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

Interactions of circulating estradiol and progesterone on pituitary responsiveness to GnRH and changes in endometrial area

Jéssica Cristina Lemos Motta

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

Piracicaba
2019

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Veterinarian

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

Advisor:
Prof. Dr. **ROBERTO SARTORI FILHO**

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DEDICATION

To my parents, Ronaldo and Eliane, you are my inspiration, I love you.

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happiness in each of my victories. I love and I miss you both. Hopefully we will see each other again, even in another life.

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Thank you, Lord! Although there is no scientific explanation for what you are and for what you do, I do believe and trust you. Thank you for everything that you did and will do in my life.

“Try to understand the difficulties of the others.
Do not conserve resentments.
Forgive the offenses, whatever they are, forgetting the unpleasant events.
Work as much as you can, being helpful as much as you can.
Spend the free time serving your Neighbor.
Adopt simplicity to have Peace.
Continue learning always.
Forget yourself, creating happiness for the others.
Live in Peace with yourself and let the others live their own existence.
Cultivate patience without anxiety, being to the others what you hope from them,
thus you will always be in the way of the true happiness.”

Translated from Andre Luiz

SUMMARY

RESUMO

Interações do estradiol e da progesterona circulantes na responsividade pituitária ao GnRH e nas mudanças da área endometrial

As mudanças na progesterona (P4) e estradiol (E2) circulantes durante o proestro produzem alterações dinâmicas na função endometrial e na liberação das gonadotrofinas pela adenohipófise. Efeitos independentes e combinados da P4 e do E2 no endométrio e adenohipófise foram avaliados. No Exp.1, foi elaborado um modelo de proestro usando apenas hormônios exógenos a partir da remoção do CL e folículos ≥ 5 mm seguido por remoção gradual de dois dispositivos intravaginais contendo P4 durante 18 h e tratamento com doses crescentes de benzoato de estradiol (BE) durante 48 h para mimetizar o proestro, usando AltoE2 (n = 9) ou BaixoE2 (n = 9). Redução da P4, aumento do E2, e aumento da área endometrial (AE) simularam valores similares ao proestro em vacas do grupo AltoE2, o qual foi usado subsequentemente. No Exp. 2 foi realizado um arranjo fatorial 2x2 contendo: AltoE2&BaixaP4 (n = 11); AltoE2&AltaP4 (n = 11); BaixoE2&AltaP4 (n = 11); BaixoE2&BaixaP4 (n = 10). Após 48 h do início dos tratamentos com BE, foi avaliada a liberação de LH e FSH após desafio com GnRH. As variáveis foram analisadas usando o PROC MIXED do SAS e área sob a curva (AUC) foi calculada com MESS. A AE aumentou durante 18 h apenas nas vacas HighE2&LowP4. Para FSH, animais do AltoE2 apresentaram maior AUC e FSH no pico após GnRH comparados aos grupos BaixoE2, com efeitos negativos discretos da AltaP4. Para LH, concentração ao pico e AUC foram 2 vezes maiores nas vacas de AltoE2 comparados a BaixoE2, com AltaP4 reduzindo essas variáveis em uma proporção similar quando comparado aos grupos de BaixaP4. Assim, máximas mudanças no útero e na adenohipófise durante o proestro dependem de ambos, BaixaP4 e AltoE2, mas E2 e P4 apresentam diferentes ações. Alterações no endométrio dependem de BaixaP4 e AltoE2, enquanto que a liberação de FSH induzida por GnRH depende primariamente de AltoE2, e a liberação de LH induzida por GnRH é independentemente aumentada pelo AltoE2 ou reduzida pela AltaP4.

Palavras-chave: FSH, LH, Proestro, Útero

ABSTRACT

Interactions of circulating estradiol and progesterone on pituitary responsiveness to GnRH and changes in endometrial area

Changes in circulating progesterone (P4) and estradiol (E2) during proestrus produce dynamic changes in endometrial function and pituitary release of gonadotropins. Independent and combined effects of P4 and E2 on endometrium and pituitary were evaluated. In Exp.1, an exogenous hormone model of proestrus was created by removal of CL and follicles ≥5 mm followed by gradual removal of intravaginal P4 implants during 18h and treatment with increasing doses of estradiol benzoate during 48h to mimic proestrus using HighE2 (n=9) or LowE2 (n=9). Decreased P4, increased E2, and increased endometrial area (EA) simulated proestrus values in HighE2 cows and this was used subsequently. Experiment 2 used a 2x2 factorial design with: HighE2&LowP4 (n=11); HighE2&HighP4 (n=11); LowE2&HighP4 (n=11); LowE2&LowP4 (n=10). At 48h, GnRH-induced LH and FSH release was determined. Variables were analyzed using PROC MIXED of SAS and AUC calculated with MESS. The EA increased dramatically during 48h only in HighE2&LowP4 cows. For FSH, HighE2 cows had greater AUC and FSH peak after GnRH than LowE2 groups, with mild negative effects of HighP4. For LH, concentration at peak and AUC were 2-fold greater in HighE2 compared to LowE2 groups, with HighP4 reducing in a similar proportion compared to LowP4 groups. Thus, maximal changes in uterus and pituitary during proestrus depend on both LowP4 and HighE2 but E2 and P4 have different actions. Changes in endometrium depend on LowP4 and HighE2, whereas GnRH-induced FSH secretion primarily depends on HighE2, and GnRH-induced LH secretion is independently increased by HighE2 or reduced by HighP4.

Keywords: FSH, LH, Proestrus, Uterus

1 INTRODUCTION

The dramatic changes in circulating steroid concentrations throughout the estrous cycle have been well-characterized in many different species including various breeds of cattle [1-3]. Circulating estradiol (E2) concentrations are dependent upon the E2 precursor production by the theca cells followed by the aromatization by the granulosa cells from the ovarian follicles in a process that is termed the two-cell, two-gonadotropin model of E2 synthesis [4]. Following the LH surge and ovulation, the follicular granulosa and thecal cells become the large and small steroidogenic luteal cells, respectively, with greatly increased circulating progesterone (P4) during the luteal phase and pregnancy, due to induction of the P4 synthesis pathways in these cells [5, 6]. The proestrous period is the time of the most dynamic changes in steroid hormone concentrations. There is a decrease in circulating P4 from peak to nadir concentrations occurring during a 24-32 hour period with a contrasting increase in circulating E2 from minimal to peak concentrations during a 48-72 hour period [7]. Thus, the hormonal milieu changes from a P4-dominated to an E2-dominated environment during a two-day interval. These dynamic changes in circulating steroid hormones have marked consequences for the physiology of a variety of different tissues, including the uterus and anterior pituitary gland.

The dramatic effects of ovarian hormones on endometrial morphology were first described in the early 1900s [8] with current studies focused on the molecular actions of E2 and P4 in the uterus [9]. Using ultrasonography, substantial oscillations in endometrial thickness (ET) have been described throughout the cycle of several species including bovine [10] and primates [11]. In humans, there is an increase in endometrial area (EA) from 50-150% relative to D0 of the cycle (day of the highest LH concentration) that occurs during the follicular/proliferative phase of the menstrual cycle when circulating P4 is low and E2 is elevated. In contrast, there is a corresponding reduction in EA during the luteal phase when circulating P4 concentrations are increasing [12]. Similarly in cattle, ET increases during the follicular or proestrous phase when circulating P4 is decreasing and E2 is increasing, whereas ET is lower during the luteal phase when P4 is elevated [10]. Souza et al. [13] evaluated ET in the uterine horns during an induced proestrus and observed dramatic changes in ET during a 48 hour period as circulating P4 decreased and E2 increased. Nevertheless, no previous study was designed to differentiate the role of decreasing P4 separately from the role of increasing E2 in the endometrial changes that occur during proestrus.

In addition to the uterine actions, ovarian steroids have effects on many other tissues including the anterior pituitary gland, regulating the gonadotropins secretion. Studies in both beef and dairy cattle and using either *Bos taurus* or *Bos indicus* breeds have demonstrated a dramatic reduction in the GnRH-induced LH surge in the presence of high circulating P4, compared to a lower P4 environment [14-17]. However, the distinct effects of ovarian steroids on GnRH-induced LH or FSH release were confounded in these previous studies because high circulating P4 environments were also marked by low circulating E2. Conversely, the low circulating P4 environments with the greatest response to GnRH treatment also had elevated circulating E2. Previous studies using cultured pituitary cells [18] or laboratory rodents [19] have suggested differential effects of E2 and P4 on pituitary release of gonadotropins, however these distinct effects of E2 and P4 on pituitary gonadotropin release have not yet been clearly differentiated during in vivo manipulative studies.

