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LOCAL AND SYSTEMIC ACTIONS OF GnRH RELATING TO ESTRADIOL AND ASSOCIATED REPRODUCTIVE PARAMETERS IN BEEF CATTLE

BY

JERICA J. J. RICH

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Animal Science

South Dakota State University

2017

LOCAL AND SYSTEMIC ACTIONS OF GnRH RELATING TO ESTRADIOL AND ASSOCIATED REPRODUCTIVE PARAMETERS IN BEEF CATTLE

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Animal Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ABBREVIATIONS

AI	artificial insemination
3βHSD	3β hydroxysteroid dehydrogenase
˚C	degrees celsius
cAMP	cyclic adenosine monophosphate
cc	cubic centimeter
CIDR	controlled internal drug release device
CL	corpus luteum
d	day(s)
DHEA	dihydroepiandrostenedione
DNA	deoxyribonucleic acid
ERα	estrogen receptor α
FSH	follicle stimulating hormone
FTAI	fixed-time artificial insemination
g	grams
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GnRH	gonadotropin releasing hormone
h	hour(s)
IGF	insulin-like growth factor
IGF-BP	insulin-like growth factor binding protein
mm	millimeter
min	minutes
M	molar
mRNA	messenger RNA
ng	nanogram

P450 scc	cytochrome P450 side chain cleavage enzyme
P450 17α	cytochrome P450 17α-hydroxylase
P450 arom	cytochrome P450 aromatase
PCR	polymerase chain reaction
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PKC	protein kinase C
RIA	radioimmunoassay
RNase	ribonuclease
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
s	seconds
SAS	statistical analysis system
sd	standard deviation
se	standard error
StAR	steroidogenic acute regulatory protein
TAI	timed artificial insemination

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CHAPTER 1

ABSTRACT

LOCAL AND SYSTEMIC ACTIONS OF GnRH RELATING TO ESTRADIOL AND ASSOCIATED REPRODUCTIVE PARAMETERS IN BEEF CATTLE

JERICA J. J. RICH

2017

In cattle, estradiol is responsible for modulating many mechanisms involved in successful reproduction. Specifically estradiol is the primary signal for the initiation of standing estrus (Allrich, 1994), and cattle expressing estrus prior to fixed time AI (FTAI) have been reported to have increased preovulatory estradiol concentrations (Perry and Perry, 2008) and increased pregnancy success compared to animals that did not exhibit estrus (Richardson et al., 2016). Gonadotropin releasing hormone, classically, stimulates estradiol production via the two-cell two-gonadotropin hypothesis (Fortune and Quirk, 1988), and GnRH administered systemically in small doses (5 μg) has been reported to elicit an LH pulse similar to a physiological pulse (Ginther et al., 1996). In a study where multiple small doses (5 μg) of GnRH were administered following CIDR removal, concentrations of estradiol were increased (Larimore et al., 2016). These studies demonstrate that estradiol production from ovarian follicles could be stimulated with systemically administered GnRH. In addition, to the classical pathway, GnRH receptor mRNA has been characterized in bovine granulosa cells (Ramakrishnappa et al., 2003), and GnRH has been reported to have local stimulatory and inhibitory actions on steroidogenesis within the ovary (Sharpe, 1982; Janssens et al., 2000). The role of GnRH in bovine follicles as it pertains to estradiol production and/or the regulation of estradiol production has not been well characterized. Therefore, the objective of the subsequent

studies were to investigate the relationship of GnRH and its impact on estradiol production and other reproductive parameters by conducting field trials where GnRH was administered systemically (Chapters 3 and 4), as well as the local abundance of GnRH-I and GnRH-II mRNA within granulosa cells of bovine antral follicles (Chapter 5).

In Chapter 3, beef cows and heifers (n = 1620) were synchronized using the 7-day CIDR protocol, and randomly assigned to one of three treatments (0, 5, and 10 μg of a GnRH analog at CIDR removal). Animals were visually observed for estrus and inseminated following detection in estrus. Interval to estrus was calculated for each animal that exhibited estrus (INTERVAL 1). Animals that did not exhibit estrus were given 100 μg of GnRH at the time of AI and their interval to estrus was designated at 120 h (INTERVAL 2). There was an effect of age ($P < 0.01$) and a treatment by age interaction ($P = 0.05$) on INTERVAL 1. Heifers had a shorter interval to estrus than cows (50 h vs 54 h respectively). Furthermore, heifers given 5 μg of GnRH tended to have a shorter interval to estrus ($P = 0.07$; 47 ± 1.4 h) compared to 0 μg (50 ± 1.5 h) and did have a shorter interval compared to 10 μg ($P < 0.01$; 52 ± 1.5 h). There were no differences among treatments in interval to estrus among cows ($P \geq 0.34$). When animals that did not exhibit estrus were included in the analysis at 120 h there was no treatment by age interaction ($P = 0.49$). This is likely due to the fact that treatment ($P < 0.01$), but not age ($P = 0.96$) or treatment by age ($P = 0.74$) influenced expression of estrus, with 5 μg tending to have more animals in estrus ($P = 0.10$; 79 ± 4%) compared to 0 μg (74 ± 5%), and 10 μg having fewer animals in estrus compared to either other treatment ($P < 0.04$; 68 ± 6%). Estrus ($P < 0.01$) and age ($P < 0.01$) influenced pregnancy success with heifers having greater pregnancy success compared to cows (49 ± 5% vs 38 ± 4%,

respectively) and animals exhibiting estrus having greater pregnancy success compared to animals that did not exhibit estrus (57 ± 4% vs 32 ± 4%, respectively). However, there was no difference in pregnancy success between treatments among animals that exhibited estrus ($P > 0.30$). Among animals that did not exhibit estrus 0 μg had increased pregnancy success ($P \leq 0.05$; 40 ± 6%) compared to 5 μg and 10 μg which did not differ (27 ± 6% and 29 ± 5%, respectively). This experiment demonstrated that small doses of GnRH following CIDR removal can shorten the interval to estrus and increases the percentage of animals exhibiting estrus prior to AI.

In Chapter 4, beef cows and heifers (n = 247) were synchronized using the 7-day CO-Synch + CIDR protocol and were assigned to one of three treatment groups [0 μg GnRH at CIDR removal (0 μg); 10 μg GnRH at CIDR removal (10 μg), or 5 μg at CIDR removal plus 5 μg 12 h later (5 + 5 μg)]. Animals were visually observed for estrus and artificially inseminated 55 (heifers) or 60 (cows) hours following CIDR removal (FTAI). Blood samples were collected beginning at CIDR removal and every 12 hours until time of AI. Concentrations of estradiol were not influenced by treatment ($P = 0.66$) or a treatment by time interaction ($P = 0.87$), but were influenced by time ($P < 0.0001$). Estradiol concentrations increased from CIDR removal to 48 hours after CIDR removal and then were decreased at the time of AI. Expression of estrus was not influenced by age ($P = 0.31$), or treatment ($P = 0.60$), however, there was a treatment by age interaction ($P < 0.01$) for expression of estrus. For animals administered the 5 + 5 μg treatment, expression of estrus was increased ($P < 0.01$) among heifers compared to cows (84 ± 3% vs 70 ± 3%, respectively). Pregnancy success was influenced by estrus ($P < 0.01$), treatment ($P = 0.02$), and a treatment by estrus ($P < 0.01$) interaction, but was not

influenced by age ($P = 0.91$), treatment by age ($P = 0.83$), or treatment by estrus by age ($P = 0.94$). Animals that exhibited estrus had increased pregnancy success compared to animals that did not exhibit estrus (67 ± 2% vs 41 ± 7% respectively). Animals administered the 5 + 5 µg treatment had increased pregnancy success ($P \leq 0.05$; 69 ± 6%) compared to animals administered the 0 µg and 10 µg treatments which did not differ (53 ± 6% vs 41 ± 8%, respectively). Pregnancy success was similar ($P > 0.27$) between animals that did and did not exhibit estrus for animals administered the 0 µg and 5 + 5 µg treatments. For animals administered the 10 µg treatment, conception rates were decreased ($P < 0.01$) among animals that did not exhibit estrus compared to animals that exhibited estrus. This study demonstrated that the 5 + 5 µg treatment of GnRH following CIDR removal, when implementing a fixed-time AI protocol, positively influenced conception rates.

