, similar to their role in Ovsynch-type protocols. Unfortunately, factors such as drug price (expensive) and unsatisfying results of previous studies including this hormone in synchronization protocols stimulated the use of P4/E2-based protocols in *Bos indicus*. Previous studies using GnRH-PGF-GnRH (Ovsynch-type) protocols in *Bos indicus* reported low ovulation rate [15] and poor fertility [16]. Thereafter, including a P4 implant during the interval between first GnRH and PGF [17-19], or associating to temporal weaning of calves targeted for fertility improvements [20, 21]. However, the unavailability of E2 in some countries requires alternative strategies. Then GnRH- and/or only P4-based protocols must be refined. In addition, 7 d P4-based FTAI protocols have been studied as a new option

for synchronization of ovulation in *Bos indicus* [22, 23], and adjustment of drugs and doses must be studied.

Thus, our proposal was to understand reproductive outcomes (follicle and luteal function, and fertility) related to distinct synchronization strategies in *Bos indicus*, varying on the drug administered at the start of the protocol, its length (5, 7, or 9 d), and ovulation inducers at the end of the protocol. Three studies were performed: the first consisted of three experiments, and the objective was to monitor daily ovarian function to improve the knowledge of P4-based FTAI protocols, and also to validate adaptations to improve fertility; the second study included ovarian dynamics and fertility outcomes related to a 7 d FTAI protocol, and the aim was to compare GnRH vs. estradiol benzoate (EB) at the start, and using or not GnRH at the time of AI in heifers and cows; the third study focused on adjustment of EC doses during a 7 d FTAI protocol and their effects on ovarian function and fertility.

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2. OVARIAN FUNCTION OF *Bos indicus* SUBMITTED TO PROGESTERONE-BASED FIXED-TIME AI PROTOCOLS

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ABSTRACT

Three experiments evaluated ovarian dynamics and circulating progesterone (P4) during P4-based protocols initiated with GnRH, estradiol benzoate (EB), or no treatment in Nelore (*Bos indicus*) cattle. Different protocol lengths (5, 7, or 9 d) and methods of ovulation induction at end of protocol (estradiol cypionate [EC] and/or GnRH) were chosen to match each protocol. In Exp 1 (n = 59 cows), a 9d EB-based protocol (D0: 2 mg EB and intravaginal P4 [1 g]; D9: P4 removal, 0.5 mg EC, 0.526 mg cloprostenol [PGF], and 300 IU equine chorionic gonadotropin [eCG]) was compared to a 7d GnRH protocol (D0: 16.8 µg buserelin acetate [GnRH] and P4 implant; D6: PGF and eCG; D7: PGF and P4 removal; D9: 8.4 µg GnRH), or to a 5d protocol (D0: P4 implant alone; D5: P4 removal, PGF, eCG, and EC; D7: GnRH). In Exp 2 (n = 55 cows), a 7d EB-based protocol was compared to a 7d GnRH-based protocol. In Exp 3 (n = 64 heifers), comparison of 7d EB-based, 7d GnRH-based, or 5d protocol was done. For all experiments, ovarian ultrasonography was performed daily from D0 until 4 d after implant withdrawal, and blood samples collected at D0 and first PGF. Effects were considered significant when $P \leq 0.05$. In Exp 1, both GnRH and EB synchronized emergence of a new follicular wave in 90% of cows, whereas the 5d protocol synchronized only 26.3%. The ovulatory follicle (OF) had slower growth (mm/d) in the 5d protocol (0.8 ± 0.07) compared to GnRH-7d (1.3 ± 0.07) and EB-9d (1.2 ± 0.07). Ovulation rate (~90%) and diameter of OF (13-14 mm) were similar. In Exp 2, G-7d cows had greater ovulation rate after D0, and number of CL and circulating P4 at PGF than EB-7d (50.0%, 1.1 ± 0.2 CL, 4.6 ± 0.5 ng/mL vs. 20.0%, 0.4 ± 0.08 CL, 1.9 ± 0.2 ng/mL). Day of OF emergence, growth rate of OF, ovulation rate (~87%), and OF size were similar between G-7d and EB-7d. In Exp 3, G-7d heifers had greater ovulation rate to D0 treatment than EB-7d or P-5d (59.1 vs. 9.5 vs. 0.0%), more CL at PGF than EB-7d (0.82 ± 0.2 vs. 0.25 ± 0.1) but not P-5d (0.48 ± 0.1), greater P4 at PGF than EB-7d or P-5d (3.8 ± 0.7 vs. 1.6 ± 0.4 vs. 1.9 ± 0.3 ng/mL), similar OF growth rate, OF diameter, and ovulation rate to protocol (~80%). In conclusion, the P-5d protocol did not synchronize follicle wave but produced similar ovulation rate at the end, whereas, GnRH and EB protocols had similar follicle dynamics but differences in CL number and P4. Based on ovarian and hormonal dynamics, each of these synchronization methods seem promising for use in FTAI, although fertility still needs to be evaluated.

Keywords: Artificial insemination, Beef cattle, GnRH, Nelore, Synchronization

2.1 Introduction

Artificial insemination (AI) in beef cattle primarily utilize fixed-time AI (FTAI) protocols that attempt to synchronize the time of ovulation at the end of the protocol [1–4]. Development of these protocols has been based on an understanding of the reproductive physiology that underlies the normal estrous cycle, particularly the regulation of bovine follicular waves, and the hormonal and follicular dynamic responses to specific hormonal treatments [5–7]. Synchronization of ovulation protocols have been based on three key aspects of ovarian physiology: synchronization of follicle wave emergence using treatments at the initiation of the protocol; control of circulating progesterone (P4) concentrations provided by the corpus luteum (CL) or from a P4 implant to regulate growth patterns of the preovulatory follicle (OF), uterine environment, and timing of estrus/ovulation; and finally a method to synchronize the time of ovulation in order to allow FTAI at the end of the protocol.

In many countries, particularly in *Bos indicus* cattle, follicle wave emergence is synchronized by treatment with estradiol (E2) products, predominantly estradiol benzoate (EB), together with treatment with an intravaginal P4 implant to inhibit circulating FSH and LH, and thereby induce atresia of growing follicles followed by a subsequent surge in FSH that induces a new follicular wave [8]. Another strategy to synchronize the emergence of a new follicular wave is treatment with gonadotropin-releasing hormone (GnRH) to induce an LH surge and ovulation of the dominant follicle (DF) followed by an FSH surge that initiates the synchronous emergence of a new follicular wave [9], despite not widely applied and achieving unsatisfactory responses in the past in *Bos indicus* [10–14]. Recently, it has also been proposed that P4 released from intravaginal implants or from an injection of P4 may also synchronize the emergence of a new follicle wave [15].

Emergence of a new follicle wave after treatment with E2 and P4 is expected to occur after ~3 d [16–20]. In contrast, treatment with GnRH in the presence of a DF larger than ~9 mm of diameter can induce ovulation within ~28 h with earlier follicle wave emergence [21,22]. However, the percentage of animals that ovulate to GnRH treatment is highly variable depending on day of the estrous cycle when GnRH is given, dose of GnRH, functional state of the potentially ovulatory follicle, and circulating P4 concentration at the time of GnRH treatment [23–25]. Daily follicular dynamics and fertility in *Bos indicus* have been reported for EB-based FTAI protocols but much less is known about their response to GnRH-based FTAI protocols.

Other aspects of FTAI protocols have also been varied in previous research trials. For example, the length of treatment with the P4 implant has varied from 5 to 9 d with few

detailed comparisons to provide the rationale for a given protocol length. The number and timing of prostaglandin F2 α (PGF) treatments have also been varied in order to assure that complete CL regression occurs at an optimal time (within the range of PGF2 α -responsiveness of the CL), in relation to other treatments in the protocol [26]. In some experiments, particularly in protocols that are initiated with GnRH, the use of two doses of PGF has been employed to assure complete regression of the CL in all animals. This strategy may be particularly important in FTAI protocols that have 7 or fewer days between the initial GnRH and final PGF treatments, due to the potential for a younger CL that may be more difficult to regress with a single PGF treatment [27–29]. Finally, two strategies have generally been employed to induce ovulation at the end of the protocol, either treatment with E2 products to induce an endogenous GnRH/LH surge or treatment with GnRH to directly induce an LH surge. The E2 compound estradiol cypionate (EC) has been commonly employed because of the delayed circulating E2 peak after EC treatment, allowing it to be used concomitant with P4 implant removal, making a more convenient protocol with the need for fewer animal handlings [30,31]. It has also been suggested that E2 supplementation may produce a more optimal hormonal environment during the proestrous period (i.e., interval between decrease of P4 and the beginning of estrus) [32–35]. In addition, treatment with GnRH at the time of AI has been used as a strategy to assure ovulation in beef cattle FTAI protocols [36,37].

Thus, the objective of the present study was to evaluate ovarian dynamics and circulating P4 profiles of *Bos indicus* heifers and non-lactating cows during P4-based FTAI protocols differing in initial treatments to synchronize follicle wave emergence (EB, GnRH, or P4 implant only), each treatment with an adjusted length and final treatment to induce ovulation. Two main hypotheses tested were: 1. The developmental profile of the OF would differ among treatments performed on D0; and 2. Despite different profiles, all protocols tested would induce a synchronized ovulation of the OF in a high percentage of cows and heifers.

2.2 Material and methods

The experiments were conducted in a commercial beef farm, located in Itatinga, SP, Brazil. Each one of the three experiments had two replicates. The females were kept on pasture (*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 # 2017.5.1618.11.9).

2.2.1 Experiment 1

A total of 59 non-lactating multiparous Nelore (*Bos indicus*) cows with an average body condition score (BCS, scale 1-5 points, using 0.25 increments) 3.0 ± 0.1 , were randomly assigned to one of three treatment groups (Fig. 1). The EB-9d (n = 20) group received 2.0 mg estradiol benzoate (EB) i.m. (Syncrogen; GlobalGen Vet Science, Jaboticabal, Brazil) and a new intravaginal implant with 1.0 g of P4 (Repro neo 1.0 g; GlobalGen Vet Science) on D0. Nine d later (D9), 0.526 mg sodium cloprostenol (PGF; Induscio; GlobalGen Vet Science), 300 IU equine chorionic gonadotropin (eCG; eCGen; GlobalGen Vet Science) and 0.5 mg EC (Cipion, GlobalGen Vet Science) were administered i.m., concomitant to the implant removal. The G-7d (n = 20) group was treated on D0 with 16.8 µg of buserelin acetate (GnRH; Maxrelin; GlobalGen Vet Science) i.m. and a new intravaginal implant containing 1.0 g P4. Six d later (D6), cows received 0.526 mg PGF and 300 IU eCG i.m. (administration of this hormone in G-groups was anticipated to D6 for prolonged time of action). After 24 h (D7), a second PGF was administered and the implant was removed. On D9, cows were treated with 8.4 µg GnRH i.m. The last group, P-5d (n = 19), received only an intravaginal implant containing 1.0 g P4 on D0. Five d later (D5), 0.526 mg PGF, 300 IU eCG and 0.5 mg EC were administered i.m., concomitant to the implant withdrawal. On D7, a final treatment with 8.4 µg GnRH was performed.

Figure 1. Design of experiment 1 with non-lactating Nelore cows submitted to three groups: P-5d (n = 19), G-7d (n = 20) or EB-9d (n = 20). Implant: 1 g of progesterone (P4); GnRH 1: 16.8 µg buserelin acetate; EB: 2 mg estradiol benzoate; PGF: 0.526 mg cloprostenol sodium; eCG: 300 IU; EC: 0.5 mg estradiol cypionate; GnRH 2: 8.4 µg buserelin acetate.

2.2.2 Experiment 2

A total of 55 non-lactating multiparous Nelore cows with BCS 3.2 ± 0.1 were randomly assigned to one of two groups (Fig. 2). The EB-7d cows ($n = 25$) were submitted to the same protocol as EB-9d from Exp. 1, however D9 treatments (implant withdrawal; PGF, eCG and EC administration) were carried out on D7. Further, an additional treatment of GnRH (8.4 μ g, i.m.) was performed on D9. The G-7d group ($n = 30$) was similar to G-7d from Exp. 1, but with administration of EC (0.5 mg, i.m.) on D7.

Figure 2. Design of experiment 2 with non-lactating Nelore cows submitted to two groups: G-7d ($n = 30$) or EB-7d ($n = 25$). Implant: 1 g of progesterone (P4); GnRH 1: 16.8 μ g buserelin acetate; EB: 2 mg estradiol benzoate; PGF: 0.526 mg cloprostenol sodium; eCG: 300 IU; EC: 0.5 mg estradiol cypionate (only used in EB-7d); GnRH 2: 8.4 μ g buserelin acetate.