The long-term goal of this research is to understand the physiological actions of ovarian steroids on steroid-responsive reproductive tissues. For this specific study, we chose to evaluate the effects of physiologic concentrations of P4 and E2 on the uterus and anterior pituitary gland. To have a consistent physiologic model, we first developed a program treating cows with exogenous hormones that produced changes in circulating E2 and P4 concentrations that simulated the changes that occur during the bovine proestrous period (Experiment 1). This model was then used to determine the independent and interactive roles of circulating E2 and P4 on the steroid-induced changes in the endometrium and anterior pituitary during proestrus. Endometrial thickness and area (ET and EA) were used as a proxy for steroid-induced changes in uterine function since it has been shown to dramatically change during proestrus and it can be accurately monitored on a frequent basis using transrectal ultrasound. To monitor steroid-induced changes in anterior pituitary function, a GnRH challenge was performed and the dynamic changes in circulating FSH and LH concentrations were monitored. Therefore, the main objective of Exp. 1 was to develop a proestrus-like model of the changes in circulating E2 and P4 using only exogenous hormonal treatments. The main objective of Exp. 2 was to determine the independent and combined roles of the increase in circulating E2 and the decrease in circulating P4 on three physiologic changes that occur during proestrus, increased EA and increased GnRH-induced LH and FSH secretion. Our first hypothesis was that both the decrease in P4 and the increase in circulating E2 would be required for complete proestrus-type responses in all three physiologic responses. Our second hypothesis was that the relative roles of changes in E2 and P4 would vary for the three different physiologic measures. The bovine model was used since it has previously been

utilized as an excellent animal model for the proestrus period [13], optimal changes in circulating steroids during proestrus have been found to correlate with fertility in cattle [20], and techniques have been well validated for frequent evaluation of endometrial (transrectal ultrasonography) and pituitary (blood sampling after GnRH challenge) functions that dramatically change during proestrus.

2 MATERIAL AND METHODS

In order to study the influence of circulating P4 and E2 on EA and pituitary responsiveness to GnRH, two sequential experiments were developed. In the first study we developed a proestrus-like protocol using only exogenous hormones. Based on the results from this experiment, in the subsequent study we developed four distinct hormonal environments by combining different circulating E2 and P4 concentrations. These studies were conducted from October of 2017 to July of 2018 at the University of São Paulo, Piracicaba, SP, Brazil. All procedures were approved by the Animal Research Ethics Committee of Escola Superior de Agricultura "Luiz de Queiroz" (CEUA - 2017.5.1619.11.5). Nonlactating and nonpregnant multiparous Holstein cows were used in both experiments and were kept on pasture (*Cynodon ssp*.) with haylage (*Cynodon dactylon*) supplementation twice a day and with free access to water and mineral salt.

2.1 Experiment 1

2.1.1 Proestrus protocol

A total of 18 cows (BCS: 3.43 ± 0.12; scale: 1 to 5 [21]) were used to develop a hormonal milieu similar to proestrus using only exogenous hormones. In order to have all cows at a similar hormonal status at the first day of the proestrus-protocol, cows were presynchronized using an intravaginal P4 implant (1g P4, Repro neo, GlobalGen Vet Science, Jaboticabal, SP, Brazil) and treatment with 2 mg estradiol benzoate (EB) i.m. (Syncrogen, GlobalGen Vet Science). Seven days later (D0) the implant was removed and the proestrus protocol was initiated (Figure 1). Thus, on D0 two intravaginal implants (2g P4 each, Repro sync, GlobalGen Vet Science) were inserted and 0.526 mg cloprostenol sodium (PGF; Induscio, GlobalGen Vet Science) i.m. was administered. A second treatment with PGF was performed 24 hours later. On D4 and D5 all follicles ≥ 5 mm were aspirated (ASP) in order to remove the endogenous source of E2. On D5, after ASP, cows were randomly assigned to two groups: LowE2 vs. HighE2. The total dose of EB (0.4 mg for LowE2; 0.8 mg for HighE2) was distributed into eight increasing i.m. injections 6 hours apart (LowE2: 0.02; 0.02; 0.04; 0.04; 0.06; 0.06; 0.08; 0.08 mg; HighE2: 0.04; 0.04; 0.08; 0.08; 0.12; 0.12; 0.16; 0.16 mg) to mimic the gradual increase of E2 throughout proestrus. The first EB treatment was performed on D5 after ASP. The EB was diluted in sterilized filtered peanut oil (provided by GlobalGen

Vet Science) to achieve the concentration of 0.04 and 0.08 mg/mL, for the LowE2 and HighE2 treatments, respectively. Gradual reduction of circulating P4 concentration was provided by removing the first implant on D5 concomitant to the first EB treatment and removing the second one 18 hours later.

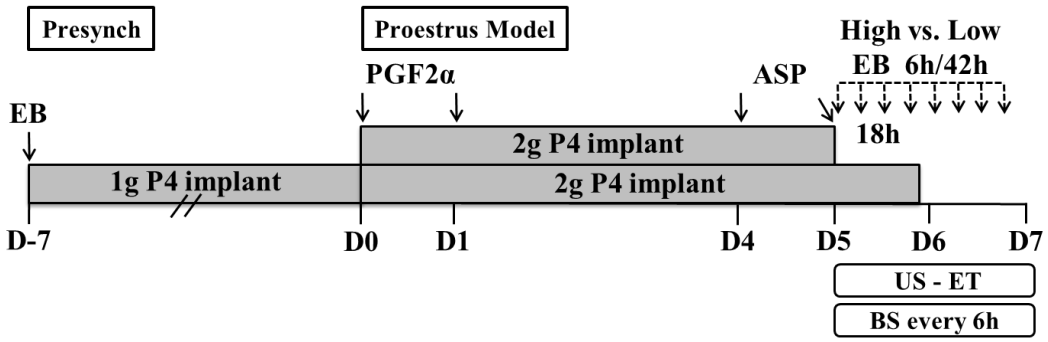

Figure 1. Schematic representation of experiment 1 procedures. Presynchronization: D-7: 2 mg estradiol benzoate (EB) i.m. and one intravaginal implant containing 1 g progesterone (P4). Proestrus model: D0: 1 g implant withdrawal; insertion of two implants containing 2 g P4 each; 0.526 mg cloprostenol sodium i.m. (PGF2α); D1: second dose of PGF2α; D4: follicular aspiration (ASP) of all follicles ≥ 5 mm. D5: second ASP; one P4 implant was removed; second implant was removed 18 hours later; from D5 to 42 hours later EB was administered every 6 hours in the HighE2 group (n = 9; 0.04; 0.04; 0.08; 0.08; 0.12; 0.12; 0.16; 0.16 mg) and Low E2 group (n = 9; 0.02; 0.02; 0.04; 0.04; 0.06; 0.06; 0.08; 0.08 mg); US - ET: Transrectal ultrasound examination on D5 and 12, 24, and 48 hours later for endometrial thickness. BS: Blood samples for estradiol (E2) every 6 hours just before each EB treatment and 6 hours after the last treatment; for P4 at 0, 12, 18, 24, 30, and 48 hours after the first P4 implant removal.

2.1.2 Endometrial dynamics

Endometrial dynamics were evaluated just after ASP and before the first EB treatment on D5 and 12, 24, and 48 hours later using the technique previously described [13]. Briefly, a 7.5 MHz linear-array transducer (Medison, Sonoace PICO, Seoul, Korea) was placed transrectally ~2 cm from the uterine bifurcation in order to generate a transversal image of the uterine horn. Then, the image was frozen and saved, one uterine horn at a time. Later, saved images were analyzed in the ultrasound machine. Endometrial thickness corresponded to the distance from endometrial lumen to the endometrium/myometrium interface. Both uterine horns were measured and the average value was used to calculate the EA, considering the endometrial image as a circle (πXR2; R [radius] was the ET). All ultrasound examinations were performed by the same technician.

