DANILO ZAGO BISINOTTO

Impact of 17β-estradiol addition at the moment of timed-AI in Nelore cows

Pirassununga

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Dissertation submitted to the Postgraduate Program in Animal Reproduction of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Master's degree in Sciences.

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Prof. Guilherme Pugliesi, Ph.D.

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CERTIFICADO : EMENDA v24/07/2023

Certificamos que a EMENDA (versão de 24/07/2023) da proposta intitulada "Impacto da adição de 17?-estradiol no momento da inseminação artificial em tempo fixo em vacas Nelore.", CEUA nº 1646060721 (ID 034871), sob a responsabilidade de **Guilherme Pugliesi** *e equipe; Danilo Zago Bisinotto* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos vigentes para sua apresentação, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), sendo assim **APROVADO** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia Universidade de São Paulo (CEUA/FMVZ) em 02/08/2023.

Pedido apresentado à CEUA: Venho por meio dessa emenda notificar a redução dos grupos experimentais propostos e solicitar inclusão de maior número de animais a serem avaliados no experimento 3 deste projeto. Inicialmente seriam utilizados apenas 2.400 fêmeas bovinos, Bos Indicus. Com a mudança dos grupos experimentais após discussão no exame de qualificação foram usadas apenas 731 fêmeas que não apresentaram estro e assim somente estas receberam os tratamentos propostos. Contudo, solicita-se a inclusão de mais animais que expressaram estro para que os dados sejam analisados e incluídos para se ter uma avaliação retrospectiva mas comparativa com os grupos experimentais já que os animais estavam no manejo normal da fazenda e de forma contemporânea nos mesmos lotes dos animais tratados. Assim, solicita-se a mudança de número de animais para que o total de animais usados no experimento 3 oriundos da Agropecuária Nelore Paranã - Iaciara - GO seja 3.850 fêmeas bovinas. Salientamos ainda que os animais que expressaram estro e que estamos solicitando a inclusão não receberam nenhum tratamento ou manipulação adicional ao que a fazenda já realiza, mas que por serem um grupo de comprovada fertilidade poderia ser incluído como referência para os animais tratados.

Considerações da CEUA: O pesquisador solicita alteração do número de animais do terceiro experimento, que inicialmente era de 2400 animais, para 3850. Deste modo, serão incluídas fêmeas que expressaram estro. Não há óbices para a alteração solicitada.

Término previsto: 08/2022

Origem:	Prefeitura do Campus da USP de Pirassununga				
Espécie:	Bovinos	sexo: Fêmeas	idade: 1 a 8 anos	Quantidade mantida:	0
Linhagem:	Nelore (Bos indicus)		Peso: 300 a 500 kg		
Origem:	Animais de proprietários				
Espécie:	Bovinos	sexo: Fêmeas	idade: 2 a 8 anos	Quantidade solicitada:	3850
Linhagem:	Nelore (Bos indicus)		Peso: 400 a 500 kg		

ANIMAIS UTILIZADOS

		Total Aprovado	Quantidade Utilizada
Bovinos	Fêmeas	6250	0
Bovinos	Fêmeas	60	0

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

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EVALUATION FORM

Author: BISINOTTO, Danilo Zago

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Committee Members

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

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Resumo

Bisinotto, D, Z. **Impacto da adição de 17β-estradiol no momento da inseminação artificial em tempo fixo em vacas Nelore**. 2023. 58p. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2023.

Objetivo do estudo foi avaliar os efeitos do tratamento com estradiol (17β-E) no momento da Inseminação Artificial em Tempo Fixo (IATF) na expressão gênica uterina, taxa de expressão de estro (TEE) e taxa de prenhez (P/IATF) em vacas Nelore com um pequeno folículo dominante (FD) ou que não mostraram estro até o momento da IATF. Nos Experimentos 1 e 2, vacas não lactantes foram submetidas a um protocolo a base de P4/17β-E, começando no início do diestro (D0). No D7, 12,5mg de dinoprost trometamina e 1mg de cipionato de 17β-E foram administrados, e no D9 as vacas com FD<11,5 mm foram distribuídas em diferentes grupos experimentais. No Experimento 1 (n=16/grupo): Controle (C, sem tratamento), E-2 (i.m., 2mg de 17β-E) e E-4 (i.m., 4mg de 17β-E); enquanto no Experimento 2: C (sem tratamento; n=12); E-2 (2mg de 17β-E, n=14); GnRH (0,1mg de acetato de gonadorelina, n=13); e E-2+GnRH (associação de GnRH e 17β-E, n=13). Em ambos os experimentos, entre os dias 9 e 11, as vacas foram submetidas a ultrassonografia transretal a cada 12 (Exp1) ou 6 (Exp2) horas para detecção da ovulação, determinação da espessura endometrial (EE) e adesivo um adesivo marcador de estro foi colocado na base da cauda para TEE. No Experimento 1, foi coletada uma amostra de citologia uterina 4 horas após o tratamento para avaliar a expressão de receptores para 17β-E (*E2R*), ocitocina (*OXTR*) e P4 (*PGR*). No Experimento 3, 3829 vacas lactantes com ECC de 2,9 (escala de 1 a 5) foram submetidas à IATF. No D0, as vacas receberam um dispositivo de P4 (0,6g) e 2mg de benzoato de 17β-E. No D9, os dispositivos foram removidos e as vacas receberam 300UI de eCG, 0,39mg de cloprostenol sódico, 1mg de cipionato de 17β-E e um bastão marcador na base da cauda para avaliar TEE foi aplicado. No D11, o diâmetro do FD foi determinado, a IATF foi realizada e as vacas que não

apresentaram estro receberam 0,1mg de acetato de gonadorelina e foram alocadas em 2 grupos: GnRH (n=368) e E-2+GnRH (2mg de 17β-E; n=363). No Experimento 1, os dados foram separados por contrastes ortogonais (C1: C *vs*. 17β-E; e C2: E-2 *vs*. E-4) e o Experimento 2 em um fatorial 2x2. As variáveis foram avaliadas por ANOVA ou regressão logística usando o software SAS. No Experimento 1, a EE reduziu (P<0,05) 12 horas após o tratamento nas vacas tratadas com 17β-E, além de apresentarem maior (P<0,05) abundância de transcritos para *OXTR* e menor (P<0,05) para *ESR1* e *ESR2*. Para o contraste C2, nenhuma diferença significativa (P>0,1) foi observada. No Experimento 2, a TEE não diferiu (P>0,1), mas o intervalo entre o tratamento e a ovulação (h) foi maior (P<0,05) nas vacas do grupo E-2 (40±1,6) em comparação com os outros grupos (C: 31±2; GnRH: 28±0,9; E-2+GnRH: 29±0,1). Ao avaliar as taxas de ovulação, houve um efeito tanto do GnRH (P>0,01), onde os grupos tratados apresentaram uma maior taxa de ovulação (88,9%) quando comparado a grupos não tratados (35,4%), quanto grupos que receberam 17β-E (P>0,01) teve uma menor taxa (43,9%) quando comparado aos que não recebeu (77,8%), até 36 horas. No Experimento 3, a P/IATF foi de 55% para as vacas em estro. Para aquelas sem sinais de estro, não foi observado diferença (P>0,1) na P/IATF entre os grupos, GnRH (34%) e E-2+GnRH (31%). Independentemente do tratamento com E-2, as vacas com um FD≥11 mm (n=192) tiveram uma P/IATF maior (P<0,05) (49%) do que aquelas com FD<11 mm (n=377; 29%). Assim, a administração de 17β-E no momento da TAI modula a expressão de receptores uterinos, mas retarda a ovulação, contudo não impactando a P/TAI quando associada ao tratamento com GnRH em vacas Nelore lactantes com um pequeno FD ou que não mostraram estro na IATF.

Palavras-chave: Estradiol. Modulação Uterina. Folículo Dominante. Inseminação Artificial em Tempo Fixo.

Abstract

Bisinotto, D, Z. **Impact of 17β-estradiol addition at the moment of timed-AI in Nelore cows**. 2023. 58p. Dissertation (Master of Sciences) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2023.

This study aimed to evaluate the effects of administering estradiol (E-17β) at the time of TAI on uterine gene expression, estrous expression rate (EER), and pregnancy rate (P/TAI) in Nelore cows with a small dominant follicle (DF) or not showing estrus until the time of TAI. In Experiments 1 and 2, non-lactating cows were submitted to a P4/ E-17β -based protocol for starting at early diestrus (D0). On D7, 12.5mg dinoprost tromethamine and 1mg E-17β cypionate were injected. On D9, cows with DF<11.5 mm were assigned to different experimental groups. In Exp1 (n=16/group): Control (C, no treatment), E-2 (i.m., 2mg E-17β) and E-4 (.i.m, 4mg E-17β); whereas in Exp2: C (no treatment; n=12); E-2 (2mg E-17β, n=14); GnRH (0.1mg gonadorelin acetate, n=13); and E-2+GnRH (association of GnRH and E-17β, n=13). In both experiments, between D9 and D11 cows were submitted to transrectal ultrasonography every 12 (Exp1) or 6 (Exp2) hours (h) for ovulation detection, determination of endometrial thickness (ET), and a patch was fitted halfway between the hip and tail head for EER. In Exp1, a uterine cytological sample was collected 4h after treatment to evaluate the transcript expression of receptors for E-17β (*E2R*), oxytocin (*OXTR*), and P4 (*PGR*). In Exp3, 3,829 suckled cows with a BCS of 2.9 (scale 1 to 5) were submitted to a TAI. On D0, cows received a P4-device (0.6g) and 2mg E-17β benzoate. On D9, devices were removed and cows received 300IU eCG, 0.39mg sodium cloprostenol, 1mg E-17β cypionate, and a marker stick at the base of the tail for EER. On D11, TAI was performed and cows that did not demonstrate estrus had the DF diameter determined, received 0.1mg gonadorelin acetate, and were allocated into two groups: GnRH (n=368) and E-2+GnRH (2mg E-17β; n=363). In Exp1, data were separated by orthogonal contrasts (C1; C vs. E-17β; and C2: E-2 vs. E-4) and Exp2 in a 2x2 factorial. Variables were evaluated by ANOVA or logistic regression using SAS software. In Exp1, ET was reduced (P<0.05) 12h after treatment in E-17β-treated cows. The E-17β-treated

cows had greater (P<0.05) transcript abundance for OXTR and lesser (P<0.05) for *ESR1* and *ESR2*. For C2 contrast, no significant difference (P>0.1) was observed. In Exp2, the EER did not differ (P>0.1), but the interval from treatment to ovulation (h) was longer (P<0.05) in cows from the E-17β (40±1.6) compared to others (C: 31±2; GnRH: 28±0.9; E-2+GnRH: 29±0.1). When evaluating ovulation rates, there was an effect of both GnRH (P>0.01), where treated groups showed higher ovulation rates (88.9%) when compared to untreated groups (35.4%), as groups that received E-17β (P>0.01) had a lower rate (43.9%) when compared to those that did not receive it (77.8%), up to 36 hours. In Exp3, the P/TAI was 55% for cows in estrus. For those not showing estrus, no difference (P>0.1) in pregnancy rate was observed between GnRH (34%) and E-2+GnRH (31%) groups. Regardless of the E-17β-treatment, cows with a DF≥11 mm (n=192) had a greater (P<0.05) P/TAI (49%) than those with DF<11 mm (n=377; 29%). In conclusion, E-17β administration in the moment of TAI modulates the mRNA expression of uterine receptors in cows with a small DF but does not impact the P/TAI compared with GnRH treatment in suckled Nelore not showing estrus previous to TAI.