2.2.3 Experiment 3

A total of 64 pubertal nulliparous Nelore heifers (~ 2 yr old) with BCS 3.0 ± 0.1 and body weight 336.9 ± 38.9 were randomly assigned to one of three groups (Fig. 3). Heifers from the EB-7d group ($n = 21$) were treated with 1.5 mg EB i.m. and an intravaginal implant containing 0.5 g P4 (Repro one 0.5 g; GlobalGen Vet Science) on D0. Seven d later (D7), 0.526 mg PGF, 200 IU eCG and 0.5 mg EC were administered i.m., concomitant to implant removal. On D9, heifers were treated with 8.4 μ g GnRH i.m. The G-7d group ($n = 22$) received 16.8 μ g GnRH i.m. and an intravaginal implant with 0.5 g P4. Six d later (D6), heifers received 0.526 mg PGF and 200 IU eCG i.m. After 24 h (D7), 0.526 mg PGF was administered i.m. and the implant was removed. On D9, heifers were treated with 8.4 μ g GnRH i.m. Finally, the P-5d group ($n = 21$) received only an intravaginal implant with 0.5 g P4 on D0. Five d later (D5), 0.526 mg PGF, 200 IU eCG and 0.5 mg EC were administered i.m., concomitant to implant withdrawal. On D7, 8.4 μ g GnRH was administered.

Figure 3. Design of experiment 3 with pubertal Nelore heifers submitted to three groups: P-5d (n = 21), G-7d (n = 22), or EB-7d (n = 21). Implant: 0.5 g of progesterone (P4); GnRH 1: 16.8 µg buserelin acetate; EB: 1.5 mg estradiol benzoate; PGF: 0.526 mg cloprostenol sodium; eCG: 200 IU; EC: 0.5 mg estradiol cypionate; GnRH 2: 8.4 µg buserelin acetate.

2.2.4 Combined data from experiments in which animals were treated with GnRH on D0

Data from G-7d groups during all experiments were combined in order to study the outcomes related to the use of GnRH at the onset of FTAI protocols in *Bos indicus*. In this analysis, females that ovulated after D0 of the protocol (Ovulated, n = 38) were compared to those that did not ovulate (Not ovulated, n = 34) to investigate patterns of follicular and luteal development, and circulating P4 concentrations.

2.2.5 Individual, typical and atypical patterns

Excluding cows (Exp. 1 and 2) and heifers (Exp. 3) that did not ovulate at the end of the protocol, the distribution of individual patterns of each OF within synchronization strategies was initially described. Thereafter, we selected typical and atypical patterns of cows or heifers to illustrate a variety of physiological responses that have occurred throughout the protocols.

2.2.6 Ultrasound examinations, blood sampling and P4 assay

Transrectal ultrasound ovarian examinations in B-mode with a 7.5 Mhz linear transducer (DP-2200 VET, Mindray, Shenzhen, China) were performed daily (24 h interval), from the beginning of the protocol (D0) until 4 d after P4 implant removal. All follicles and CL presenting diameter ≥ 5 mm and ≥ 10 mm, respectively, were measured and recorded.

Thus, ovulation rate after D0 was determined by the disappearance of the DF and the development of a new CL. At the end of the protocol, ovulation rate was considered by disappearance of the DF. The day of follicle wave emergence was defined by a retrospective evaluation of the DF to the time when it was ~4 mm. Turn-over of the follicle wave was considered in cases that a DF emerged between D0 and D5 of the protocol, then it became atretic, followed by a new follicle wave emergence.

On D0 and at the first PGF treatment (D5, D6, D7, or D9 depending on the experiment and group) of the synchronization protocol, blood samples were collected by puncture of the jugular vein into evacuated tubes containing heparin sodium (Vacutainer, Dickinson, Franklin Lakes, NJ). Immediately after collection, the tubes were placed on ice and kept refrigerated until processing. Blood samples were centrifuged right after the end of collections at 1,700 x g for 15 min and aliquots of plasma were frozen and stored in duplicates at -20°C until assayed for P4.

Concentrations of P4 were determined using a solid-phase RIA kit containing antibody-coated tubes and ¹²⁵I-labeled P4 (ImmuChem Coated Tube P4 125 RIA Kit, MP Biomedicals, Costa Mesa, CA) validated for bovine plasma in our laboratory as reported [29]. The intra- and inter-assay CVs and the sensitivity were 5.3%, 8.6%, and 0.08 ng/mL, respectively.

2.2.7 Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC), and all experiments were done in a completely randomized design.

Discrete variable responses were analyzed using the generalized linear mixed model (GLIMMIX procedure) fitting a binary (ovulation rate after D0, follicle wave emergence between D0 and D5, ovulation rate at the end of the protocol, and time of ovulation after the end of the protocol) and exponential distribution (number of CL at PGF, and day of follicle wave emergence). Continuous variable responses were analyzed using the linear mixed models (MIXED procedure). All variable responses (plasma P4 concentration on D0 and at PGF, and growth rate and maximum diameter of the DF) were tested for normality of the residuals using the Shapiro-Wilk statistic method obtained by PROC UNIVARIATE procedure of SAS. When non-normality of the data was detected (it happened just for circulating P4 concentration data), data was transformed by log. If the normality through the

previously mentioned transformation method was not achieved, then nonparametric analysis for ranked transformed data was performed with the RANK procedure of SAS.

The selection of the model that best fitted each variable response of interest was performed by finding the model with the lowest value for the *Akaike Information Criterion Corrected* (AICC) using the backward elimination procedure that removed independent variables with $P > 0.10$ from the model. Treatment was considered as fixed effect, and tested covariates were replica, BCS on D0 of the protocol, and presence of CL on D0.

Differences were considered significant for $P \leq 0.05$, whereas a tendency was designated when $P \leq 0.10$ and $P > 0.05$. The results are expressed as least squares means $\pm$ standard error of the mean (LSM $\pm$ SEM), unless otherwise indicated.

2.3 Results

2.3.1 Experiment 1

Results regarding Exp. 1 are described on Table 1. Cows from G-7d group presented greater ovulation rate after D0 than EB-9d and P-5d groups, and greater number of CL at PGF compared to EB-9d, but both were similar to P-5d. Circulating P4 concentration on D0 and at PGF were similar among groups, despite a tendency for lower P4 concentration at PGF for EB-9d. When we analyzed the G-7d group separately, circulating P4 concentration on D0 and at PGF was similar for cows that ovulated or not after D0. The percentage of cows presenting follicle wave emergence between D0 and D5 was lower for P-5d compared to G-7d and EB-9d groups. The G-7d group tended to emerge the follicle wave earlier than the EB-9d. Despite the lower growth rate of the ovulatory follicle (OF) for P-5d compared to G-7d (regardless of ovulation or not after D0) and EB-9d, the maximum diameter of the OF was similar among groups. In addition, ovulation rate at the end of the protocol was similar among the three protocols. No differences were detected for time of ovulation between groups in 48 to 72 and 73 to 96 h intervals (Fig. 4). One cow from G-7d [5.6% (1/18)] and three from EB-9d group [16.7% (3/18)] presented turn-over of the DF.

Table 1. Ovarian dynamics and circulating P4 concentration of non-lactating Nelore (*Bos indicus*) cows from experiment 1 (P-5d: only P4-based protocol with 5-d of P4 implant; G-7d: GnRH-based protocol with 7-d of P4 implant; EB-9d: EB-based protocol with 9-d of P4 implant).

	P-5d	G-7d	EB-9d	P-value
Ovulation rate after D0, % (n/n)	5.3 ^b (1/19)	50.0 ^a (10/20)	5.0 ^b (1/20)	0.005
P4 at D0, ng/mL (n)	3.1 ± 0.9 (19)	2.3 ± 0.7 (19)	1.3 ± 0.5 (16)	0.3
P4 at D0 according to ovulation after D0, ng/mL (n) ¹	-	Yes (10) 2.0 ± 1.0	No (9) 2.6 ± 1.1	0.2
CL number at PGF (n)	0.74 ± 0.2 ^{ab} (19)	1.0 ± 0.2 ^a (20)	0.45 ± 0.1 ^b (20)	0.05
P4 at PGF, ng/mL (n)	3.2 ± 0.5 (19)	3.3 ± 0.7 (19)	2.6 ± 0.6 (17)	0.06
P4 at PGF according to ovulation after D0, ng/mL (n) ¹	-	Yes (10) 4.3 ± 1.1	No (9) 2.2 ± 0.6	0.4
Follicle wave emergence between D0 and D5, % (n/n)	26.3 ^b (5/19)	90.0 ^a (18/20)	90.0 ^a (18/20)	0.0003
Day of follicle wave emergence (n) ²	1.6 ± 0.7 (5)	1.4 ± 0.3 (18)	2.7 ± 0.6 (18)	0.1
Day of follicle wave emergence according to ovulation after D0 (n) ¹	-	Yes (10) 1.5 ± 0.5	No (8) 1.3 ± 0.4	0.7
Growth rate of the ovulatory follicle, mm/d (n) ³	0.8 ± 0.07 ^b (18)	1.3 ± 0.07 ^a (18)	1.2 ± 0.07 ^a (17)	<0.0001
Maximum diameter of the OF, mm (n) ³	13.0 ± 0.4 (18)	14.0 ± 0.4 (18)	13.4 ± 0.4 (17)	0.2
Maximum diameter of the OF according to ovulation D0, mm (n) ¹	-	Yes (9) 13.6 ± 0.6	No (9) 14.5 ± 0.6	0.3
Ovulation rate at the end of the protocol, % (n/n)	94.7 (18/19)	90.0 (18/20)	85.0 (17/20)	0.6

¹Analysis considering only G-7d group, comparing cows that ovulated or not after D0 of the protocol.

²Analysis of cows with follicle wave emergence between D0 and D5.

³Analysis of cows that ovulated at the end of the protocol; OF = ovulatory follicle.

Figure 4. Proportion of non-lactating Nelore (*Bos indicus*) cows from experiment 1 (P-5d: only P4-based protocol with 5-d of P4 implant; G-7d: GnRH-based protocol with 7-d of P4 implant; EB-9d: EB-based protocol with 9-d of P4 implant) with ovulation at the end of the protocol between 48 to 72 and 73 to 96 h after P4 implant removal.

2.3.2 Experiment 2

Results related to Exp. 2 are described in Table 2. Ovulation rate after D0 and number of CL at PGF was greater for G-7d group. Despite similar circulating P4 on D0 between protocols, cows from G-7d group that ovulated after D0 had lower P4 concentration on D0 compared to G-7d cows without ovulation. Circulating P4 at PGF was more than two fold greater for G-7d compared to EB-7d. There was no difference between P4 concentration at PGF for G-7d cows that ovulated or not after D0. The percentage of cows emerging the follicle wave between D0 and D5, as well as the day of follicle wave emergence were similar between both protocols. Also, no difference was found for growth rate of the OF and its maximum diameter among groups. As expected, cows from G-7d group that ovulated after D0 presented earlier day of follicle wave emergence and greater OF compared to cows without ovulation after D0. Ovulation rate at the end of the protocol for G-7d and EB-7d was similar. No difference was detected for time of ovulation (Fig. 5). Three cows from G-7d [10.7% (3/28)] and none from EB-7d group [0% (0/24)] had turn-over of the DF.

Table 2. Ovarian dynamics and circulating P4 concentration of non-lactating Nelore (*Bos indicus*) cows from experiment 2 (G-7d: GnRH-based protocol with 7-d of P4 implant and without estradiol cypionate at implant removal; EB-7d: EB-based protocol with 7-d of P4 implant).

	G-7d		EB-7d	P-value
Ovulation rate after D0, % (n/n)	50.0 (15/30)		20.0 (5/25)	0.02
P4 at D0, ng/mL (n)	3.8 ± 0.7 (29)		3.0 ± 0.7 (20)	0.4
P4 at D0 according to ovulation after D0, ng/mL (n) ¹	Yes (15) 2.3 ± 0.8	No (14) 5.3 ± 0.9	-	0.009
CL number at PGF (n)	1.1 ± 0.2 (30)		0.4 ± 0.08 (25)	0.0007
P4 at PGF, ng/mL (n)	4.6 ± 0.5 (29)		1.9 ± 0.2 (24)	<.0001
P4 at PGF according to ovulation after D0, ng/mL (n) ¹	Yes (15) 4.3 ± 0.7	No (14) 4.8 ± 0.8	-	0.6
Follicle wave emergence between D0 and D5, % (n/n)	93.3 (28/30)		96.0 (24/25)	0.7
Day of follicle wave emergence (n) ²	2.0 ± 0.4 (28)		2.2 ± 0.5 (24)	0.8
Day of follicle wave emergence according to ovulation after D0 (n) ¹	Yes (14) 1.2 ± 0.3	No (14) 2.9 ± 0.8	-	0.03
Growth rate of the ovulatory follicle, mm/d (n) ³	1.2 ± 0.04 (23)		1.2 ± 0.05 (23)	0.6
Maximum diameter of the OF, mm (n) ³	12.9 ± 0.3 (23)		12.5 ± 0.3 (23)	0.4
Maximum diameter of the OF according to ovulation D0, mm (n) ¹	Yes (10) 13.7 ± 0.3	No (13) 12.3 ± 0.4	-	0.02
Ovulation rate at the end of the protocol, % (n/n)	83.3 (25/30)		92.0 (23/25)	0.4

¹Analysis considering only G-7d group, comparing cows that ovulated or not after D0 of the protocol.

