Role of progesterone concentrations during early follicular development in beef cattle: I. Characteristics of LH secretion and oocyte quality

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ABSTRACT

Objective was to investigate the effect of different progesterone (P4) concentrations during early follicular development on luteinizing hormone (LH) secretion and oocyte characteristics in beef cows. Primiparous cows ($n = 24$) were estrous pre-synchronized and follicular ablation was performed (d 0) 6 days following the time of ovulation. At the time of follicular ablation, cows were assigned to either: 1) high P4 treatment - HiP4; a new CIDR was inserted on d 0 to supplement P4 from the existing corpus luteum [CL], or 2) low P4 treatment - LoP4; a previously-used CIDR and two doses of PGF 8 to 12 h apart were given on d 0. Concentrations of P4 were greater ($P < 0.01$) in the cows of the HiP4 than LoP4 group on d 1.5, 2.5, and 3.5. Peripheral concentrations of E2 were greater ($P < 0.05$) in the cows of the LoP4 than HiP4 group on d 2.5 and 3.5. Frequency of LH pulses was greater ($P < 0.05$) in the LoP4 than HiP4 group on d 2.5, but mean LH concentration and pulse amplitude did not differ between treatments. Number of follicles aspirated per cow, total oocytes recovered, recovery rate, percentage of oocytes graded 1 to 3, oocyte diameter, percentage BCB+ oocytes, and relative abundance of oocyte mRNA for FST did not differ ($P > 0.10$) between treatments. In conclusion, lower P4 concentrations during early follicular development resulted in increased LH pulse frequency and E2 concentrations, but did not affect characteristics of oocyte developmental competence.

1. Introduction

Luteinizing hormone (LH) stimulates growth and maturation of the dominant follicle (Savio et al., 1993; Gong et al., 1995) and the oocyte contained in the follicle (Gong et al., 1995). It has been observed that the enhanced pregnancy rates after is presumably due to the greater LH stimulation during the latter stages of dominant follicle growth. In these studies, LH stimulation of the ovulatory follicle was increased by either inducing onset of proestrus earlier in follicular development (Peters and Pursley, 2003;
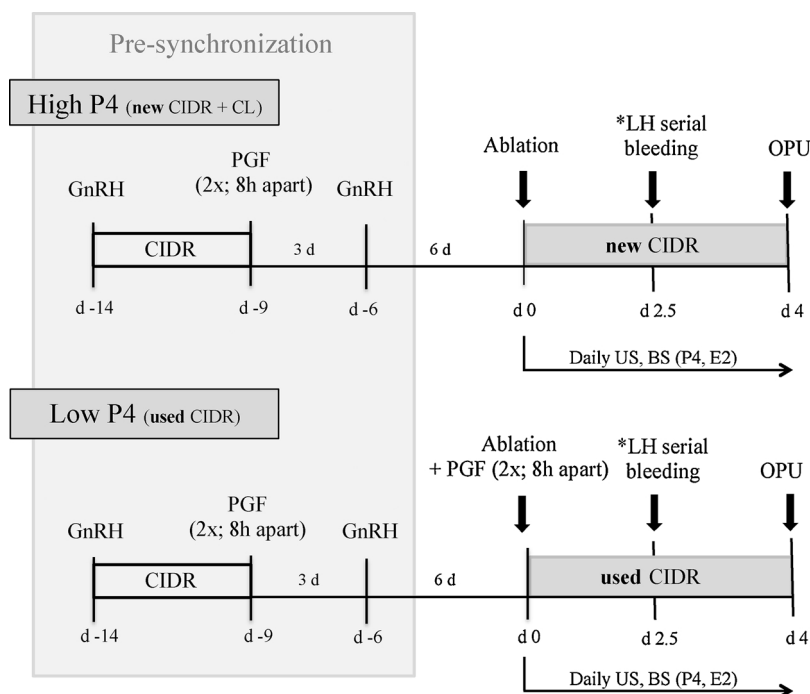
Fig. 1. Diagram of treatments (HiP4 = high progesterone; LoP4 = low progesterone), ultrasonography (US) and blood samples (BS). CIDR = intravaginal progesterone device (new = never used; used = previously used for 5 d). GnRH = gonadotropin release hormone. PGF = prostaglandin F2α. CL = corpus luteum. LH = luteinizing hormone OPU = ovum pick-up. *LH serial bleeding was performed every 15 min for 12 h.

Bridges et al., 2008, 2010) or by decreasing the amount of exogenous progesterone (P4) used during estrous synchronization programs (Dias et al., 2009; Claro et al., 2010).