Keywords: Estradiol. Uterus Modulation. Dominant Follicle. Timed Artificial Insemination.

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Summary

1. Introduction

In the beef cattle industry, reproductive efficiency directly impacts the profitability of cow-calf systems. To improve reproductive efficiency, timed artificial insemination (TAI) has been widely implemented in South America to increase the number of pregnant cows without the need to detect estrus. Moreover, the hormonal protocols used for TAI reduce the interval between parturition and conception (DAVIS & WHITE, 2020), particularly in suckled *Bos indicus* cows in postpartum anestrus (BARUSELLI et al., 2004). In the Brazilian cattle industry, the most used hormonal protocols in *Bos indicus* beef breeds include both 17β-estradiol (E-17β) and progesterone (P4).

A fundamental step in the hormonal protocol for TAI is the treatment to induce and synchronize ovulation. Ovulation is typically induced by the administration of E-17β at or after the removal of the P4 device, or by using GnRH analogs 12-18 hours before TAI. Among the exogenous E-17β options, E-17β benzoate (EB) and E-17β cypionate (EC) are the most commonly used. The administration of 1 mg EC at 48 hours before TAI in *Bos indicus* cows increases the proportion of cows in estrus at the time of TAI compared to the administration of GnRH analog at TAI (SÁ FILHO et al., 2011a). However, around 20-40% of the females receiving EB or EC to induce ovulation still have a small DF (<11.5 mm of diameter) and/or do not express estrus at TAI, which is negatively associated with rates of pregnancy per TAI (P/TAI) (SÁ FILHO et al., 2011a; PUGLIESI et al., 2016; NISHIMURA et al., 2018). Moreover, in females bearing a small DF and with low circulating E-17β (PERRY et al., 2005) or

not showing estrous behavior (HILL et al., 2014), the ovulation induced only by GnRH analog is associated with a low P/TAI and an increased rate of pregnancy loss. On the other hand, in females previously treated with EC at the time of P4 device removal, the addition of GnRH treatment at TAI improves ovulation rate and subsequent P/TAI in cows with small DF (ALVES et al., 2021).

Therefore, the reduced pregnancy success in cows with small DF or not showing estrous at TAI is associated with reduced circulating E-17β and may be mitigated by an additional E-17β treatment around TAI. In this regard, in dairy cows receiving GnRH analog for ovulation induction, the treatment with 1 mg E-17β at 24 (SOUZA et al., 2007) or 8 hours (SOUZA et al., 2011) before TAI improved the proportion of cows exhibiting estrus. Moreover, cows that had an endometrial thickness <8 mm and were treated with E-17β had greater P/TAI compared to untreated cows (SOUZA et al., 2011). The injection of E-17β in Holstein's cows promoted an earlier E-17β peak in circulation compared to EB and EC (SOUZA et al., 2005). The circulating E-17β peak occurs 2 hours after treatment, followed by a gradual reduction, regardless of the dose (BÓ et al., 2000). In addition, the dose of E-17β administered increases the luteinizing hormone (LH) within 18 to 24 hours (BÓ et al., 1994).

Therefore, the use of E-17β at TAI may improve ovulation synchrony and benefit the modulation of the uterine environment in beef females with small DF or not showing estrus. The central hypothesis of the current experiment is that administration of E-17β at TAI increases the proportion of females exhibiting estrus, the synchrony of ovulation and modulates the uterine environment in beef females with small DF or that do not demonstrate estrus, ultimately resulting in an increase

of P/TAI. The general aim of this experiment was to evaluate the effects of E-17β administration at TAI on the time of ovulation, uterine environment, estrous expression, and P/TAI, in Nelore cows with small DF or that did not demonstrate estrus at TAI.

2. Material and methods

2.1 Experimental designs

Experiments were submitted and approved by the ethics committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo institution, protocol number 1646060721.

2.1.1 Experiment 1

In Experiment 1, we aimed to characterize the effects of administering different doses of E-17β in cows with small DF (<11.5 mm) on the plasma concentrations of E-17β, ovulation induction, and endometrial thickness and gene expression. Based on a preliminary experiment from our group indicating an improved endometrial thickness growth (FELTRIN et al., 2023) and the known improvement in P/TAI (SÁ FILHO et al., 2011a) in Nelore cows treated with 1 mg EC at P4 device removal, we aimed to evaluate the treatment of different doses of E-17β in females previously treated with EC.

Animals and experimental design

Forty Nelore cows, weighing an average of 560 kg, from the experimental herd of the Fernando Costa campus of the University of São Paulo in Pirassununga – SP, Brazil, were used in two replicas. The cows were allocated in paddocks of *Brachiaria*

decumbens pastures with *ad libitum* access to mineralized salt, water and supplemented with corn silage.

Using a follicular control model to induce a small DF at TAI as described in detail in previous studies (MESQUITA et al., 2014; PUGLIESI et al., 2016), cows were submitted to a pre-synchronization of the estrous cycle (Figure 1). The objective was to promote an early corpus luteum (CL) at the beginning of the protocol for synchronization of ovulation, resulting in more circulating P4 and reduced LH pulsatility, which consequently leads to a slower DF growth (COLAZO et al., 2008) and reduced circulating E-17β (SILVA et al., 2023).

For the pre-synchronization protocol, seven days before the beginning of the synchronization protocol (day -7; day 0 = beginning of synchronization), all cows received two doses of 12.5 mg dinoprost (PGF2α; 2.5 mL, i.m., Lutalyse®, São Paulo, SP, Brazil) 12 hours apart and were submitted to an ultrasonography evaluation. On day -5, 0.25 mg gonadorelin was administered (2.5 mL, i.m., Fertagyl®, MSD, São Paulo, SP, Brazil). On day 0, cows were evaluated by color-Doppler ultrasonography, and those that did not have an active CL, as described by Pugliesi et al (2014), were removed from the experiment. In sequence, the animals received an intravaginal P4 device (1.9 g, CIDR®, Zoetis) and 2 mg EB (2 mL, i.m., Gonadiol®, Zoetis). On day 7, the CIDR was removed, the CL was evaluated by color-Doppler ultrasonography for the exclusion of cows without an active CL, cows received an i.m. administration of 12.5 mg PGF2α and 1 mg EC (0.5 mL, i.m., E.C.P.®, Zoetis), and an estrous detection patch (Boviflag®, ABS Pecplan, Uberaba, MG, Brazil).

On day 9, the DF diameter was determined, and cows with a DF diameter between 8.5 to 11.5 mm were maintained in the experiment. So, from the 80 synchronizations (40 cows used twice), data from 48 cows met the criteria for inclusion in the experiment. Cows meeting criteria for inclusion were randomly divided within replicate into three experimental groups: **Control**: no additional treatment (n=16); **E-2**: received 2 mg E-17β on day 9 (n=16); and **E-4**: received 4 mg E-17β on day 9 (n=16). The E-17β used in this experiment was a commercially available production with a concentration of 10 mg/mL (17 Beta®, Botupharma, Botucatu, SP, Brazil). The base product was diluted in sesame oil to obtain a dilution of 1 mg E-17β per mL, and 2 and 4 mL of this mixture was administered i.m., to cows from E-2 and E-4 groups, respectively.

Cows were evaluated by ultrasonography between days 7 and 11 to detect ovulation and determine uterine characteristics. Also, 4 hours after the treatment on day 9, cows were submitted to a cytological sample collection by cytobrush to determine gene expression.

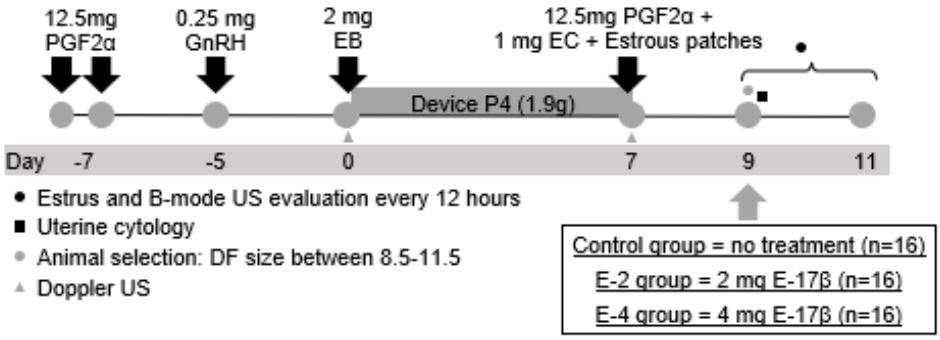

Figure 1. Experimental design: Experiment 1. Nelore cows were submitted to a pre-synchronization. On day -7, all animals received a double dose of 12.5 mg PGF2α in intervals between 12 hours; on day -5, 0.25 mg gonadorelin (GnRH) was administered; on day 0, only animals that had a functional CL started synchronization, and received a P4 device and 2 mg EB; on day 7, the device was removed, 12.5 mg PGF2α and 1 mg EC were administered, and presence of functional CL was confirmed; on day 9, the dominant follicle (DF) size was determined and only cows with a DF diameter between 8.5 and 11.5 mm were used. Estrous and ultrasound evaluations were performed every 12 hours until day 11; a uterine cytological sample was collected 4 hours after treatment.

2.1.2 Experiment 2

Based on the results obtained in Experiment 1, 2 mg of E-17β was chosen for the following experiments. In Experiment 2, we aimed to compare the effects of E-17β associated or not with GnRH administration for ovulation induction and uterine modulation.

In this experiment, 37 Nelore cows from the same herd enrolled in Experiment 1 were used in three replicas. The cows were allocated in paddocks of *Brachiaria decumbens* pastures with *ad libitum* access to mineralized salt, water and supplemented with corn silage.