2.1.3 Blood sample collection and hormone assay:

Blood samples were collected by coccygeal venipuncture into heparinized evacuated tubes (Vacutainer; 9 mL, Becton Dickinson, Franklin Lakes, NJ, EUA) for later E2 and P4 analyses. Tubes were centrifuged at 1,700 x g at 4oC for 15 minutes and plasma samples stored at -20^{o} C until assayed. For P4, samples were taken on D5 just before the first implant removal and 12, 18, 24, 30, and 48 hours later. From D5 to D7 blood samples were collected every 6 hours just before each EB treatment (0 to 42 hours) and 6 hours after the last treatment for E2 analyses. Concentrations of P4 were determined using a solid phase radioimmunoassay (RIA) kit (ImmuChem Progesterone Coated Tube, MP Biomedicals, Santa Ana, CA, EUA) according to the manufacturer's instructions, except for times of incubation, as described by Melo et al. [22]. Plasma E2 was evaluated by a hand-made RIA [23] with some modifications [24]. Sensitivity, and intra- or inter-assay coefficients of variation for P4 assay were 0.01 ng/mL, 8.2 and 5.5%, respectively. For the E2 assay, the coefficients of variation were < 5% and the sensitivity was 0.05 pg/mL.

2.2 Experiment 2:

2.2.1 Hormonal protocol and endometrial dynamics:

In order to study the influence of circulating P4 and E2 concentrations on EA and pituitary responsiveness to GnRH, 43 cows were randomized into four treatment groups using a 2X2 factorial experimental design: HighE2&LowP4 (n = 11); HighE2&HighP4 (n = 11); LowE2&HighP4 (n = 11); LowE2&LowP4 (n = 10). All groups received similar treatments as described in experiment 2 from D-7 to D5 (D-7: 2 mg EB and 1g P4 implant; D0: 1g P4 implant withdrawal, 0.526 mg PGF and two 2g P4 implants insertion; D1: 0.526 mg PGF; D4 and D5: ASP). On D5, groups with low P4 (HighE2&LowP4 and LowE2&LowP4) had one implant removed and 18 hours later the second implant was removed (same as done in experiment 1). In the high P4 groups, both P4 implants were maintained. The EB treatments were performed as described in the HighE2 (0.8 mg of EB) group from experiment 1 from D5 to D7 in both the HighE2&LowP4 and HighE2&HighP4. Groups with low E2 did not receive EB treatments. On D7, after the last ET evaluation and blood collection for E2 and P4, all cows were treated with 8.4 µg buserelin acetate (GnRH; Maxrelin, GlobalGen Vet Science). The EA was evaluated as described in experiment 1, following the same schedule (Figure 2).

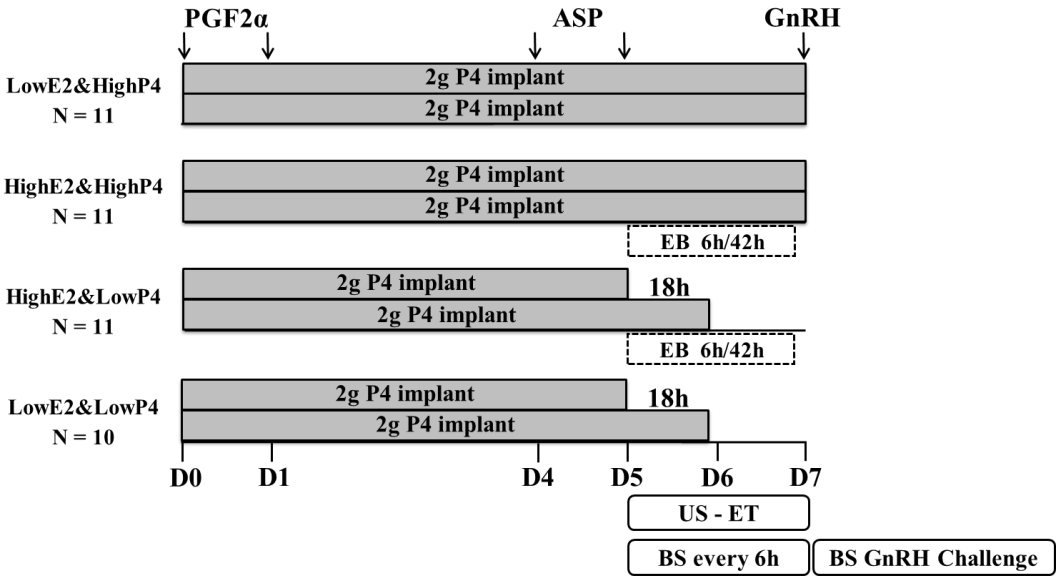

Figure 2. Schematic representation of experiment 2 procedures. All cows were submitted to the same presynchronization protocol described on experiment 1 (not shown). From D0 to D5 the procedures were similar for all groups. D0: insertion of two intravaginal implants containing 2g progesterone (P4) each; 0.526 mg cloprostenol sodium (PGF2α); D1: second dose of PGF2α; D4: follicular aspiration (ASP); D5: second ASP; cows were randomized into four groups. LowE2&HighP4: no further treatments; HighE2&HighP4: estradiol benzoate (EB) every 6 hours for 42 hours (0.04; 0.04; 0.08; 0.08; 0.12; 0.12; 0.16; 0.16 mg); HighE2&LowP4: one implant was removed on D5, followed by the removal of the second implant 18 hours later, EB every 6 hours for 42 hours (0.04; 0.04; 0.08; 0.08; 0.12; 0.12; 0.16; 0.16 mg); LowE2&LowP4: one implant was removed on D5, followed by the removal of the second implant 18 hours later. US – ET: Transrectal ultrasound examination on D5 and 12, 24, and 48 hours later for endometrial thickness (ET). BS: Blood samples for estradiol (E2) every 6 hours just before each EB treatment and 6 hours after the last treatment; for P4 at 0, 18, 30, and 48 hours after the first P4 implant removal. GnRH challenge: on D7 (after last ET evaluation) 8.4 µg buserelin acetate (GnRH) was administrated i.m.; BS just before and 0.5, 1, 2, 3, 4, 5, and 6 hours after GnRH.

2.2.2 Blood samplings and hormonal analysis:

Blood sample collection followed the procedures described in experiment 1. For P4 analyses, samples were collected on D5, just before the first P4 implant removal and 18, 30, and 48 hours later. For E2 analyses, samples were collected every 6 hours from D5 to D7 just before the EB treatments and 6 hours after the last EB treatment. Samples for LH and FSH analyses were collected from the jugular vein just before the GnRH treatment and 0.5, 1, 2, 3, 4, 5, and 6 hours after GnRH treatment. Plasma P4 and E2 concentrations were determined as described in experiment 1. Sensitivity, intra- and inter-assay coefficients of variation for the P4 assay were 0.01 ng/mL, 5.4 and 8.7%, respectively. For the E2 assay, the sensitivity, intra-

and the inter-assay coefficients of variation were 0.05 pg/mL, 15.0% and 19.0%, respectively. The LH and FSH analyses were performed by RIA [25, 26] with some modifications for FSH [27] and for LH [28]. Sensitivity, and intra- and inter-assay coefficients of variation for LH were 0.07 ng/mL, 8.2 and 12.6% and for FSH 0.02 ng/mL, 11.3 and 9.4%, respectively.

2.2.3 Statistical analyses

All continuous data were analyzed with the MIXED procedure of Statistical Analysis System (SAS, Version 9.4 for Windows, SAS Institute Inc., Cary, NC). All data were tested for normality of studentized residuals using the UNIVARIATE procedure of SAS according to the Shapiro-Wilk test. The homogeneity of variances was evaluated with the Levene test using the Hovtest and Welsh methods. Non-normally distributed data were transformed to logarithm, square root, or inverse scale before analysis if residual distribution was improved. In addition, outliers were removed when necessary. Nonparametric analysis was performed with the Wilcoxon test using the NPAR1WAY procedure of SAS when data did not normalize even after transformation.

Hormonal concentrations and EA throughout time in experiment 1 were analyzed using the MIXED procedure as repeated measures using time as the repeated statement. The model included effects of group, time, and group*time interaction. In order to evaluate E2 and P4 concentrations generated by the treatments (high or low), comparisons between high E2 (HighE2&LowP4 and HighE2&HighP4) vs. low E2 (LowE2&LowP4 and LowE2&HighP4) and high P4 (HighE2&HighP4 and LowE2&HighP4) vs. low P4 groups (HighE2&LowP4 LowE2&LowP4) were performed. For these analyses, the model included effects of group (high vs. low), time, and group*time interaction.

