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Effect of Standing Estrus Prior to an Injection of GnRH on Steriodogenic Enzyme Expression in Luteal Tissue

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ABSTRACT

Cows detected in estrus around the time of fixed-time AI had increased pregnancy success and progesterone concentrations. Additionally, GnRH following onset of estrus influenced LH pulse frequency and CL formation/function. Therefore our objective was to determine steriodogenic enzyme expression within luteal tissue of cows that were or were not detected in standing estrus prior to an injection of GnRH. Cows were synchronized with the CO-Synch protocol (day -9 100 mg GnRH; day -2 25 mg PGF₂ α ; day 0 100 mg GnRH). Estrus was detected with the HeatWatch system. Location and size of the ovulatory follicle was determined on day 0 at time of GnRH by transrectal ultrasonography; blood samples were collected on day 3,4,5,7, and 9; and luteal tissue was collected on d 10 (n = 3 estrus and n = 8 no estrus) from CL originating from similar sized follicles (13.5 to 16 mm). Total cellular RNA was extracted and relative mRNA levels were determined by real-time RT-PCR and corrected for GAPDH. There was no effect of estrus on CL weight (P = 0.83). There was no effect of estrus by time (P = 0.17) or estrus (P = 0.97) on progesterone concentrations, but there was an effect of time (P < 0.01). In addition, there was no effect of estrus, follicle size, or CL weight on LH receptor expression (P = 0.97, 0.94, and 0.85), StAR expression (P = 0.87, 0.92, and 0.86), CYP11A1 expression (P = 0.49, 0.27, and 0.99), or 3HSD expression (P = 0.49, 0.61, and 0.91). However, there was a correlation between follicle size and CL weight (P = 0.01; R² = 0.51); for every increase of 1 mm in follicle size, CL weight increased by 1.1 g. In addition, there was an effect of CL weight by time (P = 0.01) on progesterone concentrations and an effect of time (P < 0.01) with a tendency for an effect of CL weight (P = 0.06). In summary, estrus did not influence CL weight, progesterone concentrations, or expression of steriodogenic enzymes. However, as follicle size increased, CL weight increased, and CL weight influenced progesterone concentrations.

INTRODUCTION

Progesterone is essential for the establishment and maintenance of pregnancy (McDonald et al., 1952). Therefore, several studies have investigated techniques to increase fertility by increasing corpus luteum (CL) function. One proposed method is to give an injection of GnRH at time of insemination. Kaim et al. (2003) reported that an injection of

GnRH given within 3 hours of the onset of standing estrus increased the ovulatory LH surge, and LH is involved in CL development and function (Peters et al., 1994; Quintal-Franco et al., 1999; Kaim et al., 2003). Approximately 80% of progesterone secreted by corpora lutea is believed to be secreted by large luteal cells (Niswender et al., 1985), and Farin et al. (1988) reported when ewes were treated with LH or hCG on day 5 through 10 of the estrous cycle, more large luteal cells and fewer small luteal cells were present in the corpus luteum compared to untreated controls. However, when LH was blocked around the time of ovulation, animals had decreased subsequent concentrations of progesterone compared to controls (Peters et al., 1994; Quintal-Franco et al., 1999). Furthermore, when GnRH was administered to cows that did and did not exhibit standing estrus; cows that initiated standing estrus had greater subsequent concentrations of progesterone compared to cows that did not initiate estrus (Fields 2008).

Moreover, research has reported cows detected in standing estrus around the time of the GnRH injection during a fixed-time artificial insemination (FTAI) protocol had increased pregnancy rates compared to cows not detected in estrus (Perry et al., 2005; 2007). However, there was no difference in the rate of increase in progesterone between animals that initiated standing estrus and those that did not initiate standing estrus, and no effect of standing estrus on area under the LH curve, average concentrations of LH, or LH pulse frequency (Fields et al., 2008).

Therefore, the objective of this experiment was to determine steroidogenic enzyme expression within luteal tissue of cows that were or were not detected in standing estrus prior to an injection of GnRH.