²Analysis of cows with follicle wave emergence between D0 and D5.

³Analysis of cows that ovulated at the end of the protocol; OF = ovulatory follicle.

Figure 5. Proportion of non-lactating Nelore (*Bos indicus*) cows from experiment 2 (G-7d: GnRH-based protocol with 7-d of P4 implant and without estradiol cypionate at implant removal; EB-7d: EB-based protocol with 7-d of P4 implant) with ovulation at the end of the protocol between 48 to 72 and 73 to 96 h after P4 implant removal.

2.3.3 Experiment 3

Results from Exp. 3 are described in Table 3. Ovulation rate after D0 was greater for G-7d heifers than for EB-7d and P-5d. Circulating P4 concentration on D0 was similar among groups, regardless if ovulation occurred or not on G-7d. Number of CL at PGF was greater for G-7d compared to EB-7d, but both were similar to P-5d heifers. Circulating P4 concentration at PGF was greater for G-7d compared to other groups, with no difference between heifers with or without ovulation after D0 in G-7d group. Heifers with follicle wave emergence between D0 and D5 was lower for P-5d compared to G-7d and EB-7d groups. The day of follicle wave emergence, the growth rate of the OF and its maximum diameter were similar among protocols. Heifers from G-7d group that ovulated or not after D0 had similar day of follicle wave emergence and diameter of the OF. Ovulation rate at the end of the protocol was similar for G-7d, EB-7d and P-5d. There was a tendency for greater ovulation rate at 48 to 72 h interval for P-5d compared to G-7d group, as well as at 73 to 96 h interval for G-7d compared to P-5d group (Fig. 6). One heifer from EB-7d [5.3% (1/19)] and none from the other groups [G-7d: 0% (0/20); P-5d: 0% (0/7)] had turn-over of the DF.

Table 3. Ovarian dynamics and circulating P4 concentration of pubertal Nelore (*Bos indicus*) heifers from experiment 3 (P-5d: only P4-based protocol with 5-d of P4 implant; G-7d: GnRH-based protocol with 7-d of P4 implant; EB-7d: EB-based protocol with 7-d of P4 implant).

	P-5d	G-7d		EB-7d	P-value
Ovulation rate after D0, % (n/n)	0.0 (0/21)	59.1 ^a (13/22)		9.5 ^b (2/21)	0.01
P4 at D0, ng/mL (n)	2.1 ± 0.5 (21)	2.4 ± 0.5 (22)		2.9 ± 0.6 (21)	0.8
P4 at D0 according to ovulation after D0, ng/mL (n) ¹	-	Yes (13) 2.7 ± 0.7	No (9) 2.1 ± 0.8	-	0.6
CL number at PGF (n)	0.48 ± 0.1 ^{ab} (21)	0.82 ± 0.2 ^a (22)		0.25 ± 0.1 ^b (21)	0.001
P4 at PGF, ng/mL (n)	1.9 ± 0.3 ^b (21)	3.8 ± 0.7 ^a (22)		1.6 ± 0.4 ^b (21)	<.0001
P4 at PGF according to ovulation after D0, ng/mL (n) ¹	-	Yes (13) 3.8 ± 1.1	No (9) 3.9 ± 1.0	-	0.7
Follicle wave emergence between D0 and D5, % (n/n)	33.3 ^b (7/21)	90.9 ^a (20/22)		90.5 ^a (19/21)	0.0004
Day of follicle wave emergence (n) ²	2.1 ± 0.4 (7)	2.3 ± 0.2 (20)		2.2 ± 0.2 (19)	1.0
Day of follicle wave emergence according to ovulation after D0 (n) ¹	-	Yes (11) 1.6 ± 0.5	No (9) 3.0 ± 1.0	-	0.2
Growth rate of the ovulatory follicle, mm/d (n) ³	1.0 ± 0.1 (16)	1.1 ± 0.1 (18)		1.1 ± 0.1 (16)	0.4
Maximum diameter of the OF, mm (n) ³	13.4 ± 0.4 (16)	12.8 ± 0.3 (18)		12.2 ± 0.4 (16)	0.07
Maximum diameter of the OF according to ovulation D0, mm (n) ¹	-	Yes (12) 13.0 ± 0.3	No (6) 12.3 ± 0.6	-	0.4
Ovulation rate at the end of the protocol, % (n/n)	81.0 (17/21)	81.8 (18/22)		76.2 (16/21)	0.9

¹Analysis considering only G-7d group, comparing heifers that ovulated or not after D0 of the protocol.

²Analysis of heifers with follicle wave emergence between D0 and D5.

³Analysis of heifers that ovulated at the end of the protocol; OF = ovulatory follicle.

Figure 6. Proportion of pubertal Nelore (*Bos indicus*) heifers from experiment 3 (P-5d: only P4-based protocol with 5-d of P4 implant; G-7d: GnRH-based protocol with 7-d of P4 implant and without estradiol cypionate at implant removal; EB-7d: EB-based protocol with 7-d of P4 implant) with ovulation at the end of the protocol before 48, between 48 to 72, and 73 to 96 h after P4 implant removal.

2.3.4 Combined data from experiments in which animals were treated with GnRH on D0

Comparing ovulated and not ovulated outcomes from all G-treatments (Table 4), there was a tendency for higher circulating P4 on D0 for not ovulated group, not differing at PGF. The percentage of animals with follicle wave emergence between D0 and D5 was similar between groups, being higher than 90%. Ovulated group had greater number of CL at PGF and earlier follicle wave emergence compared to not ovulated group. The OF tended to be smaller for not ovulated animals.

Table 4. Ovarian dynamics and circulating P4 concentration of G-treatment from all experiments (combined data).

	Ovulated	Not ovulated	P-value
P4 at D0, ng/mL (n)	2.4 ± 0.5 (38)	3.6 ± 0.6 (32)	0.09
CL number at PGF (n)	1.3 ± 0.2 (38)	0.7 ± 0.1 (34)	0.01
P4 at PGF, ng/mL (n)	4.1 ± 0.5 (38)	3.8 ± 0.5 (32)	0.5
Follicle wave emergence between D0 and D5, % (n/n)	92.1 (35/38)	91.2 (31/34)	0.9
Day of follicle wave emergence (n) ¹	1.4 ± 0.2 (35)	2.5 ± 0.4 (31)	0.03
Maximum diameter of the OF, mm (n) ²	13.5 ± 0.3 (31)	12.8 ± 0.3 (28)	0.08

¹Analysis of heifers and cows with follicle wave emergence between D0 and D5.

²Analysis of heifers and cows that ovulated at the end of the protocol; OF = ovulatory follicle.

2.3.5 Individual, typical and atypical patterns

All individual patterns throughout each synchronization protocol were illustrated (Fig. 7) for an overview of the different synchronization strategies and the development of the OF.

Figure 7. Individual patterns of the ovulatory follicle observed throughout different synchronization protocols. A: patterns for P-5d groups (only P4-based protocol with 5-d of P4 implant). B: patterns for G-7d (GnRH-based protocol with 7-d of P4 implant). C: patterns for EB-7d (EB-based protocol with 7-d of P4 implant). D: patterns for EB-9d (EB-based protocol with 9-d of P4 implant). Gray lines represent pubertal heifers, and black line represents non-lactating cows.

The P-5d protocol was based on the maintenance of the growing DF until ovulation at the end (Fig. 8A). Atypical patterns reported for this protocol were: 1. Synchronization of follicle wave emergence by ovulation after D0 (Fig. 9A); 2. Synchronization of follicle wave emergence by atresia of a DF (Fig. 9B).

Figure 8. Typical patterns observed throughout different synchronization protocols. Corpus luteum (gray lines with blanc squares), follicular development (first follicle [black line with solid circle], second follicle [black line with solid triangle] and third follicle [black line with solid square]) and P4 concentration (grey column on D0 and at PGF administration) were illustrated. A: typical pattern for P-5d groups (only P4-based protocol with 5-d of P4 implant). B: typical pattern for G-7d (GnRH-based protocol with 7-d of P4 implant). C: typical pattern for EB-7d (EB-based protocol with 7-d of P4 implant). D: typical pattern for EB-9d (EB-based protocol with 9-d of P4 implant).

Figure 9. Atypical patterns observed throughout P-5d protocol (only P4-based protocol with 5-d of P4 implant). Corpus luteum (gray lines with blanc squares), follicular development (first follicle [black line with solid circle], second follicle [black line with solid triangle] and third follicle [black line with solid square]) and P4 concentration (grey column on D0 and at PGF administration) were illustrated.

In GnRH-based protocols it was considered typical when cattle ovulated after D0 and had a new follicle wave emerging between 0 and 5 d after (Fig. 8B). Ovarian atypical patterns were reported: 1. No synchronization of follicle wave emergence (Fig. 10A); 2. No synchronization of follicle wave through the recovery of the subordinate follicle (Fig. 10B); 3. Synchronization of follicle wave through atresia of a growing DF (Fig. 10C).

Figure 10. Atypical patterns observed throughout G-7d protocol (GnRH-based protocol with 7-d of P4 implant). Corpus luteum (gray lines with blanc squares), follicular development (first follicle [black line with solid circle], second follicle [black line with solid triangle] and third follicle [black line with solid square]) and P4 concentration (grey column on D0 and at PGF administration) were illustrated.

Typical pattern for protocols based on EB and P4 (EB-7d and EB-9d) was atresia of the growing DF, followed by follicle wave emergence ~3 d (0 to 5 d) after of the beginning of the protocol (Fig. 8C and 8D). Atypical patterns described were: 1. Synchronization of follicle wave emergence by ovulation after D0 (Fig. 11A and 12A); 2. No synchronization of follicle wave emergence (Fig. 11B and 12B); 3. Turn-over of the synchronized follicle wave (Fig. 11C and 12C).

Figure 11. Atypical patterns observed throughout EB-7d (EB-based protocol with 7-d of P4 implant). Corpus luteum (gray lines with blanc squares), follicular development (first follicle [black line with solid circle], second follicle [black line with solid triangle] and third follicle [black line with solid square]) and P4 concentration (grey column on D0 and at PGF administration) were illustrated.

Figure 12. Atypical patterns observed throughout EB-9d (EB-based protocol with 9-d of P4 implant). Corpus luteum (gray lines with blanc squares), follicular development (first follicle [black line with solid circle], second follicle [black line with solid triangle] and third follicle [black line with solid square]) and P4 concentration (grey column on D0 and at PGF administration) were illustrated.

2.4 Discussion

Our first hypothesis consisted of different developmental patterns of the OF produced by the synchronization strategies. The hypothesis was supported due to the expected distinct pharmacological action of each treatment confirmed by daily evaluation of ovarian dynamics. Impacted by the age of the OF, females from G-groups from all experiments presented a satisfactory ovulation rate ($\geq 50\%$) after D0 treatment. These results might be explained based on the dose of GnRH that was administered on D0 of the protocol (16.8 μg buserelin acetate). Considering that a higher dose of GnRH induces a greater LH peak even under high circulating P4, it is expected to have an adequate ovulatory response [23,24]. Even though, during Exp. 2, cows that did not ovulate to this GnRH dose had higher circulating P4 on D0 than cows that ovulated, suggesting the blockage of ovulation even when higher GnRH dose is used. Although ovulation to the treatment on D0 was not expected for the other groups, $\sim 10\%$ of the females ovulated. It could be due to the day of the estrous cycle at the beginning of the protocol, or in EB-groups, caused by an inducer effect of the E2 [39,40]. As the number of studies related to GnRH-based protocols in *Bos indicus* are limited, these results will improve the knowledge in this area.

Number of CL at PGF was greater for G compared to EB-groups for the three experiments due to greater ovulation rate after D0 for G-groups. In addition, it is important to consider that administration of EB on D0 of the protocol can induce luteolysis, reducing the number of CL at PGF [39,40]. Consistent with this idea, the P-groups had an intermediate number of CL at PGF, probably because in this protocol there was no administration of EB on D0 and no luteolytic effect of this hormone. Besides the number of CL, circulating P4 concentration at PGF was greater for G-groups on Exp. 2 and 3, but not on Exp. 1. It might be due to the increase in P4 concentration between D0 and PGF for EB group (1.3 ± 0.5 to 2.6 ± 0.6 ng/mL) on Exp. 1. Curiously, only in Exp. 1 the P4 concentration at PGF was higher for cows from G-group that ovulated after D0 compared to the ones that did not ovulate. It can be partially explained in function of the higher P4 on D0 for cows that did not ovulate after D0 on Exp. 2, and for Exp. 3, it may be related to the phase of the estrous cycle that heifers were on D0 of the protocol.