Area under the curve (AUC) was calculated for FSH and LH from experiment 2 using the package called “MESS”, which can be used through the statistical software R (R Core Team, 2016). To calculate the AUC a spline interpolation was used, and the numerical integral was calculated for each mathematical function. Then, AUC, concentration at peak and time of the peak for LH and FSH were evaluated by factorial analysis using the PROC MIXED. The final model included E2 effect, P4 effect, and E2*P4 interaction. An additional analysis was performed in order to evaluate the total change in EA by removing the basal from the maximal area for each cow. Then, the same factorial analysis was applied.

For LH and FSH concentrations and EA measurements changes over time (experiment 2), data were analyzed as repeated measures using time as the repeated statement in the factorial analysis. The final model was composed by E2 effect, P4 effect, time, and their interactions. The covariance structure selected was the unstructured (UN), which provided the best adjustment for the analyses based on the lowest value of corrected Akaike's information criterion. In order to present, in a different way, the effects of high or low E2/P4 on LH and FSH concentrations over time, an additional analysis was performed, in which the four treatments (HighE2&LowP4 vs. HighE2&HighP4 vs. LowE2&LowP4 vs. LowE2&HighP4) were compared. For this analysis, the model included effects of treatment, time, and treatment*time interaction.

Tukey honest significant difference post hoc test was performed to determine differences. Significant differences were declared when $P \leq 0.05$, whereas tendencies were considered when $0.10 \geq P > 0.05$. Data are presented as means ± SEM.

3 RESULTS

3.1 Experiment 1

The E2 concentration was greater for HighE2 compared to LowE2 at 42 (5.6 ± 0.8 vs. 3.0 ± 0.3 pg/mL) and 48 hours (6.8 ± 0.6 vs. 2.9 ± 0.5 pg/mL) after first EB treatment (Figure 3A). The HighE2 group had a detectable increase in E2 concentrations after 18 hours, whereas an increase in circulating E2 was only detected after 36 hours in the LowE2 group. In addition, E2 concentration at peak was greater for the HighE2 than for the LowE2 group (7.2 ± 0.5 vs. 3.5 ± 0.4 pg/mL). Thus, the higher dose was selected to be used in the subsequent experiment.

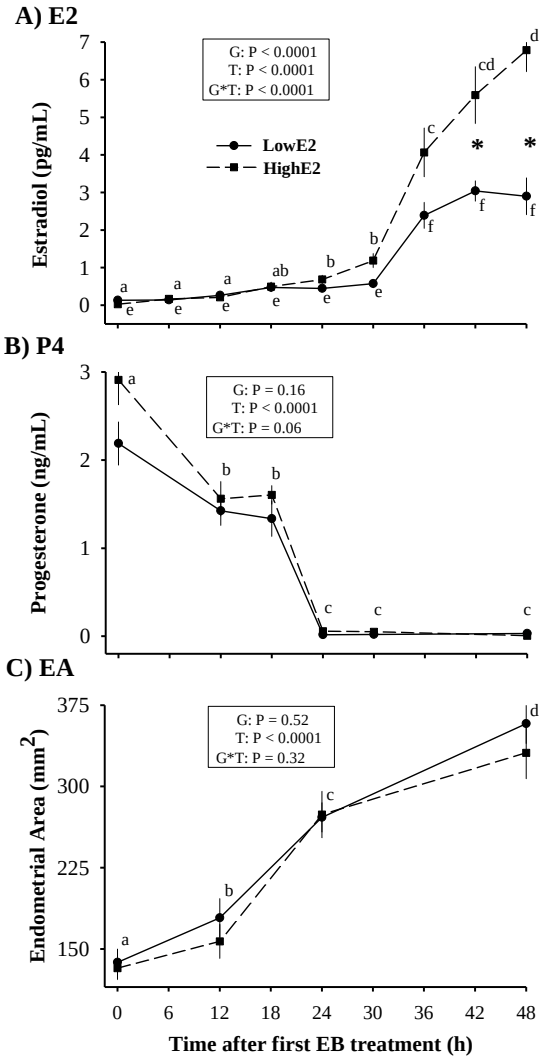

Figure 3. Means ± SEM of estradiol (E2; panel A) and progesterone (P4; panel B) concentrations and endometrial area (EA; panel C) from experiment 1. LowE2 group (n = 9; estradiol benzoate [EB] total dose 0.4 mg) is represented by the continuous line with solid circles and the HighE2 group (n = 9; EB total dose 0.8 mg) by the dashed line with

solid squares. For E2, different letters [(a-d or e-f)] mean difference ($P \leq 0.05$) through time within the same group and the symbol (*) means difference between groups. For P4 [(a-c)] and ET [(a-d)] different letters denote difference ($P \leq 0.05$) between groups.

As expected, there was no effect of group ($P = 0.16$) for P4 concentration but a time effect was present ($P < 0.0001$). As shown in Figure 3B, a clear decrease was observed after removing the first implant (time 0 to time 12; from 2.6 ± 0.3 to 1.5 ± 0.2 ng/mL) and the second implant (time 18 to time 24; from 1.5 ± 0.0 to 0.2 ± 0.0 ng/mL). Circulating P4 concentrations were relatively constant between 12 and 18 hours (one P4 implant present) and between 24 and 48 hours (no P4 implants present).

Although the EA did not differ between groups, it increased over time. At 12, 24, and 48 hours after the first EB treatment, the EA was ~1.2, ~2, and ~2.5 fold greater compared to time 0, respectively (Figure 3C). In addition, all cows (9/9) from the HighE2 group expressed estrus compared to 66.7% (6/9) of the cows from the LowE2 group.

3.2 Experiment 2

Circulating P4 concentrations did not differ within the P4 treatment groups for Low (HighE2&LowP4 and LowE2&LowP4; $P = 0.72$) or High (HighE2&HighP4 and LowE2&HighP4; $P = 0.36$) P4 treatments. Therefore, the results for P4 concentration from these groups were combined (Figure 4B). As expected, the LowP4 groups had decreases in circulating P4 throughout the protocol, as previously observed in experiment 1, whereas the HighP4 groups maintained elevated P4 concentrations (Figure 4B). In addition, the two groups treated with HighE2 had similar circulating E2 and were combined in subsequent analyses, as were the two groups assigned to LowE2 and their results were combined (Figure 4A). As expected, from 12 hours to the end of the analysis (48 hours) HighE2 groups had circulating E2 greater compared to LowE2 groups ($P < 0.0001$).

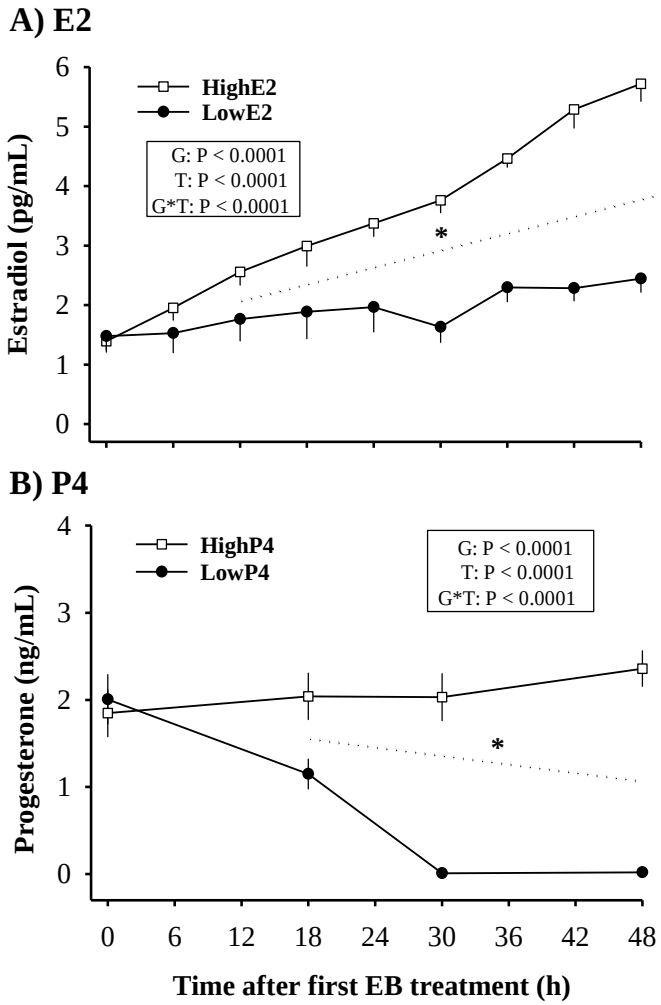

Figure 4. Means ± SEM of estradiol (E2; Panel A) and progesterone (P4; Panel B) concentrations from experiment 2. For E2 the results are comparing HighE2 (HighE2&LowP4 and HighE2&HighP4) vs. Low E2 (LowE2&HighP4 and LowE2&LowP4) groups; whereas for P4 results are comparing HighP4 (HighE2&HighP4 and LowE2&HighP4 combined) vs. LowP4 (HighE2&LowP4 and LowE2&LowP4 combined) groups. High steroid groups (n = 22) are represented by open squares and the Low steroids (n = 21) by solid circles. The dotted line containing the symbol (*) means difference ($P \leq 0.05$) between groups.