The mechanism by which increased LH stimulation during follicular development enhances fertility in cattle is likely multifactorial. In the dominant follicle, LH upregulates expression of its receptor gene and other genes and abundance of proteins is greater that stimulate proliferation of follicular cells and increase functionality and steroidogenesis including the production of estradiol (E2; Luo et al., 2011). Within the follicle, E2 enhances mitosis (Goldenberg et al., 1972) and formation of gap junctions (Merk et al., 1972), and there is a greater relative abundance of mRNA for FSH and LH receptors in the granulosa cells (Richards et al., 1976). In the oocyte, LH induces nuclear maturation, consequently initiating cytoplasmic maturation (Blondin and Sirard, 1995; Fair et al., 1995; Arlotto et al., 1996) and enhances developmental competence of the oocyte for successful fertilization and early embryo development (Savio et al., 1993; Gong et al., 1995). It, therefore, is plausible that the increased LH pulse frequency throughout follicular development may improve oocyte competence in cattle. The objective of the present study was to investigate the effect of decreased P4 concentrations during early follicular growth on LH secretion, follicular development and oocyte characteristics in beef cows. It was hypothesized that decreasing circulating concentrations of P4 during the early stages of follicular development would increase LH pulse frequency and, consequently, increase E2 concentration and enhance oocyte competence.

2. Materials and methods

2.1. Animals and treatments

All procedures involving animals used in this research were approved by The Ohio State University Agricultural Animal Care and Use Committee (IACUC #2012A00000151). Suckled Angus and Angus-Crossbred primiparous cows (n = 24) were estrous pre-synchronized with the 5 d CO-Synch + CIDR program (Fig. 1; CIDR [Zoetis, New York, NY, USA] + GnRH [Cystorelin®, 100 µg, i.m., Merial, Inselin, NJ, USA] followed 5 d later with CIDR removal and 2 doses of PGF, 8 h apart [Lutalyse®, 25 mg each, i.m., Zoetis] and GnRH [100 µg, i.m.] 3 d after PGF). Transvaginal ultrasonic-guided follicular aspiration occurred 6 d following the GnRH-induced ovulation (ablation; d 0 of the experiment). At the time of follicular ablation, cows were stratified by days postpartum (85.1 ± 1.0 d), and assigned to receive either: 1) high P4 treatment (HiP4): a new CIDR was inserted to supplement P4 from the existing corpus luteum (CL) or 2) low P4 treatment (LoP4): a CIDR that had previously been used for 5 d was inserted and two doses of prostaglandin F2α (PGF; 8 to 12 h apart) were given to induce luteal regression (Fig. 1). Blood samples were collected on d 0, 1.5, 2.5, and 3.5 to measure P4 concentrations and on d 2.5, and 3.5 to measure E2 concentrations. On d 2.5, from a subset of cows (HiP4, n = 6; LoP4, n = 7) serial blood collections occurred (every 15 min for 12 h) for LH determination. On d 4, CIDR were removed, and ovum pick-up

(OPU) was performed.

2.2. Used CIDR sanitation

At the time of removal from a cow enrolled in a 5 d CO-Synch + CIDR program, CIDR were immediately rinsed in water to remove debris followed by a final rinsing in 2% Novalsan® Solution (Zoetis, New York, NY, USA). The devices were then allowed to air dry, and later packaged and stored at room temperature for future use. At time of insertion, CIDR were first submerged in a 0.1% Betadine® Solution (Purdue Frederick, Norwalk, CT, USA) followed by insertion into the cranial vagina using manufacturer recommended procedures.

2.3. Ultrasonography

Trans-rectal ultrasonography (US; Fig. 1) was performed using a 7.5 MHz linear array transducer (Aloka 500 V; Aloka, Wallingford, CT) to characterize ovarian structures in all cows on the following days: 1) d 0 (ablation): to verify the response of the pre-estrous synchronization program through the presence of a CL; 2) d 1.5: to identify emergence of the new follicular wave (detection of a cohort of follicles $\geq$ 4 mm in diameter; Knopf et al., 1989); 3) d 2.5: to confirm luteolysis and CL regression in the LoP4 treatment group; confirm emergence of the development of a new wave of follicular growth in cows that were not identified on d 1.5; and to determine the size of the largest follicle in the ovaries as well as follicular development; 4) d 4: determine the number of follicles present in the ovaries and the occurrence of follicular deviation (dominant follicle, DF $\geq$ 8.5 mm; Ginther et al., 2001).

2.4. Follicle ablation procedure

Prior to follicle ablation, cows were properly restrained in a cattle squeeze-chute, epidural anesthesia was performed with 5 mL of 2% lidocaine (Sparhawk Laboratories, Inc., Lenexa, TX), and the rectal and vaginal areas were cleaned and sanitized using water and a scrub solution of 7.5% povidone iodine. Ablation was performed by ultrasonic-guided transvaginal aspiration using a 7.5-MHz convex transducer (Aloka 500 V, Corometrics Inc. Wallingford, CT) and a 17-gauge needle (Bergfelt et al., 1994). The procedure was performed to remove the DF of the first follicular wave and any other follicles with a diameter equal or greater than 5 mm, to synchronize the time of emergence of a wave of new follicular development (Mussard et al., 2007; Bridges et al., 2010).

2.5. Blood collection and radioimmunoassay (RIA)

Blood samples were collected (Fig. 1) via jugular venipuncture into 10 mL EDTA Vacutainer tubes (BD Vacutainer®, Franklin Lakes, NJ) on d 0, 1.5, 2.5, and 3.5 for P4 and on d 2.5 and 3.5 for E2 concentrations. Samples were stored on ice immediately after collection for < 2 h and then centrifuged at 1500 x g at 4 °C for 20 min. Plasma was decanted and stored at – 20 °C until analysis. Plasma concentrations of P4 were determined using a Coat-a-Count® RIA kit (Siemens, Los Angeles, CA) as previously described (Burke et al., 2001). Average intra-assay CV was 7.1%, and inter-assay CV (two assays) for pooled plasma samples containing 1.3 and 3.4 ng/mL of P4 were 9.7% and 5.4%, respectively. The average sensitivity of the assay was 0.06 ng/mL.