Although the E2-based protocols are widely applied especially in South America for beef cattle, in several countries this hormone is not used. Therefore, development of efficient protocols that replace E2 by another product, such as GnRH, or even just not use any of them on D0, such as the protocol with 5 d of implant, is necessary for *Bos indicus*. In the present study, synchronization strategies based on EB or GnRH administration at the onset of the

protocol had similar efficiency regarding to induction of follicle wave emergence ($\geq 90\%$). As expected, P-groups were not able to induce follicle wave emergence, except in females that were at their natural time for follicle emergence on D0 of the protocol ($\sim 30\%$). The increase in circulating P4 caused by the insertion of one implant containing 0.5 or 1.0 g of P4 was not sufficient to induce atresia of the DF. In contrast, Cavalieri [15] induced follicle wave emergence by atresia of the DF using intravaginal implants in *Bos indicus* heifers. However, the implants used in this study contained 3.12 g of P4 and reached a peak of ~ 50 ng/mL of circulating P4.

On average, the day of follicle wave emergence was similar between G and EB-groups, except on Exp. 1 in which G-group tended to have emergence earlier than EB-group (1.4 ± 0.3 vs. 2.7 ± 0.6 d; $P = 0.1$). Despite of that, cows from G-group of Exp. 2 that ovulated after D0 had earlier follicle wave emergence compared to those that did not. Thus, the mean value was similar between G and EB-groups, probably in function of the cows with follicle wave emergence that was not promoted by ovulation after D0. On Exp. 3, no difference was detected for day of wave emergence, including the analysis of heifers that ovulated or not from G-group (mean day 1.6 ± 0.5 vs. 3.0 ± 1.0 , respectively), maybe due to the variation observed in heifers that did not ovulate after D0.

Follicle growth rate in P-group from Exp. 1 was lower compared to the other groups, whereas heifers from this group on Exp. 3 had follicle growth rate similar to the others. This result may be associated to the early follicle wave emergence (1.6 ± 0.7 d) combined to the low number of cows with wave emergence [26.3% (5/19)], reducing the follicle growth rate on Exp. 1. In addition, during all experiments, no difference was detected for the maximum diameter of the OF. This result was expected in function of the similar day of follicle wave emergence. When G-groups were analyzed based on ovulation rate after D0 on Exp. 2, an earlier day of follicle emergence promoted a greater OF. This effect was not observed during Exp. 1 and 3 because the day of follicle wave emergence was similar for cows with or without ovulation on Exp. 1, and on Exp. 3 maybe in function of the low number of heifers that did not ovulate after D0 [with ovulation ($n = 12$) vs. without ovulation ($n = 6$)].

Our second hypothesis it was supported due to the similar ovulation rate and time of ovulation at the end of the synchronization protocols. This result is especially important for the P-groups, because it is a novel and shorter duration protocol, which does not require EB or GnRH on D0. However, the fertility of the OF that emerged before the onset of P-based protocol is unknown and needs to be tested. Although in *Bos taurus*, it has been shown that persistent follicles result in poor embryo quality, and reduced fertility and CL lifespan

[25,41–44], preliminary data from our lab is not showing low fertility in Nelore cows that ovulated persistent follicles (13-d old follicle developed during a controlled experimental design).

To confirm the patterns observed during GnRH-based protocols, combined data from G-treatment of all experiments (Table 4) resulted in a complete evaluation of the efficacy of GnRH-based protocols in this study. By comparing females ovulated or not ovulated after D0, it was possible to identify that not ovulated group had a tendency ($P = 0.09$) to higher P4 concentration on D0. Therefore, it might explain the similar P4 concentration at PGF for both groups, despite an increase in P4 concentration from D0 to PGF for ovulated group. Although the high dose of GnRH administered on D0 was sufficient to promote a satisfactory ovulation rate, the P4 concentration on D0 still seems to interfere in this process. As expected, ovulated group had greater number of CL at PGF in function of ovulation of the DF after D0. Furthermore, these animals had an earlier follicle wave emergence compared to not ovulated because administration of GnRH in females with a growing DF on D0 induces LH and FSH peak approximately 2 h after administration [45]. Regarding OF, ovulated animals presented a tendency ($P = 0.08$) for greater OF, probably in function of the earlier follicle wave emergence in this group, resulting in more time for the growth of the OF.

It is important to remind that all synchronization strategies had distinct responses to the pharmacologic association that was applied, as proved during our first hypothesis. However, atypical patterns were detected in specific females during each protocols and despite of the low incidence of atypical patterns, to study these cases may help in adjustments of them. Thus, at first, individual patterns of each female that ovulated the OF at the end of the protocol were included into a figure to illustrate the “overview pattern” of the protocols (Fig. 7) and it is clear that despite the high incidence of some OF patterns, all treatments provided individual responses that did not represent what it was expected for the respective protocol.

Exploring P-5d responses during Exp. 1 and 3, typical patterns were observed (Fig. 8A). In this example, despite of high P4 concentration, this short protocol kept the DF growing, resulting in its ovulation at the end. In addition, atypical patterns as synchronization of the follicle wave emergence by a natural ovulation (Fig. 9A), probably due to the phase of the estrous cycle at the start of this protocol, were reported. Specifically, in this case, despite ovulation at the end of the protocol, sub-luteal circulating P4 could decrease the chance to become pregnancy, if this female it was inseminated. Other example of atypical pattern is the atresia of the DF (Fig. 9B) followed by follicle wave emergence, showing that even during a

short protocol (5 d of P4 implant), the current growing follicle can undergo atresia causing new follicle wave emergence. In addition, typical patterns of G-7d groups was represented by ovulation of the DF and early follicle wave emergence after D0, as shown in Fig. 8B. However, in the same protocol, it was observed a cow that did not synchronize the follicle wave emergence between D0 and D5 (Fig. 10A) because of the absence of a responsive DF at GnRH administration on D0. At this moment there was a regressing DF and a second follicle that was growing but still small for ovulation induced by GnRH. Despite of that, this cow ovulated an older follicle at the end of this protocol. Furthermore, it was reported a heifer that ovulated after D0 but did not had follicle wave emergence between D0 and D5 (Fig. 10B). This pattern had been discussed by Sartori et al. [22] and it seems likely that LH may reduce circulating E2 concentration allowing a new FSH surge to recover the subordinate follicle of the last follicle wave. Finally, there was another cow that synchronized the follicle wave emergence by atresia of the DF instead by ovulation (Fig. 10C), probably due to the high circulating P4 on D0 that may have negatively interfered in ovulation at the start of this protocol.

Regarding to EB-9d, from Exp. 1, or EB-7d from Exp. 2 and 3, it was expected that the EB associated to P4 would induce atresia of the growing DF and stimulating, in this way, a new follicle wave emergence around D3 of the protocol (Fig. 8C and 8D). Atypical patterns for these protocols were reported, as the synchronization of follicle wave emergence by ovulation of the DF at the start of this protocol (Fig. 11A and 12A). This ovulation could be caused by the phase of the estrous cycle at the beginning of the protocol, or by an inducer effect of the EB. Despite of that, in both cases the follicle wave was synchronized and the cow ovulated at the end of the protocol. In contrast, it was observed cows that did not synchronize the follicle wave emergence and remained with the current DF until ovulation at the end of the protocol (Fig. 11B) or until D6 of the protocol, when it was detected a new follicle wave emergence (Fig. 12B). The control of the growing DF may be due to the circulating P4, and in the first example, low circulating P4 associated to a shorter protocol (7 d of P4 implant) supported the development of the DF until the end of the protocol, and high circulating P4 associated to a longer protocol (9 d of P4 implant), on the second example, caused his atresia and consequently the start of a new follicle wave. Of particular interest, two examples of turn-over of the synchronized follicle wave were represented (Fig. 11C and 12C). Curiously, the first example illustrates a heifer with low circulating P4 reporting the turn-over of the synchronized follicle wave, and failing to ovulate at the end of the protocol. However, the high P4 concentration in the second example, especially during a long protocol (9 d of P4

implant), may reduce the capacity of the growing DF to reach the end of the protocol and ovulate.

In conclusion, the present study has described the physiology behind GnRH, EB/P4 and P4-based protocols. Moreover, despite each of the protocols evaluated provided distinct ovarian function, supporting our first hypothesis, all showed to be promising for FTAI in beef cattle due to the synchronized ovulation at the end of the protocols, also confirming our second hypothesis. Of particular interest, P-5d protocol did not synchronize follicle wave emergence but produced similar and synchronized ovulation rate at the end of the protocol as others. Also, G-7d protocol had similar outcomes compared to EB-P4 protocols. Nevertheless, the fertility of these protocols should be evaluated.

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3. HORMONAL ASSOCIATIONS FOCUSED ON OPTIMIZING FERTILITY IN *Bos indicus* SUBMITTED TO 7-D PROGESTERONE-BASED FIXED-TIME AI PROTOCOLS

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ABSTRACT

The aim was to study ovarian dynamics and fertility of Nelore cattle submitted to 7-d progesterone (P4)-based fixed-time AI (FTAI) protocols using different hormonal associations. In Exp.1, 2 yr old heifers (n = 973) were randomly assigned to one of four treatments: EB-0 (estradiol benzoate, EB on D0 and no GnRH at AI), EB-G (EB on D0 and GnRH at AI), G-0 (GnRH on D0 and no GnRH at AI), or G-G (GnRH on D0 and at AI). On D0, heifers received an intravaginal P4 implant (0.5 g) for 7 d and EB (1.5 mg) or GnRH (16.8 µg). On D7, implant was withdrawn and heifers received cloprostenol (PGF; 0.530 mg) and estradiol cypionate (EC, 0.5 mg). Heifers in G groups also had a PGF and eCG (200 IU) on D6, whereas EB heifers received eCG on D7. At FTAI on D9, only EB-G and G-G groups received GnRH (8.4 µg). In Exp. 2, cows (n = 804) received the same treatments (EB-0, EB-G, G-0, or G-G) using different P4 implants (1.0 g) and doses of EB (2.0 mg) and eCG (300 IU). Therefore, for both experiments, when groups were denominated EB, it means EB on D0 and PGF, EC, and eCG on D7. When groups were called G, it means they were treated with GnRH on D0, PGF and eCG on D6 and PGF and EC on D7. Effects were considered significant when $P \leq 0.05$. After treatment on D0, GnRH had more ovulations than EB in heifers [60.3 (287/476) vs. 12.7% (63/497)] and cows [73.7 (83/112) vs. 24.4% (28/113)]. Luteolysis after D0 was greater in EB than G in heifers [39.2 (159/406) vs. 20.0% (77/385)] and cows [25.5 (14/55) vs. 1.6% (1/64)]. Heifers in G had larger follicles (mm) than EB on D7 (10.3 ± 0.2 vs. 9.2 ± 0.2) and D9 (11.9 ± 0.2 vs. 11.3 ± 0.2). Cows had larger follicles in G than EB on D7 (11.0 ± 0.3 vs. 9.9 ± 0.3) but not at AI. Estrus was greater in G than EB for heifers [80.3 (382/476) vs. 69.6% (346/497)] and cows [67.6 (270/400) vs. 56.2% (227/404)]. There were no treatment effects on pregnancy per AI (P/AI) in heifers [EB-0 = 56.7 (139/245), EB-G = 53.6 (135/252), G-0 = 52.6 (127/241), and G-G = 57.5% (135/235)]. However, cows from EB-G had greater P/AI than EB-0 [69.5 (142/204) vs. 60.2% (120/200)] with intermediate P/AI for G-0 [62.7% (127/203)] and G-G [60.9% (120/197)]. For P/AI in heifers, there was no interaction of GnRH at AI with estrus expression, however cows that did not display estrus had greater P/AI if they received GnRH at AI [GnRH = 59.1 (91/154) vs. No GnRH = 48.2% (78/162)]. Thus, protocols evaluated in this study, based on EB or GnRH for *Bos indicus* heifers and cows had differing ovarian dynamics but similar overall fertility, enabling the use of any of them for reproductive programs. Treatment with GnRH at time of AI increased fertility in some instances in *Bos indicus* cows but not in heifers.

Keywords: Beef cattle, Estrus, Fertility, Nelore, Ovulation

3.1 Introduction

Synchronization protocols that result in fixed-time AI (FTAI) have become an important part of strategies for improving management of reproduction in cattle operations in many parts of the world including the USA and Brazil [1–3]. These strategies allow controlled breeding seasons, increased reproductive efficiency, and improved genetic progress [4]. In beef cattle operations, FTAI protocols have been tailored to the hormones that are approved for use in the specific country and that match the management style of the operation and the physiology of the animals that are being bred. Programs for FTAI in *Bos indicus* have generally utilized estradiol (E2) products, such as E2 benzoate (EB), although many countries with *Bos indicus* do not currently have approval for use of E2 products in FTAI protocols. It is generally assumed that E2 protocols are more efficient in *Bos indicus* beef cattle than protocols that are initiated with gonadotropin-releasing hormone (GnRH), although direct comparison of these protocols are needed. Therefore, this study compared ovarian responses and fertility in FTAI protocols that utilize a conventional EB or an adapted GnRH-based protocol, both in association to progesterone (P4), in *Bos indicus* heifers and cows.