For EA there was a significant interaction of P4 with E2, with the increase in EA occurring only in the group with both high E2 concentrations combined with low P4 concentrations (Figure 5B and 5C). At 24 and 48 hours the EA from HighE2&LowP4 was greater than HighE2&HighP4 (Figure 5B) and LowE2&LowP4 (Figure 5C). Furthermore, no changes were detected in low E2 groups (Figure 5A) and high P4 groups (Figure 5D). In an additional analysis performed by calculating the numeric change from basal to maximal area, this interaction was also present, with low P4 having a change ~14 fold greater than high P4

only in the presence of high E2, whereas high E2 had a change almost 4 fold greater than low E2 but only during low P4 concentrations (Figure 6).

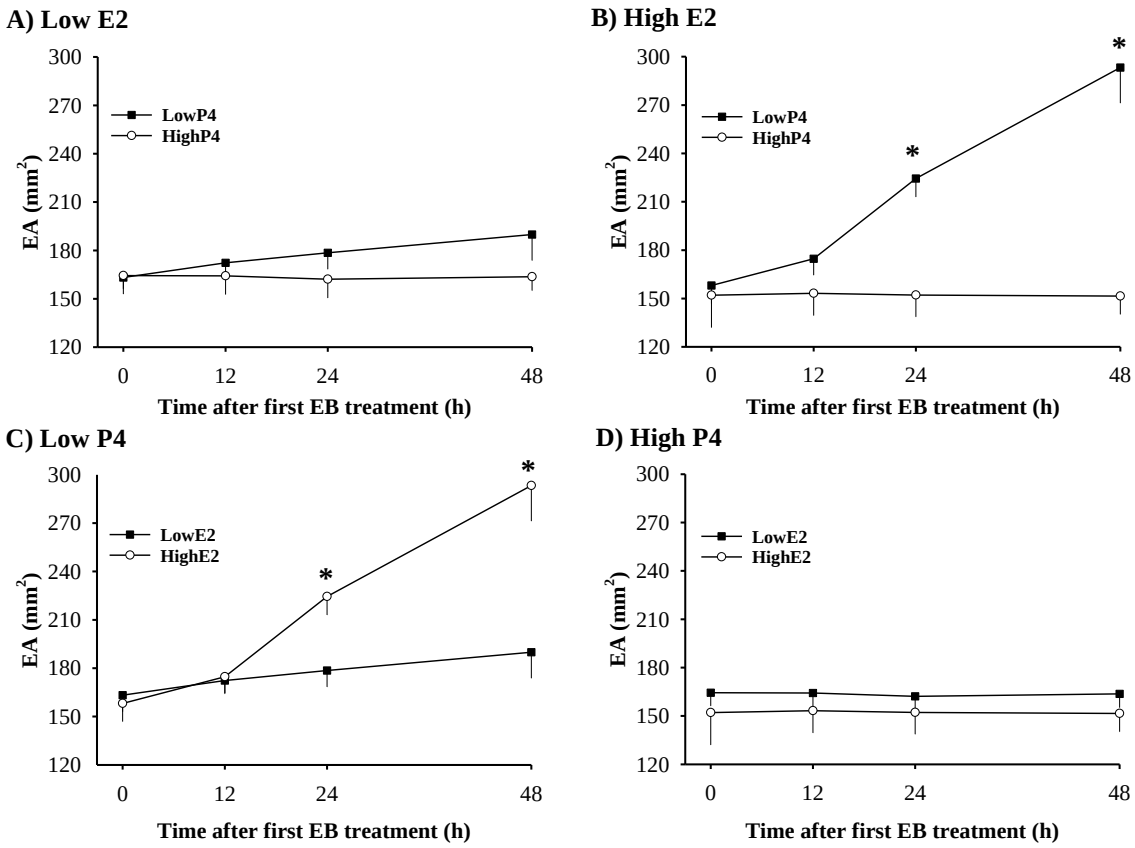

Figure 5. Means ± SEM for endometrial area (EA) normalized for the time of first estradiol benzoate (EB) treatment from experiment 2. Panel A: effect of progesterone [P4; low (n = 10 vs. high (n = 11)] in a low estradiol (E2) milieu. Panel B: effect of P4 [low (n = 11) vs. high (n = 11)] in a high E2 milieu. Panel C: effect of E2 [low (n = 10) vs. high (n = 11)] in a low P4 milieu. Panel D: effect of E2 [low (n = 11) vs. high (n = 11)] in a High P4 milieu. Low steroid concentrations are represented by the black squares and high steroid concentrations by the open circles. EA was affected by P4 ($P < 0.0001$), time ($P = 0.002$), P4 by time interaction ($P = 0.009$) and P4 by E2 interaction ($P = 0.0007$), but not by E2 ($P = 0.93$) and E2 by time interaction ($P = 0.07$). Symbol (*) means difference ($P \leq 0.05$) between groups within time.

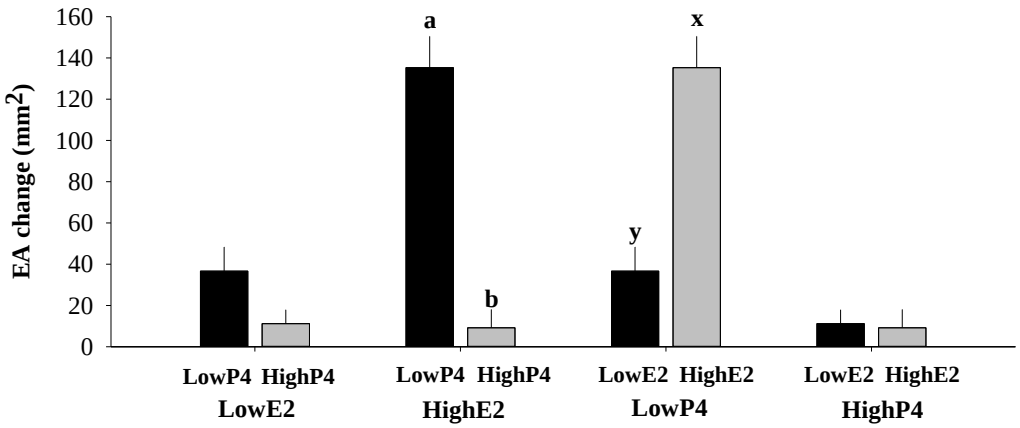

Figure 6. Total change on endometrial area (EA) from basal to maximum area from experiment 2. In each data set it is represented separately the effect of progesterone (P4; low vs. high) under low and high estradiol (E2) concentrations and the effect of E2 (low vs. high) under low and high P4 concentrations. Low steroid concentrations are represented by black bars and high steroid concentrations by the grey bars. EA was affected by E2 (P = 0.005), P4 (P < 0.0001) and E2 and P4 interaction (P < 0.0001). [(a, b)]Different letters mean difference (P ≤ 0.05) between low and high P4 under high E2 environment, or [(x, y)] difference (P ≤ 0.05) between low and high E2 under low P4 environment.