Plasma concentrations of E2 were determined by using a double-antibody, I^{125}-based assay that was previously described by Burke et al. (2003) but modified in the laboratory where the assays were conducted. Standards were prepared by solubilizing purified E2 (Sigma®; St. Louis, MO) into phosphate buffered saline (PBS) containing 0.1% gelatin (Sigma®; 0.1% PBS-gel, p = 7.4) then diluted according to the concentration of standards desired. For standard curve concentrations, 200 μL of standard and 400 μL of charcoal stripped steer plasma were used per tube. The tubes into which the pools were placed and the samples to be analyzed received 400 μL of the unknown sample or pools, and 200 μL of 0.1% PBS gel. All contents of tubes were extracted by adding 4 mL of diethyl ether anhydrous (Sigma-Aldrich Co. LLC, Milwaukee, WI, USA), re-suspended into 200 μL of 0.1% PBS gel and incubated overnight at 4 °C. The E2 labeled with I^{125} (E2D2), E2 antiserum (E2D1) and precipitating solution (N6) were acquired from a RIA kit available commercially (Coat-a-Count® Double Antibody Estradiol, Siemens, Los Angeles, CA) and used in volumes of 100 μL, 60 μL and 1 mL, respectively, for each 400 μL sample extracted. The E2 RIA was validated comparing the curves of samples containing a pre-determined known to that of samples of unknown concentration using similar dilutions in 0.1% PBS gel. The intra-assay CV was 4.3% and inter-assay for low (2.6 pg/mL), medium (7.7 pg/mL) and high (16.1 pg/mL) pools were 19.6%, 17.5%, and 16.6%, respectively. The sensitivity of the assay was 0.49 pg/mL.

Serial blood samples were collected to assess patterns of LH secretion on d 2.5 of the experiment in a subset of randomly selected cows from each treatment (HiP4, $n = 6$; LoP4, $n = 7$). Indwelling jugular catheters were placed in the cows, and blood samples were collected at 15 min intervals for 12 h. Blood samples for LH analysis were allowed to clot for 48 h at 4 °C, and then centrifuged at 2785 x g for 20 min. Serum were stored at −20 °C until analyses were performed. Serum concentrations of LH were determined using a double-antibody system previously validated in the laboratory where assays were performed (Anderson et al., 1996). The intra-assay CV was 8.2% and the inter-assay CV for low and high pools was 16.7% and 12.8%, respectively. The sensitivity of the assay was 0.17 ng/mL. An LH pulse was defined as an increased concentration that occurred within 30 min of the previous nadir that exceeded three standard deviations of the nadir, and with a decrease of at least 30 min to the subsequent nadir. Pulse amplitude of LH was defined as the concentration of LH at the peak minus the concentration at the previous nadir, and mean LH concentration was determined as the average of the LH concentration during the 12 h of sampling for each cow.

2.6. OPU procedure

Procedures for OPU were similar to those previously described for ovarian follicle ablation. During OPU, all follicles ≥ 3 mm in diameter were aspirated into a conical tube containing embryo recovery medium (Vigro Flush Media; Bioniche Life Sciences, Belleville, ON, Canada) supplemented with 50 USP heparin/mL (H3393; Sigma 101 Aldrich, St. Louis, MO). The follicular aspirate was collected via the aspiration tubing attached to the needle passed through a rubber stopper in a 50 mL conical tube (Falcon; Thermo Scientific, Waltham, MA) that was kept warm at 35 ± 2 °C in a heating block (Multi-Blok Heater; Lab-Line Instruments, Melrose Park, IL). The aspiration was performed using a foot-operated pump (V-MAR-5100; Cook Veterinary Products, Queensland, Australia) set at 80 mm Hg and a flow rate of 15 mL/min. The aspirate from the OPU was transported immediately to the laboratory. Number of follicles aspirated per cow and size of follicles aspirated were recorded.