Cows were submitted to the same pre-synchronization and synchronization protocols used in Experiment 1 to obtain a small DF on day 9 (day 0= beginning of synchronization). The inclusion criteria for this experiment were the same as for Exp. 1; cows that had an active CL at the P4 device removal, and a DF between 8.5 to 11.5 mm in diameter on day 9 were included in the study). A total of 52 synchronization met the inclusion criterial, and within replicate cows were randomly divided into four experimental groups in a 2x2 factorial arrangement, as follows: **Control group**: no additional treatment (n=12); **E-2 group**: received 2 mg E-17β on day 9 (n=13); **GnRH group**: received 0.1 mg GnRH (gonadorelin) on day 9 (n=14); and **GnRH + E-2 group**: received 0.1 mg GnRH plus 2 mg E-17β on day 9 (n=13).

Cows were evaluated by ultrasonography between days 7 and 11 to detect ovulation and determine uterine characteristics.

2.1.3 Experiment 3

Based on the results obtained in Experiment 2, where the effectiveness of the treatment with GnRH analog for anticipation of ovulation of small DF was sustained, we aimed in Experiment 3 to determine the effects of the additional E-17β treatment in GnRH-treated beef cows that did not exhibit estrus at TAI on P/TAI and pregnancy loss. The use of cows not showing estrus was based on the association of the presence of smaller follicles in cows without estrus expression at TAI compared to those in estrus (MADUREIRA et al., 2020).

The study was carried out between December 2021 to March 2022 (breeding season 2021-2022) on a commercial farm located in Iaciara, Goiás, Brazil. A total of 3,839 *Bos indicus* suckled beef cows (multiparous [>2 calves], n=2,892 and secondiparous, n=934) with an average of 48 days post-partum (calving to first TAI) and body condition score of 2.9 at TAI (BCS; on a scale of 1 to 5, in which 1 [emaciated] and 5 [obese]; Ayres et al., 2008) were used. Cows were kept in breeding groups of 80-130 cows each on *Brachiaria decumbens*, *Andropogon gayanus*, *Panicum maximum* (cv Mombaça), and *Panicum maximum* (cv Massai) pastures with *ad libitum* access to water and a mineralized salt.

Cows were submitted to an ovulation synchronization. On day 0 of the protocol, cows received an intravaginal P4 device (0.6 g; Fertilcare 600®; MSD Saúde Animal, São Paulo, Brazil) and administration of 2 mg EB (Fertilcare Sincronização®; 2 mL; i.m.; MSD Saúde Animal). On day 9, the device was withdrawn, and 0.4 g sodium cloprostenol (Ciosin®,1.5 mL; i.m.; MSD Saúde Animal), 1 mg EC (Fertilcare Ovulação®, 2 mL; i.m.; MSD Saúde Animal) and 300 IU eCG (Folligon®; 1.5 ml; i.m.; MSD Saúde Animal) were injected. Also on day 9, cows were painted with chalk

marker (RAIDEX®, Germany) halfway between the hip and tail head to determine the occurrence of estrous at the TAI moment. On day 11, cows were inseminated by 11 operators with semen from 16 sires (1-2 operators and 2-4 sires per breeding group).

At TAI, estrous behavior was evaluated based on the detection of the chalk marker. Thus, cows with chalk marker still present were considered as not showing estrus (19%; 731/3,839) and were selected to receive one of the treatments: **GnRH group**: received 0.1 mg GnRH (gonadorelin) on day 9 (multiparous, n=259 and secundiparous, n=109); and **GnRH+E-2 group**: received 0.1 mg GnRH plus 2 mg E-17β on day 9 (multiparous, n=262 and secundiparous, n=101). A single operator determined through transrectal ultrasonography the diameter of DF and the presence of fluid in the uterine lumen. The remained animals (81%, 3,108/3,829) were considered in estrus and were kept without further treatment.

A pregnancy check was performed 35 days (PC35) after TAI and 65-170 days (confirmatory PC). Pregnant cows on PC35 detected as non-pregnant in the confirmatory PC were considered as undergoing a pregnancy loss. Also, on PC35, the presence or absence of CL in animals that were not pregnant was determined.

2.2 Ultrasonography and palpation exams

In Experiments 1 and 2 the evaluations were performed by a single operator with a duplex B-mode and pulse-wave Color-Doppler ultrasound instrument (MyLab Delta, Esaote Healthcare, Italy), with a linear array multifrequency transducer (3.5-7.5 MHz) in B mode (RES-A, gain 50%, P 74mm, X/M, PRS 1) and Color-Doppler mode (gain 61%, PRF 730Hz, frequency 6.3 MHz, WF 4, PRS 3, PRC M/2). For the Color-Doppler ultrasonography evaluations on days 0 and 7, an active CL was

considered when the luteal tissue area had blood perfusion covering > 25% of its area, as proposed by Pugliesi et al. (2014).

From days 9 to 11, the ovaries were scanned every 12 hours, in Experiment 1, and every 6 hours, in Experiment 2, for determination of the DF size and occurrence/day of ovulation. The follicular diameter was calculated by the mean of the maximum distance between the follicular wall and the perpendicular using the caliper function. Ovulation was considered to have occurred when a previously-identified DF was not detected in a subsequent evaluation and confirmed by observation of a corpus luteum the site of follicle disappearance. The time of ovulation was determined considering the average time between the last evaluation when DF was present and the subsequent evaluation.

Endometrial thickness was also evaluated every 12 hours between days 9 and 11, as described by Pierson and Ginther (1987). Endometrial thickness was determined in the body of the uterus using the caliper function, right after the cervix, measuring the maximum distance between the dorsal and ventral borders of the endometrium when the two ends were as parallel as possible. When fluid was present in the lumen of the uterus, the fluid pocket was measured and the diameter subtracted from overall diameter to obtain endometrial thickness. The differences in endometrial thickness between days 9 and 9.5 and between days 9.5 and 10 was also determined.

In Experiment 3, the ultrasound used for evaluations was an ExaPad (IMV imaging, France) coupled with a linear array multifrequency transducer (3.5-7.5 MHz) in B mode (configuration: depth: 60 mm, frequency: 7.5 Mhz, focus: 15 mm, dynamic range: 70 dB). The DF diameter was determined at TAI as reported in

Experiment 1 in a subgroup of animals (n=569). Only follicles with a diameter from 6 to 18 mm were considered as DF (GIMENES et al., 2008; SÁ FILHO et al., 2010b) and were subsequently used in the analysis (GnRH group, n=260; and GnRH+E-2, n=268). The presence or absence of fluid in the uterine lumen was recorded as previously described (SILVA et al., 2021).

2.3 Detection of estrous behavior

In Experiments 1 and 2, cows received an estrous detection patch applied halfway between the hip and tail head to detect animals showing estrous behavior (received mounting). The patch was evaluated every 12 hours. In Experiment 3, cows were painted with chalk marker halfway between the hip and tail head for evaluation at TAI on day 11. In both methods when the patch or paint had been removed more than 50%, the cow was considered in estrus.

2.4 Blood collections and plasma E-17β assay

In Experiment 1, blood samples were collected from the jugular vein 4h and 12h after treatment for determination of plasma E-17β concentrations. Samples were collected into 9 mL heparinized evacuated tubes (Firstlab, Curitiba, PR, Brazil) and immediately placed in a bath with cooled water and ice. Tubes were centrifuged at 3100 x g for 15 min at 4 °C and the plasma was stored at –20 °C for subsequent measurements.

For the E-17β assay, six samples per group were randomly chosen. Plasma samples (2 mL) were extracted using diethyl ether, as reported by Miura et al. (2020). A commercial ELISA kit was used (Estradiol ELISA Kit, Cayman Chemical Company, EUA) in a single assay. Six standards were used for the generation of the standard

curve (9.76, 39.06, 156.25, 625, 2,500, and 10,000 pg/mL), and samples were run in duplicate. The sensitivity of the assay was 9.8 pg/mL and the intra-assay coefficient of variation was 7.6%.

2.5 Uterine cytology

In Experiment 1, endometrial epithelial cell samples were collected using the cytobrush technique at 4 hours after treatment, as described by Cardoso et al. (2017).

To perform the technique, an adaptation of a non-sterile cervical brush was used (Viamed Ltd, West Yorkshire,UK). The brush was coupled to the tip of a conventional TAI applicator, covered by a disposable sheath, and protected by a sanitary sheath. Then, guided by transrectal palpation, the applicator was introduced into the female's vagina, and after passing the cervix, the sanitary sheath was removed, leading to exposure of the cervical brush in the lumen of the uterine body and rotated to recover epithelial cells. Thereafter, the brush was returned to the sheath to prevent contamination by contact with other portions, and the whole apparatus was withdrawn. The sample was placed in a cryotube containing 1 mL of Trizol (Life Technologies) and then in liquid nitrogen at –196°C, and then stored in a freezer at –80°C. All cows underwent the procedures, but two samples were not taken as the collection device could not pass the cervix, and 11 samples were discarded due to the presence of blood on the cytological brush.

2.6 RNA extraction and RT-PCR quantification

Total RNA was extracted using samples obtained by the cytobrush technique as described by Ferraz et al. (2021). For this, the samples were extracted with Trizol reagent (Life Technologies, Frederick, USA) according to the manufacturer's instructions. Spectrophotometer (Nanovue™ Plus, Spectrophotometer, GE Healthcare, UK, absorbance at 260 nm) was used for measuring the concentration of total RNA on extracts. The ratio of absorbance at 260 and 280 nm was used for estimating the purity of total RNA, and when the samples presented a 260/230 ratio ranging from 1.7 to 2.0, were used for transcript abundance analyses. To avoid genomic DNA contamination, RNA samples were treated with DNAse (DNaseI, Amplification Grade; Life Technologies), as per manufactures instructions. The isolated RNA was submitted to reverse transcription using a High-Capacity cDNA RT Kit (Life Technologies) following the manufacturer's instructions and the cDNA was stored at −20°C until quantitative PCR analysis. The relative abundance of transcripts was carried out using SYBR Green PCR Master Mix (Life Technologies) for the amplification reactions in Step One Plus thermocycler (Applied Biosystems Real-Time PCR System; Life Technologies). Specific primers for each selected gene (Table 2) were designed using Primer Quest (IDT; http://www.idtdna.com/Primerquest/Home/Index) or previous studies reported (Table 2). GeNorm software (https://genorm. cmgg. be) was used to select reference genes, and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and β-actin (ACTB) were shown to be the most stable genes. Relative abundance of transcripts was obtained after normalization of the target genes' Cq values by the geometric

mean of the transcript abundance of reference genes, according to the methodology described by Pfaffl (2001).

Table 1. The primers used for each target gene are in the table below.