The FSH concentration at peak was affected by E2 (P < 0.0001) and P4 (P = 0.02) concentrations, with a strong positive effect of high E2, which had a peak 1 ng/mL greater than low E2 groups (2.0 ± 0.20 vs. 1.0 ± 0.10 ng/mL), and a mild negative effect of high P4, which had a peak only ~0.5 ng/mL lower than low P4 groups (1.2 ± 0.12 vs. 1.8 ± 0.24 ng/mL). This positive effect of high E2 concentration was even more evident in the AUC (P < 0.0001), while only a tendency (P = 0.06) was observed for the negative effect of high circulating P4 (Table 1). In addition, cows treated with high E2 had an earlier (P = 0.03) FSH peak than low E2, with no effect of P4 (P = 0.45; Table 1). The effects of E2 and P4 on the timing and magnitude of the GnRH-stimulated FSH concentrations is graphically shown in Figure 7. At time 0 FSH concentrations were similar for low and High E2 cows but after GnRH treatment there was an effect of E2 treatment, time, and an estradiol by time interaction with FSH being greater at all times after time 0 for high compared to low E2 (Figure 7A). Moreover, there was an effect of P4, time, and a P4 by time interaction with High P4 cows having lower FSH concentrations at 1 hour and a tendency at 2 hours compared to low P4 cows (Figure 7B). There was no E2*P4 interaction (P = 0.64). Then, an additional analysis was performed, comparing the groups by time (Figure 8). The circulating FSH concentrations

were similar at time 0 and 0.5 hour followed by significant effects of group ($P < 0.0001$), time ($P < 0.0001$), and a group*time interaction ($P < 0.0001$). Groups with HighE2 (HighE2&LowP4 and HighE2&HighP4) had a much greater increase in circulating FSH throughout time compared to the two LowE2 groups (LowE2&LowP4 and LowE2&HighP4). Therefore, for most times analyzed and for most measures of GnRH-stimulated FSH secretion, the E2 effects were much greater compared to the minor P4 effects.

Table 1. Results (means ± SEM) of circulating FSH and LH concentration at peak, time of peak and area under the curve (AUC) after buserelin acetate treatment in nonlactating Holstein cows (n = 43) under different steroid concentrations from experiment 2.

		High E2		Low E2		P - value		
		LowP4 (n = 11)	HighP4 (n = 11)	LowP4 (n = 10)	HighP4 (n = 11)	E2	P4	E2*P4
FSH	Peak (ng/mL)	2.4 ± 0.35	1.6 ± 0.11	1.1 ± 0.13	0.8 ± 0.14	< 0.0001	0.02	0.29
	Time to Peak (min)	109.1 ± 10.91	130.9 ± 7.32	144.0 ± 9.80	136.4 ± 8.45	0.03	0.45	0.12
	AUC (ng^2)	7.3 ± 0.89	5.9 ± 0.40	3.9 ± 0.36	3.0 ± 0.39	<0.0001	0.06	0.69
LH	Peak (ng/mL)	12.2 ± 1.57	8.1 ± 1.35	7.2 ± 1.43	2.6 ± 0.43	0.0002	0.001	0.85
	Time of Peak (min)	109.1 ± 10.91	120.0 ± 0.00	138.0 ± 9.17	147.3 ± 12.44	0.005	0.29	0.93
	AUC (ng^2)	36.9 ± 5.10	19.9 ± 2.35	19.4 ± 2.72	8.3 ± 0.93	<0.0001	<0.0001	0.36

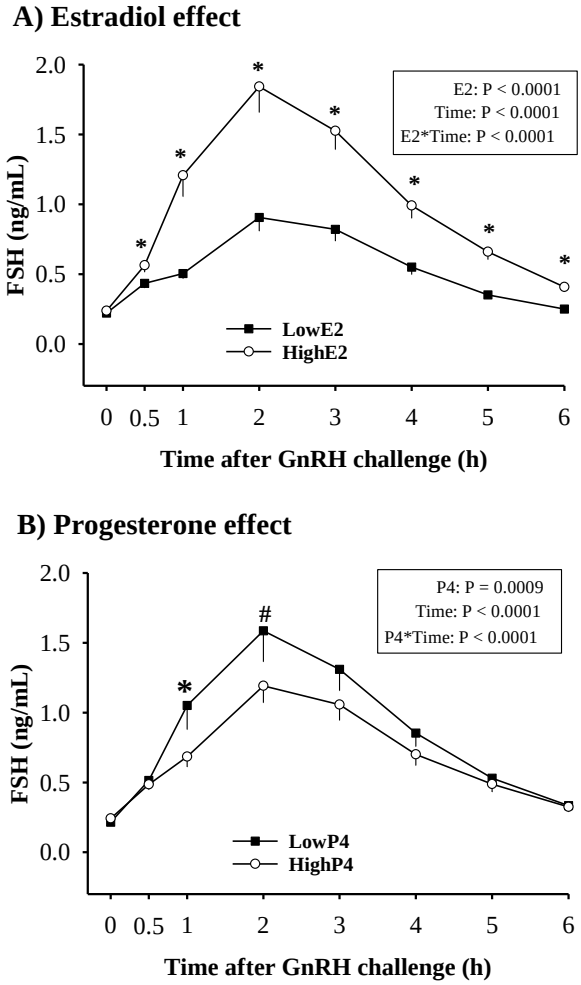

Figure 7. Means ± SEM of the main effects of estradiol [E2; Panel A; low (n = 21) vs. high (n = 22)] and progesterone [P4; Panel B; low (n = 21) vs. high (n = 22)] on FSH release normalized for the time of buserelin (GnRH) treatment from experiment 2. Time 0 corresponds to the time just before GnRH administration. Low steroid concentrations are represented by the black squares and high steroid concentrations by the open circles. Symbol (*) means difference ($P \leq 0.05$) or tendency ($^{\#}$; $0.05 < P \leq 0.1$) between groups within time.

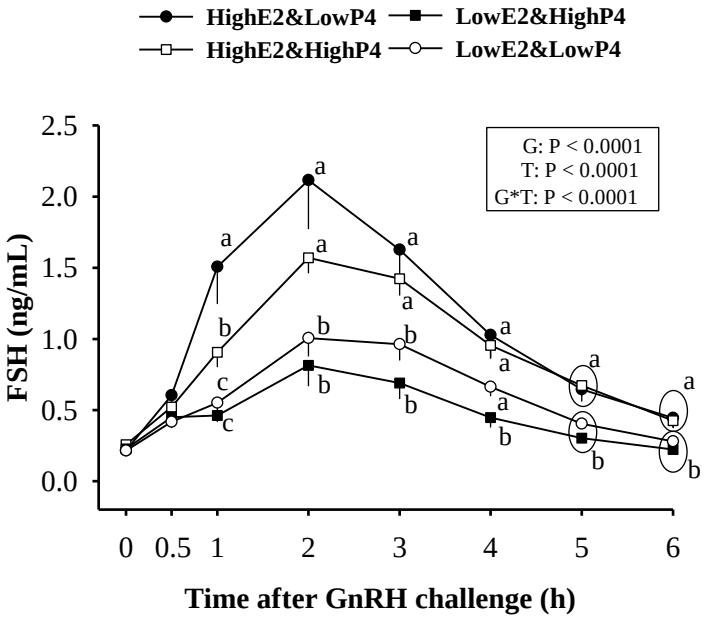

Figure 8. Means ± SEM of circulating FSH concentration normalized for the time of buserelin (GnRH) treatment in cows under different hormonal milieu from experiment 2. Time 0 corresponds to the time just before GnRH administration. HighE2&Low P4 (n = 11) is represented by solid circles, LowE2&HighP4 (n = 11) by solid squares, HighE2&HighP4 (n = 11) by open squares and LowE2&LowP4 (n = 10) by open circles. $^{(a\text{-}c)}$Different letters mean difference (P < 0.05) among groups in each hour.

The LH concentrations at peak and the AUC were markedly affected by E2 and by P4 with comparable effects for each hormone. For example, the High E2 cows had a peak of LH ~5 ng/mL greater than the low E2 groups (10.2 ± 1.11 vs. 4.8 ± 0.87), while peak LH was ~4.5 ng/mL lower in the High P4 groups compared to the low P4 groups (5.4 ± 0.91 vs. 9.9 ± 1.18). The time to the LH peak was only affected by E2 (P = 0.005) and not by P4 (P = 0.29) with average time to the LH peak about 30 min earlier in the HighE2 than LowE2 treatments (Table 1). The inhibitory effect of high P4 and the stimulatory effect of E2 on the LH profile after GnRH treatment were of a similar magnitude with HighE2 having greater LH than LowE2 from 30 minutes until 5 hours after GnRH and HighP4 having lower LH concentrations than LowP4 at all sampling times (Figure 9). Because there was no E2*P4 interaction (P = 0.64), as seen for FSH, an additional analysis was performed, comparing the groups by time (Figure 10). The basal concentrations of LH (time 0 before GnRH) was greater for the LowP4 compared to HighP4 groups (0.7 ± 0.1 vs. 0.3 ± 0.1 ng/mL). After GnRH treatment, the HighE2&LowP4 had the greatest increase in circulating LH with more than a 10-fold increase, the HighE2&High P4 and LowE2&LowP4 groups were similar, but with an intermediate response for more than a 6-fold increase, and the LowE2&HighP4 group

was much lower with a minimal increase in circulating LH after GnRH treatment. Thus, the LH release after GnRH treatment was influenced by both circulating P4 and E2 concentrations with similar effects of each hormone.