2.7. Cumulus-oocyte complexes handling

Cumulus-oocytes complexes (COCs) were identified using a zoom stereomicroscope (Nikon® SMZ 475 T, Philadelphia, PA) at 50x magnification, and quality graded on a 1 to 6 scale (1 = ≥ 5 layers compact cumulus and homogenous oocyte cytoplasm, 6 = denuded) according to Blondin and Sirard (1995). Brilliant cresyl blue (BCB) staining was used to identify oocytes in the growing phase (Alm et al., 2005). Each COC was washed three times in Vigro-complete flush media (Bioniche Life Sciences, Belleville, ON, Canada), treated with 26 μM BCB diluted in PBS, and incubated for 90 min at 38.5 °C in humidified atmosphere. The oocytes were then washed again in Vigro-complete flush media and the color of the cytoplasm was evaluated under the stereomicroscope at 50x magnification. Those oocytes detected with blue ooplasm were designated as BCB+, and the oocytes without blue coloration in the cytoplasm were designated as BCB-. The concentration of 26 μM has been previously used for goat (Rodriguez-Gonzalez et al., 2002), pig (Roca et al., 1998) and cattle (Alm et al., 2005) oocytes, without any subsequent detrimental effect on *in vitro* maturation of oocytes. Following BCB staining and evaluation, cumulus cells were removed by continuous pipetting of the COCs, and diameters of denuded oocytes were measured using a micrometer attached to the stereomicroscope. Oocytes were evaluated using 50x magnification. All subjective parameters were performed blind to treatment to avoid any bias of the data. Cumulus cells and oocytes from individual cows were pooled and stored in 100 μl of lysis buffer (RNAqueous micro kit, Ambion®) at −80 °C until further analyses.

2.8. Relative abundance of follistatin

Total RNA was extracted from each pool of denuded oocytes from a single cow, and residual genomic DNA was removed by DNase I digestion using the RNAqueous micro kit (Ambion) according to the manufacturer's instructions (Patel et al., 2007). Preceding RNA extraction, samples were individually spiked with 250 g of Green Fluorescent Protein (GFP) synthetic RNA as an exogenous control and 50 μg tRNA as a carrier (Bettegowda et al., 2005; Patel et al., 2007). The oligonucleotide primers for bovine FST were obtained from Dr. George Smith (Michigan State University) and can be found in GenBank (GenBank accession number for FST: BF774514). Each reaction mixture consisted of 1 μL of cDNA, 0.75 μL each of forward and reverse primers, 3.75 μL of nuclease-free water, and 6.25 μL of SYBR Green PCR Master Mix into a total reaction volume of 12.5 μL. Reactions were performed in duplicate and changes in relative abundance of mRNA were determined by real-time PCR using the CFX96 touch real-time PCR machine (Biorad). The amount of mRNA of interest was normalized relative to the abundance of the endogenous control 18 s rRNA in cumulus cells and GFP in oocytes (Patel et al., 2007; Bettegowda et al., 2008).

2.9. Statistical analyses

Data were analyzed using a model that included treatment only. Concentrations of P4 and E2 over time were analyzed as repeated measures using the MIXED procedure of SAS (SAS Instutute Inc. Cary, NC, USA, version 9.3). Frequency of LH pulses, mean LH concentration and pulse amplitude, and P4 and E2 concentrations on d 2.5 were analyzed in the subset group of cows that received the serial bleeding ($n = 13$) using the MIXED procedure of SAS. For variables related to ovarian activity, such as number of follicles aspirated, size of the largest follicle at d 2.5 and at OPU, number of oocytes recovered, oocyte recovery rate (total oocytes/total follicles), and the relative abundance of mRNA for FST, cow ($n = 24$) within treatment was the experimental unit and was analyzed using the MIXED procedure of SAS. Occurrence of follicular deviation prior to OPU (DF ≥ 8.5 mm) was analyzed using the GLIMMIX procedure of SAS. Oocyte was the experimental unit for assessing oocyte quality, and percentage of oocytes that stained BCB+ ($n = 49$) were analyzed by treatment using the GLIMMIX procedure of SAS. Oocyte size was analyzed using the MIXED procedure in SAS. Mean values were analyzed using the MEANS procedure of SAS. Data are expressed as the mean ± SEM.

The relationship between 1) P4 concentrations and E2 concentrations on d 2.5 and d 3.5; 2) E2 and follicular diameter on d 3.5; and 3) E2 and occurrence of follicular deviation on d 3.5 were assessed in a separate analysis across treatments. Regression analysis using the general linear models procedure of SAS (PROC GLM) was used to determine if the relationship was linear, quadratic, or cubic. Individual equations for each parameter were generated based on the intercept and slope estimates according to maximum likelihood estimates from each significant effect.

3. Results

Concentrations of P4 differed (treatment x d, $P < 0.01$) between HiP4 and LoP4 treatments, being lower in the LoP4 than HiP4 on
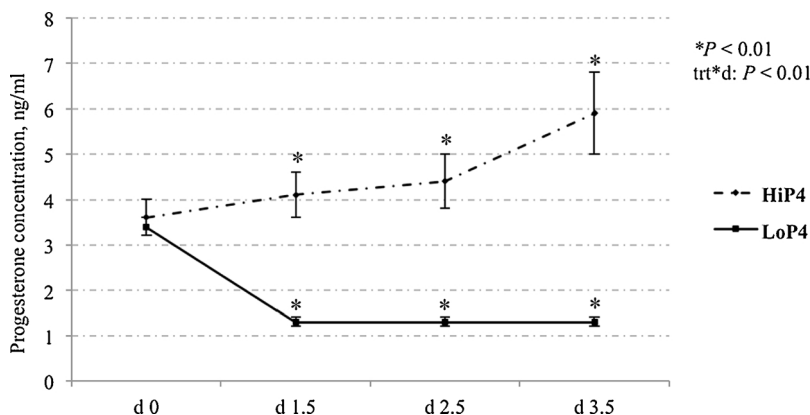
Fig. 2. Mean (± SEM) progesterone concentrations on days 0, 1.5, 2.5, and 3.5 of the experiment between high progesterone (HiP4) and low progesterone (LoP4) treatment groups. *Treatments differ within days ($P < 0.01$).