Gene	Primer sequences	Reference
α-estradiol receptor	5'-GAATCTGCCAAGGAGACTCG-3' 5'-ATCATCTCTCTGGCGCTTGT-3'	(PERRY et al., 2020)
β-estradiol receptor	5'-ACCTGCTGAATGCTGTGAC-3' 5'-GTTACTGGCGTGCCTGAC-3'	(PERRY et al., 2020)
Progesterone receptor	5'-CAGTGGTCAAGTGGTCTAAATC-3' 5'-TCTCCATCCTAGTCCAAATACC-3'	(CONNOR et al., 2005)
Oxytocin receptor	5'-CGTGCAGATGTGGAGTGTCT-3' 5'-CCTATCAGTCACAGCGTGGA-3'	(PERRY et al., 2020)
β-actine	5'- GGATGAGGCTCAGAGCAAGAGA-3' 5'- TCGTCCCAGTTGGTGACGAT-3'	(ARAÚJO et al., 2016)
GAPDH	5'-GATTGTCAGCAATGCCTCCT-3' 5'-GGTCATAAGTCCCTCCACGA-3'	(HAN et al., 2006)

2.7 Statistical analysis

Data were analyzed with the Statistical Analysis System (SAS, Version 9.4 for Windows, SAS Institute Inc., Cary, NC). In Experiment 1, data were analyzed as orthogonal contrast, contrast 1 (C1) evaluated the E-17β effect (Control *vs.* E-2 and E-4 group) and contrast 2 (C2) effect of dose E-17β (E-2 *vs.* E-4 group). In Experiment 2, data were analyzed as 2x2 factorial: factor 1: GnRH and factor 2: E-17β. Furthermore, an additional analysis was conducted that combined the results of Control and E-2 groups from Experiment 1 and 2, the ovulation rate was evaluated up to 36 and 48 hours post-treatment, increasing the experimental number. In both

experiments, the continuous dependent variables (day and time of ovulation and estrus, DF diameter, endometrial thickness, E-17β concentration, and abundance of transcripts) were evaluated for the normality of residues by the Shapiro-Wilk test, and non-normally distributed data were transformed to logarithm, square root, or inverse scale before analysis if the residual distribution was improved. Data were analyzed using ANOVA with the MIXED procedure considering the main effects of GnRH and E-17β, and time for endometrial thickness and E-17β concentration. For endometrial thickness, data from the first evaluation (day 9) were used as a covariate. For the binomial data in both and additional analysis (ovulation and estrus rate), the Fisher's Exact test was performed by the FREQ procedure of SAS.

In Experiment 3, a first analysis was performed to compare the results between cows with estrus or without estrus at TAI (cows showing estrus *vs.* no estrus [GnRH, and GnRH+E-2 groups]). Then, the effect of E-17β in cows without estrus and treated with GnRH was evaluated. For both analyses, the dependent variables, pregnancy rate (PC35 and confirmatory PC) pregnancy loss, and presence of CL on D35 were analyzed by the GLIMMIX procedure using a binomial distribution. Multivariable models were built, and a backward stepwise elimination method was applied, considering Wald's criterion, where factors with $P > 0.2$ are excluded from the model. The initial statistical model was composed of the fixed effects of estrus behavior (n=2), treatment group (n=2), categories of BCS based on the median BCS (n=2, BCS < 3 or BCS ≥ 3), and their possible interactions. For the random effects, the parity category (secondiparous or multiparous), sire (n=16), and technician (n=11) nested in the breeding group were included in the model. The subgroup of cows with ultrasonography evaluation at TAI (n=528) was submitted to a secondary

analysis using the GLIMMIX procedure and the statistical model was composed by the effects of treatment group (n=2), BCS (n=2), class of DF diameter (n=2, DF< 11mm or DF≥ 11mm) and presence of fluid in the endometrial lumen (n=2, with or without fluid). The relationship between the DF diameter and the probabilities was analyzed separately in each experimental group (GnRH or GnRH+E-2) using the LOGISTIC procedure, where regression curves were created using the coefficients provided by the interactive data analysis. The formula obtained for the relationship between DF and pregnancy probability for GnRH and GnRH+E-2, respectively, were =EXP(0,2856*(DF)+-3,5458)/(1+EXP(0,2856*(DF)+-3,5458)) and =EXP(-0,0404*(DF^2)+1,0998*(DF)+-7,6792)/(1+EXP(-0,0404*(DF^2)+1,0998*(DF)+-7,6792)).

In all experiments, the probability of P ≤0.05 indicated that a difference was significant, and 0.05 < P ≤ 0.1 indicated a tendency of significance. Data are presented as the mean ± SEM.

3. Results

3.1 Experiment 1

Ovarian characteristics

For follicular characteristics (Table 2), as expected, no difference in the pre-treatment DF size (day 9) was observed for both contrasts (Control *vs.* E-2 and E-4 group [C1], and E-2 *vs.* E-4 group [C2]). There was no difference in DF diameter (mm) at the evaluation preceding ovulation between Control and E-17β-treated animals, or between E-17β doses (Table 2). There was a tendency for a greater ovulation rate in cows receiving E-17β compared to Control 48 (75% *vs.* 50%) and

36 hours (25% *vs*. 46.9%) after treatment, but no differences were observed between E-2 and E-4 groups in both moments (Table 2). For the time of ovulation, there was no significant difference in either C1 or C2 contrasts (Table 2).

Table 2. Characteristics of the dominant follicle (DF), estrous expression, ovulation, and endometrial thickness in cows treated or not with 2 or 4 mg of E-17β (Experiment 1).

	Treatment[1]			P value[2]	
	Control (n=16)	E-2 (n=16)	E-4 (n=16)	C1	C2
DF diameter (mm)					
On day 9	9.4 ± 0.2	9.5 ± 0.2	9.3 ± 0.2	0.80	0.40
Before ovulation	9.9 ± 0.3	9.9 ± 0.3	9.8 ± 0.3	0.30	0.70
Estrus					
Expression rate (%)	81.3	81.3	93.4	0.27	0.24
Interval from day 9 to estrus (h)[3]	15 ± 2.8	15 ± 2.5	14.6 ±2.1	0.65	0.89
Ovulation rate (%)					
Until 48 hours after treatment	50	75	75	**0.06**	0.31
Until 36 hours after treatment	25	50	43.8	**0.08**	0.25
Interval from day 9 to ovulation (h)[3]	37.2 ± 2.8	33 ± 2.1	33 ± 2.6	0.24	0.58
Development in endometrial thickness (%)					
Between days 9 and 9.5	-2.4 ± 1.4	-8±3	-8.5 ± 2.1	**<0.01**	0.74
Between days 9.5 and 10	3.3 ± 1.8	11.6 ± 3.7	3.6 ± 2.7	0.26	0.16

[1]E-2 and E-4 indicate the group of cows receiving 2 or 4 mg of 17β-estradiol on day 9 of a synchronization protocol (day 0= beginning of protocol).

[2] An orthogonal contrast was considered in the analyses: C1 (E-17β effect; Control *vs.* Treatment), C2 (dose effect; E-2 group *vs.* E-4 group). Significant differences were declared at P≤0.05 and a tendency was declared when 0.05 < P ≤ 0.1.

[3] Only those who ovulated and had estrus detected until day 11

Abbreviations: DF= dominant follicle; ET= endometrial thickness; h = hours.

Estrous expression

No differences were observed between the Control and the E-17β groups (81.3% *vs.* 87.5%, respectively), nor between E-2 and E-4 groups (Table 2) in proportion of cows exhibiting estrus. In addition, no difference was observed in the time of estrous behavior between contrasts C1 and C2 (Table 2).

Plasma E-17β concentrations

For plasma E-17β concentrations, a significant interaction between dose and time was observed in both contrasts ($P<0.01$). Concentrations of E-17β were greater in cows receiving E-17β (701.1 ± 262.0 pg/mL) at 4 hours after treatment when compared to control cows (59.6 ± 11.8 pg/mL), whereas no difference was observed at 12 hours (Figure 2, Panel A). For the comparison of doses, concentrations of E-17β were greater at 4 hours for cows receiving 4 mg (1,173.6 ± 460.0 pg/mL) than 2 mg (228.6 ± 56.0 pg/mL), but no difference between doses was observed at 12 hours (Figure 2, Panel B).

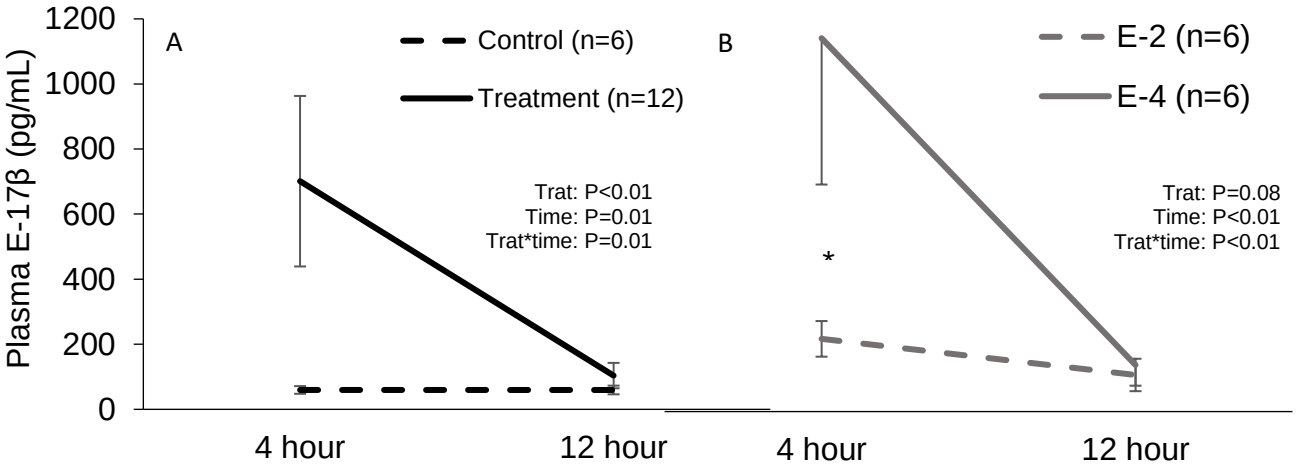

Figure 2. Plasma estradiol concentrations at 4 and 12 hours after treatment. E-2 and E-4 acronyms indicate the group of cows receiving 2 or 4 mg of 17β-estradiol on day 9 of a synchronization protocol (day 0= beginning of protocol). The main effects of treatment (Trat) and time, and interaction (Trat*time) are shown. The asterisk (*) indicates differences (P ≤ 0.05). Panel A: Orthogonal contrast: C1 (E-17β effect) Control vs. Treatment. Panel B: Orthogonal contrast: C2 (dose effect) E-2 group vs. E-4 group. Significant differences were declared at P≤0,05, indicated by asterisk (*), and a tendency was declared when 0.05 < P ≤ 0.1.