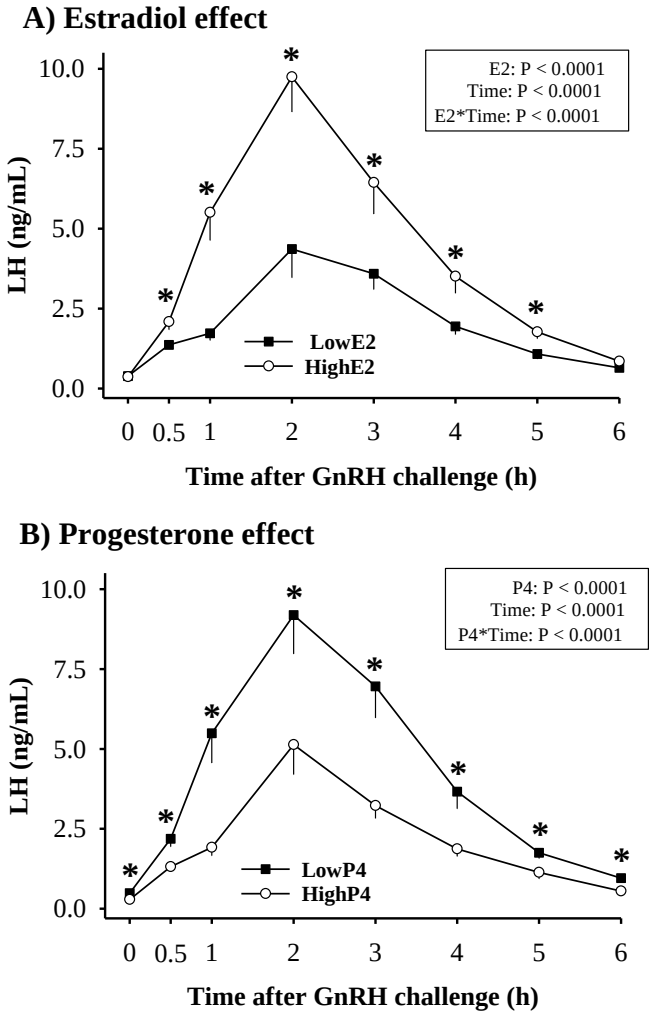

Figure 9. Means ± SEM of the main effects of estradiol [E2; Panel A; low (n = 21) vs. high (n = 22)] and progesterone [P4; Panel B; low (n = 21) vs. high (n = 22)] on LH release normalized for the time of buserelin (GnRH) treatment from experiment 2. Time 0 corresponds to the time just before GnRH administration. Low steroid concentrations are represented by the black squares and high steroid concentrations by the open circles. Symbol (*) means difference ($P \leq 0.05$) between groups within time.

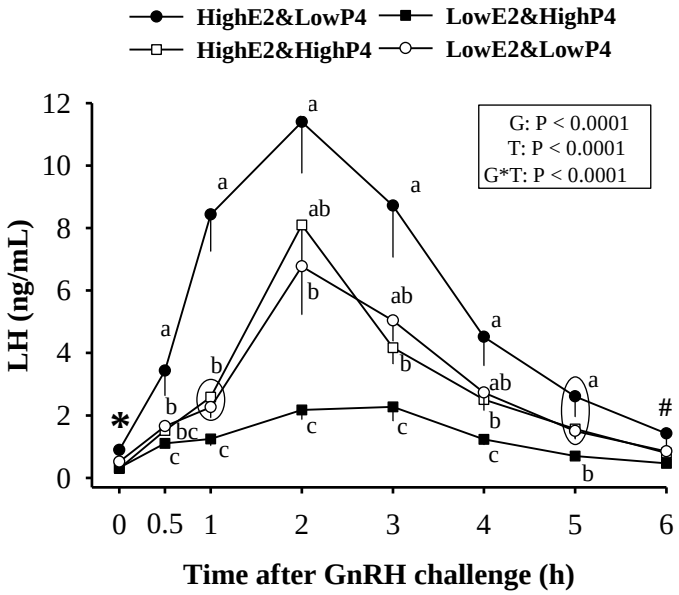

Figure 10. Means ± SEM of circulating LH concentration normalized for the time of buserelin (GnRH) treatment in cows under different hormonal milieu from experiment 2. Time 0 corresponds to the time just before GnRH administration. HighE2&Low P4 (n = 11) is represented by solid circles, LowE2&HighP4 (n = 11) by solid squares, HighE2&HighP4 (n = 11) by open squares and LowE2&LowP4 (n = 10) by open circles. $^{(a\text{-}c)}$Different letters mean difference (P < 0.05) among groups in each hour. *LH concentration at 0 hour: for groups with low P4 (HighE2&LowP4; LowE2&LowP4) LH was higher than for groups with high P4 (HighE2&HighP4; LowE2&HighP4). # LH concentration at 6 hours: HighE2&LowP4 was similar to LowE2&LowP4, but higher than the others; HighE2&HighP4 was similar to LowE2&HighP4 and to LowE2&LowP4; LowE2&LowP4 was higher than LowE2&HighP4.

4 DISCUSSION

This research has introduced a new model for studying the effects of the dramatic changes in circulating E2 and P4 concentrations during proestrus on the dynamic modification of the uterine endometrium by measuring EA by ultrasound and on two pituitary responses, GnRH-stimulated LH and FSH secretion. The natural proestrus is marked by a decrease in circulating P4 from peak to nadir concentrations during a 32 hour period and a gradual increase in circulating E2 during a 48 h period. Our model removed the endogenous ovarian sources of P4 and E2 and provided a pattern of treatments with exogenous P4 and E2 that mimicked various aspects of the natural proestrous period. A nadir of circulating P4, < 0.01 ng/mL, was achieved during a 30 hour period, whereas circulating E2 increased from a nadir to about 6 pg/mL during a 48 h period. This model produced estrus in all cows (experiment 1), a typical increase in uterine EA (experiment 1 and 2), and a typical proestrous increase in GnRH-stimulated LH and FSH responsiveness (experiment 2). Thus, this animal model using exogenous hormones seemed appropriate for studying the independent and combined effects of the dramatic changes in circulating steroid concentrations during proestrus on uterine and pituitary function.

The changes in ET have been previously examined in cattle using ultrasound [10, 13] and the timing of our evaluations were based on a previous experiment that evaluated the endometrium every 6 hours with gradual incremental changes (unpublished results) that were also clearly observed in our data. In the present experiment we chose to calculate EA instead of reporting only ET, as previously done, since we anticipated that it would be more representative of the dramatic increase in uterine volume and thickness of the endometrium that occurs during the proestrous period. In the present study, the decrease in circulating P4 and the increase in circulating E2 produced over a 2 fold enlargement of the endometrium (average EA increase of 2.17-fold in the HighE2&LowP4 group in two experiments) during a 48 hour period. Results from previous studies can be compared to the present results by conversion of measurements of uterine body thickness or ET to values of EA. A study during the natural estrous cycle reported an increase of 1.6 fold within 72 hours [10] and a study during an induced proestrus had a 1.8-fold increase in EA within 24 hours and maintenance of the increased area during the following 3 days [13]. Any potential differences in timing or magnitude of the increase in EA are likely to be explained by differences in ultrasound quality and experimental model. A recent study compared the endometrial changes under natural and

induced proestrus and reported a more rapid increase in endometrium size in the induced than in the natural proestrus [29]. Our results appeared to be more similar to the natural proestrus, with a gradual and constant increase in the size of the endometrium during a 48 h period rather than the rapid increase followed by a plateau that has been reported during an induced proestrus [13, 29]. It is likely that the more gradual changes in circulating E2 and P4 that are produced during the exogenous hormone treatments in the present model are responsible for the gradual changes in EA during the 48 period.