d 1.5, 2.5, and 3.5 (Fig. 2). Concentrations of E2 were greater (treatment x d, $P < 0.01$) in the LoP4 than HiP4 on d 2.5 and 3.5 (Fig. 3). In the subset of cows sampled for LH ($n = 13$), P4 and E2 differences were consistent with the overall group, with P4 being less and E2 greater in the LoP4 than HiP4 treatment groups (Table 1; Fig. 4). Pulse frequency of LH was greater ($P < 0.05$) in the LoP4 than HiP4 treatment, whereas mean LH concentration and pulse amplitude did not differ between treatment groups (Table 1).

Size of the largest follicle on d 2.5 and at OPU on d 4, number of follicles aspirated per cow, number of oocytes recovered per cow, and oocyte recovery rate did not differ between treatments (Table 2). Percentage of cows with a DF $\geq$ 8.5 mm at OPU tended to be greater in the LoP4 than in HiP4 treatment groups (follicular deviation; Table 2). The percentage of oocytes (graded 1–3), percentage of oocytes staining BCB +, oocyte size, and the relative abundance of oocyte mRNA for FST (pooled by cow) were similar ($P > 0.10$) between treatment groups (Table 3).

A negative linear relationship was detected between P4 and E2 concentrations on d 2.5 ($P = 0.03$; Fig. 5), and on d 3.5 ($P = 0.05$; Fig. 6). A positive linear relationship was detected between E2 on d 3.5 and follicle diameter on d 3.5 ($P = 0.03$; Fig. 7); and between E2 on d 3.5 and probability of follicular deviation occurrence by d 3.5 ($P = 0.02$).

4. Discussion

The effects of decreased P4 concentrations during early follicular development on LH and E2 concentrations, follicular development, and oocyte characteristics were investigated in primiparous beef cows in the present experiment. The animal model used was successful in maintaining distinct concentrations of progesterone throughout follicular development in both groups (LoP4 and HiP4). Relatively lesser P4 concentrations during early follicular development resulted in a relatively greater LH pulse frequency during early follicular growth. In addition, follicles subjected to relatively lesser concentrations of P4 and consequently increased pulsatile LH stimulation had greater estrogenic capacity based on the increased plasma E2 concentrations during early follicular development, as well as the negative relationship between P4 and E2 concentrations on d 2.5 and 3.5. There, however, were no treatment differences in indicators of developmental competence of oocytes (oocyte grade, size, BCB + staining, or relative abundance of FST).

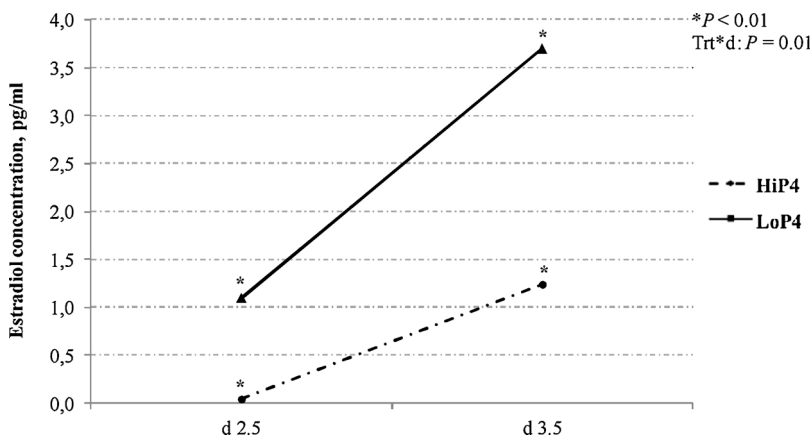In the current experiment, LH pulse frequency in the LoP4 was almost twice that of the HiP4 treatment (0.46 ± 0.06 and

Fig. 3. Mean (± SEM) estradiol concentrations on days 2.5 and 3.5 of the experiment between high progesterone (HiP4) and low progesterone (LoP4) treatment groups. *Treatments differ within days ($P < 0.01$).

Table 1

Characteristics of luteinizing hormone (LH), and progesterone and estradiol concentrations on d 2.5 of the experiment (mean ± SEM).

Variable	HiP4 (n = 6)	LoP4 (n = 7)	P value
LH pulse frequency, pulses/h	0.27 ± 0.05	0.46 ± 0.06	< 0.05
Mean LH concentration, ng/mL	1.17 ± 0.15	1.58 ± 0.28	> 0.1
Mean LH pulse amplitude, ng/mL	0.93 ± 0.15	0.95 ± 0.14	> 0.1
Progesterone concentration, ng/mL	3.80 ± 0.95	1.29 ± 0.16	< 0.05
Estradiol concentration, pg/mL	0.08 ± 0.08	1.34 ± 0.50	< 0.05