In Chapter 5, beef cows/heifers were synchronized using the CO-Synch protocol and artificially inseminated. On day 16 after insemination animals were transported to a local abattoir. Following slaughter ovaries were collected and all follicles were classified as small (< 5 mm), medium (5 to 10 mm), or large (> 10 mm). Follicles were aspirated to collect follicular fluid and granulosa cells. Follicles were pooled by size within animal (n = 23, 16, and 18 for small, medium, and large, respectively). Follicular fluid concentrations of estradiol were determined by radioimmunoassay. Total cellular RNA was extracted from the granulosa cells and RT-PCR was performed to determine relative abundance of mRNA for GnRH-I, GnRH-II, and GAPDH. Data were analyzed using the mixed procedure in SAS. Follicle size influenced concentration of estradiol. Large follicles had increased estradiol ($P < 0.0001$) compared to small and medium follicles

(18,626 ± 2,650 vs 1,270 ± 2,307 and 8,925 ± 2,763 pg/mL, respectively). There was no difference ($P = 0.31$) in relative abundance of GnRH-I mRNA among small, medium, or large follicles (3.8 ± 0.78, 3.40 ± 0.85, and 1.95 ± 0.94; respectively). Relative abundance of GnRH-II mRNA was influenced by follicle size ($P < 0.05$), with greater abundance in small follicles (40.97 ± 9.27) compared to medium (6.32 ± 11; $P = 0.02$) or large (7.85 ± 11.11; $P = 0.02$) follicles. However, there was no difference ($P = 0.92$) in relative abundance between medium and large follicles. When follicles were classified by concentration of estradiol, follicles with the lowest 25% of estradiol had decreased (LOWE2; $P < 0.0001$) concentrations of estradiol (434 ± 1,074 pg/mL) compared to the middle 50% (MIDE2; 7,029 ± 961 pg/mL) which was decreased ($P < 0.0001$) compared to the greatest 25% (HIGHE2; 46,423 ± 2,025 pg/mL). LOWE2 had greater ($P < 0.01$; 5.37 ± 0.67) abundance of GnRH-I mRNA compared to MIDE2 and HIGHE2 which did not differ (1.85 ± 0.55 and 1.23 ± 1.65, respectively). Relative abundance of GnRH-II mRNA was greater ($P < 0.01$) in LOWE2 (42.33 ± 9.56) compared to MIDE2 (5.98 ± 8.90). HIGHE2 was similar ($P \geq 0.12$) to the other two groups (8.90 ± 19.12). Results from this study demonstrate a relationship between granulosa cell GnRH-I and II mRNA abundance with estradiol concentrations within the follicle, where decreased GnRH-I and GnRH-II may be playing a role in increased estradiol production.

These three experiments demonstrate GnRH directly impacting estradiol, and/or reproductive performance of beef cows and heifers through interval to estrus, expression of estrus, and increasing conception rates when used with FTAI protocols. The mechanisms by which GnRH is having its actions remains to be elucidated.

CHAPTER 2

LITERATURE REVIEW

INTRODUCTION

Estradiol plays a critical role in fertility among cattle, specifically it has been reported that preovulatory estradiol regulates reproductive processes that result in increased pregnancy success, such as uterine pH (Perry and Perry, 2008), endometrial gene expression (Bauersachs et al., 2005) and sperm transport (Larimore et al., 2015) that impact embryo development and survival. Estradiol is also critical within the ovary as it is associated with growth, divergence, ovulation, termination, and eventual regression/atresia of follicles, atresia may also be attributed to decreased steroidogenesis by specific follicles (Bodensteiner et al., 1996b). Estradiol from the follicle is believed to be produced via the two-cell two-gonadotropin hypothesis (Figure 1, Fortune and Quirk, 1988). Classically, GnRH from the hypothalamus is transported to gonadotrope cells in the anterior pituitary via hypophyseal portal vessels, these gonadotrope cells synthesize and release luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the systemic circulation that transports them to their endocrine target, the gonads. Specifically they act on granulosa and theca cells to regulate processes involved with steroidogenesis and folliculogenesis (Schally et al., 1971; Liu and Hsueh, 1986).

Cows that exhibit estrus prior to FTAI have increased preovulatory concentrations of estradiol and greater conception rates compared to those that do not exhibit estrus (Perry et al., 2005; Perry et al., 2007; Perry and Perry, 2008; Pereira et al., 2016; Richardson et al., 2016). Previous research indicated that multiple small (5 μg)

intramuscular injections of GnRH after CIDR removal increased circulating concentrations of estradiol (Larimore et al., 2016), likely through the classical pathway.

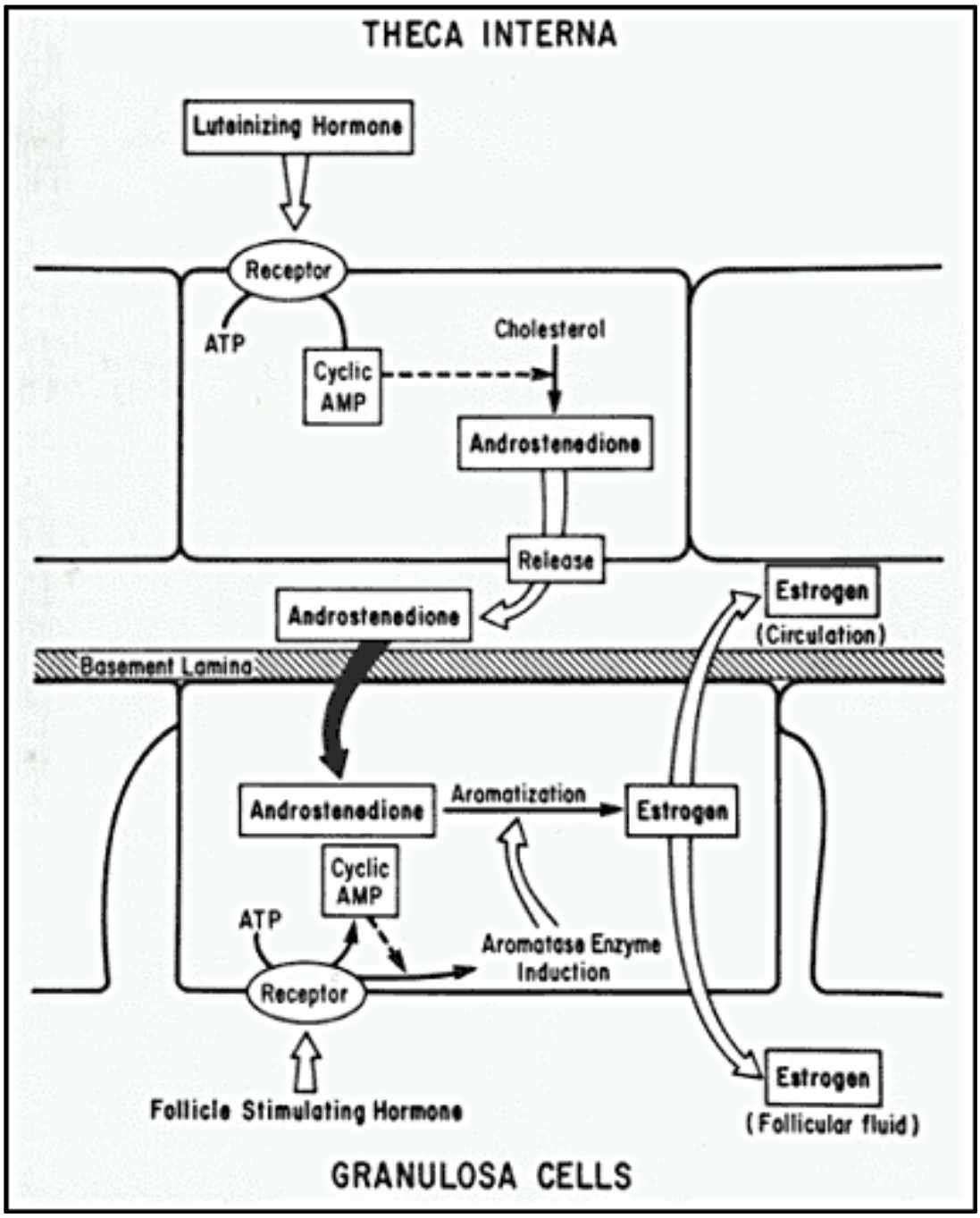

Figure 1. Representation of the two-cell, two-gonadotropin theory. The two cells being granulosa and theca cells and the two gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Adapted from Erickson, 1978).

Gonadotropin releasing hormone has been reported to have both inhibitory and stimulatory actions on steroidogenesis in the ovary (Sharpe, 1982) and GnRH receptor mRNA expression has been characterized in the bovine ovary (Ramakrishnappa et al., 2003). However, the specific systemic and local actions of GnRH are not well known. This review will delve into literature regarding the bovine estrous cycle, follicle development, steroid hormone production, and GnRH actions via the classical hypothalamo-pituitary-gonadal axis, as well as, local actions of GnRH at the ovary.

BOVINE ESTROUS CYCLE

An estrous cycle is characterized by the events that occur between stages of sexual receptivity and/or ovulations; the cycle is divided into two phases, the luteal phase and the follicular phase, and the average length of the cycle is 21 days (Fortune, 1994; Forde et al., 2011). During the luteal phase (days ~1-17) of the cycle, the dominant ovarian structure is the corpus luteum which secretes progesterone, and during the follicular phase (days ~18-21), growing follicles secrete estradiol (Senger, 2003). Understanding the components of the estrous cycle allows for manipulation and intervention of management practices in order to be successful in cattle reproduction.