Protocols that are initiated with progestogen treatment, such as intravaginal P4 implants, and E2 esters (mainly EB), are termed E2/P4-based protocols and employ distinct physiology to initiate a follicular wave compared to GnRH-based protocols. Treatment with EB and P4 at the initiation of the protocol causes suppression of the circulating gonadotropins, FSH and LH, and lead to inhibition of the current follicular wave [5]. There is a subsequent surge in FSH that initiates a new follicular wave [6,7]. Recent physiological experiments from our lab using daily ovarian ultrasonography have demonstrated that protocols initiated with EB produce synchrony in emergence of the new follicular wave resulting in subsequent ovulation of a single dominant follicle at the end of the protocol in *Bos indicus* heifers and cows (Madureira et al., Companion paper), to results that were previously reported for EB treatments [5,8,9]. Use of GnRH at the initiation of the protocol caused ovulation in ~55% of *Bos indicus* heifers and cows and had synchronized emergence of a new follicular wave (from D0 to D5 of the protocol) in a high percentage of cows, similar to EB treatment [92.4% (61/66) for GnRH and 91.4% (66/72) for EB]. Ovulation to GnRH at the initiation of the protocol requires a large enough LH surge to induce ovulation of a dominant follicle with ovulatory capacity (i.e. LH receptors in the granulosa cells) [10–12]. It is known that the magnitude of the LH surge can be negatively influenced by circulating P4 concentrations and positively influenced by increasing the GnRH dose [13,14]. In our recent comparisons of GnRH and EB protocols, the number of CL that were present at the time of

prostaglandin F2 α (PGF) treatment was much greater in animals treated with GnRH than EB due to GnRH-induced ovulation at protocol initiation (Madureira et al., Companion paper) and EB-induced regression of CL during the protocol [15,16]. Nevertheless, initiation of the protocol with EB or GnRH produced a high percentage of heifers or cows that ovulated at the end of the protocol (~90%). In addition, the presence of younger CL at the time of P4 implant removal of the GnRH-initiated protocol necessitated the use of two PGF treatments to assure CL regression to the protocol [17–19]. Studies are now needed employing FTAI at the end of these protocols to determine if there is a difference in fertility with these two types of protocols.

In all FTAI protocols, expression of estrus at the end has been associated with increased fertility [20–22]. Cattle that express estrus are more likely to ovulate to the protocol because the hormonal environment that induced estrus (high circulating E2 in the absence of P4) is also likely to induce a GnRH surge from the hypothalamus and an LH surge from the pituitary [23]. One strategy that has been employed during FTAI protocols, particularly in animals that do not express estrus prior to AI, is to give GnRH at the time of AI to assure that all animals have an LH surge and avoiding too late ovulations. However, studies using GnRH at AI have not had consistent results, with some studies finding no interaction between GnRH treatment and expression of estrus on fertility [22] and others reporting a positive effects of GnRH at AI on fertility, particularly in animals that do not show estrus [24,25].

Thus, the main objectives of this study were to further explore the physiology of conventional EB or adapted GnRH FTAI protocols, and to compare fertility of these protocols in *Bos indicus* heifers and cows. This research employed P4-based FTAI protocols with the P4 device implanted for 7 d since this strategy has previously been found to yield satisfactory pregnancies per AI (P/AI) [24,26]. In addition, the effect of GnRH treatment at the time of AI was investigated in these two types of protocols. Our hypotheses were: 1) Using EB or GnRH strategies at the beginning of a P4-based protocol would produce different follicle and luteal dynamics; 2) Both conventional EB or adapted GnRH protocol could produce similar P/AI; 3) Administration of GnRH at the time of AI would increase P/AI but only in females that were not detected in estrus by the time of AI.

3.2 Material and methods

The experiments were conducted at the Experimental Station “Hildegard Georgina Von Pritzelwiltz”, located in Londrina, PR, Brazil. All females were kept on pasture (*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 University of São Paulo (ESALQ/USP) approved all procedures involving heifers and cows (Protocol # 2017.5.1618.11.9).

3.2.1 Experiment 1

A total of 973 nulliparous heifers were used that were 26.0 ± 2.0 months old with body condition score (BCS) of 3.0 ± 0.01 (1-5 scale) and average body weight of 307.1 ± 22.6 kg. 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 [D-24: insertion of a 7-d used intravaginal P4 implant with 0.5 g (Repro one, GlobalGen Vet Science, Jaboticabal, Brazil); D-12: P4 implant withdrawal and administration of 0.5 mg E2 cypionate i.m. (EC; Cipion, GlobalGen Vet Science)]. After 12 d (D0), all heifers, regardless of CL presence, were randomly assigned to the treatments (Fig. 1): EB-0 (EB at D0 + no GnRH at AI; $n = 245$), EB-G (EB at D0 + GnRH at AI; $n = 252$), G-0 (GnRH at D0 + no GnRH at AI; $n = 241$), or G-G (GnRH at D0 and AI; $n = 235$). On D0, all heifers received a new P4 implant with 0.5 g (Repro one, GlobalGen Vet Science) and EB-groups were treated with 1.5 mg EB i.m. (Syncrogen, GlobalGen Vet Science), whereas G-groups were treated with 16.8 μ g buserelin acetate i.m. (GnRH; Maxrelin, GlobalGen Vet Science). On D7, all heifers had their P4 implant removed and received 0.530 mg cloprostenol sodium i.m. (PGF; Induscio, GlobalGen Vet Science), 0.5 mg EC and had tail-chalk spread on the base of their tailhead. Heifers from G-groups also received an extra PGF and 200 IU eCG i.m. (ECGen, GlobalGen Vet Science) treatment on D6, 24 h before the procedures done on D7 (administration of eCG in G-groups was anticipated to D6 for prolonged time of action). The EB-groups were untreated on D6 and received the 200 IU eCG on D7. At AI (48 h after P4 implant withdrawal), only EB-G and G-G groups received 8.4 μ g GnRH, and all heifers were checked for estrus. Therefore, when groups were denominated EB, it means EB on D0 and PGF, EC, and eCG on D7. When groups were called G, it means they were treated with GnRH on D0, PGF and eCG on D6 and PGF and EC on D7.

Figure 1. Experimental design from Exp. 1 and Exp. 2 (Nelore heifers and cows, respectively) submitted to a progesterone (P4)-based fixed-time AI protocol initiating with buserelin acetate (GnRH¹), or estradiol benzoate (EB²), and receiving GnRH¹ (G-G or EB-G) or not (G-0 or EB-0) at the time of AI. Ultrasound evaluations were performed for CL presence at D0, D6, D7, and D16, and for diameter of the biggest follicle at D7 and D9 in a subset of animals.

¹GnRH dose was 16.8 µg at the beginning of the protocol and 8.4 µg at the time of AI.

²EB dose was 1.5 mg for heifers and 2.0 mg for cows.

When initiating with GnRH, the protocol also had anticipating equine chorionic gonadotropin (eCG; heifers: 200 IU; cows: 300 IU), and an extra cloprostenol sodium (PGF; 0.530 mg) on D6. When initiating with EB, they received eCG on D7. All groups had an intravaginal P4 implant on D0 (heifers: 0.5 g; cows: 1.0 g) and were also treated with PGF and estradiol cypionate (EC; 0.5 mg) on D7.

3.2.2 Experiment 2

A total of 804 Nelore cows with BCS of 3.0 ± 0.01 (1-5 scale) and 67.2 ± 23.1 d postpartum were used, divided into parity: multiparous ($n = 504$; BCS 2.9 ± 0.01), primiparous ($n = 188$; BCS 3.0 ± 0.02), and non-lactating ($n = 112$; BCS 3.3 ± 0.02). Most of the cows were at first AI of the breeding season ($n = 579$), and the remaining were at the second AI ($n = 225$). All cows were submitted to the same experimental treatments as described for heifers (Fig. 1), with some adjustments in hormone doses: on D0, a new intravaginal P4 implant with 1.0 g (Repro neo, GlobalGen Vet Science) was used and 2.0 mg of EB was administered i.m. rather than the 1.5 mg used for heifers. The dose of eCG for cows was 300 IU i.m.

In both experiments, heifers and cows were inseminated by one of three technicians using 20×10^6 frozen/thawed proven semen of six Nelore sires (Genex, São Carlos, Brazil).

3.2.3 Ultrasound examinations

For all heifers ($n = 973$; Exp. 1), transrectal ultrasound examinations of the ovaries in B-mode with a 7.5 MHz linear transducer (DP-2200 VET, Mindray, Shenzhen, China) were performed on D0 and D6 for G-groups, and on D0 and D7 for EB-groups to evaluate the presence of CL. Also, approximately 15.0% of all heifers from EB ($n = 84$) and G-groups ($n = 71$) were evaluated on D7 and D9 for diameter of the largest follicle (mm). During Exp. 2, only cows that were being submitted to the second FTAI ($n = 225$) were evaluated on D0 for presence of CL, but all cows ($n = 804$) were evaluated on D6 (G-groups) and D7 (EB-groups) for CL presence. Similar to Exp. 1, approximately 10.0% of cows from EB ($n = 42$) and G-groups ($n = 43$) were evaluated on D7 and D9 for diameter of the largest follicle. All follicle measures were performed by the same operator.

Seven d after FTAI (D16), ultrasound evaluation was performed in a subset of the heifers from Exp. 1 ($n = 173$) and cows from Exp. 2 ($n = 313$) to check for CL presence, in order to determine the percentage of animals that ovulated to the protocols.

Pregnancy diagnosis was performed by the same equipment and operator at 30 to 35 d after FTAI.

3.2.4 Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC), and all experiments were done in a completely randomized design.

Discrete responses of measured variable were analyzed using the generalized linear mixed model (GLIMMIX procedure) fitting a binary (ovulation rate after D0, luteolysis between D0 and PGF, estrus expression, and ovulation rate at the end of the protocol) or exponential distribution (CL number at PGF). Continuous variable responses were analyzed using the linear mixed models (MIXED procedure). All variable responses (diameter of the largest follicle on D7 and D9) were tested for normality of the residuals using the Shapiro-Wilk statistic method obtained by PROC UNIVARIATE procedure of SAS.

Selection of the model that best fit each variable response of interest was determined by finding the model with the lowest value for the *Akaike Information Criterion Corrected* (AICC) using the backward elimination procedure that removed independent variables with P

> 0.10 from the model. Treatment was considered a fixed effect and the tested covariates were semen batch and BCS on D0 of the protocol for all analyses, and bull and inseminator for fertility analyses.

Differences were considered significant for $P \leq 0.05$, whereas a tendency was designated when $P \leq 0.10$ and $P > 0.05$. The results are expressed as least squares means $\pm$ standard error of the mean (LSM $\pm$ SEM), unless otherwise indicated.

3.3 Results

3.3.1 Experiment 1

At the beginning of the breeding season, 14.9% (145/973) of heifers were cycling (presence of CL at the first ultrasound evaluation). After the protocol for induction of cyclicity, 78.0% (646/828) of the induced heifers had a CL on D0 of the FTAI protocol.

Results regarding ovarian dynamics are presented in Table 1. As expected, G groups had much greater ovulation rate after D0 and greater number of CL at PGF compared to EB. Also, more heifers underwent luteolysis between D0 and PGF when the protocol was initiated with EB than G. The size of the largest follicle on D7 and D9 of the protocol was greater for heifers receiving G than EB. In addition, more heifers from G than EB were detected in estrus after P4 implant withdrawal. In spite of these differences, the percentage of heifers that ovulated after D9 of the protocol was above 90.0% in both groups and not different between groups. Also, no differences were detected for double ovulation after AI [$P = 0.9$; G groups: 0 (0/74), and EB groups: 2.4% (2/84)].

Table 1. Ovarian dynamics and estrus expression of Nelore heifers (Exp. 1) submitted to 7-d progesterone (P4)-based FTAI protocols initiating with buserelin acetate (GnRH), anticipating equine chorionic gonadotropin (eCG), and giving an extra cloprostenol sodium (PGF) on D6 or initiating with estradiol benzoate (EB) and receiving eCG on D7. For all groups, heifers had an intravaginal P4 insert on D0 and were also treated with PGF and estradiol cypionate on D7 at P4 implant removal.