Uterine characteristics

For endometrial thickness between days 9 and 11, no effects (P>0.1) of treatment (C1; Figure 3, Panel A) or dose (C2; Figure 3, Panel B) were observed. However, a significant time effect was observed in both contrasts, which reflected an increase in endometrial thickness on day 10 (12.9 mm), followed by a progressive reduction through day 11 (12.4 mm). When evaluating the change in endometrial thickness, a greater rate of decrease in endometrial thickness was observed between day 9 today 9.5 (12 hours after treatment) in the E-17β-treated groups than in the Control group (C1; Table 2). For the comparison of E-17β doses (C2), there was no significant difference. When evaluating the change in endometrial thickness

between days 9.5 and 10 (Table 2), no significant difference was observed for any of the contrasts.

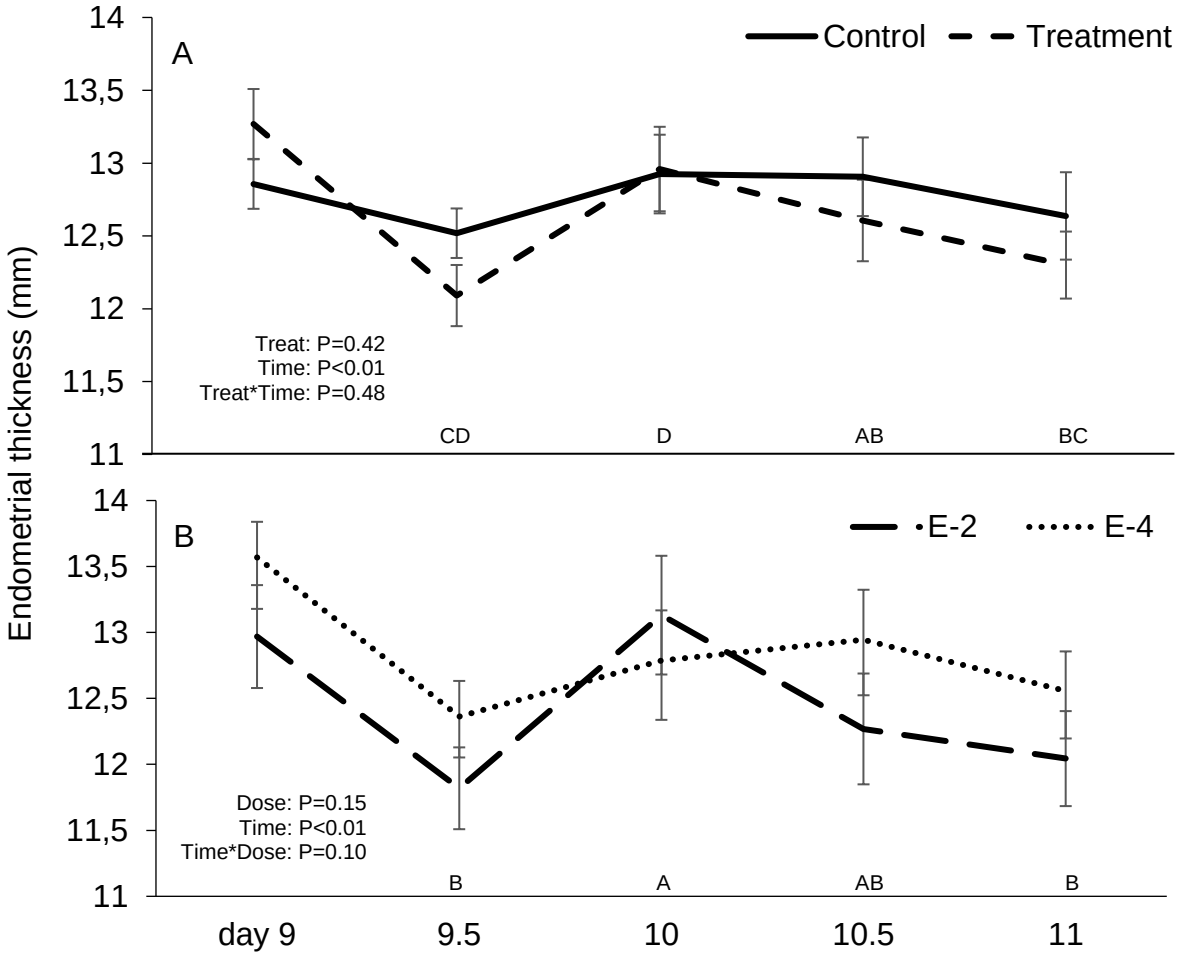

Figure 3. Endometrial thickness over the different evaluation days. E-2 and E-4 acronyms indicate the group of cows receiving 2 or 4 mg of 17β-estradiol on day 9 of a synchronization protocol (day 0= beginning of protocol). The day 9 measurement was considered as a covariate (Cov) in the analysis. The main effects of treatment (Trat), dose, and time and the interactions of treatment and time (Trat*time) and dose and time (Time*Dose) are shown. Panel A: Orthogonal contrast: C1 (E-17β effect) Control vs. Treatment. Panel B: Orthogonal contrast: C2 (dose effect) E-2 group vs. E-4 group. Significant differences were declared at P≤0.05, indicated by capital letters (ABCD), and a tendency was declared when 0.05 < P ≤ 0.1.

Regarding the abundance of transcripts for the genes evaluated, no significant difference was observed in C1 and C2 contrasts for *PGR* expression (Figure 4). For *OXTR*, the abundance was 1.7 times greater in the E-17β-treated groups compared to the Control group (C1), and a trend for greater abundance was observed for the

cows that received 4 mg of E-17β compared to 2 mg (Figure 4). For the transcripts of E-17β receptors, cows in the Control group showed an expression of *ESR1* and *ESR2*, respectively, 2.8 and 2 times greater compared to Controls, whereas no difference between E-17β doses was observed (C2) (Figure 4).

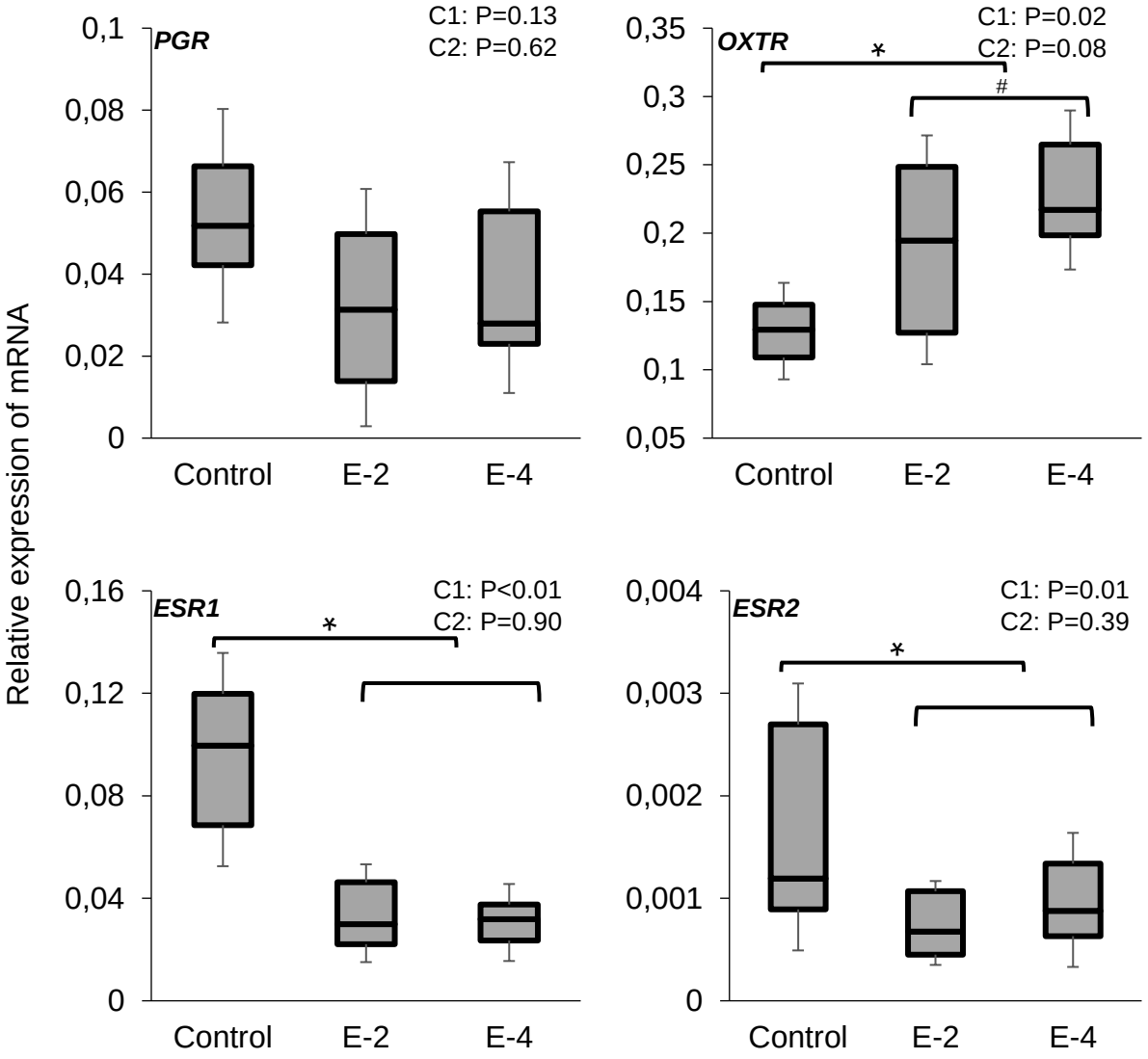

Figure 4. Box plotting of relative transcript abundance of receptors for P4 (*PGR*), oxytocin (*OXTR*), and E-17β (*ESR1* and *ESR2*) 4 hours after cows received or not (Control; n=13) 2 or 4 mg of E-17β (E-2 group; n=12 and E-4 group; n=10). E-2 and E-4 acronyms indicate the group of cows receiving 2 or 4 mg of 17β-estradiol on day 9 of a synchronization protocol (day 0= beginning of protocol). The main effects of orthogonal contrast: C1 (E-17β effect) Control vs. Treatment and C2 (dose effect) E-2 group vs. E-4 group are shown. Significant differences were declared at P≤0.05, indicated by asterisk (*), and a tendency was declared when 0.05 < P ≤ 0.1, indicated by pound sign (#).

3.2 Experiment 2

Ovarian and estrous expression characteristics

No significant (P>0.1) interaction between E-17β and GnRH treatments was observed for ovarian and estrous expression end points, except for the interval from treatment (day 9) to ovulation.