Luteal Phase

Ovulation is a traumatic process to the ovarian follicle and results in the rupture of blood vessels within the follicular wall. At this stage the theca cells and any remaining granulosa cells luteinize to become either small or large luteal cells to form the corpus luteum (CL) (Smith et al., 1994; Howles, 1997; Forde et al., 2011). The CL is

responsible for production of progesterone, the major hormone of the luteal phase (Smith et al., 1994). During the luteal phase follicular waves still occur, but there is a disparity in estradiol to progesterone concentrations. The ratio of low estradiol to high progesterone concentration exerts negative feedback to the hypothalamus and inhibits GnRH release, collectively preventing increased production of estradiol, expression of estrus, and ovulation (Adams et al., 1992a; Baratta et al., 1994; Janovick and Conn, 1996; Nett et al., 2002).

Follicular Phase

Following regression of the CL, progesterone concentrations decline. In the absence of progesterone, the inhibitory feedback to the hypothalamus is removed and GnRH is released into the hypophyseal portal vessels resulting in the synthesis and release of LH and FSH from gonadotrope cells (Schally et al., 1971; Walters and Schallenberger, 1984). Luteinizing hormone and FSH stimulate growth of the dominant follicle and estradiol production. Elevated concentrations of estradiol, in the absence of progesterone, act on the brain to cause behavioral estrus which lasts approximately 12 to 16 hours (Vailes et al., 1992; Allrich, 1994). Furthermore, the increased production of estradiol from the dominant follicle feeds back positively to the gonadotrope cells to increase their responsiveness to GnRH, this allows for frequent LH pulses to occur for a period of two to three days that causes a prolonged growing and maintenance of the dominant follicle. This increased responsiveness coupled with increased LH pulse frequency, allows for a surge release of LH following a surge of GnRH, resulting in ovulation of the dominant follicle, 24 to 30 hours after the onset of estrus (Chenault et al.,

1975; Christenson et al., 1975; Lemon et al., 1975; Adams et al., 1992a; Allrich, 1994; Roche, 1996).

Estrus

Estrus refers to the period of time when the female is sexually receptive to the male. Standing estrus is the visual sign that a female will stand to be mounted by herd mates and/or the bull, and typically lasts 12 to 16 hours (Allrich, 1994; Forde et al., 2011). Estradiol is the primary signal to the hypothalamus to initiate the expression of estrus (Vailes et al., 1992; Diskin and Sreenan, 2000).

There is a positive relationship between expression of estrus at the time of artificial insemination, pregnancy success, and embryo survival until day 30 in beef cattle (Vasconcelos et al., 1999; Perry et al., 2005; Perry et al., 2007; Jinks et al., 2013; Pereira et al., 2016; Richardson et al., 2016). Variations in interval to estrus and pregnancy success in FTAI protocols have been hindrances to the successful use of protocols to synchronize estrus (Roche and Boland, 1991; Bo et al., 1994). Investigation in implementing tools (i.e. hormones) to manipulate the estrous cycle in a way to enhance expression of estrus presents an imperative area of research.

There are many factors that impact the expression of estrus. Specifically, increased circulating cortisol has been shown to delay and/or block the preovulatory LH surge and expression of estrus (reviewed by Stevenson, 2001). Another factor to consider when managing a cow/calf beef herd would be postpartum anestrous, as it is compounded by other factors such as body condition score at calving, nutrition, calf presence, and previous dystocia (Short et al., 1990; Stagg et al., 1998). Postpartum beef

cows or first-calf heifers are dealing with decreased GnRH-induced LH release (Roche et al., 1992), which is inhibitory to estrus (Vailes et al., 1992). Furthermore these postpartum cows are raising a calf, and in instances where the animal is in a negative plane of nutrition, reproductive traits are lower in priority ranking in the nutrient partitioning hierarchy (Short and Adams, 1988); in discussing nutrient availability and partitioning, special consideration must be made for first calf heifers that are still growing themselves while raising their new calf. All of the aforementioned conditions pose hindrances in re-initiating cyclicity and successful expression of estrus.

Fertilization

Increased estradiol concentrations in the absences of progesterone, positively feedback to induce the surge release of LH and the subsequent ovulatory cascade (Allrich, 1994), resulting in an oocyte being released into the oviduct (Senger, 2003). Approximately 12 hours after the initiation of standing estrus is the optimal time for spermatozoa to be introduced into the tract via natural service or artificial insemination (Chenault et al., 1975; Christenson et al., 1975; Lemon et al., 1975; Adams et al., 1992a; Allrich, 1994; Roche, 1996). Once in the female tract, spermatozoa begin their journey to the oviduct, during this time the sperm are undergoing a process known as capacitation, which results in the removal of seminal plasma proteins from the sperm head to allow for binding to the oocyte, this process is supported by secretions of the female reproductive tract, containing Na^{+}, K^{+}, and Ca^{2+} (Fraser, 1992, 1998). Next, the spermatozoa free of seminal plasma proteins and cholesterol make contact with the zona pellucida and must undergo the acrosome reaction in order to fertilize the oocyte

(Yanagimachi, 1988). The acrosome reaction commences when the spermatozoa makes contact with the zona pellucida of the oocyte (Ward and Kopf, 1993). The zona pellucida consists of three glycoproteins (ZP1, ZP2, and ZP3) (Wassarman, 1988; Wassarman and Mortillo, 1991). Once the sperm has bound, and fertilization takes place, the zona block occurs which prevents additional sperm from penetrating the zona pellucida (Braden et al., 1954).

In order to achieve successful ovulation of oocytes, that can later be fertilized and develop into offspring there are strict requirements. Ovarian steroidogenesis and folliculogenesis are the necessary precursors to successful reproduction. Therefore, the purpose of the following sections is to review literature regarding follicular growth, and steroid hormone production, specifically estradiol and progesterone.

FOLLICULOGENESIS

Follicular development in cattle occurs in wave like patterns, and there are typically two to three waves per estrous cycle (Rajakoski, 1960; Ireland and Roche, 1983; Roche and Boland, 1991; Fortune, 1994; Forde et al., 2011). Evidence for this style of growth has been reported in calves as early as two weeks of age (Evans et al., 1994; Ginther et al., 1996). The initiation of follicular waves is characterized by a small cohort of follicles beginning to grow under the influence of increasing concentrations of FSH (Adams et al., 1992b; Evans et al., 1994). During these follicular waves a dominant follicle emerges. Dominant follicles then have one of two fates, atresia or ovulation (Bodensteiner et al., 1996b; Quirk et al., 2004). This section will review the process of folliculogenesis in more detail.

Follicle Populations/Classification

Follicles contained within the ovary can be classified into one of two classes; preantral follicles: primordial, primary, and secondary, and antral follicles: tertiary. The dynamics of preantral-secondary and antral follicles involve four processes: recruitment, selection, dominance, and ovulation or atresia (Fortune, 1994; Bodensteiner et al., 1996a; Bodensteiner et al., 1996b; Howles, 1997; Senger, 2003). Cattle are born with approximately 133,000 total primordial (non-growing) follicles, whose numbers remain fairly consistent until four years of age when they begin to decrease until there are few to none remaining at approximately fifteen to twenty years of age (Erickson, 1966). These primordial follicles are the pool from which cohorts of growing follicles are derived. Growing follicles (preantral and antral) rapidly increase in number from one month of age until almost four months of age, then continue to increase in number gradually and then remain constant up until puberty, at this time growing follicle numbers begin to decrease in a fashion similar to what is observed among primordial follicles (Erickson, 1966). A transient rise in FSH stimulates each wave of recruitment (Adams et al., 1992b; Sunderland et al., 1994). There are three proposed models by which a dominant follicle is selected for continued growth and ovulation or atresia. The three models are, 1) LH receptor expression in granulosa cells resulting in a shift from FSH to LH dependency (Xu et al., 1995; Bao et al., 1997), 2) Increased concentrations of estradiol preceding the gonadotropin dependence shift from FSH to LH (Evans and Fortune, 1997), and 3) Increased availability of insulin-like growth factors as a result of degradation of IGF-BP acting synergistically with gonadotropins to stimulate follicular cell growth and differentiation (Giudice, 1992; Spicer and Echternkamp, 1995). The single dominant

follicle within the cohort of antral follicles, can more effectively bind gonadotropins, has increased estradiol to a low progesterone ratio and is the one that continues to grow to preovulatory status (Ireland and Roche, 1983; Quirk et al., 2004).