	GnRH (n = 476)	EB (n = 497)	P-value
Ovulation rate after D0, % (n/n)	60.3 (287/476)	12.7 (63/497)	<0.0001
Luteolysis between D0 and PGF, % (n/n)	20.0 (77/385)	39.2 (159/406)	<0.0001
CL number at PGF (n)	1.1 ± 0.06 (476)	0.5 ± 0.02 (497)	<0.0001
Diameter of the biggest follicle on D7, mm (n)	10.3 ± 0.2 (71)	9.2 ± 0.2 (84)	<0.0001
Diameter of the biggest follicle on D9, mm (n)	11.9 ± 0.2 (71)	11.3 ± 0.2 (84)	0.01
Estrus expression, % (n/n)	80.3 (382/476)	69.6 (346/497)	0.0002
Ovulation rate after D9, % (n/n)	90.2 (74/82)	92.3 (84/91)	0.6

The P/AI were similar between treatments (Fig. 2A). However, there was an effect of cyclicity ($P = 0.01$), with P/AI greater in cyclic heifers (62.0%; 90/145) or in heifers with a CL present after the induction protocol (56.8%; 367/646) than in heifers without a CL after the protocol for induction of cyclicity (46.2%; 84/182). There was no interaction between expression of estrus and treatment with GnRH at the time of AI on P/AI (Fig. 3A), but heifers that expressed estrus had greater P/AI than those that did not show estrus [57.4 (428/745) vs. 48.1% (110/228)].

3.3.2 Experiment 2

Results on ovarian dynamics and expression of estrus in cows (Exp. 2) are presented in Table 2. There was a much greater percentage of cows that ovulated to GnRH, 73.7%, than EB with only 24.4%. In addition, over 25.0% of the cows from the EB groups underwent luteolysis between D0 and PGF compared to less than 2.0% in G groups. These results produced a much greater number of CL at PGF in G than in EB. Moreover, at D7 the diameter of the largest follicle was greater in the G than EB-groups, whereas there was no difference in follicle diameters at time of AI (D9). In addition, more cows from the G-groups were detected

in estrus than in the EB-groups. However, there were no differences in percentage of cows that ovulated to the protocol, which was high in both groups (93.3%) or in percentage of cows that double ovulated to the protocols [$P = 0.9$; G groups: 8.2 (12/147), and EB groups: 7.6% (11/145)].

Table 2. Ovarian dynamics and estrus expression of Nelore cows (Exp. 2) submitted to 7-d progesterone (P4)-based FTAI protocols initiating with buserelin acetate (GnRH), anticipating equine chorionic gonadotropin (eCG), and giving an extra cloprostenol sodium (PGF) on D6 or initiating with estradiol benzoate (EB) and receiving eCG on D7. For all groups, cows had an intravaginal P4 insert on D0 and were also treated with PGF and estradiol cypionate on D7 at P4 implant removal.

	GnRH (n = 400)	EB (n = 404)	P-value
Ovulation rate after D0, % (n/n)	73.7 (83/112)	24.4 (28/113)	<0.0001
Luteolysis between D0 and PGF, % (n/n)	1.6 (1/64)	25.5 (14/55)	0.004
CL number at PGF (n)	1.1 ± 0.06 (400)	0.3 ± 0.02 (404)	<0.0001
Diameter of the biggest follicle on D7, mm (n)	11.0 ± 0.3 (43)	9.9 ± 0.3 (42)	0.01
Diameter of the biggest follicle on D9, mm (n)	13.0 ± 0.3 (43)	12.7 ± 0.3 (42)	0.5
Estrus expression, % (n/n)	67.6 (270/400)	56.2 (227/404)	0.001
Ovulation rate after D9, % (n/n)	93.6 (147/157)	93.0 (145/156)	0.8

In regard to fertility, EB-G had greater P/AI than EB-0 ($P = 0.05$), but G-0 and G-G were intermediate and not different from the EB-groups (Fig. 2B). There was an effect of parity and multiparous (64.5%; 325/504) and non-lactating cows (69.8; 78/112) had greater P/AI than in primiparous cows (55.3%; 104/188). There was a tendency ($P = 0.1$) for an interaction between expression of estrus and GnRH treatment at AI (Fig. 3B) with GnRH at AI having a positive effect in cows without expression of estrus [$P = 0.05$; 10.9% absolute increase; 22.6% relative increase (10.9/48.2)], but no effect of GnRH at AI in cows that expressed estrus. Cows that were detected in estrus had greater P/AI compared to those that did not express estrus [69.1 (337/488) vs. 53.5% (169/316)].

Figure 2. Pregnancy per AI (P/AI) of Nelore heifers (A) and cows (B) submitted to a progesterone (P4)-based FTAI protocol initiating with buserelin acetate (GnRH; called G) and also with anticipated equine chorionic gonadotropin (eCG), and an extra cloprostenol sodium (PGF) on D6, or initiating with estradiol benzoate (EB) and receiving eCG on D7. All groups had an intravaginal P4 implant on D0 and were also treated with PGF and estradiol cypionate on D7 at P4 insert removal. In addition, receiving GnRH (G-G or EB-G) or not (G-0 or EB-0) at the time of AI. In heifers, there was no interaction between treatments ($P = 0.2$), or principal effects for treatments on D0 ($P = 0.9$) and D9 ($P = 0.8$). However, in cows there was a tendency for interaction between treatments ($P = 0.1$), and EB-G had greater P/AI compared to EB-0 ($P = 0.05$).

Figure 3. Pregnancy per AI (P/AI) according to estrus expression and GnRH treatment at the time of AI in Nelore heifers (A) and cows (B) submitted to a progesterone (P4)-based fixed-time AI protocol initiating with buserelin acetate (GnRH; called G) and also with anticipated equine chorionic gonadotropin (eCG), and an extra cloprostenol sodium (PGF) on D6, or initiating with estradiol benzoate (EB) and receiving eCG on D7. All groups had an intravaginal P4 implant on D0 and were also treated with PGF and estradiol cypionate on D7 at P4 insert removal. In addition, receiving GnRH (G-G or EB-G) or not (G-0 or EB-0) at the time of AI. In heifers, there was no interaction between treatments ($P = 0.6$), or principal effect of GnRH ($P = 0.9$), but heifers in estrus had greater P/AI compared to heifers without estrus expression ($P = 0.01$). However, in cows there was a tendency for interaction between treatments ($P = 0.08$), and cows without estrus that received GnRH at the time of AI had greater P/AI compared to cows without estrus and not receiving GnRH ($P = 0.05$).

3.4 Discussion

In general, most of the 24 mo old Nelore heifers do not have CL at the beginning of the breeding season, especially due to nutrition and genetic factors [27,28]. Thus, the strategy to induce cyclicity has been stimulated and it is based on the exposure of non-cycling heifers (with adequate body weight and age) to circulating P4 for a period of time, usually 12 d, and an inducer of ovulation, generally with EC on the last day of the induction protocol. During Exp. 1, few heifers were cycling at the beginning of the breeding season, and non-cycling heifers were submitted to a cyclicity induction protocol, with a very satisfactory response (78%) that was similar or above results reported by others [29,30].

Our first hypothesis was supported due to the different mechanisms that GnRH and EB act on the reproductive system. Therefore, variables such as ovulation rate after D0 and D9, luteolysis between D0 and PGF, number of CL at PGF, diameter of the biggest follicle on D7 and D9, and expression of estrus had distinct responses based on the treatment at the start of the protocol. Greater ovulation rate after GnRH administration on D0 of the protocol was observed during Exp. 1 and 2 (~67%), and this result can be justified by the dose of GnRH that was used (16.8 µg of buserelin acetate). It is known that a higher dose of GnRH can promote an adequate LH peak even in the presence of high circulating P4 concentrations, increasing ovulation rate [13,14]. Another possible explanation for the high ovulation rate outcome after D0 could be the time of the estrous cycle that heifers and cows were on D0 of the FTAI protocol, because an important requirement for high ovulation rate it is the presence of a growing dominant follicle with adequate diameter [31,32]. The majority of heifers [heifers with CL after the induction protocol (n = 646)] were expected to be at around day 9 of the estrous cycle when the FTAI protocol was taking place. Also, cows were near the same day, because it was expected that most of them (~90%) ovulated 24 h after the first AI, and non-pregnant cows would have initiated a new follicle wave at ~21 d after the first AI, which corresponds to 9 d before the pregnancy diagnosis (day 30). Therefore, it is expected that cows had a growing dominant follicle at the time of GnRH treatment [33].

As expected, heifers and cows from G-group had greater number of CL at PGF, and this result can be explained by the higher ovulation rate in females receiving GnRH at the beginning of the protocol, and also by a lower luteolysis between D0 and PGF in G-group compared do EB-group. During both experiments there was more luteolysis between D0 and PGF for EB-group, especially in heifers from Exp. 1. It is very likely that EB treatment on D0 induced a luteolytic effect due to the presence of E2 receptors during early diestrus. In sheep, administration of 0.750 mg EB in mid-cycle (day 9) caused an increase in endometrial

oxytocin receptors and luteolysis [34]. In addition, Robinson et al. [35] evaluating uterine biopsy samples during estrous cycles of non-lactating Holstein-Friesian cows identified an increase in E2 receptor α mRNA in the luminal epithelium and epithelial cells of the superficial glands during early luteal phase (between days 4 and 10).

During Exp. 1 and 2, G-groups had a bigger dominant follicle on D7 of the protocol. There are two factors related to this result. The first one is associated to the higher ovulation rate after D0 in GnRH-treated females, which leads to an earlier follicle wave emergence in females that ovulated after D0 compared to females responding to E2/P4 association from EB-groups. The other factor is the anticipation of eCG and first PGF treatment on D6 for G-group. Despite of that, regarding size of the follicle at the time of AI (D9), only heifers (Exp. 1) from G-groups had bigger follicles on D9 compared to EB-heifers. In Exp 2. (cows), the size of the greatest follicle on D9 was similar between experimental groups. Corroborating those results, Madureira et al. (Companion paper) detected earlier emergence of the follicle wave during GnRH-based 7-d FTAI protocols in Nelore heifers and cows that ovulated after GnRH administration. Also, Melo et al. [36] during treatment with GnRH plus EB on D0 of a protocol for dairy cows observed more cows that ovulated after D0 initiating a new follicle wave earlier than those that did not ovulate, and cows with ovulation had bigger follicles at the end the protocol.

Heifers and cows from G-groups expressed more estrus at the end of the protocol compared to EB-groups. It may be due to the bigger dominant follicle on D7 of G-groups, producing more circulating E2 between D7 and D9 of the FTAI protocol [37], and promoting more expression of estrus [38]. This finding can also be explained by the anticipated PGF treatment (D6 of the protocol) for G-group, probably causing reduction of circulating P4 and increasing the development of the dominant follicle [25-27]. In addition, G-groups had an earlier treatment with eCG (D6; one day before EB-groups) as mentioned before, having more time for action of this hormone, contributing for better follicle development [28-30].

A high ovulation rate after D9 was obtained during this study. This is an important result that shows an efficient capacity to ovulate a high number of females submitted to P4-based protocols that started either with GnRH plus adjustments on D6, or conventional EB protocols for *Bos indicus*. Also, no differences were detected between G and EB-groups for double ovulation rate. As expected, both synchronization strategies (with GnRH or EB) promoted an emergence of a new follicle wave in an adequate moment (at least 3 d prior to eCG administration), sufficient to not increase double ovulation, because of follicle deviation had already occurred [7, 31-33].

The second hypothesis was related to fertility outcomes of conventional EB or GnRH-adapted protocols. It has been shown that P4-based protocols starting with GnRH and with eCG plus an extra PGF on D6 can achieve similar P/AI in *Bos indicus* when compared to P4-based protocols starting with EB, being an alternative strategy for synchronization programs, especially in countries where E2-based treatments are not available. However, it is worthy to mention that the protocol starting with GnRH requires one extra cow or heifer handling in order to guaranty efficient luteolysis [17–19]. In addition, our third hypothesis was partially supported, showing that only during a P4-based 7-d FTAI protocol based on EB administration at the start, cows that do not have expression of estrus are benefited with GnRH administration at the time of AI, and this effect was not identified in heifers.

During Exp. 1, treatments had similar P/AI. Then, in this study, heifers could be submitted to a conventional EB or an adapted GnRH 7-d P4-based FTAI protocol, and with no benefit of GnRH at the time of AI, making the protocol cheaper. However, during Exp. 2 it was detected greater P/AI for EB-G compared to EB-0. This can be explained because of the lower estrus expression in cows from the EB-group. In addition, it was observed an important increment on P/AI for cows that were not detected in estrus but received GnRH at AI, compared to those that were not detected in estrus and did not receive GnRH. Based on the information that ovulation rate was similar between cows not showing estrus that received or not GnRH at AI [90.5% (57/63) vs. 88.2% (75/85), respectively], this increment in fertility could be related to a more synchronous ovulation induced by GnRH, i.e. preventing a delayed LH peak, especially when we consider the ideal time for LH peak is 16 h before AI [46]. Treatment with GnRH concomitant with AI did not improve P/AI in heifers (Exp. 1) not showing estrus, however it should be mentioned that estrus expression of heifers was greater for both groups in relation to cows.

In summary, the P4-based 7-d FTAI protocols promoted satisfactory P/AI in both heifers and Nelore cows. Despite ovarian dynamics differences between strategies (EB or GnRH), both were efficient and promoted adequate synchronization of the follicle wave, dominant follicle size, and high ovulation rate at the end of the protocol, resulting in above average P/AI for these beef cattle parity and breed. Of particular interest, this study contributed to increase the knowledge of using GnRH for FTAI protocols in *Bos indicus*.