MATERIALS & METHODS

Experimental Design

Thirty-three Angus-cross non-pregnant, non-lactating, cycling mature beef cows were synchronized with the CO-Synch protocol. Cows were injected with GnRH (100 g as 2 mL of Cystorelin i.m.; Merial, Diluth, Ga) on day -9. An injection of prostaglandin F_{2α} (PGF_{2α} 25 mg as 5 mL of Lutalyse i.m., Pfizer Animal Health, New York, NY) was given on day -2. Forty-eight hours after the PGF_{2α} injection cows were given an injection of GnRH (100 g as 2 mL of Cystorelin i.m.). The HeatWatch electronic estrous detection system was used to determine initiation of standing estrus. Onset of estrus was determined as the first of 3 mounts within a 4-hour period of time lasting 2 seconds or longer in duration. Transrectal ultrasonography was performed using an Aloka 500V ultrasound with a 7.5 MHz transrectal linear probe (Aloka, Wallingford, CT). Both ovaries of each cow were examined at time of the second GnRH injection. All follicles > 8 mm in diameter were recorded.

Blood samples were collected by venipuncture of the Jugular Vein into 10 mL Vacutainer tubes (Fisher Scientific, Pittsburgh, PA) on day 3, 4, 5, 7, and 9 after GnRH treatment. All blood was allowed to coagulate at room temperature then stored at 4° C for 24 h. Samples were centrifuged at 1,200 x g for 30 min, and the serum was harvested and frozen at -20° C until analyzed by radioimmunoassay (RIA). On day 10 after the second GnRH injection cows were transported to a local abattoir. Only animals (n = 11) that had a similar

dominant follicle size (14.8 ± 0.39 and 15.0 ± 0.24 mm for estrus and no estrus, respectively) were analyzed in the study.

Radioimmunoassay

Blood samples were analyzed for serum concentrations of progesterone by RIA using methodology described by Engel et al. (2008). All samples were analyzed in a single assay and intra-assay coefficients of variation was 5.39%. Assay sensitivity was 0.4 ng/mL.

Blood and Tissue Collection

Ovaries were collected within 30 minutes of slaughter and immediately placed on ice. Location of CL were confirmed with location of dominant follicle prior to GnRH. Corpra lutea were dissected from the ovary and divided into equal part. Each section of the CL was placed in RNase free tubes (USA Scientific) and snap frozen in liquid nitrogen. Samples were stored at -80°C until total RNA was extracted.

RNA isolation

A SV Total RNA Isolation System (Promega Corporation) was used to extract RNA from the corpra lutea samples. An empty 1.5 ml eppendorf tube (USA Scientific) was weighed and $\frac{1}{4}$ of the corpus luteum was inserted into the tube and measured again to find the weight of the sample. The sample was then placed in a tube with lysis buffer and homogenized. After homogenization, lysis buffer was added to sample in order to bring the concentration to the recommended 171 mg/ml, and 175 μl of sample was then transferred to a new 1.5 ml eppendorf tube. The remaining sample was inserted into a 2.0 ml eppendorf tube and frozen at -80°C . To the sample, 350 μl of RNA dilution buffer was added and mixed. Samples were then incubated in a 70°C heating block for 3 min and then centrifuged at $13,000 \times g$ for 1 minute at room temperature. The supernatant was then transferred to a new 1.5 ml eppendorf tube and 200 μl of 95% ethanol was added and mixed. The mixture was transferred to a Spin Column and centrifuged at $13,000 \times g$ for 1 minute at room temperature. The liquid was removed from the collection tube and collected as waste. To the spin column, 600 μl of RNA wash solution was added and centrifuged at $13,000 \times g$ for 1 minute at room temperature. After removing the liquid from the collection tube, 50 μl of DNase incubation mix was added to the membrane of the spin basket and incubated at room temperature for 15 minutes, then 200 μl of DNase stop solution was added. The spin basket was centrifuged for 1 minute at $13,000 \times g$ at room temperature. The spin column assembly was washed twice with RNA wash solution centrifuged for 1 minute at $13,000 \times g$ at room temperature. The RNA was eluted off the spin basket membrane by adding 100 μl of nuclease-free water and centrifuged at $13,000 \times g$ for 1 minute at room temperature. After purification RNA concentrations were measured at 260nm and 280nm by spectrophotometry.

Quantitative Real-Time PCR

Prior to quantitative real-time RT-PCR, all RNA samples were diluted to 30ng/ μl with RNase/DNase free water (MP Biomedicals), and concentration was determined by spectrophotometry. A single-step SYBR Green reaction was performed using the iScript One-step RT-PCR Kit with SYBR Green (Bio-Rad Laboratories, Inc) and a Stratagene MX

3000P QPCR System. The SYBR Green reaction was performed for genes with the reverse transcription at 42°C for 30 minutes and 95°C for 10 minutes to inactivate reverse transcription. For all of the genes of interest, transcription was followed by 40 cycles of: 30 seconds at 95°C for melting; 1 minute at the annealing temperatures (61°C for StAR, CYP11A1, 3HSD, LH receptor, and GAPDH); and 30 seconds at 72°C for extension.