For follicular characteristics (Table 3), no difference in DF size on day 9 was observed between treatments. However, a tendency of smaller DF before ovulation was observed in cows treated with GnRH (10.0 ± 0.2 mm) compared to those not treated (10.7 ± 0.2 mm). No difference (P>0.1) in ovulation rate until 48 hours after treatment was observed among treatment groups, but when evaluating proportion of cows that ovulated by 36 hours after treatment, significant main effects of GnRH and E-17β were detected. A greater ovulation rate until 36 hours after treatment was detected in cows receiving GnRH (P<0.01) compared to those not receiving GnRH (69.2% *vs.*19.2%) and in the cows not receiving E-17β (P<0.01) compared to those receiving E-17β (60% *vs.* 29.6%). There were no significant differences (P>0.1) among groups for estrus expression and interval from treatment to estrus expression (Table 3).

Table 3. Characteristics of the dominant follicle (DF), estrous expression, ovulation, and endometrial thickness in cows treated or not with 2 mg of E-17β or 0.1 mg GnRH analog (Experiment 2).

	Treatment[1]				P value[2]		
Endpoints	Control (n=12)	E-2 (n=13)	GnRH (n=14)	GnRH+ E-2 (n=13)	GnRH	E-17β	GnRH* E-17β
DF diameter (mm)							
On day 9	10 ± 0.2	9.8 ± 0.3	9.4 ± 0.2	9.8 ± 0.3	0.41	0.8	0.38
Before ovulation	10.7 ± 0.3	10.8 ± 0.3	9.7 ± 0.3	10.2 ± 0.2	**0.05**	0.39	0.61
Estrous characteristics							
Interval from day 9 to estrus (h) [3]	10.7 ± 2.5	19 ± 3.8	11 ± 2	14.2 ± 2.8	0.58	0.11	0.86
Ovulation							
Interval from day 9 to ovulation (h)[3]	32 ± 2.5[A]	40.7 ± 1.7[B]	27.6 ± 0.7[A]	27.7 ± 0.7[A]	**<0.01**	**<0.01**	**<0.01**

[1]E-2 indicates the group of cows receiving 2 of 17β-estradiol, the GnRH group of cows receiving 0.1 mg of GnRH analog, and GnRH+E-2 receiving both on day 9 of a synchronization protocol (day 0= beginning of protocol).

[2] Was considering a 2x2 factorial arrangement: GnRH, E-17β or interaction (GnRH*E-17β) effect. Significant differences were declared at P≤0,05, and a tendency was declared when 0.05 < P ≤ 0.1. Capital letters A and B means statistical difference.

[3] Only those who ovulated and had estrus detected until day 11.

Abbreviations: DF= dominant follicle; ET= endometrial thickness; h = hours.

For the time of ovulation, a significant interaction of GnRH and E-2 treatments was observed (Table 3). In cows not receiving GnRH, the interval to ovulation was greater in those treated with E-17β (E-2 group); whereas no difference was observed in cows receiving GnRH and treated or not with E-17β (GnRH *vs.* E-2+GnRH group). Considering the inconsistent results of the E-17β effect on ovulation rate between Experiments 1 and 2 and aiming to gain statistical power, the data of ovulation rate in the common Control and E-2 groups from Experiments 1 and 2 were combined (n=28 for Control, and n=30 for E-2 group). In the combined results, no significant difference was observed between cows from the Control and E-2 groups for the ovulation rate up to 36 hours after treatment (39% *vs*. 33%, respectively; P=0.70), total ovulation rate until 48 hours (64% *vs*. 70%, respectively; P=0.70) and time of ovulation (33.3 ± 1.5 *vs*. 36.1 ± 1.6 hours, respectively; P=0.16).

Endometrial characteristics

For endometrial thickness, no significant effects of GnRH, E-17β or their respective interaction were observed. However, a time effect indicated that endometrial thickness being similar between days 9.5 and day 10.5, but decreasing on day 11 (Figure 5). Likewise, no significant difference was observed in the endometrial development rate between days 9 and 9.5 or between days 9.5 and 10.

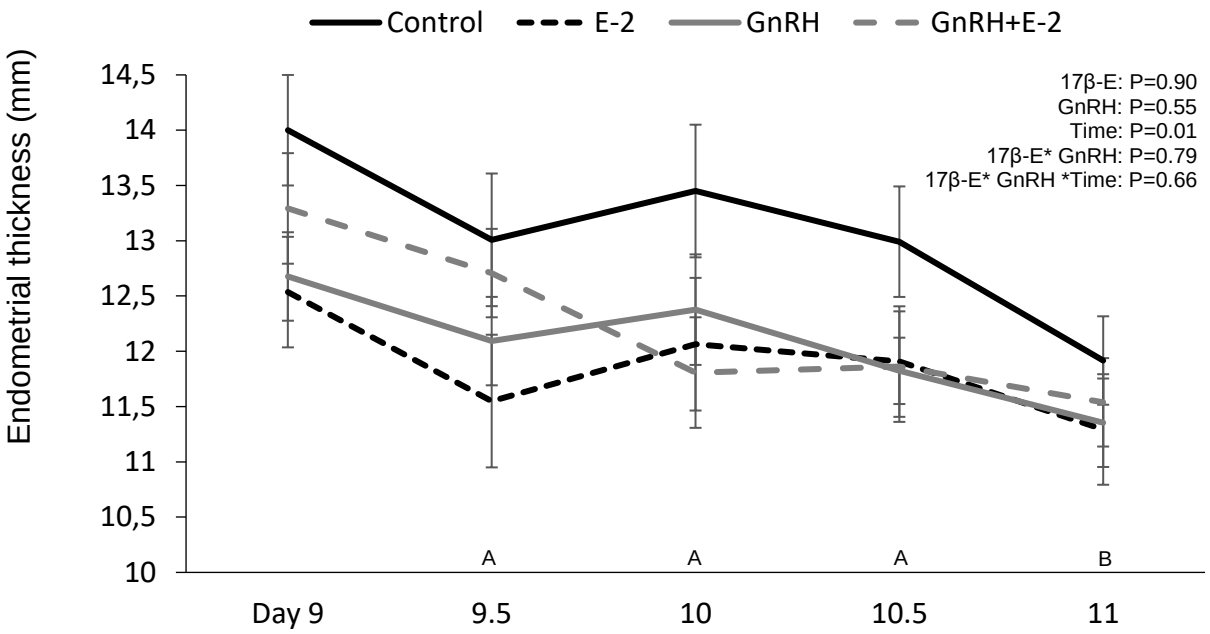

Figure 5. Endometrial thickness over the different evaluation days. E-2 indicates the group of cows receiving 2 of 17β-estradiol, the GnRH group of cows receiving 0.1 mg of GnRH analog, and GnRH+E-2 receiving both on day 9 of a synchronization protocol (day 0= beginning of protocol). The day 9 measurement was considered a covariate in the analysis. The main effects of 17β-estradiol (17β-E), GnRH, and time and the interactions of 17β-estradiol and GnRH (17β-E*GnRH) and 17β-estradiol, GnRH and time (17β-E* GnRH *Time) are shown. It was considered a 2x2 factorial arrangement: GnRH, E-17β, or interaction (GnRH*E-17β) effect. Significant differences were declared at P≤0.05, indicated by capital letters (ABCD), and a tendency was declared when 0.05 < P ≤ 0.1.

3.3 Experiment 3

The overall P/TAI at PC35 and confirmatory PC were 50% (1,947/3,839) and 47% (1,808/3,837), respectively. Greater rates of P/TAI at PC35, and confirmatory PC and CL on PC35 were observed in cows detected in estrus than in those not detected in estrus (Table 4). Also, an effect of BCS on P/TAI and the presence of CL was detected. Cows with high BCS (score ≥3) had greater P/TAI at PC35 and confirmatory PC, and greater presence of CL at PC35, however no change in pregnancy loss (Table 4).

Table 4. Pregnancy per timed-artificial insemination (P/TAI) at pregnancy check on day 35 (PC35) and between 65 and 170 days (confirmatory PC), pregnancy loss, and CL on PC35 according to the expression of estrus at TAI, body condition score and parity order.

	Main effects					
	Estrus[1]			BCS[2]		
End-point	With	Without	P Value[3]	<3	≥3	P Value[3]
PC35 (%)	55 (1,706/3,107)	33 (241/732)	**<0.01**	47 (756/1,612)	53 (1,191/2,227)	**<0.01**
Confirmatory PC (%)[4]	51 (1,592/3,107)	30 (216/732)	**<0.01**	43 (696/1,612)	50 (1,112/2,227)	**<0.01**
Pregnancy loss (%)	7 (112/1,704)	10 (25/241)	0.35	8 (58/754)	7 (79/1,191)	0.30
CL on PC35 (%)	40 (554/1,401)	29 (140/491)	**<0.01**	30 (257/856)	42 (437/1,036)	**<0.01**

[1] Estrus was evaluated at TAI on day 11, cows were painted with chalk marker halfway between the hip and tail head.
[2] BCS was evaluated in at TAI on day 11, on a scale of 1 to 5, in which 1 [emaciated] and 5 [obese]; (AYRES et al., 2009).
[3] Significant differences were declared at P≤0,05, and a tendency was declared when 0.05 < P ≤ 0.1.
[4] Data from two cows were not recorded at the confirmatory pregnancy check.
Abbreviations: PC35 = pregnancy check performed 35 days after TAI; Confirmatory PC = pregnancy check performed 65-170 days after TAI; CL= corpus luteum

For the cows not detected in estrus at TAI, the P/TAI at PC 35 and the confirmatory PC did not differ (P>0.1) between GnRH and GnRH+E-2 groups (Figure 6). In addition, the rates of pregnancy loss and presence of CL on the day of PC35 in non-pregnant cows did not differ (P>0.1) between groups (Figure 6).