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4. ADJUSTMENT OF ESTRADIOL CYPIONATE DOSE FOR *Bos indicus* COWS SUBMITTED TO A PROGESTERONE-BASED FIXED-TIME AI PROTOCOL

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ABSTRACT

Fixed-time AI (FTAI) protocols for *Bos indicus* based on estradiol (E2) and progesterone (P4) commonly include E2 as ovulation inducer at the end, mainly estradiol cypionate (EC), because it synchronizes ovulation time, facilitates management, has low cost, and stimulates more females to express estrus by the time of AI. However, literature data is controversial regarding EC dose, which also has never been tested in shorter protocols (7 d of P4 implant). Therefore, primiparous (n = 216) and multiparous (n = 453) Nelore cows with 91.0 ± 20.4 d postpartum, diagnosed as not pregnant at ~32 d after the first AI were submitted to a FTAI protocol [D0: intravaginal implant with 1.0 g P4, and 2.0 mg estradiol benzoate (EB, im); D7: implant withdrawal, 0.526 cloprostenol sodium (PGF, im), and 300 IU eCG (im); D9: estrus detection and FTAI]. On D7, cows were randomly assigned to receive either 0.5 (EC0.5; n = 337) or 1.0 mg EC (EC1; n = 332). Measurements of the largest follicle (LF) on D7 and D9, and presence of corpus luteum (CL) on D16 were done by ultrasound only in multiparous cows. Pregnancy per AI (P/AI) was evaluated 35 d after AI. Effects were considered significant when $P \leq 0.05$. Despite similar LF diameter (mm) on D7 [EC0.5: 9.6 ± 0.2 (n = 56) vs. EC1: 9.2 ± 0.2 (n = 49)], on D9 the LF was bigger in EC0.5 than EC1 cows (12.6 ± 0.3 vs. 11.3 ± 0.3). In addition, EC0.5 cows had higher ovulation rate on D16 (94.0%; 156/166) than EC1 (85.9%; 134/156). For estrus expression, there was no interaction between EC dose and parity, however more EC1 cows were detected in estrus than EC0.5 [83.0% (276/332) vs. 73.4% (247/337)] and multiparous had more estrus expression than primiparous cows [82.8% (375/453) vs. 73.6% (159/216)]. When EC dose, parity, and estrus expression were analyzed for fertility, there was an interaction between estrus and parity. Primiparous that did not express estrus had lower P/AI (19.0%; 11/58) than primiparous in estrus (50.6%; 80/158), or multiparous with (60.7%; 227/374) or without estrus (49.4%; 39/79). There was an interaction between EC dose and parity on fertility. Primiparous from EC0.5 group had lower P/AI (30.2%; 32/106) than primiparous from EC1 (53.6%; 59/110), or multiparous from EC0.5 (58.4%; 135/231) or EC1 groups (59.0%; 131/222). Thus, treatment with 1.0 mg EC induced more cows to express estrus, but produced smaller follicles at AI as well as lower ovulation rates after AI (evaluated only in multiparous cows). Despite that, EC1 improved fertility in primiparous cows.

Keywords: Artificial insemination, Nelore, estradiol cypionate, estrus expression, primiparous

4.1 Introduction

Synchronization protocols for fixed-time AI (FTAI) are used in beef cattle operations to improve efficiency and profitability by adding genetics, controlling the breeding season, simplifying routine managements and optimizing reproductive outcomes [1,2]. In this way, several studies have been done to understand physiological mechanism and practical applications that underline the synchronization of ovulation in beef [3,4] and dairy herds [5,6]. Focusing in beef cattle programs for FTAI, very common strategies for synchronization of the ovulation are the estradiol (E2) and progesterone (P4)-based protocols. Variations of E2/P4-based protocols have been developed and tested, such as the period of exposure to P4 supplementation, varying from 5 to 9 days [7–10 plus Madureira et al. (in preparation)], with satisfactory fertility results in most of them [11,12]. In addition, E2 esters, such as E2 benzoate (EB) or E2 cypionate (EC) have also been used at the end of these protocols.

Despite variations among E2/P4-based protocols, primarily they must fulfill specific requirements to ensure a synchronized ovulation and enable an efficient large scale FTAI program. Besides synchronizing an emergence of the follicle wave by several ways [13,14, Madureira et al REF] and controlling circulating P4 [15], the ideal protocol must ensure an adequate proestrus period (i.e., interval between P4 decrease and the onset of estrus), with enough circulating E2 for proper endometrial function to support conceptus growth and pregnancy maintenance [16–18]. Furthermore, E2 esters induce more expression of estrus that are associated with improved pregnancy per AI (P/AI) [19]. In addition, females in estrus during FTAI protocols has also an adequate preovulatory follicle [20,21] and subsequently luteal function [22].

Comparing physiology and fertility outcomes between E2 esters (1.0 mg EC at the moment of the P4 implant removal vs. 1.0 mg EB administered 24 h later) during an 8 d FTAI protocol in ovariectomized *Bos indicus* heifers (n = 10), Sales et al. [23] detected differences on circulating LH profile for EC and EB [time of LH surge after administration (50.5 ± 3.6 vs. 19.6 ± 1.2 h), magnitude (9.4 ± 2.2 vs. 20.5 ± 1.9 ng/mL) and duration (16.5 ± 1.0 vs. 8.6 ± 0.2 h) of the LH peak, and area under the curve (339.4 ± 36.4 vs. 158.6 ± 26.1 ng/mL/72 h), respectively]. Despite that, both were effective for synchronization of the ovulation and P/AI. In dairy cows, either 1.0 mg EC or 1.0 mg EB as ovulation inducers achieved similar P/AI at 32 and 60 d after FTAI. There was no treatment effect on pregnancy loss either [24]. In addition, Souza et al. [25] compared circulating E2 after treatment with 1.0 mg E-17 β , 1.0 mg EB, or 1.0 mg EC in lactating Holstein cows (n = 12) after aspiration of all follicles >5 mm, and reported differences for interval from treatment (E-17 β , EB and EC, respectively) until

peak concentration (4.0 ± 0.0^a vs. 16.0 ± 6.1^{ab} vs. 30.7 ± 3.5^b h), interval from treatment until return to nadir (22.7 ± 4.8^a vs. 30.7 ± 7.1^{ab} vs. 50.7 ± 4.8^b h), maximum circulating E2 concentration (12.8 ± 4.0^a vs. 9.6 ± 3.5^b vs. 3.4 ± 0.2^c pg/mL), and area under the curve (187.9 ± 31.2^a vs. 222.1 ± 51.3^a vs. 118.7 ± 34.8^b pg²).

Despite particular circulating E2 and LH patterns related to the type of E2 administered at the end of FTAI protocols, EC is largely included into E2/P4-based protocols and its best dose for *Bos indicus* cows is still controversial, since there are reports of successful use of either 0.5 mg EC in cows [8,26 plus Madureira et al. (in preparation)] and heifers [12,27,28], or 1.0 mg EC in cows [23,29]. In addition, using 0.5 mg EC in cows may prevent preovulatory follicle turn-over because of the hormonal milieu at the time of circulating E2 peak after EC administration (residual circulating P4). Exploring this argument, the fact that E2 peak occurs ~31 h after administration of 1.0 mg EC (lactating Holstein cows data) [25], and also emphasizing that *Bos indicus* (Nelore) have a delayed catabolism of steroid hormones compared to *Bos taurus* (Holstein) [30], and complete luteolysis (circulating $P4 \leq 0.5$ ng/mL by 56 h after PGF and remaining for 3 d) occurring ~30 h (ranging from 18 to 40 h) in lactating Holstein cows [31] supports our hypothesis that residual circulating P4 at the time of EC-induced E2 peak in *Bos indicus*, may cause follicle turn-over before AI. On the other hand, 1.0 mg EC may induce a more synchronized ovulation at the end of the protocol compared to 0.5 mg [29].

Thus, the objective of this experiment was to compare doses of EC as ovulation inducers and their influence on follicle development and fertility outcomes during a P4-based 7 d FTAI protocol in Nelore (*Bos indicus*) cows. Our hypotheses were: 1. The higher dose of EC (1.0 mg) would reduce the largest follicle (LF) diameter at the time of AI, consequently reducing ovulation rate after AI, indicating turn-over of the follicle wave between D7 and AI; 2. Despite less expression of estrus, cows receiving 0.5 mg EC would achieve higher P/AI compared to 1.0 mg EC.

4.2 Material and methods

The experiments were conducted at the Experimental Station “Hildegard Georgina Von Pritzelwiltz”, located in Londrina, PR, Brazil. All females were kept on pasture (*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 University of São Paulo (ESALQ/USP) approved all procedures involving heifers and cows (Protocol # 2017.5.1618.11.9).

4.2.1 Cow management

A total of 669 Nelore (*Bos indicus*) cows, diagnosed not pregnant at ~32 d after the first AI and 91.0 ± 20.4 d postpartum, were used during a resynchronization protocol. Primiparous ($n = 126$) and multiparous ($n = 453$) cows had an average body condition score (BCS, scale 1-5 points, using 0.25 increments) of 2.9 ± 0.1 and 3.1 ± 0.2 , respectively.

4.2.2 Experimental design

At the start of the FTAI protocol (D0), all cows received an intravaginal P4 implant with 1.0 g (Repro neo, GlobalGen Vet Science, Jaboticabal, Brazil), 2.0 mg EB i.m. (Syncrogen, GlobalGen Vet Science) and were randomly assigned to one of the two treatments described below (Fig. 1). Seven d later (D7), all cows were treated with 0.530 mg cloprostenol sodium (PGF) i.m. (Induscio, GlobalGen Vet Science) and 300 IU eCG i.m. (eCGen, Globalgen Vet Science). In addition, on D7, two treatments of EC (Cipion, GlobalGen Vet Science) were administered i.m.: EC0.5 (0.5 mg, $n = 337$) or EC1 (1.0 mg, $n = 332$). On D9 (48 h after implant removal), cows were inseminated by one of three technicians using 20×10^6 frozen/thawed proven semen of six sires (GENEX, São Carlos, Brazil). For estrus evaluation, all cows had the base of their tailhead painted with tail-chalk on D7 and, at the time of AI, they were checked based on the disappearance of the tail-chalk signaling for positive activity of estrus.

Figure 1. Experimental design to evaluate the effect of the dose of estradiol cypionate (EC) during a progesterone (P4)-based fixed-time AI (FTAI) protocol in Nelore (*Bos indicus*) cows. On day 0 (D0) cows received an intravaginal implant containing 1.0 g P4 and were treated with 2.0 mg estradiol benzoate (EB) i.m. After 7 d (D7) the P4 implant was removed and 0.530 mg cloprostenol sodium (PGF), 300 IU equine chorionic gonadotropin (eCG), and

0.5 (EC0.5 treatment) or 1.0 mg EC (EC1 treatment) were administered i.m. On day 9 (D9), 48 h after P4 implant removal, cows were inseminated.

4.2.3 Ultrasound examination

Only cows diagnosed not pregnant after a first post-partum FTAI were enrolled in this study. Thus, after pregnancy diagnosis by transrectal ultrasound examination in B-mode using a 7.5 MHz linear transducer (DP-2200 VET, Mindray, Shenzhen, China) at ~32 d after first AI, non-pregnant cows were submitted to the experimental protocols. In addition, as the beginning of the experimental FTAI protocol match with pregnancy diagnosis of the last AI, partial data related to presence of CL at this time were registered.

During the synchronization protocol, approximately 20.0% of multiparous cows ($n = 105$) were randomly submitted to ultrasound examination of the ovaries to evaluate the diameter (mm) of the LF on D7 and at AI. All measurements were conducted by the same operator and based on the average between measurements of LF's two perpendicular axes. On the same way, ovulation rate was calculated by presence of corpus luteum (CL) at 7 d after FTAI (D16) only in multiparous cows ($n = 322$).

Pregnancy diagnosis (pregnancy per AI; P/AI) was conducted by the same procedure, equipment, and operator 30-35 d after FTAI.

4.2.4 Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC), and all experiments were done in a completely randomized design.

Discrete variable responses were analyzed using the generalized linear mixed model (GLIMMIX procedure) fitting a binary (estrus expression, ovulation rate at the end of the protocol, and P/AI). Continuous variable responses were analyzed using the linear mixed models (MIXED procedure). All variable responses (diameter of the largest follicle on D7 and at AI) were tested for normality of the residuals using the Shapiro-Wilk statistic method obtained by PROC UNIVARIATE procedure of SAS.

The selection of the model that best fitted each variable response of interest was performed by finding the model with the lowest value for the *Akaike Information Criterion Corrected* (AICC) using the backward elimination procedure that removed independent variables with $P > 0.10$ from the model. Treatment was considered fixed effect, and tested

covariates were semen batch and BCS on D0 of the protocol for all analyses, and bull, inseminator and parity for fertility analysis.