Statistics

Influence of expression of standing estrus on CL weight was analyzed by the GLM procedure of SAS. Plasma concentrations of progesterone were analyzed by repeated measures using the Mixed procedures of SAS as described by Littell et al., (1998). All covariance structures were modeled in the initial analysis. The indicated best fit covariance structure, Ante-dependence, was used for the final analysis. The model included the independent variables of treatment (estrus or no estrus; and CL weight), day, and treatment by day. Relative expression of StAR, CYP11A1, 3HSD, and LH receptor were analyzed using the GLM procedure of SAS with expression of GAP-DH as a covariate. The correlation between dominant follicle size and CL weight was analyzed by the PROC CORR procedure of SAS.

RESULTS

There was no effect of standing estrus prior to an injection of GnRH on day 10 CL weight ($P = 0.83$; Figure 1). In addition there was no effect of estrus by time ($P = 0.17$) or estrus ($P = 0.97$) on progesterone concentrations on days 3, 4, 5, 7, and 9 after the GnRH injection. However, there was an effect of time ($P < 0.01$, Figure 2), with circulating concentrations of progesterone increasing from day 3 through day 9.

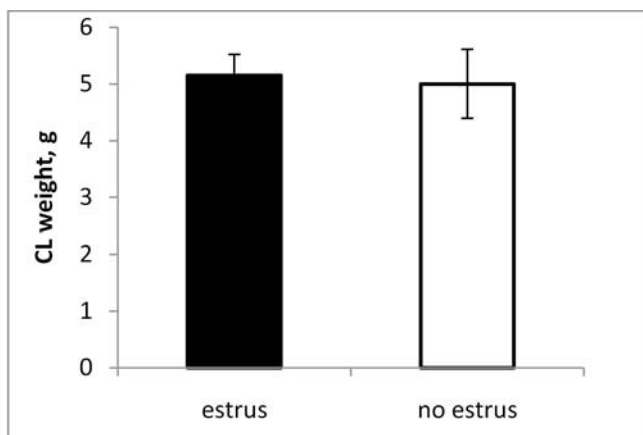


Figure 1. Influence of detection in standing estrus prior to an injection of GnRH on day 10 luteal weight ($P = 0.83$).

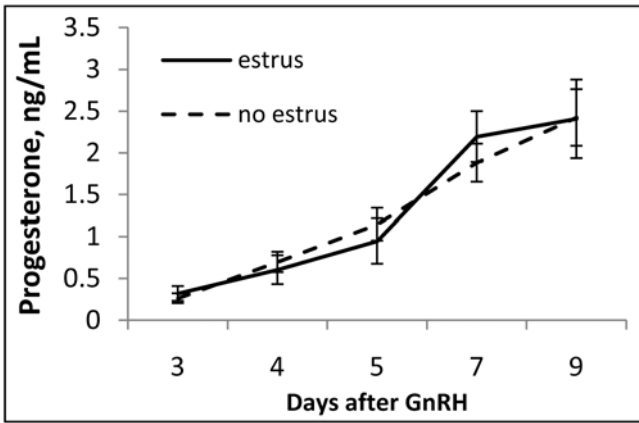


Figure 2. Influence of detection in standing estrus prior to an injection of GnRH on circulating concentrations of progesterone. Estrus by time ($P = 0.17$), estrus ($P = 0.97$), time ($P < 0.01$).

There was no effect of estrus, follicle size, or day 10 CL weight on LH receptor expression ($P = 0.97, 0.94,$ and 0.85 , Figure 3). There was no effect of estrus, follicle size, or day 10 CL weight on expression of StAR ($P = 0.87, 0.92,$ and 0.86 , Figure 4), CYP11A1 ($P = 0.49, 0.27,$ and 0.99 , Figure 5), or 3HSD ($P = 0.49, 0.61,$ and 0.91 , Figure 6).