Antral Follicles

Ovarian follicles are the main functional unit of the ovary. They are responsible for producing steroid hormones, ovulating oocytes, and luteinizing into corpora lutea to assist in the maintenance of pregnancy. Antral/tertiary follicles are composed of two outer layers of theca cells, the theca interna and the theca externa. The theca cell layers are supplied by a vasculature network and separated from the other components of the follicle by a basement membrane. Theca interna cells play a role in steroid production and contain LH receptors, while the theca externa provide structural support for the follicle. Within the basement membrane are the avascular granulosa cells which also play a role in steroid production and have FSH receptors (Senger, 2003; Rodgers and Irving-Rodgers, 2010). Preantral and early antral bovine follicles that are between 2 and 4 mm in diameter are believed to be gonadotropin-independent, while the growth of bovine antral follicles greater than 4 mm in diameter is considered to be gonadotropin dependent (Bao and Garverick, 1998; Orisaka et al., 2009). The central cavity (antrum) of the follicle is filled with follicular fluid, which is derived from secretions of the surrounding cells as well as a result of fluid movement from the extra-follicular space/vasculature (Rodgers and Irving-Rodgers, 2010). A specialized group of granulosa cells exists that crowns the oocyte, this group of cells is termed the cumulus oophorus (Senger, 2003; Rodgers and Irving-Rodgers, 2010). The follicular fluid is kept separate

from the rest of the body/circulation, and maintains an environment rich in steroids, proteins, and growth factors conducive to follicular and oocyte maturation (Smith et al., 1994; Roche, 1996; Howles, 1997). Antral follicles vary by size and are often classified as either small (less than 5 mm), medium (5 to 10 mm) or large (greater than 10 mm; (Matton et al., 1981).

STEROIDOGENESIS

Steroid production is reliant on the transfer of cholesterol into the inner mitochondrial membrane via steroidogenic acute regulatory protein (StAR) that has been localized to the theca interna. Both theca and granulosa cells contain cytochrome P450 side chain cleavage enzyme (P450scc) which is needed for the conversion of cholesterol to pregnenolone, pregnenolone is then converted to 17 hydroxy-progesterone and then androstenedione via 17 α-hydroxylase cytochrome P450 enzyme (P45017α), androstendione is converted to testosterone via 3β-hydroxysteroid dehydrogenase enzyme (3βHSD) (Bao et al., 1997). Testosterone within the theca cells can then cross the basement membrane and enter the granulosa cells. Granulosa cells bind FSH and convert testosterone into estradiol by working in concert with the enzyme cytochrome P450 aromatase (P450arom) (Liu and Hsueh, 1986; Fortune and Quirk, 1988; Bodensteiner et al., 1996b; Bao and Garverick, 1998). Maximal steroid hormone concentrations are observed in the follicular fluid as a result of increased expression of mRNA for gonadotropin receptors, steroidogenic enzymes and StAR (Bao and Garverick, 1998).

Estradiol

Cytochrome P450 scc and cytochrome P450 aromatase enzymes are responsible for the steroidogenic pathway leading to the production of estradiol, and have been detected in granulosa cells as early as recruitment (Figure 2; Bao and Garverick, 1998). However, among preantral and antral follicles less than 4 mm in diameter, granulosa cell expression of cytochrome P450 aromatase was undetectable, this suggesting that these follicles are unable to convert testosterone to estradiol within the granulosa cells and giving rise to the conclusion that follicles less than 5 mm in diameter are estrogen-inactive (Ireland and Roche, 1983; Bao and Garverick, 1998). Estradiol in bovine follicles has a positive feedback effect on its own production by inhibiting progesterone secretion by granulosa cells (Fortune and Quirk, 1988), and by positively feeding back to the hypothalamus to stimulate GnRH to induce LH release from the anterior pituitary (Baratta et al., 1994) by increasing the number of GnRH receptors at the level of the anterior pituitary (Gregg et al., 1991). Estradiol and FSH in increasing concentrations induced increased LH receptor binding sites on granulosa cells in rats (Bodensteiner et al., 1996b). Estradiol may also be modulating its own synthesis, as an *in vivo* pretreatment of Estradiol 17β was shown to inhibit 17α-hydroxylation *in vitro* by rat theca interstitial cells (Magoffin and Erickson, 1981) thus preventing the conversion of progestins to androgens, leaving insufficient androgen substrate that subsequently inhibits estrogen synthesis (Liu and Hsueh, 1986).

Culturing of human granulosa-luteal cells with estradiol resulted in a decrease in GnRH and GnRH-R mRNA (Nathwani et al., 2000; Kang et al., 2001a). Specifically at concentrations as low as 1 nM of estradiol, GnRH mRNA abundance was reduced by

45% compared to control, and at an estradiol concentration of 100 nM a 70% decrease in GnRH mRNA was observed relative to the control. Similar results were observed for GnRH-R mRNA with a 40% and 65% decrease in GnRH-R mRNA for the 1 nM and 100 nM concentration of estradiol, respectively (Nathwani et al., 2000).

Furthermore there was a positive relationship between follicle size and peak estradiol concentration among cows that exhibited estrus (Perry et al., 2014). Previous research has also reported a relationship with follicles size, circulating concentrations of estradiol, and pregnancy success to AI among beef cattle (Perry et al., 2005). When employing synchronization protocols for the use of artificial insemination, cattle are sometimes forced to ovulate smaller follicles, with the administration of GnRH, in these instances, animals experience decreased pregnancy success due to decreased estradiol production of that smaller follicle (Perry et al., 2005).

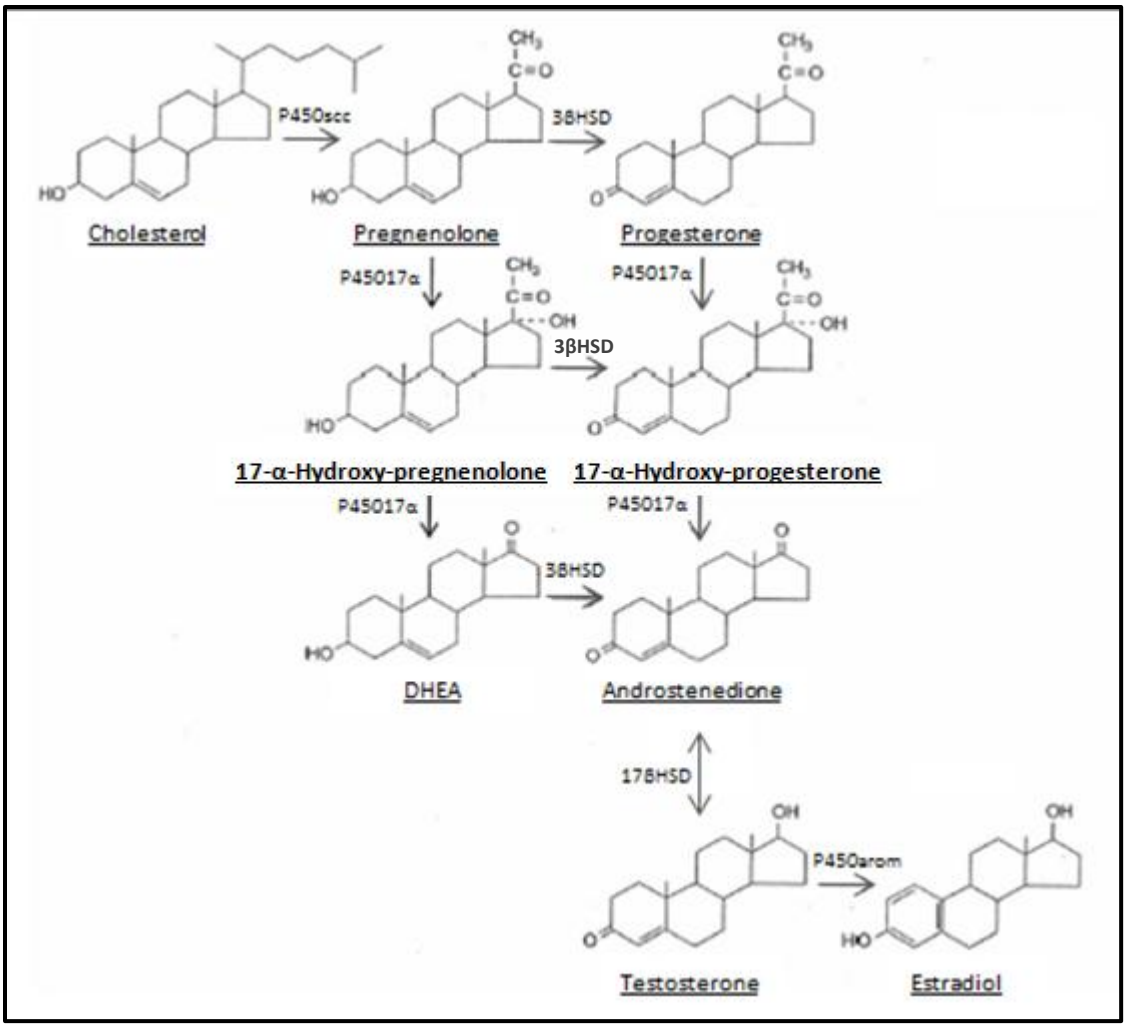

Figure 2. Ovarian biosynthesis of Estradiol-17β. The steroidogenic pathway with its steroid hormone intermediates and respective enzymes are illustrated. Dehydroepiandrosterone (DHEA), Cytochrome P450 side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3βHSD), 17α-hydroxylase cytochrome P450 (P45017α), 17β-hydroxysteroid dehydrogenase (17βHSD), and cytochrome P450 aromatase (P450arom) (Adapted from Hadley and Levine, 2007).