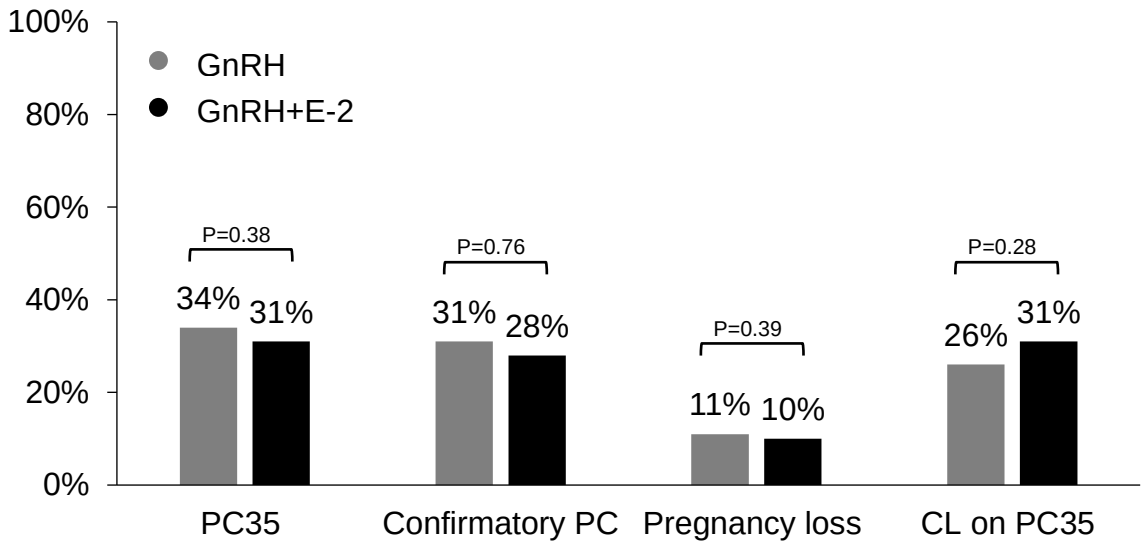

Figure 6. Pregnancy rates at 35 (PC35) and between 65 and 170 days (confirmatory PC) after TAI, pregnancy loss, and presence of CL on the day of PC35 in non-pregnant cows. GnRH indicates the group of cows receiving 0.1 mg of GnRH analog, and GnRH+E-2 receiving both on day 11 of a synchronization protocol (day 0= beginning of protocol). In the statistical analysis, only animals that did not demonstrate estrous were considered. Significant differences were declared at P≤0,05, and a tendency was declared when 0.05 < P ≤ 0.1.

When the P/TAI was analyzed only in the subgroup of cows that was evaluated by ultrasonography at TAI, the overall P/TAI was 37% at PC35 (195/528). The P/TAI was only affected by the DF size. For the GnRH group, a linear relationship indicated that the pregnancy probability increased with the DF diameter increase; whereas for the GnRH+E-2 group, the pregnancy probability increased until DF reaches 13 mm and then decreased after it reached 15 mm (P=0.01; Figure 7).

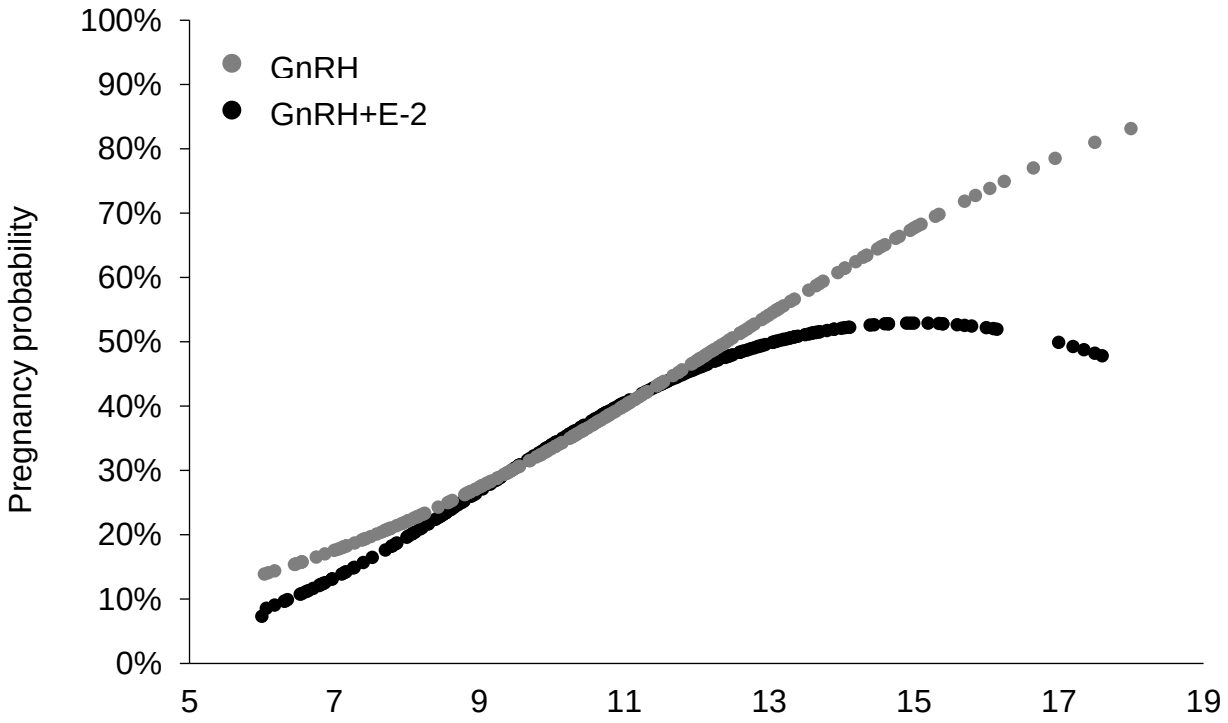

Figure 7. Pregnancy probability (%) according to DF diameter in a subgroup of animals (n=569) evaluated in GnRH (linear function) and GnRH+E-2 (quadratic function) groups. GnRH (n=260) indicates the group of cows receiving 0.1 mg of GnRH analog, and GnRH+E-2 (n=268) receiving both on day 11 of a synchronization protocol (day 0= beginning of protocol). Only cows with follicles 6 to 18 mm in diameter were considered. GnRH indicates the group of cows receiving 0.1 mg of GnRH analog, and GnRH+E-2 receiving both on day 11 of a synchronization protocol (day 0= beginning of protocol). Pregnancy probability to GnRH = EXP (0.2856*(DF)+-3.5458)/(1+EXP(0.2856*(DF)+-3.5458)), to GnRH+E-2 = EXP(-0.0404*(DF^2)+1.0998*(DF)+-7.6792)/(1+EXP(-0.0404*(DF^2)+1.0998*(DF)+-7.6792)). Significant differences were declared at P≤0,05 in both functions.

A separate analysis was performed based on the DF diameter and animals were divided into two categories: ≥ 11 mm, with an average of 13.6 mm, and < 11 mm, with an average of 9.5 mm (Figure 8), similar division used in the previous experiment considering DF larger than ≥ 11.5 mm. A significant interaction between the DF category and treatment group (GnRH or GnRH+E-2) was not detected, but cows with DF≥11 mm had a greater pregnancy rate than DF<11 mm at PC35 (50%, 93/185 *vs.* 30%,102/343), and confirmatory PC (46%, 86/185 *vs.* 26%, 88/343).

However, no significant difference between cows with DF ≥11 or <11 mm was observed for pregnancy loss (Figure 8).

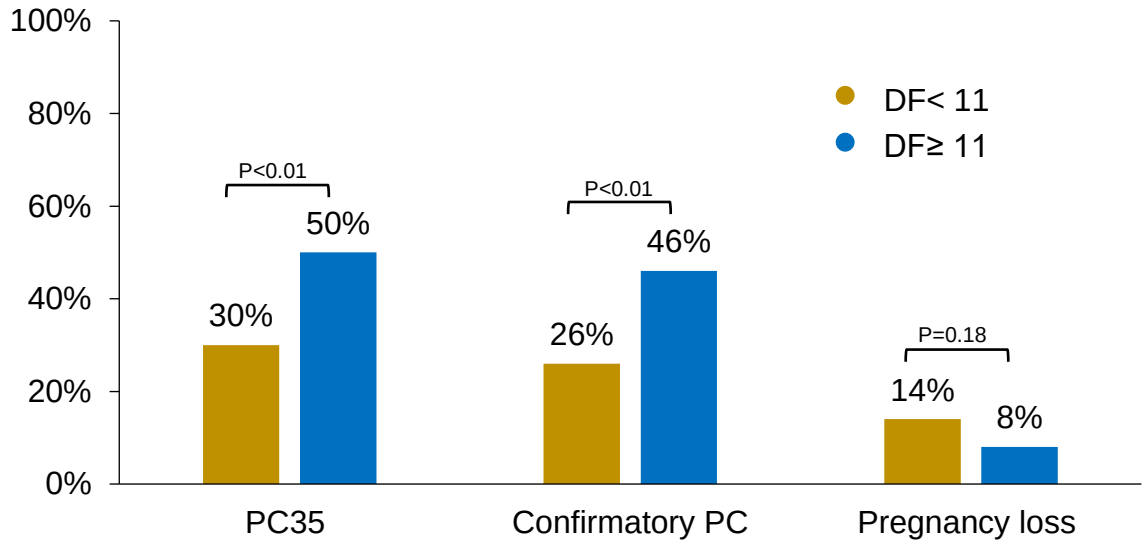

Figure 8. Pregnancy rates at 35 (PC35) and between 65 and 170 days (confirmatory PC) after TAI, pregnancy loss, and presence of CL on the day of PC35 in non-pregnant cows. On day 11 of a synchronization protocol (day 0= beginning of protocol), after treatment, the diameter of the dominant follicle (DF) of a subgroup of animals was measured and divided into two subgroups DF<11mm (n=343) and DF≥11mm (n=185), only follicles with 6 to 18 mm in diameter were considered. Only animals that did not demonstrate estrous were evaluated. Significant differences were declared at P≤0,05, and a tendency was declared when 0.05 < P ≤ 0.1.

In addition, for the cows evaluated at TAI, a separate analysis was performed to evaluate the effect of fluid presence in the uterine lumen at the ultrasonography evaluation at TAI on P/TAI. No significant effect of uterine fluid presence was observed in P/TAI or pregnancy loss, but the DF diameter was larger in cows with fluid present in the uterus at the time of AI than in cows without uterine fluid present (Table 6).

Table 5. Size of the dominant follicle (DF) at TAI, the pregnancy rate per timed-artificial insemination (P/TAI) at day 35 (PC35) and between 65-170 days (confirmatory PC) after TAI and pregnancy loss in a subgroup of animals (n=528) that had their uterus evaluated for presence of luminal fluid at TAI.

	Uterine luminal fluid[1]		P value[2]
End-point	With	Without	
DF diameter (mm)	11.2 ± 0.13	10.4 ± 0.18	**P<0.01**
PC35	40% (138/346)	31% (57/182)	P=0.22
Confirmatory PC[3]	36% (123/346)	28% (51/182)	P=0.26
Pregnancy loss	11% (15/138)	11% (6/57)	P=0.99

[1] On day 11 of a synchronization protocol (day 0= beginning of protocol), after treatment, uterine luminal fluid of a subgroup of animals was measured and divided into two subgroups with (n=346) and without (n=182) uterine luminal fluid
[2] Significant differences were declared at P≤0,05, and a tendency was declared when 0.05 < P ≤ 0.1.
[3] Data from two cows were not recorded at the confirmatory pregnancy check.
Abbreviations: DF = dominant follicle; PC35 = pregnancy check performed 35 days after TAI; Confirmatory PC = pregnancy check performed 65-170 days after TAI; CL= corpus luteum

4. Discussion

A high circulating concentration of E-17β is associated with increased pregnancy success in cattle (JINKS et al., 2013; CIERNIA et al., 2021). Beyond the effects on oocyte and embryo, circulating E-17β during pro-estrus and estrous phases directly affects endometrial function by regulating gene expression (BAUERSACHS et al., 2005; PERRY et al., 2020). In this study, a transient increase in plasma concentrations of E-17β after treatment with 2 mg of 17β-E at TAI modulated the uterine environment in Nelore cows with small DF; however, it was unable to improve ovulation synchrony, the manifestation of estrus, or P/TAI.