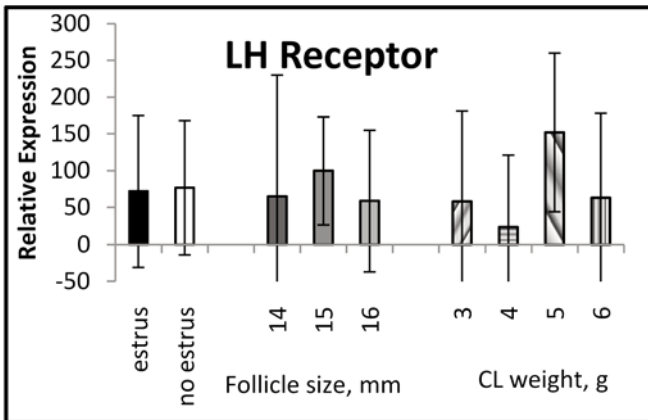


Figure 3. Influence of standing estrus prior to an injection of GnRH, ovulatory follicle size at time of GnRH injection, and luteal weight on day 10 on expression of LH receptor ($P > 0.85$).

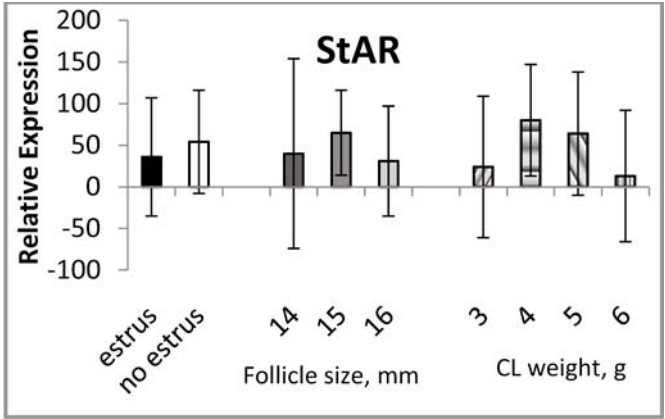


Figure 4. Influence of standing estrus prior to an injection of GnRH, ovulatory follicle size at time of GnRH injection, and luteal weight on day 10 on expression of StAR ($P > 0.86$).

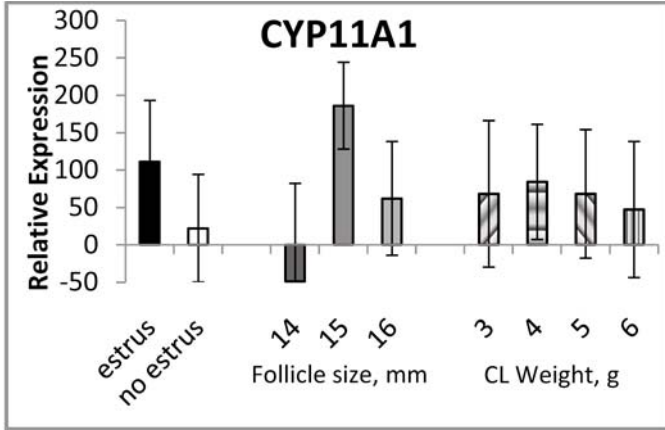


Figure 5. Influence of standing estrus prior to an injection of GnRH, ovulatory follicle size at time of GnRH injection, and luteal weight on day 10 on expression of CYP11A1 ($P > 0.27$).

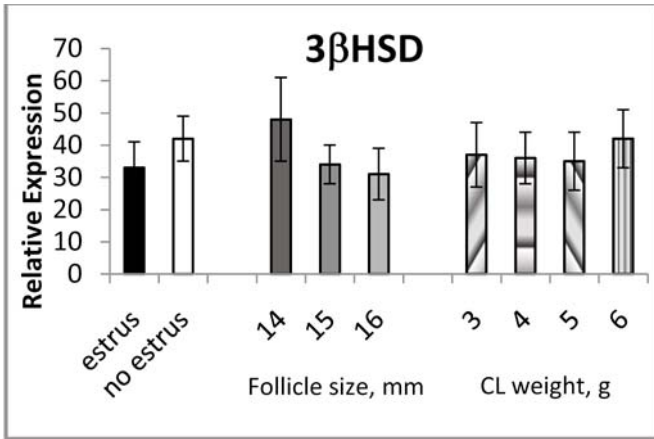


Figure 6. Influence of standing estrus prior to an injection of GnRH, ovulatory follicle size at time of GnRH injection, and luteal weight on day 10 on expression of 3HSD ($P > 0.85$).