Progesterone

During the mid-luteal phase of the estrous cycle endogenous progesterone concentrations are increased due to production by the CL. As mentioned above, in employing synchronization protocols when an animal is forced to ovulate smaller follicles, estradiol concentrations peak at a lower concentration and those smaller follicles have fewer granulosa and theca cells, which luteinize to form an inferior CL. Lishman and Inskeep (1991) summarized that inferior CL function can arise from a variety of situations 1) Deficiencies in maturation of the preovulatory follicle and/or in the ovulatory stimulus, 2) Inadequate support of the CL once it has formed, or 3) Premature activation of the luteolytic process. These inferior CLs lead to decreased pregnancy success as well (Perry et al., 2005).

During the luteal phase, follicular waves still occur, but due to high progesterone concentrations inhibiting surge releases of GnRH, LH, and FSH, the dominant follicles do not ovulate, but becomes atretic (Adams et al., 1992a; Baratta et al., 1994; Janovick and Conn, 1996; Nett et al., 2002). When Adams et al. (1992a) injected early-luteal phase, nulliparous Holstein heifers with varying doses of progesterone, they found that animals injected with higher doses of progesterone had hastened follicular wave emergence and suppression of growth of the dominant follicle. Thus demonstrating the negative feedback of progesterone on LH pulsatile secretion. However, FSH was not suppressed, resulting in hastened emergence of subsequent anovulatory follicular waves.

Prolongation of the growing phase of the ovulatory follicle was observed with low and declining concentrations of progesterone after the cessation of treatment (Adams et al., 1992a), this added to the hypothesis that increased LH pulse frequency resulting from

low circulating progesterone is responsible for promoting continued growth and maintenance of the dominant follicle (Adams et al., 1992a). In conclusion, in the presence of progesterone and decreased estradiol, follicular waves still occur in cows but ovulation does not take place.

GONADOTROPIN RELEASING HORMONE

Gonadotropin releasing hormone is a decapeptide which was originally isolated from 150,000 porcine hypothalami by the work of three scientists, Schally, Guillemin, and Yalow, who won the Nobel Prize in 1977. Since its discovery, 24 different forms of GnRH have been identified (Figure 3), among those, three distinct isoforms have been characterized (GnRH-I, GnRH-II, and GnRH-III). Only GnRH-I and GnRH-II have been found in mammals, specifically GnRH-I is thought to work predominantly through the classical hypothalamo-pituitary-gonadal axis while GnRH-II actions have been reported predominantly in extra-pituitary sites (Millar, 2005), making it of special interest to researchers in the field of reproductive physiology. Gonadotropin releasing hormone-II, specifically was discovered from 10,000 chicken hypothalami, and was 30% as potent as mammalian GnRH (GnRH-I) in its ability to cause the release of gonadotrophs in a bioassay (rat anterior pituitary cells; (Miyamoto et al., 1984). The carboxy and amine terminal residues of the GnRH peptide are highly conserved across species with amino acids 5-8 being more variable. Amino acid 8 of the GnRH peptide is thought to be the most variable amino acid and allows for ligand-receptor specificity (Millar, 2005).

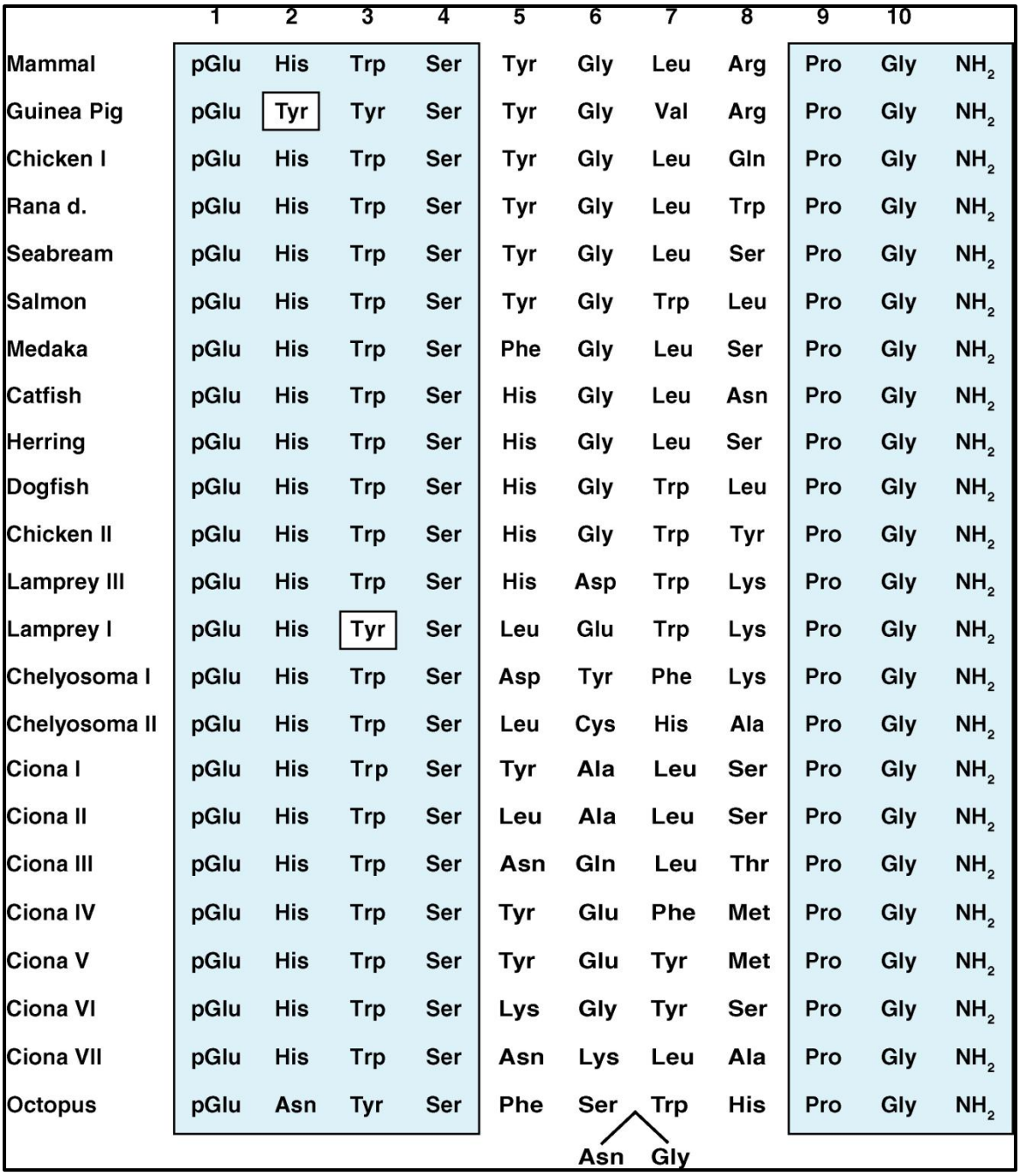

Figure 3. Primary amino acid sequence of GnRH forms named for the species in which they were discovered. The boxed regions show conserved carboxy and amine terminal residues. The non-conserved amino acids are thought to be either unimportant or responsible for ligand-selectivity of different GnRH receptors (Adapted from Millar, 2005).

Gonadotropin releasing hormone, or longer lasting GnRH analogs are used extensively for reproductive management in cattle. Specifically, GnRH can be used to induce postpartum ovulations, reversibly suppress ovarian activity, and more commonly can be used to synchronize estrus and ovulation (D'Occhio and Aspden, 1999). Gonadotropin releasing hormone injections (100 μg i.m.) administered during synchronization protocols can induce ovulation of inferior/smaller follicles, resulting in decreased estradiol concentrations. Therefore investigation into methods to increase estradiol production in synchronization protocols presents an important area of research (Jinks et al. 2013; Perry et al., 2014).

When injecting animals with GnRH it is difficult to distinguish whether changes are a result of local actions of GnRH or if they are secondary to the pituitary release of LH and FSH. In the following sections the local actions of GnRH in mammalian species will be discussed further, and special considerations for employing GnRH in cattle reproductive management systems will be discussed.

Localization of GnRH

Gonadotropin releasing hormone, like other hypothalamic peptides, has extra-pituitary actions (Ortmann and Diedrich, 1999; Millar, 2005; Ramakrishnappa et al., 2005). Considerable research has been focused on actions of GnRH at the ovarian level. Expression of GnRH in the ovary was first characterized in rats via ligand binding (Clayton et al., 1979; Jones et al., 1980; Reeves et al., 1980). Expression was next localized to primary, secondary, and tertiary follicles of rats (Clayton et al., 1979; Whitelaw et al., 1995). Both GnRH-I and GnRH-II have been demonstrated in the

human ovary via RT-PCR and southern blot analysis (Peng et al., 1994; Kang et al., 2000; Choi et al., 2001).

Expression of GnRH mRNA has also been characterized in granulosa cells of small, medium, and large bovine antral follicles and luteal tissue (Ramakrishnappa et al., 2003), and in human and rat granulosa and luteal cells (Minaretzis et al., 1995; Olofsson et al., 1995; Kang et al., 2001b). A GnRH-like molecule was first identified in bovine follicles, and suppressed the LH-stimulated accumulation of cAMP in a dose dependent manner (Aten et al., 1987). Furthermore this GnRH-like molecule was in greatest concentrations in bovine granulosa cells (Ireland et al., 1988).