A **1430 - HiP4**

B **1316 - LoP4**
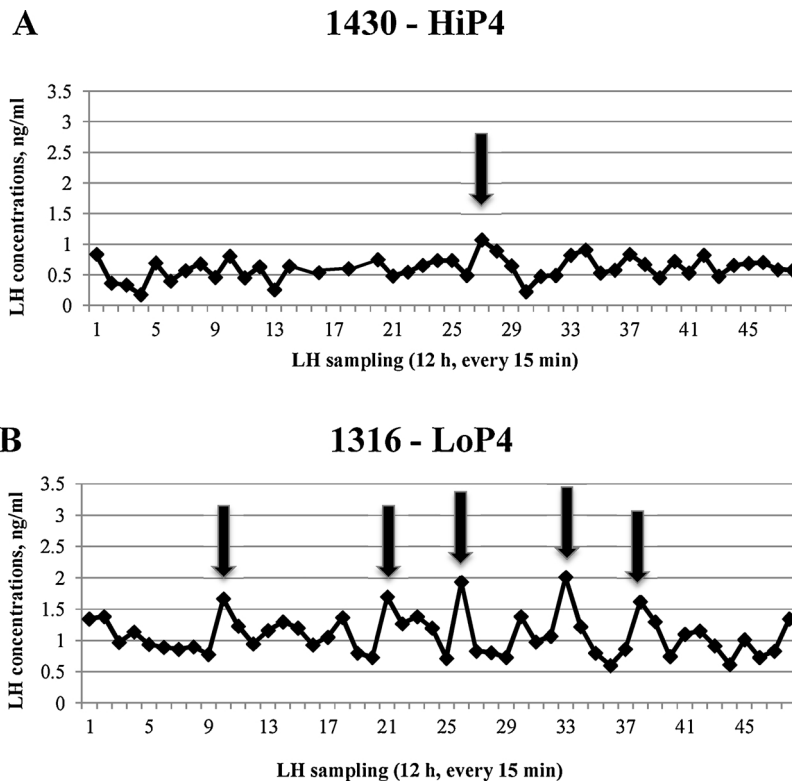
Fig. 4. Representative LH secretion pattern throughout the serial bleeding; A: high progesterone (HiP4) treatment; B: low progesterone (LoP4) treatment; Arrows represent each pulse identified per animal.

Table 2

Follicular characteristics of oocytes recovered on d 4 from cows in the HiP4 and LoP4 groups (mean ± SEM).

	HiP4 (n = 11 cows)	LoP4 (n = 13 cows)
Follicles aspirated per cow, n	4.55 ± 0.49	5.23 ± 0.46
Size of the largest follicle at d 2.5, mm (range)	5.8 ± 0.3 (4.2 to 7.3)	6.3 ± 0.3 (4.4 to 8.5)
Size of the largest follicle at OPU, mm (range)	7.3 ± 0.6 (5.0 to 9.6)	8.2 ± 0.4 (6.2 to 11.1)
Follicular deviation[*], %	30[b]	69[a]
Total oocytes recovered per cow, n	1.82 ± 0.48	2.23 ± 0.34
Oocyte recovery rate, %	40	43

[*]Follicular deviation was considered when the dominant follicle (DF) ≥ 8.5 mm in diameter (Ginther et al., 2001).
[a,b]Values tend to differ (P = 0.08).

0.27 ± 0.05 pulses/h, respectively), which is consistent with previous results (Rahe et al., 1980; Schallenberger and Prokopp, 1985). Previous studies demonstrated that extending the period of sub-luteal P4 concentrations resulted in development of a DF that grew larger than the normal ovulatory diameter and persisted for a longer period of time (Sirois and Fortune, 1990; Stock and Fortune, 1993; Anderson and Day, 1994), negatively impacting fertility (Savio et al., 1993; Mihm et al., 1994; Ahmad et al., 1995). In the present study, oocytes were harvested around the time of expected follicular deviation and thus, the effects of decreased P4

Table 3

Effects of different concentrations of progesterone during early follicular phase on oocyte characteristics (mean ± SEM).

	HiP4 (n = 20 oocytes)	LoP4 (n = 29 oocytes)
Oocytes graded 1 to 3, %	40	38
Oocyte size, μm	155.4 ± 2.1	154 ± 1.8
BCB + staining, %	45	38
FST, relative expression[1]	1.0 ± 0.51	0.34 ± 0.11