A correlation was found between follicle size and CL weight ($P = 0.01$; $R^2 = 0.51$, Figure 7), for every increase of 1 mm in follicle size, CL weight increased by 1.1 g. In addition, there was an effect of CL weight by time ($P = 0.01$) on concentrations of progesterone and an effect of time ($P < 0.01$) with a tendency for an effect of CL weight ($P = 0.06$, Figure 8). As CL weight increased circulating concentrations of progesterone tended to increase.

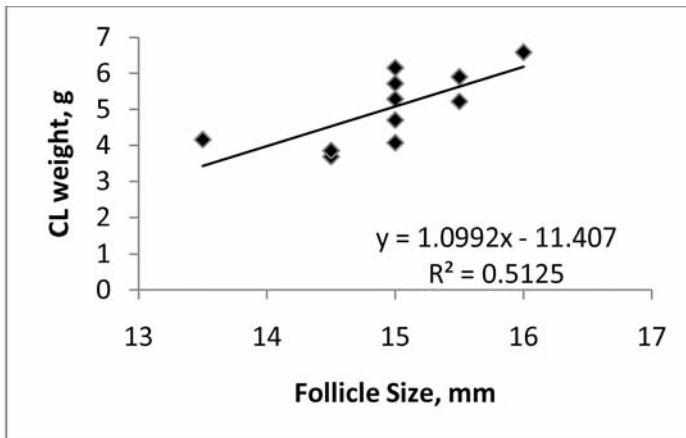


Figure 7. Correlation between ovulatory follicle size at time of GnRH injection and luteal weight on day 10 ($P = 0.01$).

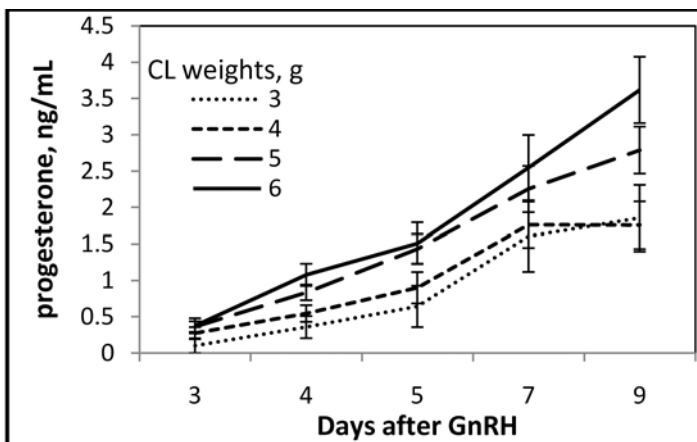


Figure 8. Influence of day 10 luteal weight on changes in circulating concentrations progesterone following induced ovulation. Luteal weight by time ($P = 0.01$), time ($P < 0.01$), luteal weight ($P = 0.06$).

DISCUSSION

Luteinizing hormone (LH) plays a vital role in the development and function of the corpus luteum. Previous work has reported that increasing the ovulatory LH surge increased CL function (Kaim et al., 2003). Furthermore, when GnRH was administered to cows that did and did not exhibit standing estrus; cows that initiated standing estrus had greater subsequent concentrations of progesterone compared to cows that did not initiate estrus (Fields 2008). However in the present study, detection of standing estrus prior to an injection of GnRH had no effect on CL weight, progesterone concentrations, or expression of steroidogenic enzymes. In the present study cows that did and did not exhibit standing estrus were selected to have similar sized ovulatory follicles. Among dairy cows, induced ovulation of small follicles (11.54 ± 0.22 mm) resulted in smaller CL that secreted less progesterone compared to cows induced to ovulate larger follicles (14.47 ± 0.39 mm, Vasconcelos et al., 2001), and ovine follicles induced to ovulate 12 hours after luteal regression had fewer granulosa cells and formed smaller CL that secreted less progesterone than follicles induced to ovulate 36 hours after luteal regression (Murdoch and Van Kirk, 1998). This is important since granulosa cells are generally believed to differentiate into large luteal cells (Smith et al., 1994) and approximately 80% of progesterone secreted by the corpora lutea is believed to be secreted by large luteal cells (Niswender et al., 1985). In the present study, there was a correlation between follicle size and CL weight; as follicle size increased, CL weight increased, and CL weight influenced progesterone concentrations. However, there was no difference in steroidogenic enzyme expression. Therefore, larger follicles had more cells and resulted in a heavier CL, and a larger CL was capable of producing more progesterone. This

is important because greater production of progesterone by the CL could lead to higher conception rates, and an increase in pregnancy success.

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