GnRH Actions on Folliculogenesis

Gonadotropin releasing hormone antibody infusion into prepubertal porcine ovarian tissue resulted in a decreased number of follicles greater than 0.5 mm in diameter, and increased incidence of atresia in follicles with greater than 4 layers of granulosa cells. However, there were no reported differences in number of follicles less than 0.5 mm in diameter nor were circulating concentrations of estradiol, LH, and FSH affected, likely due to the fact that only one ovary was infused (Patton et al., 1991); thus providing evidence of direct action of GnRH as follicles at this stage are gonadotropin independent (Orisaka et al., 2009). Other methods of GnRH administration may have impacted folliculogenesis either through direct actions on the ovary or through regulation of LH release. When small doses (5 µg and 10 µg) of GnRH agonist were injected (i.m.) into heifers twice daily for three weeks, pulsatile secretion of LH was suppressed and development of follicles beyond 9 mm was blocked, the combined effects prevented

increased estradiol production to allow for the preovulatory LH surge and ovulation (Gong et al., 1995). Upon cessation of the treatment dominant follicles reached ovulatory size and ovulated (Gong et al., 1995). Deslorelin (a GnRH analog) implants of 700 µg slightly delayed the emergence of the third dominant follicle in non-lactating Holstein cows implanted 4 days after ovulation, while Deslorelin implants of 2100 µg drastically delayed the selection of the third dominant follicle, and extended the estrous cycle (Rajamahendran et al., 1998). The number of medium antral follicles was reduced in the 700 µg and 2100 µg Deslorelin implanted groups, and overall the 700 µg Deslorelin group had the fewest large antral follicles compared to control cows between days 13 and 19 following ovulation (Rajamahendran et al., 1998). All of the aforementioned studies reported inhibitory actions of GnRH on follicular growth.

When nulliparous Holstein heifers were treated with GnRH approximately 32 hours before ovulation (100 µg i.m., Cystorelin) variability in interval from GnRH treatment to ovulation was reduced, ovulation occurred at 32 hours in GnRH treated heifers versus 48 to 56 hours for the control group; GnRH treatment also allowed for quicker attainment of: maximal FSH concentrations, emergence of a dominant follicle, and ovulation of the dominant follicle in the subsequent wave (Bodensteiner et al., 1996a). In the same study, GnRH treatment increased the number of follicles greater than 5 mm per wave by 53%, all of these showing a stimulatory effect of GnRH on follicular growth (Bodensteiner et al., 1996a).

In regards to one of the fates of growing follicles, atresia, GnRH has been reported to have direct effects on apoptosis in the ovary. The initiation of apoptosis in granulosa cells is one of the earlier signs of atresia, and is a process in which unwanted

cells can be selectively eliminated (Tilly and Hsueh, 1993; Yuan and Giudice, 1997). Furthermore research exists that supports the direct effects of GnRH in regulating follicular atresia and induction of cellular apoptosis (Hsueh et al., 1984; Piquette et al., 1991; Billig et al., 1994; Whitelaw et al., 1995; Motomura, 1998; Sridaran et al., 1998). High expression of GnRH-R mRNA has been found in atretic follicles of rats during the follicular phase (Whitelaw et al., 1995) and *in vitro* studies with GnRH inhibited DNA synthesis and stimulated the induction of apoptosis, with the actions of GnRH being confined to the rat granulosa cells (Billig et al., 1994; Saragueta et al., 1997). This stimulatory effect of GnRH in inducing apoptosis has also been observed in porcine and human granulosa cells (Zhao et al., 2000).

GnRH Actions on Steroidogenesis

Gonadotropin releasing hormone has been reported to have both stimulatory and inhibitory actions on ovarian function and subsequently steroidogenesis (Sharpe, 1982; Janssens et al., 2000). In cultured rat granulosa cells the use of a GnRH analog inhibited ovarian growth by blocking estradiol secretion, by inhibiting the induction of LH receptors via FSH and the aromatase enzyme (Hsueh et al., 1980). Gonadotropin releasing hormone has also been reported to decrease expression of granulosa FSH and LH receptors (Piquette et al., 1991; Tilly et al., 1992), and luteal cytochrome P450 scc (P450 scc) and 3βHSD (Sridaran et al., 1999a; Sridaran et al., 1999b) in both *in vitro* and *in vivo* experiments using rats. In a study by Larimore et al. (2016) beef cows that did not exhibit estrus prior to fixed-time AI treated with intramuscular injections of GnRH (5 μg), every eight hours from the time of prostaglandin administration (hour 0) to 33 hours,

had decreased abundance of mRNA for 3βHSD and P450 scc in granulosa cells, indicating decreased function of the steroidogenic pathway. Administration of GnRH (100 µg i.m.) results in an LH surge, and following an LH surge, aromatase activity is down regulated resulting in decreased circulatory and follicular estradiol concentrations (Komar et al., 2001).

Studies on human ovaries indicated that GnRH-II also inhibited LH and FSH receptor expression (Kang et al., 2001b). Gonadotropin releasing hormone agonists added *in vitro* have a dose-dependent stimulatory effects on aromatase activity, the enzyme responsible for the conversion of androgens to estrogens, and progesterone production in monkey granulosa cells (Janssens et al., 2000). When a GnRH agonist was added to cultured bovine granulosa cells at a concentration of 200 ng/mL it had a stimulatory effect on estradiol production, however, at concentrations of 500 ng/mL and 1000 ng/mL it had an inhibitory effect on estradiol production (Ramakrishnappa et al., 2005). In *in vitro* human granulosa-luteal cells, GnRH-I mRNA abundance was down regulated by 17β-estradiol (Nathwani et al., 2000; Kang et al., 2001a). Gonadotropin releasing hormone-I suppressed expression of estrogen receptor α and β via a PKC-dependent pathway in human granulosa cells, thus providing a way for GnRH to counteract the inhibitory actions of estradiol on its own expression (Chiang et al., 2000). The aforementioned studies show the various mechanism by which GnRH and estradiol can regulate one another via the classical pathway- secondary to gonadotropin sensitivity or locally, and the resulting stimulatory and inhibitory actions that can be observed.

Continuous infusion of low doses of GnRH stimulate gonadotropin release without pituitary desensitization and down-regulation of GnRH receptors ([s.c. 20 µg/h]

(Lamming and McLeod, 1988); [i.v.2.5 µg/h] (Jagger et al., 1987). Pulsatile secretion of LH has also been shown to be blocked in studies where cattle are treated with GnRH analogs or agonists, as a result of pituitary desensitization and receptor down regulation (Vizcarra et al., 1997). Treatment of non-lactating Holstein cows with a GnRH (8 µg) intramuscular injection and Deslorelin (a GnRH analog) implants (700 µg and 2100 µg) induced a rapid LH surge (Rajamahendran et al., 1998). In the same study by Rajamahendran et al. (1998), greater progesterone concentrations were observed from days 14 to 18 after ovulation in the 700 µg and 2100 µg Deslorelin implant groups compared to controls, thus showing a stimulatory effect of GnRH on progesterone production. In another study a GnRH agonist (100 ng) had an inhibitory effect on progesterone secretion when added to bovine luteal cell cultures thus providing evidence of direct actions of GnRH on luteal cells (Milvae et al., 1984). Furthermore, in a study by Harwood et al. (1980), hCG stimulated progesterone production by collagenase-dispersed rat luteal cells via LH receptor binding was inhibited by a GnRH agonist, this inhibition was overcome as greater concentrations of hCG were used. In the same study, GnRH agonist shifted the dose response curve by an order of magnitude to the right without changing the maximal progesterone production of epinephrine stimulated collagenase-dispersed rat luteal cells (Harwood et al., 1980). From these studies it is clear that GnRH has both stimulatory and inhibitory actions on progesterone production.

In a study by Peng et al. (1994) using human granulosa-luteal cells, GnRH increased GnRH mRNA abundance at low doses, but had no effect or a slight inhibitory effect on GnRH gene expression at higher doses. Preovulatory rat granulosa cell GnRH gene expression was up-regulated when GnRH (1.18 µg/mL) was added to the culture

medium (Olofsson et al., 1995). This relationship was also reported for GnRH-II, where GnRH-II differentially regulated its own expression; treatment with GnRH-II, resulted in significant down-regulation of both GnRH-I and GnRH receptor mRNA levels in human granulosa-luteal cells (Kang et al., 2001b). From these studies, it is evident that GnRH-I and GnRH-II are modulating their own release.

The aforementioned actions of GnRH on steroidogenesis provide *in vitro* and *in vivo* evidence of how GnRH exerts direct stimulatory and inhibitory effects either on its own or in combination with other factors. Furthermore, the actions of GnRH have contradicting actions in different species, or even when employing different research methods, which, can make the prospect of studying the effects of GnRH challenging.