[1]Oocyte relative expression of mRNA for follistatin (FST) was analyzed by cow (pool of oocytes).
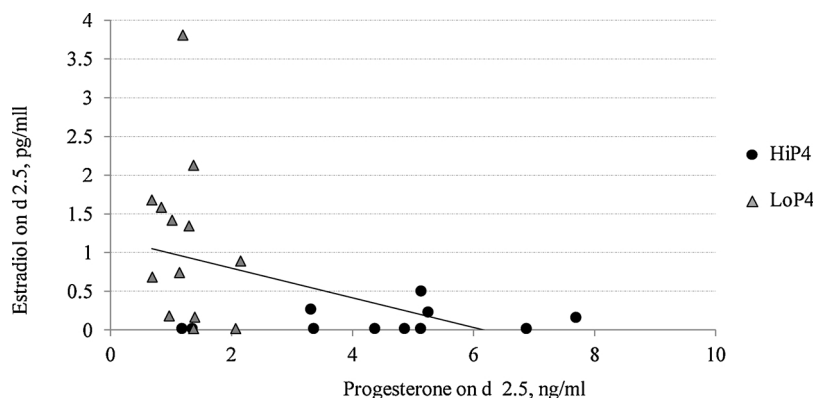
Fig. 5. Relationship between progesterone (P4) concentrations (ng/mL) on d 2.5 and estradiol (E2) concentrations (pg/mL) on d 2.5 in all cows regardless of treatment; Across treatments, decreased P4 concentrations on d 2.5 was related (*P* = 0.03) to increased E2 concentrations on d 2.5; Linear relationship was generated with the following equation: [E2 on d 2.5] = -0.1928 x [P4 on d 2.5] + 1.1776; r^2 = 0.191.
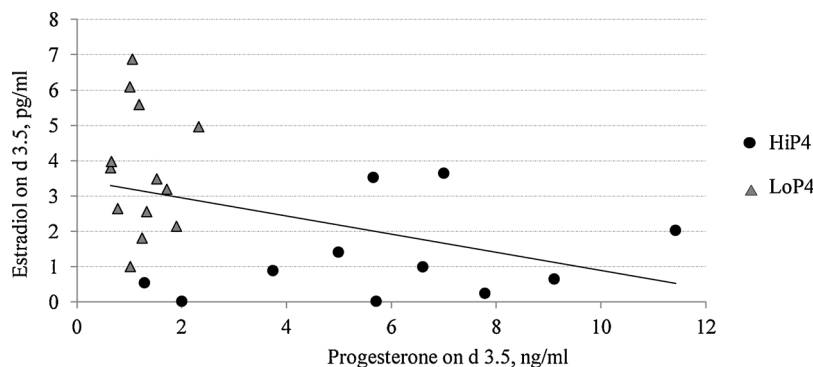
Fig. 6. Relationship between progesterone (P4) concentrations (ng/mL) on d 3.5 (12 h prior to ovum pick-up) and estradiol (E2) concentrations (pg/mL) on d 3.5 in all cows regardless of treatment; Across treatments decreased P4 concentrations on d 3.5 was related (*P* = 0.05) to increased E2 concentrations on d 3.5; Linear relationship was generated with the following equation: [E2 on d 3.5] = -0.2574 x [P4 on d 3.5] + 3.4609; r^2 = 0.1646.

concentrations and increased LH stimulation on the lifespan of the DF could not be determined.

Relatively lesser P4 concentrations during early follicular development have previously been reported to increase follicle diameter in *Bos indicus* (Carvalho et al., 2008; Dias et al., 2009; Martins et al., 2014), *Bos taurus* (Pfeifer et al., 2009; Cerri et al., 2011b) and crossbred (Mantovani et al., 2010) cattle. In the current experiment, diameter of the largest follicle present in the ovaries approximately 2.5–3.5 d after emergence of the new wave of ovarian follicular development (OPU) did not differ. Follicular diameter of the largest follicle in the ovaries, however, was positively correlated with E2 concentrations on d 3.5. Using a similar animal model, it has been reported that decreased P4 concentration for a 5 d period during early follicular development (from ablation to P4 removal) resulted in a larger DF and relatively greater E2 concentrations at P4 withdrawal; moreover, an increased percentage of these cows were in estrus before timed artificial insemination (TAI; Abreu et al., 2018). Potentially, the greater LH pulse frequency in the LoP4 group accelerated follicular growth in the present experiment, as evidenced by the fact that at OPU, follicular deviation had occurred in 69% of the LoP4 and 30% of the HiP4 treatment. Further, the greater E2 concentrations on d 3.5 were positively correlated with the probability of follicular deviation to have occurred by OPU. Greater mean peripheral E2 concentrations as early as 2.5 d after
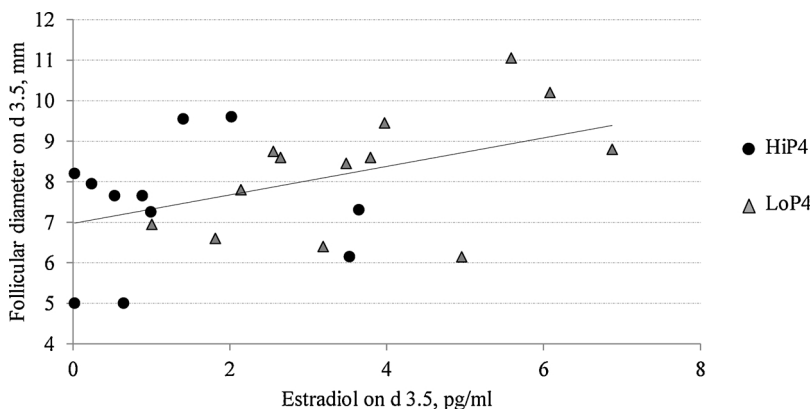
Fig. 7. Relationship between estradiol (E2) concentrations (pg/mL) on d 3.5 (12 h prior to ovum pick-up) and follicle diameter (mm) on d 3.5 in all cows regardless of treatment; Across treatments increased E2 concentrations on d 3.5 were related ($P = 0.03$) to greater follicle diameter on d 3.5; Linear relationship was generated with the following equation: Follicle diameter on d 3.5 = 0.3522 x [E2 on d 3.5] + 6.9686; $r^2 = 0.2016$.