Differences were considered significant when $P \leq 0.05$, whereas a tendency was designated when $P \leq 0.10$ and $P > 0.05$. The results are expressed as least squares means $\pm$ standard error of the mean (LSM $\pm$ SEM), unless otherwise indicated.

4.3 Results

At the time of ultrasound examination for pregnancy diagnosis after the first postpartum AI, which was not part of this experiment, it was observed that primiparous had less CL [29.8% (53/178)] than multiparous [68.7% (145/211)] cows. This time was the beginning of our experimental FTAI protocol. As expected, multiparous cows had similar diameter of the LF on D7 between EC0.5 and EC1 treatments, but on D9 it was larger in EC0.5 than EC1. In addition, ovulation rate was higher for EC0.5 treatment compared to EC1. During the P4-based 7 d FTAI protocol used in this study, only 1.2% (4/322) of multiparous cows had double ovulation after the FTAI. Ovarian dynamics outcomes are represented in Table 1.

Table 1. Measurements of the largest follicle (LF) diameter on days 7 and 9, and ovulation rate estimated by the presence of corpus luteum (CL) 7 d after FTAI (D16) in multiparous cows. Cows were submitted to a FTAI protocol with different doses of estradiol cypionate (EC) administered at the time of P4 implant removal: EC0.5 (0.5 mg) or EC1 (1.0 mg).

	EC0.5 (n = 56)	EC1 (n = 49)	P-value
LF diameter on D7, mm	9.6 $\pm$ 0.2	9.2 $\pm$ 0.2	0.2
LF diameter on D9, mm	12.6 $\pm$ 0.3	11.3 $\pm$ 0.3	0.005
Ovulation rate on D16, % (n/n)	94.0 (156/166)	85.9 (134/156)	0.02

Overall, estrus was detected in 79.5% (532/669) of the cows. There was no interaction between EC dose and parity, however multiparous had more estrus expression than primiparous cows [82.8% (375/453) vs. 73.6% (159/216)] and cows from EC1 had more estrus expression than cows from EC0.5 treatments [83.0% (276/332) vs. 73.4% (247/337)]. Expression of estrus considering dose of EC and parity is represented in Fig. 2. When EC dose, parity, and estrus expression were analyzed for P/AI, there was an interaction between

expression of estrus and parity (Fig. 3). Primiparous cows that did not express estrus had lower P/AI than primiparous that expressed estrus or multiparous cows with or without estrus. Independent of that, P/AI of cows that expressed estrus was higher than cows that did not express estrus [55.0% (293/532) vs. 32.5% (45/137)].

Figure 2. Expression of estrus (% , n/n) of primiparous and multiparous Nelore (*Bos indicus*) cows submitted to a progesterone (P4)-based 7 d fixed-time AI (FTAI) protocol using two doses (0.5 or 1.0 mg) of estradiol cypionate (EC) at the time of P4 implant removal (day 7). No interaction ($P = 0.4$) between parity (primiparous or multiparous) and treatments (EC0.5 or EC1) was detected, but there were isolated effects of parity ($P = 0.007$) and treatments ($P = 0.005$).

Figure 3. Interaction ($P = 0.05$) between parity (primiparous or multiparous) and expression of estrus (with or without estrus) on pregnancy per AI (P/AI, %, n/n) during progesterone (P4)-based 7 d fixed-time AI (FTAI) protocol in Nelore (*Bos indicus*) cows.

^{a,b}Difference between groups ($P < 0.05$).

An interaction between EC dose (0.5 or 1.0 mg) and parity (primiparous or multiparous) on fertility was observed (Fig. 4). Primiparous cows from EC0.5 treatment had lower P/AI than primiparous from EC1 treatment, or multiparous cows from both treatments.

Figure 4. Interaction Interaction ($P = 0.005$) between parity (primiparous or multiparous) and dose (0.5 or 1.0 mg) of estradiol cypionate (EC) on pregnancy per AI (P/AI, %, n/n) during a progesterone (P4)-based 7 d fixed-time AI (FTAI) protocol in Nelore (*Bos indicus*) cows.

^{a,b}Difference between groups ($P < 0.05$).

4.4 Discussion

Our first hypothesis was supported, because treatment with 1.0 mg EC reduced the size of the LF at the time of AI and ovulation after AI, compared to EC0.5 treatments in multiparous cows. Interestingly, although EC1 treatment had induced more expression of estrus, it also reduced the size of the LF on D9 (Table 1). There are two possible explanations for this effect. The first is that the increased estrus expression of this treatment promoted an anticipated LH peak and then the follicle reduced its size by the time of FTAI (D9) [Madureira REF BE vs GnRH]. The second possibility is that high circulating E2 due to the dose of 1.0 mg EC caused a turn-over of the current follicular wave and the dominant follicle became atretic, reducing its diameter on D9. Considering that ovulation rate after FTAI was lower for EC1 treatment in multiparous cows, it is most likely that the second hypothesis (turn-over of the preovulatory follicle) is responsible for the reduced size of the LF on D9 in this treatment, especially because multiparous presented ~70% of CL presence on D0, suggesting that several cows still may have high circulating P4 on the day of EC administration. Studying luteolysis in lactating Holstein cows, Martins et al. [31] induced the presence of two CL's with 13 and 7 days, and after challenged them to a usual PGF dose, it was shown that its spend ~30 h for a completely luteolysis (circulating P4 $\leq$ 0.5 ng/mL by 56 h after PGF and remaining for 3 d), ranging from 18 to 40 h. Thus, considering that *Bos indicus* (Nelore) has a delayed metabolism of steroid hormones compared to *Bos taurus* (Holstein) [30] and the estradiol peak occurs ~31 h after administration of 1.0 mg EC (lactating Holstein cows data) [25], the hypothesis of turn-over of the preovulatory follicle can be supported and must be precisely studied. Therefore, despite the EC1 treatment negatively affected the LF on D9, it also promoted more expression of estrus and did not affect fertility in multiparous cows (both EC0.5 and EC1 treatments had similar P/AI; Fig. 4).

One possible explanation for the similar P/AI of EC0.5 and EC1 treatments in multiparous cows is that despite having less cows ovulating at the end of the protocol, the dose of 1.0 mg EC may have promoted a more synchronized ovulation, as well as produced a hormonal milieu during proestrus with higher circulating E2. By comparing doses of EC, Lopes et al. [32] synchronized 29 non-lactating multiparous dairy (*Bos taurus*) cows and submitted them to four treatments (0, 0.5, 1.0, or 2.0 mg EC) administered 24 h after PGF treatment on day 7 of a FTAI protocol. The doses of 0.5 and 1.0 mg EC promoted a shorter and better synchronized ovulation (63.0 ± 5.0 and 60.0 ± 2.0 h, respectively) compared to 0 and 2.0 mg EC (83.0 ± 13 and 81.0 ± 6 h, respectively). Similarly, Bosolasco et al. [26] described two experiments comparing doses of EC during a 7 d FTAI protocol in multiparous

beef cows (*Bos taurus*). The first experiment ($n = 45$) compared 0, 0.5 and 1.0 mg EC and related a shorter interval to ovulation for 1.0 mg (58.7 ± 2.7 h) compared to 0.5 or 0 doses (66.7 ± 2.5 and 69.1 ± 2.9 h, respectively), despite the same ovulation rate among treatments. The second experiment related higher P/AI for 0.5 [60.4% (1227/2112)] compared to 1.0 mg [50.4% (1031/2044)]. Therefore, considering differences in ovarian function and circulating hormones between *Bos taurus* and *Bos indicus* cows [30,33,34] in which there is higher preovulatory peak concentrations of E2 in *Bos indicus* compared to *Bos taurus* (16.2 vs. 12.5 pg/mL), a higher EC dose may also be necessary to mimic a more physiological proestrus in Nelore cows. In addition, Torres-Júnior et al. [29] compared 0.5 or 1.0 mg EC at P4 implant removal, and 1.0 mg EB 24 h after, during a 8 d FTAI protocol in Nelore (*Bos indicus*) cows. During the first experiment, cows receiving EB ($n = 10$) had early time to ovulation after P4 implant withdraw compared to 0.5 ($n = 11$) and 1.0 ($n = 10$) mg EC (66.0 ± 2.3 vs. 78.0 ± 3.5 vs. 71.1 ± 3.6 h, respectively). Moreover, it was reported at the second experiment a greater P/AI for 1.0 ($n = 219$) than 0.5 ($n = 220$) mg EC, and no differences for EB (50.7 vs. 38.6 vs. 43.0%, respectively).

Our second hypothesis was that despite less estrus expression, cows receiving 0.5 mg EC would achieve higher P/AI compared to 1.0 mg EC. In fact, 1.0 mg EC stimulated more cows to express estrus, but 0.5 mg had lower P/AI in primiparous cows. Martins et al. [35] compared primiparous and multiparous Nelore cows receiving or not 1.0 mg EC at the time of P4 implant removal during a 8 d FTAI protocol and confirmed that there is a pharmacologic effect of EC on expression of estrus in multiparous cows [with EC: 53.4% (171/320); without EC: 16.1% (52/323)] and primiparous cows [with EC: 31.3% (31/99); without EC: 3.2% (3/94)]. Also, primiparous cows had lower expression of estrus than multiparous cows. In addition, Sá Filho et al. [36] reported more estrus expression and P/AI comparing 1.0 mg EC [64.7% (55/85) and 47.1% (40/85)] vs. no EC [44.8% (39/87) and 33.3% (29/87)], respectively, in suckled anestrous Nelore cows, and also different endometrial gene expression, suggesting that EC supplementation can modulate transcriptional profiles on proestrus.

Positive effects of expression of estrus on P/AI reported in our experiment are well known [21]. The action of E2 on behavior centers of the brain is responsible for the beginning of estrus [37], and E2 is also responsible for an adequate uterus environment to receive and support sperm for the fertilization process [38,39] and embryo development [16]. In addition, estrus expression at the end of the FTAI protocol is generally associated to adequate DF size and function at AI [19,20] and luteal function after AI [22].

The most important result (Fig. 4) observed in our study was that P/AI of primiparous cows was negatively affected by EC0.5 treatments. Thus, considering that primiparous cows had lower expression of estrus compared to multiparous cows, and that EC1 treatment improves expression of estrus (Fig. 2) due to a pharmacological effect, primiparous demand a higher EC dose to increase P/AI during a 7 d FTAI protocol. In addition, despite not comparing EC dose (all cows received 1.0 mg EC at P4 implant removal) during a 8-d FTAI protocol, Sales et al. [40] detected a more pronounced effect of eCG (300 IU) during the protocol compared to control group (no eCG) on P/AI of primiparous [45.7% (160/350) vs. 20.0% (39/195)] than multiparous cows [52.0% (517/995) vs. 39.9% (220/552)], indicating again that primiparous need greater extra stimulus for final preovulatory follicle growth.

This study demonstrated key aspects regarding estrus synchronization and ovarian dynamics in *Bos indicus* cows that were influenced by parity (primiparous or multiparous) and dose of EC (0.5 or 1.0 mg) administered at the time of P4 implant removal during a 7 d FTAI protocol. Focusing on parity, due to physiological changes associated to the first calving and subsequent lactation, concomitant with an unfinished body growth [41,42], primiparous cows usually have lower fertility and inadequate reproductive responses to a FTAI protocol compared to multiparous cows [8,27,40], and it could explain the effects associated to parity.

In conclusion, this study indicated that despite EC1 treatment produced smaller preovulatory follicles on D9 and lower ovulation rates after AI in multiparous cows, supporting our first hypothesis, this negative effect was compensated by more estrus expression. In addition, primiparous cows were negatively affected by EC0.5 treatments, although P/AI of multiparous cows had not been dose dependent, disagreeing with our second hypothesis that 0.5 mg of EC could improve P/AI compared to 1.0 mg. Thus, considering that EC1 treatment promoted more estrus expression than EC0.5, primiparous cows required the dose of 1.0 mg EC at P4 implant removal during a 7 d FTAI protocol for satisfactory fertility results.

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5. FINAL CONSIDERATIONS

Our studies were designed to provide more details on the physiology and fertility of P4-based protocols in *Bos indicus*, and despite some of them have achieved satisfactory P/AI (~55%) during this study or literature data, all of them induced specific responses related to ovarian function. It is clear that more studies related to follicle and CL development during each synchronization strategy must be done, especially when using GnRH or only P4-based protocols, that are not commonly applied. Next steps should include protocol adjustments for each female category, ciclicity status, inclusion or not of drugs in strategic moments of the protocol, as well as dose adjustments, lengths and ovulation inductors. Thus, refined approaches to P4-based protocols could promote an increase in reproductive outcomes. In addition, there was no scientific publication that had proposed a clear and careful evaluation of how protocols perform in *Bos indicus*, and suggested the use of an uncommon but effective 7 d hormonal protocol, including GnRH as an option for synchronization programs, that had not yet been clearly studied in *Bos indicus*.