The treatment with 2 or 4 mg of E-17β resulted, respectively, in a 3-fold and 19-fold increase in plasma E-17β concentrations at 4 hours after intramuscular injection but was followed by a decrease to pre-treatment levels at 12 hours. A dose-dependent increase in E-17β is expected and was also indicated by Bó et al. (2000)

using four different doses, 0.1, 0.5, 1, and 5 mg. Also, other studies have shown a peak in circulating E-17β between 2 to 6 hours after treatment with different E-17β doses (BÓ et al., 1994, 2000; GOMES et al., 2009; SOUZA et al., 2011). Although the E-17β concentrations at 4 hours after treatment were greater in cows receiving 4 mg compared to 2 mg, the effects on E-17β concentrations at 12 hours and in the endometrial gene expression and thicknesses were not different between the two doses. So, in *Bos indicus* cows, 2 mg E-17β could be an effective dose to modulate the uterine environment and estrus behavior. However, different from the observed increase of estrus expression in *Bos taurus* dairy cows (SOUZA et al., 2007), any E-17β dose improves the estrus characteristics in the present study. This difference between studies could be associated with the pre-treatment with 1mg E-17β cypionate in the present study, which resulted in a high estrus expression rate (81-87.5%) in cows with small follicles in Experiments 1 and 2, compared to a 44,4% estrus expression in the earlier study (SOUZA et al., 2007).

Modulation of endometrial function and uterine lumen environment by E-17β during pro-estrus has been well-documented to be essential for pregnancy success (PERRY et al., 2023). In a regular estrous cycle, a reduction of P4 in the bloodstream after luteolysis associated with the increase of E-17β synthesized by the dominant follicle is directly related to uterine modulation (SOUZA et al., 2011; BINELLI et al., 2022). Under these conditions, increased cellular proliferation and endometrial thickness occur before ovulation, and both characteristics are related to increased conception rates (PIERSON AND GINTHER, 1987; SOUZA et al., 2011; ARAI et al., 2013; ABDELNABY and ABO EL-MAATY, 2020). The effect of treatment with E-17β esters during TAI protocols on endometrial thickness, which is a direct indication of

endometrial cell proliferation and edema has been demonstrated by the treatment with 1 mg EC after P4 device removal in Nelore heifers (FELTRIN et al., 2023), and at different concentrations of circulating estradiol in Nelore cows (MOTTA et al., 2020). In the present study, our expectation that the additional treatment with E-17β at 48 hours after EC injection will enhance the endometrial thickness was not confirmed. Also, a decrease in endometrial thickness, regardless of the E-17β treatment in Experiment 1, suggests that the proliferation process and uterine edema may reduce close to the ovulation period, behavior not found in other studies. In addition, a high variation in endometrial thickness among the animals was noted, which could be associated with the number of pregnancies and its impact on the uterus size (BAEZ et al., 2016), since multiparous cows were used and the number of parturitions with these females was unknown. Furthermore, the study did not measure the progesterone concentration of the animals at the time of evaluation, a hormone that also influences the endometrium thickness (MOTTA et al., 2020). In addition to endometrial morphology alterations, the abundance of *ESR1* and *ESR2* in the uterine epithelium was decreased, and *OXTR* was elevated in cows that received E-17β. A similar response was observed when 3 mg E-17β was injected during late diestrus in Nelore heifers (OLIVEIRA et al., 2022). Starting from the end of diestrus, *ESR1* and *OXTR* are stimulated in the uterus (ROBINSON and MANN, 2001; MARTIN et al., 2008), and increasing circulating E-17β during pro-estrus and estrous phases affect uterine gene modulation during subsequent diestrus (BRIDGES et al., 2012; BOSOLASCO et al., 2021). A greater gene expression of the *ESR1* receptor was observed 8.5 days after embryo transfer in animals with greater concentrations of E-17β and that were not pregnant (BOSOLASCO et al.,

2021). However, the relationships between uterine gene expression and fertility are still unclear, being a response that is affected by different.

The additional treatment with GnRH at TAI in E-17β/P4-based protocols has been recently used as a strategy to promote better pregnancy rates in suckled *Bos indicus* beef cows, mainly in those that did not demonstrate estrus (SÁ FILHO et al., 2010a; MADUREIRA, et al., 2020; ALVES et al., 2021). This occurs because GnRH treatment leads to an earlier ovulation (20 to 33 hours after administration), allowing a viable window for fertilization after insemination (STEVENSON et al., 2004; BARROS et al., 2000; SÁ FILHO et al., 2010a; SÁ FILHO et al., 2010b). This effect was confirmed in females with small DF, as observed by ovulation that occurred about 9 hours earlier in GnRH-treated cows in Experiment 2. Furthermore, GnRH enhanced the proportion of cows that ovulated by 36 hours after treatment, similar to what was observed in other studies (SCHMITT et al., 1996; Alves et al., 2021). On the other hand, cows treated with GnRH had a smaller DF before ovulation, which could lead to a smaller CL size (RODRIGUES et al., 2019; SÁ FILHO et al., 2010b; VASCONCELOS et al., 2001). The enhanced chance for conception caused by the earlier ovulation may overcome this limitation on DF and CL size and benefits the pregnancy success in GnRH-treated cows; however, without altering circulating E-17β (MEE et al., 1993).

Therefore, we hypothesized that additional treatment with E-17β at the periovulatory period in Nelore cows could modulate the E-17β-related uterine characteristics and improves P/TAI in GnRH-treated cows with small DF or not showing estrus. Contrary to our hypothesis, adding 2 mg E-17β at TAI did not increase the P/TAI in GnRH-treated cows not showing estrus, as no difference in

P/TAI was observed between E-17β-treated and untreated cows. Also, the rate of pregnancy loss was similar between E-17β-treated and untreated cows. Thus, our central hypothesis was only partially supported as we did observe E-17β-induced changes in uterine gene expression, but they were not associated with improvement of uterine receptivity in cows not showing estrus submitted to TAI. The absence of improvement by adding E-17β at TAI could be related to the time of administration chosen in the present study, which was chosen based on the best moment for applied purposes in beef cattle. Souza (2011) reported that dairy cows with a thinner endometrium when treated with 1 mg of E-17β 8 hours before TAI, tended to have greater P/TAI. Therefore, for further perspectives and investigations, the study of the effects of additional doses of estradiol at different times before TAI in beef cows is indicated.

Although the positive relationship between P/TAI and DF size has been already reported elsewhere (SILVA et al., 2018), results from Experiment 3 support that Nelore cows with DF >11 mm had greater pregnancy success, even when assessing only females not showing estrus. Interestingly, the P/TAI in cows without estrous detection but with DF >11mm was equal to those females showing estrus (50%). Also, cows receiving only GnRH at TAI had an increase in the probability of P/TAI as the increase of the DF diameter; whereas for cows treated with E-17β, the probability increased only until DF reached 13 mm and then decreased when it was over 15 mm. This positive and linear relationship between DF size and probability of pregnancy has also been observed in other studies where females received GnRH at the time of TAI (RODRIGUES et al., 2019; SÁ FILHO et al., 2011a). A decrease in pregnancy probability after the DF diameter reaches 15 mm was also observed in

animals that received 1 mg with EC two days before TAI but without GnRH treatment at TAI (MARTINS et al., 2017). Therefore, it can be inferred that as the diameter of DF increases, the probability of ovulation also increases, regardless of GnRH treatment. However, in animals with a DF >15 mm, the addition of an additional source of E-17β can result in higher levels of circulating E-17β, potentially reducing the P/TAI.

The reduction in pregnancy probability could be related to an excessive circulating E-17β as a DF >15 mm has a greater ability to produce E-17β (MESQUITA et al., 2014; GONELLA-DIAZA et al., 2015). So, a greater E-17β associated with the E-17β treatment and large DF may have compromised the time of ovulation. In this regard, previous studies demonstrated that the occurrence of more than one LH surge, stimulated by DF and the application of GnRH, can lead to a decrease in the concentration of circulating P4 in diestrus (LUCY and STEVENSON, 1986; PERRY and PERRY, 2009). So, hypothesis needs further investigations to be confirmed, the combination of EC, E-17β, and GnRH treatments may have induced successive LH surges reducing of pituitary LH stores, that returns to the pre-ovulatory peak only after 1 day (NETT et al., 1987), delaying ovulation in cows with large DF or compromise the CL development. With this, defining an optimal moment of treatment that stimulates more circulant E-17β, seeking an additional stimulus in animals with small DF but not disturbing the ongoing LH surge in animals with large DF, is needed.

The relationship between uterine characteristics and pregnancy success has not yet been fully characterized, but recent evidence indicates that the periovulatory profile of E-17β and P4 are associated with uterine luminal fluid and metabolites. In

the here in results, cows with fluid present in the lumen of their uterus had a greater DF diameter at TAI, but presence of fluid in the uterus did not impact P/TAI. This result is not in agreement with those reported in dairy cows (AHMADI et al., 2019). Also, more uterine fluid is observed in animals showing estrus (SILVA et al., 2021), which leads to better fertility The differences between results of the current experiment and previous reports could be associated with the fact that the uterine fluid was only evaluated in animals that did not show signals of estrus between P4 device removal and TAI in the present study.

In summary, E-17β administration at the time of TAI promotes a transient increase in circulating E-17β, leading to reduced expression of *ESR1* and *ESR2,* and increased expression of *OXTR* within 4 hours after administration. The effect on uterine gene expression was not associated with any impact on endometrial thickness, proportion of cows exhibiting estrus, or time of ovulation in Nelore cows with DF <11.5 mm in diameter. In addition, the administration of 2 mg E-17β at TAI in suckled Nelore cows did not impact the P/TAI and pregnancy loss. Cows not showing estrus and with a large DF (≥11 mm) have greater P/TAI than those with a small DF (<11 mm) and this P/TAI is similar to that of cows that exhibited estrus. Finally, administration of E-17β at TAI is not recommended in suckled Nelore cows, but further studies are indicated to understand the effects of different strategies to increase E-17β before TAI on LH surge, uterine modulation, and pregnancy success in cows with a small DF or not showing estrus.

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