follicular ablation (∼ 1 d after new follicular emergence) in the LoP4 group may suggest the positive effect of the increased LH stimulation on follicular function. Crowe et al. (2001) also reported that heifers immunized against GnRH that were supplemented with FSH and LH had greater peripheral E2 concentrations than GnRH-immunized heifers that received either FSH or LH alone. Moreover, GnRH-immunized heifers that received FSH and LH had more medium (5–9 mm in diameter) and large (≥ 10 mm) follicles that were estrogen-active (Crowe et al., 2001).

Stewart et al. (1995) reported that LH in conjunction with insulin-like growth factor I (IGF-I) stimulates steroidogenesis in theca cells of cattle. Moreover, data from Kojima et al. (2003) suggested that LH pulse frequency in response to varying P4 concentrations may control peripheral and intra-follicular steroid concentrations. Cerri et al. (2011a) reported increased circulating and intra-follicular E2 concentrations in cows that had relatively lesser P4 concentrations. Although hormonal concentrations in the follicular fluid were not assessed in the current experiment, it is likely that intra-follicular E2 concentrations would mimic those in circulation and be greater at the time of OPU in the LoP4 than HiP4 treatment groups. Van de Leemput et al. (1998) indicated that oocytes derived from preovulatory follicles containing greater intra-follicular E2 concentrations had a greater capacity to develop to a blastocyst following *in vitro* fertilization and culture. Moreover, greater aromatase activity was observed in cells of follicles containing oocytes with a greater capacity to develop to the blastocyst stage (Driancourt et al., 1998). In view of the aforementioned benefits of relatively greater LH stimulation and E2 concentrations during follicular growth, it is somewhat surprising that oocytes in the LoP4 group did not have greater indicators of developmental competence than oocytes from the HiP4 treatment (i.e., FST and BCB+). Previous studies in the laboratory where the present research was conducted and other laboratories have resulted in reports that cows with relatively lesser P4 concentrations during early follicular development produced oocytes with greater morphological quality (grades 1 to 3; Abreu et al., 2013), and these oocytes yielded blastocysts with increased numbers of cells (Kruse et al., 2013) when compared with cows having greater P4 concentrations during the same period of follicular development.

It has been suggested that the oocyte acquires full capacity to complete maturation and be fertilized at a diameter of ≥ 120 μm (Fair et al., 1995), which occurs when follicles are approximately 2 to 3 mm in diameter (Hyttel et al., 1997). In the present study, oocytes were collected only from follicles ≥ 3 mm and oocyte diameters ranged from 133 to 173 μm, indicating that all oocytes had achieved adequate diameter to complete maturation and be fertilized regardless of the concentration of P4.Furthermore, only 41% of oocytes in all treatment groups stained positively for BCB, suggesting that more than half of the oocytes were still growing at the time of collection. The use of BCB to assess developmental capacity, however, has been questioned. Previous reports have shown that BCB + oocytes had either greater (Pujol et al., 2004; Silva et al., 2011; Janowski et al., 2012) or similar (Opiela et al., 2008) developmental capacity as BCB- oocytes. In the present experiment, a direct comparison of BCB staining patterns and oocyte development was not performed and thus, it is not possible to determine the assay's efficacy as a predictor of oocyte competence.

Oocyte-derived FST has been identified as a marker of oocyte competence. Patel et al. (2007) demonstrated that abundance of mRNA for FST was greater in oocytes with greater developmental capacity in comparison to oocytes with lesser developmental capacity. In addition, a positive relationship between FST and time to first cleavage has been observed, suggesting that this molecule may not only be associated with time to first cleavage but also normal embryonic development (Patel et al., 2007; Lee et al., 2009). It was hypothesized that the greater LH stimulation during early follicular development in the LoP4 treatment group would increase relative abundance of mRNA for FST. In the current experiment, however, the relative abundance of oocyte mRNA for FST did not differ between oocytes recovered from follicles developing when there were relatively greater or lesser P4 concentrations. The capacity of oocytes to be fertilized and develop to healthy embryos is a complex process with many redundancies to assure normal embryo development even under imperfect conditions. Thus, it is not surprising that the use of single parameters to predict oocyte developmental competence may lead to inconsistent results, as observed with BCB and FST in the present experiment. Further studies using larger numbers of parameters may help elucidate the effects of varying concentrations of P4 during early follicular growth on the oocyte developmental competence.

In conclusion, relatively lesser P4 concentrations during early follicular development resulted in greater LH stimulation and

resulted in development of antral follicles with greater estrogenic capacity. Furthermore, the negative linear relationship between plasma P4 and E2 concentrations and the positive relationship between plasma E2 concentrations and follicle diameter observed in the present study suggest that relatively lesser P4 concentrations during early follicular development accelerated follicular growth. The parameters used in the current experiment to investigate oocyte developmental competence, however, were not affected by the P4 concentration during early follicular growth.

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Conflict of interest

None.

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