GnRH Inferred-Actions on Estrus

A 5 µg dose of GnRH elicits an LH pulse similar to a physiological pulse (Ginther et al., 1996), and increased LH pulsatility increased estradiol concentrations (Fortune et al., 2001). Beef cows injected with GnRH (5 µg) intramuscularly every 8 hours from the time of prostaglandin injection (hour 0) to 33 hours after, had elevated estradiol concentrations at hours 7, 8, and 16 (Larimore et al., 2016), this was thought to occur via GnRH inducing increased LH pulsatility. Heifers treated with a GnRH agonist 24 hours after prostaglandin injection had increased diameter of the dominant follicle (Pursley et al., 1997; Taponen et al., 1999; Taponen et al., 2000), and this was associated with increased plasma concentrations of estradiol (Bergfeld et al., 1996; Maclellan et al., 1997; Dufour et al., 1999). As mentioned above, estradiol is the primary signal to the hypothalamus to initiate the expression of estrus (Vailes et al., 1992; Diskin and Sreenan,

2000). Therefore, one can infer that GnRH may positively contribute to the expression of estrus, via gonadotropin stimulation. Furthermore, a meta-analysis conducted using over 10,000 beef cattle from 22 studies reported that animals that exhibit estrus prior to fixed-time artificial insemination had greater conception rates compared to animals that did not exhibit estrus prior to insemination (Richardson et al., 2016). Therefore, the role of GnRH in inducing favorable effects (increased estradiol concentrations) when subjecting cattle to fixed-time artificial insemination programs presents an essential interaction to be investigated, and will be discussed further in the subsequent chapters.

GnRH Actions on Fertilization

Gonadotropin releasing hormone impacts fertilization both directly and indirectly. Indirect actions of GnRH that positively impact fertilization do so by changing expression of estrogen receptors at the level of the endometrium (Singh et al., 2009). Specifically, gonadotropin releasing hormone increased expression of ERα in cultured luteal phase bovine endometrium, suggesting the role it might be playing in inducing endometrial responsiveness to estradiol and the associated effects: uterine receptivity, uterine pH, implantation, and maintenance of pregnancy (Singh et al., 2009).

Gonadotropin releasing hormone has been identified in the ovary of many species (Aten et al., 1987; Oikawa et al., 1990), and more specifically rat follicular fluid (Ying et al., 1981), bovine granulosa cells (Ireland et al., 1988), and the bovine uterus and oviduct (Singh et al., 2008). Knowing this, it becomes evident that during natural service or artificial insemination, those spermatozoa are coming into contact with GnRH within the female reproductive tract, and that GnRH in turn has the opportunity to directly impact

fertilization. Specifically, in humans, GnRH and its analogs were shown to increase binding of spermatozoa to the zona pellucida (Morales and Llanos, 1996). Additionally, GnRH agonists were also reported to increase the cleavage rates of bovine oocytes *in vitro* (Funston and Seidel, 1995). Overall, GnRH seems to play a vital role in the processes involved with successful fertilization.

GnRH Receptors

As there are three distinct isoforms of GnRH, the existence of distinct receptor types is expected (Troskie et al., 1998), and presents as a formative area of research. Gonadotropin releasing hormone-I receptor is structurally and functionally different from the GnRH-II receptor (Millar, 2005; Ramakrishnappa et al., 2005). Receptors for both GnRH I and II are expressed in the gonadotrope cells of the anterior pituitary. The presence of GnRH-II receptor within the brain is thought to mediate sexual behavior, and the presence of GnRH-II receptor within reproductive tissues is indicative of the local role of GnRH-II (reviewed by Ramakrishnappa et al., 2005). Specifically, researchers have found that GnRH-II is about 30% as potent as mammalian GnRH (GnRH-I) in its ability to cause the release of gonadotropins in a bioassay of rat anterior pituitary cells (Miyamoto et al., 1984). Factors exist that are known to alter the expression of GnRH receptors and subsequently the number of GnRH receptors within the anterior pituitary, these include gonadal steroids, estradiol and progesterone, inhibin, activin, and GnRH itself in rats, sheep, and cattle (Gregg et al., 1991; Braden and Conn, 1992; Turzillo and Nett, 1999). In humans, GnRH was reported to up-regulate GnRH receptor gene expression (Peng et al., 1994).

Expression of GnRH receptors is lowest in the anterior pituitary during the luteal phase, when progesterone concentrations are increased, in sheep (Crowder and Nett, 1984; Brooks and McNeilly, 1994; Turzillo et al., 1998). Furthermore progesterone decreased the number of ovine pituitary GnRH receptors, while in the same study inhibin increased GnRH receptors (Laws et al., 1990). Inhibin and estradiol added in culture to ovine pituitary cells increased GnRH receptor numbers, while activin decreased GnRH receptor numbers (Gregg et al., 1991). Expression of GnRH receptor has been characterized in bovine granulosa cells, and was increased in granulosa cells of small follicles compared to medium and large follicles (Ramakrishnappa et al., 2005). Gonadotropin releasing hormone receptor has also been identified in human granulosa-luteal cells (Peng et al., 1994).

Special considerations in studying effects of GnRH

It is hard to distinguish direct gonadal actions from indirect actions of GnRH; those that are in response to the GnRH peptide itself versus those mediated by the hypothalamus that result in the synthesis and release of LH and FSH. Gonadotropin releasing hormone is diluted 1:500 from the time it travels from the hypophyseal portal vessels out to the systemic jugular vein (Nett et al., 1974). Furthermore, circulating levels of GnRH are known to be as low as 10 pg/mL in rats, rabbits, and rhesus monkeys (Eskay et al., 1977; Neill et al., 1977; Tsou et al., 1977). Therefore, experimental modeling becomes crucial, and Janssens et al. (2000) listed factors that may be involved in some of the contradicting results that can be observed when studying GnRH, including (1) The type and dose of GnRH analog used, (2) The different administration routes, (3)

Ovarian status at the time of exposure, (4) The ovarian cell types used in *in vitro* studies, and (5) Variation in GnRH receptor types and abundance which is dependent on species and/or ovarian status.

The effects of GnRH have been studied in a variety of ways; researchers have infused GnRH directly into ovarian tissue (Patton et al., 1991), studied the effects of GnRH in cultures (Sharpe, 1982; Milvae et al., 1984; Funston and Seidel, 1995), utilized hypophysectomized animals see review by Sharpe (1982), and GnRH has been injected systemically or implanted (Gong et al., 1995; Rajamahendran et al., 1998; Larimore et al., 2016). In implementing all of these methods to study GnRH, researchers are able to delineate multiple diverse actions of GnRH and ultimately add imperative information to the goal of understanding the mechanisms by which GnRH works systemically and locally to impact reproductive processes. That being said, there is still much to gain in exploring the actions of GnRH when introduced systemically in non-hypophysectomized animal models.

CONCLUSIONS

It is clear that there is still much to gain in improving cattle reproductive efficiency and how we implement reproductive management tools. What is causing the variation we observe in interval to estrus and pregnancy success with our FTAI protocols? Why do some growing follicles produce more estradiol than other follicles? How is GnRH having its stimulatory and inhibitory effects within the ovary? Is systemically administered GnRH working solely through gonadotrope cells in the anterior pituitary, or does it have the ability to act directly on ovarian follicles? The

following chapters will present field trials in which small doses of GnRH were systemically administered at the time of CIDR removal during a synchronization protocol and the reported effects on reproductive parameters, as well as the abundance of GnRH-I and GnRH-II mRNA in bovine granulosa cells and how that abundance related to follicular fluid estradiol concentrations of bovine antral follicles.

CHAPTER 3

INFLUENCE OF GnRH SUPPLEMENTATION AT CIDR REMOVAL ON EXPRESSION OF ESTRUS AND INTERVAL TO ESTRUS IN BEEF CATTLE

ABSTRACT

Previous research has indicated that multiple small doses of GnRH after CIDR removal increased circulating concentrations of estradiol. Thus our objective was to determine if a single small dose of GnRH (5 μg or 10 μg) at CIDR removal would impact expression of estrus and/or interval to estrus. Beef cows and heifers (n = 1620) were synchronized using the 7-day CIDR protocol, and randomly assigned to one of three treatments (0, 5, or 10 μg of a GnRH analog at CIDR removal). Animals were visually observed for estrus and inseminated following detection in estrus. Interval to estrus was calculated for each animal that exhibited estrus (INTERVAL 1). Animals that did not exhibit estrus were given 100 μg of GnRH at the time of AI and their interval to estrus was designated at 120 h. INTERVAL 2 refers to the interval when animals that did not exhibit estrus were included in the analysis. Interval to estrus was analyzed using the MIXED procedure of SAS. Expression of estrus and pregnancy success were analyzed using the GLIMMIX procedure of SAS with treatment and age as independent variables and herd as a random effect. There was an effect of age ($P < 0.01$) and a treatment by age interaction ($P = 0.05$) on INTERVAL 1. Heifers had a shorter interval to estrus than cows (50 h vs 54 h, respectively). Furthermore, heifers given 5 μg of GnRH tended to have a shorter interval to estrus ($P = 0.07$; 47 ± 1.4 h) compared to 0 μg (50 ± 1.5 h) and did have a shorter interval compared to 10 μg ($P < 0.01$; 52 ± 1.5 h). There were no differences between treatments in interval to estrus among cows ($P > 0.34$). When