The present results demonstrated for the first time that the changes in the thickness of the endometrium during the proestrous period are dependent upon the combination of high E2 and low P4, although these experiments did not allow determination of the cellular/molecular mechanisms that underlie these physiological changes. We speculate that changes in expression of uterine E2 and P4 receptors (ER and PR, respectively) may at least partially explain the present results. Kimmins and MacLaren [30] treated ovariectomized cows with exogenous E2 and P4 and evaluated endometrial mRNA concentrations for ER and PR. Cows treated with P4 alone or P4 + E2 had the lowest ER expression, whereas cows treated with E2 had the greatest ER mRNA. In addition, the cows that were not treated either with E2 or P4 had high ER and PR expression. By comparing these findings to our results, the lack of change in E2 in the HighE2&HighP4 group may be due to low expression of ER in the presence of high P4, whereas the LowE2&LowP4 group may have had high ER expression but the lack of an increase in circulating E2 may have prevented an increase in EA. Furthermore, the HighE2&LowP4 probably had the greatest ER expression and the increasing E2 induced a dramatic increase in EA. Interestingly, in experiment 1 both doses of EB were capable of inducing a similar increase in EA, suggesting that the endometrium had sufficient responsiveness to respond similarly to lower and higher circulating concentrations of E2. Future studies are needed to define the type of E2 and P4 receptors, either nuclear or plasma membrane, that are involved in the EA response and the dynamics of the process including the absolute E2/P4 concentrations that are needed to produce these changes and whether fluctuations in circulating steroids are required for these actions. In a recent study, it has been shown that the ER type 1 (ESR1) expression increases during proestus/estrus, while the ER type 2 (ESR2) expression decreases, following a similar pattern as P4 receptors, for instance (Milo C. Wiltbank, 2019, unpublished).

In addition, the precise changes in the endometrium that lead to the observed changes in EA still need to be defined. The more than doubling in EA indicates a dramatic, rapid increase in volume of the uterine horn that is unlikely to be explained by increases in cell

number through mitosis or increases in the volume of endometrial cells due to the short time intervals that are involved. It seems more likely that the increase in EA is due to vasodilation and an increase in endometrial blood flow. Intravenous treatment of ewes with estradiol-17β increased blood flow to the uterus by more than 10-fold after 2 hours [31]. Partial blockade of the E2-induced increase in uterine blood flow was achieved by treatment with L-NAME, a potent and specific inhibitor of nitric oxide production [32]. Other potential vasodilatory pathways have also been found to be regulated by E2 in the uterus. Future studies are needed to define whether changes in EA are caused by increases in uterine blood flow and the pathways involved in this induction. In addition, given the clear association of changes in ET with fertility in lactating dairy cows [13], the role of increased endometrial blood flow and increased EA in fertility need to be determined. Perhaps fertility can be increased in cattle or other species by optimizing the increase in EA during proestrus.

In general, circulating E2 had a positive effect on LH and FSH release, whereas P4 seemed to be deleterious, particularly to LH release. The negative effects of P4 on LH release after GnRH challenge in cattle have been previously described: during the estrous cycle [33, 34], using different doses of GnRH [16], using different GnRH analogues [35], and comparing beef heifers to lactating beef cows [14]. Although most of these studies did not evaluate circulating E2 concentrations, the GnRH challenges that were performed during diestrus were likely to have both higher circulating P4 and lower E2 and the evaluations that were done during low P4 were also likely to have increased circulating E2. The 4.6-fold increase in LH found in our HighE2&LowP4 group were very similar to the 4.8-fold increase in LH peak reported in Giordano et al. [16]. Similarly, treatment with E2 in the absence of P4 dramatically increases the responsiveness of the pituitary to GnRH treatment. Similar to our results, beef heifers treated with 0.25 mg EB 12 hours before the GnRH challenge had an increase in LH release, even if circulating P4 was high [15]. In anestrous ewes, prior EB treatment increased the magnitude of the GnRH-induced LH surge [36], consistent with our results. Even in primates a similar response has been observed with E2 inducing a dramatic increase in pituitary responsiveness to GnRH as evidenced by constant GnRH pulses inducing increasing LH secretion as circulating E2 increases [37, 38]. Although less studied, there is also evidence that GnRH-induced FSH secretion is increased during the E2-dominated, proestrous period compared to the P4-dominated, mid-estrous cycle [33]. The present experimental design allowed definition for the first time, in vivo, of the independent and

combined effects of the two circulating steroids, E2 and P4, on the GnRH-induced pituitary secretion of the two gonadotropins, LH and FSH.

Due to the previously described relationship between number of GnRH receptors on pituitary gonadotrophs and GnRH-induced LH secretion [39], we speculate our results could be partially explained by increased circulating E2 stimulating pituitary GnRH receptor expression, whereas greater circulating P4 could reduce or inhibit the E2-induced increase in pituitary GnRH receptor expression. Indeed, E2 has been associated with increased GnRH receptors in the pituitary gland of ewes [40], cows [41] and rats [42]. Conversely, increased circulating P4 is associated with reduced pituitary GnRH receptors in vivo [43] and P4 treatment decreased GnRH receptor mRNA concentrations in cultured pituitary cells from ewes [44]. An additional effect of P4 may be at the hypothalamic level, reducing the frequency of GnRH pulses, as reported in ewes [45], which could explain the lower basal LH (before GnRH treatment) concentrations in HighP4 compared to LowP4 groups. Furthermore, intermediate responses to GnRH may relate to intermediate levels of GnRH receptors due to potentially opposite effects of the two steroids on GnRH receptor expression. Inconsistent with this speculation are the results in ovariectomized ewes supplemented with P4 combined with E2 in which GnRH receptor mRNA was similar to ewes that were supplemented with P4 alone and GnRH receptors were lower when ewes only received E2 without P4 [46]. It seems possible that some or much of the effects of ovarian steroids on pituitary responsiveness to GnRH may be mediated through altering intracellular signal transduction pathways that are induced by GnRH rather than simply altering numbers of GnRH receptors. The present model allows a clearer delineation of the role of changes in GnRH receptors or downstream pathways in the effects of steroids on pituitary LH and FSH secretion.

Another potential participant in E2 and P4 effects on GnRH-induced LH and FSH secretion is the pituitary content of LH and FSH. Steroid-induced changes in LH and FSH synthesis could lead to changes in pituitary gonadotropin concentrations and thereby alter GnRH-induced LH or FSH release. However, treatment of ovariectomized ewes with P4 did not alter the LH/FSH content of the pituitary gland [47]. In addition, the FSH/LH content in bovine pituitary was reported to not greatly vary during the estrous cycle, although there was a sharp reduction in pituitary LH on the day of the expected LH peak, with a restoration of pituitary LH content by in the following day [48].

Taking the AUC results as an example, high E2 groups had, on average, almost a 2-fold greater FSH release compared to low E2 groups, with E2 having very similar effects in low or high P4 groups. On the other hand, the GnRH-induced LH release was stimulated in

high E2 groups (AUC was 2 fold greater than low E2 groups) and was inhibited by high P4 by a similar magnitude (AUC was 1.8 fold lower in high P4 than low P4 groups). Therefore, our data suggest that FSH release after GnRH treatment is regulated primarily by circulating E2 whereas LH secretion is regulated equivalently by both circulating E2 and P4. This results is somewhat puzzling, given the idea that LH and FSH are secreted from the same pituitary cells, the gonadotrophs, and both are present in the same secretory vesicles that fuse with the plasma membrane in response to GnRH stimulation [49]. Nevertheless, consistent with our results, a previous study also reported a different pattern of FSH and LH release after GnRH treatment in cows, especially after sequential GnRH treatments [33]. Further studies are needed to understand how GnRH-induced FSH and LH release could be regulated differentially by circulating E2 and P4. This could be important, for instance, for a better understanding of some reproductive dysfunctions, as hypothalamic amenorrhea and polycystic ovarian syndrome in women or follicular cysts in cows, and consequently leading to a more effective treatment. The precisely timed animal model developed during the present research could be useful in understanding these important biological questions.

In conclusion, an efficient proestrus-like animal model was developed using only exogenous hormones and this model was used to evaluate the differential effects of steroid hormones on three hormonally-regulated processes: the increase in endometrial size during proestrus and the changes in GnRH-induced secretion of LH and FSH from the pituitary. It seemed clear that the increase in EA during proestrus required both a decrease in circulating P4 and an increase in circulating E2. The response of the pituitary gland was more complicated with increasing E2 and decreasing P4 having equal and additive effects on GnRH-induced secretion of LH from the pituitary, whereas GnRH-induced FSH secretion from the pituitary was primarily related to the stimulatory effect of increasing E2 with surprisingly small effects of P4 on FSH secretion. Thus, steroidal regulation of uterine and pituitary function is multifaceted with distinct and complementary roles for the two steroids in different tissues during the physiological changes that occur during proestrus. The information derived from this research, as well as this animal model, may be useful for future studies of the cellular and molecular pathways involved in steroidal regulation of the diverse physiology of proestrus.

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