animals that did not exhibit estrus were included in the analysis at 120 h there was no treatment by age interaction ($P = 0.49$). This is likely due to the fact that treatment ($P < 0.01$), but not age ($P = 0.96$) or treatment by age ($P = 0.74$) influenced expression of estrus, with 5 μg tending to have more animals in estrus ($P = 0.10$; 79 ± 4%) compared to 0 μg (74 ± 5%), and 10 μg having fewer animals in estrus compared to either other treatment ($P < 0.04$; 68 ± 6%). Estrus ($P < 0.01$) and age ($P < 0.01$) influenced pregnancy success with heifers having greater pregnancy success compared to cows (49 ± 5% vs 38 ± 4%, respectively) and animals exhibiting estrus having greater pregnancy success compared to animals that did not exhibit estrus (57 ± 4% vs 32 ± 4% respectively). In addition, there tended to be a treatment by estrus interaction ($P = 0.08$). There was no difference in pregnancy success between treatments among animals that exhibited estrus ($P > 0.30$); however, among animals that did not exhibit estrus 0 μg had increased pregnancy success ($P \leq 0.05$; 40 ± 6%) compared to 5 μg and 10 μg, which did not differ (27 ± 6% and 29 ± 5%, respectively). In summary, 5 μg of GnRH at CIDR removal tended to decrease the interval to estrus and increase expression of estrus among heifers but not cows, and both 5 and 10 μg of GnRH at CIDR removal decreased pregnancy success among animals that did not exhibit estrus.

INTRODUCTION

Estradiol plays a critical role in fertility among cattle, specifically it has been reported that preovulatory estradiol regulates uterine pH (Perry and Perry, 2008), sperm transport (Larimore et al., 2015), and endometrial genes that support processes that set up the uterine environment for implantation (Bauersachs et al., 2005), all of these favorably impact embryo development and survival. Estradiol is also critical within the ovary as it is associated with growth, divergence, ovulation, and eventual regression/atresia of follicles (Bodensteiner et al., 1996b). Classically, estradiol from the follicle is believed to be produced via the two-cell two-gonadotropin theory (Fortune and Quirk, 1988). A 5 μg dose of GnRH stimulates an LH pulse similar to a physiological pulse (Ginther et al., 1996). Multiple small doses of GnRH administered systemically following CIDR removal increased circulating concentrations of estradiol (Larimore et al., 2016). Estradiol is the primary signal to the hypothalamus to initiate estrus behavior (Vailes et al., 1992; Diskin and Sreenan, 2000).

Cows and heifers that exhibit estrus prior to fixed time AI have increased concentrations of estradiol (Perry and Perry, 2008) and increased conception rates versus animals that do not exhibit estrus (Richardson et al., 2016). Variations in expression of estrus, interval to estrus, and/or pregnancy success have been hindrances to the successful use of protocols to synchronize estrus (Roche and Boland, 1991; Bo et al., 1994). As a result, investigation in ways to manipulate these protocols to favorably impact expression of estrus, interval to estrus, and/or conception rates poses an imperative area of research. Therefore, the objective of this study was to determine if a single small dose of GnRH (5

μg or 10 μg) at CIDR removal would impact expression of estrus, interval to estrus, or pregnancy success.

MATERIALS AND METHODS

All procedures were approved by the South Dakota State University Institutional Animal Care and Use Committee.

Experimental Design

Beef cows and heifers (n = 1620) from five operations in Minnesota and South Dakota were randomly assigned to one of three treatment groups (0, 5, or 10 μg GnRH analog at CIDR removal). All animals had their estrous cycle synchronized using the 7-day CO-Synch + CIDR protocol. Animals were administered GnRH (100 μg as 2 cc of Factrel i.m.; Pfizer Animal Health, Madison, NJ) on day -7, and a CIDR insert (Pfizer Animal Health, Madison, NJ) was inserted intravaginally. On day 0, $PGF_{2\alpha}$ was adminstered ($PGF_{2\alpha}$; 25 mg as 5 cc of Lutalyse i.m.; Pfizer Animal Health, Madison, NJ), CIDRs were removed, and GnRH treatments applied. The animals were visually observed for estrus, and inseminated following detection in estrus. Animals that did not exhibit estrus were given 100 μg of GnRH at time of AI, 120 hours post CIDR removal, and this was recorded as their interval to estrus. When animals that did not exhibit estrus were included in the analysis with estrus animals, this was termed INTERVAL 2. Within each location, AI bulls and AI technicians were distributed evenly among the three treatment groups. A period of at least 7 days was maintained before cows/heifers were turned out with clean-up bulls, for all five herds. Interval to estrus was calculated for

each animal that exhibited estrus (INTERVAL 1). All cows and heifers from the five respective herds were maintained as single groups throughout the entire experiment. Across the five herds, the breeding season ranged from 60 to 90 days in length.

Ultrasonography

Pregnancy diagnoses were conducted between 30 and 70 days following artificial insemination via transrectal ultrasonography using an Aloka 500V ultrasound with a 7.5 MHz transrectal linear probe (Aloka, Wallingford, CT), and crown-rump length was used to determine fetal age.

Statistical Analysis

Differences in interval to estrus were analyzed by analysis of variance using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). When the F statistic was significant ($P < 0.05$), mean separation was performed using least significant differences (Means ± SEM, Snedecor and Cochran, 1989). Differences were considered to be significant when $P \leq 0.05$ and a tendency when $P > 0.05$ but $P \leq 0.10$. Difference is pregnancy success between treatments were analyzed as binomial distributions in the GLIMMIX procedure of SAS with herd included as a random variable. The statistical model for expression of estrus included treatment, age (heifer or cow), and the treatment by age interaction. The statistical model for pregnancy included treatment, estrus and age and all 2- and 3-way interactions. Differences were considered to be significant when $P \leq 0.05$ and a tendency when $P > 0.05$ but $P \leq 0.10$.

RESULTS

Interval to Estrus (INTERVAL 1)

There was an effect of age ($P < 0.0001$; Figure 4), a weak tendency for an effect of treatment ($P = 0.11$; Figure 5), and a treatment by age interaction ($P = 0.05$; Figure 6), on INTERVAL 1. Heifers had a shorter interval to estrus compared to cows (50 h ± 1.1 h vs 54 ± 1 h, respectively). Furthermore, heifers treated with 5 μg of GnRH at CIDR removal tended to have a shorter interval to estrus ($P = 0.07$; 47 ± 1.4 h) compared to 0 μg (50 ± 1.5 h) and did have a shorter interval compared to 10 μg ($P < 0.01$; 52 ± 1.5 h). There were no differences between treatments in interval to estrus among cows ($P \geq 0.34$).

Interval to Estrus (INTERVAL 2)

When animals that did not exhibit estrus were included in the analysis at 120 hours (INTERVAL 2), there was an effect of age ($P = 0.03$; Figure 7), and treatment ($P < 0.0001$; Figure 8), however there was no treatment by age interaction ($P = 0.50$; Figure 9) on interval to estrus. Heifers had a shorter interval to estrus compared to cows (70 h vs 73 h, respectively). When all animals were included in the analysis, animals administered the 5 μg treatment had a shorter ($P = 0.05$) interval to estrus compared to the 0 μg treatment (68 ± 1.3 h vs 71 ± 1.3 h, respectively), and both the 5 μg and the 0 μg treated animals had shorter ($P \leq 0.02$) intervals to estrus compared to the 10 μg (76 ± 1.3 h) treatment of GnRH.

Expression of Estrus

Treatment ($P < 0.001$), but not age ($P = 0.96$) or treatment by age ($P = 0.74$) influenced expression of estrus. The 5 μg treatment of GnRH following CIDR removal tended to have more animals in estrus ($P = 0.10$; 79 ± 4%) compared to 0 μg (74 ± 5%), and 10 μg which had fewer animals in estrus compared to either of the other treatments ($P < 0.04$; 68 ± 6%). There was no difference in expression of estrus between heifers and cows (74 ± 1% vs 74 ± 0.5%, respectively).

Pregnancy Success

Age ($P < 0.01$) and expression of estrus ($P < 0.0001$) influenced pregnancy success with heifers having greater pregnancy success compared to cows (49 ± 5% vs 38 ± 4%, respectively) and animals exhibiting estrus having greater pregnancy success compared to animals that did not exhibit estrus (57 ± 4% vs 32 ± 4%, respectively). There was no difference in pregnancy success between treatments ($P = 0.22$; Figure 13). There tended to be a treatment by estrus interaction ($P = 0.08$; Figure 14). There was no difference in pregnancy success between treatments among animals that exhibited estrus ($P \geq 0.30$); however, among animals that did not exhibit estrus the 0 μg treatment had increased pregnancy success ($P \leq 0.05$; 40 ± 6%) compared to 5 μg and 10 μg which did not differ (27 ± 6% and 29 ± 5%, respectively).

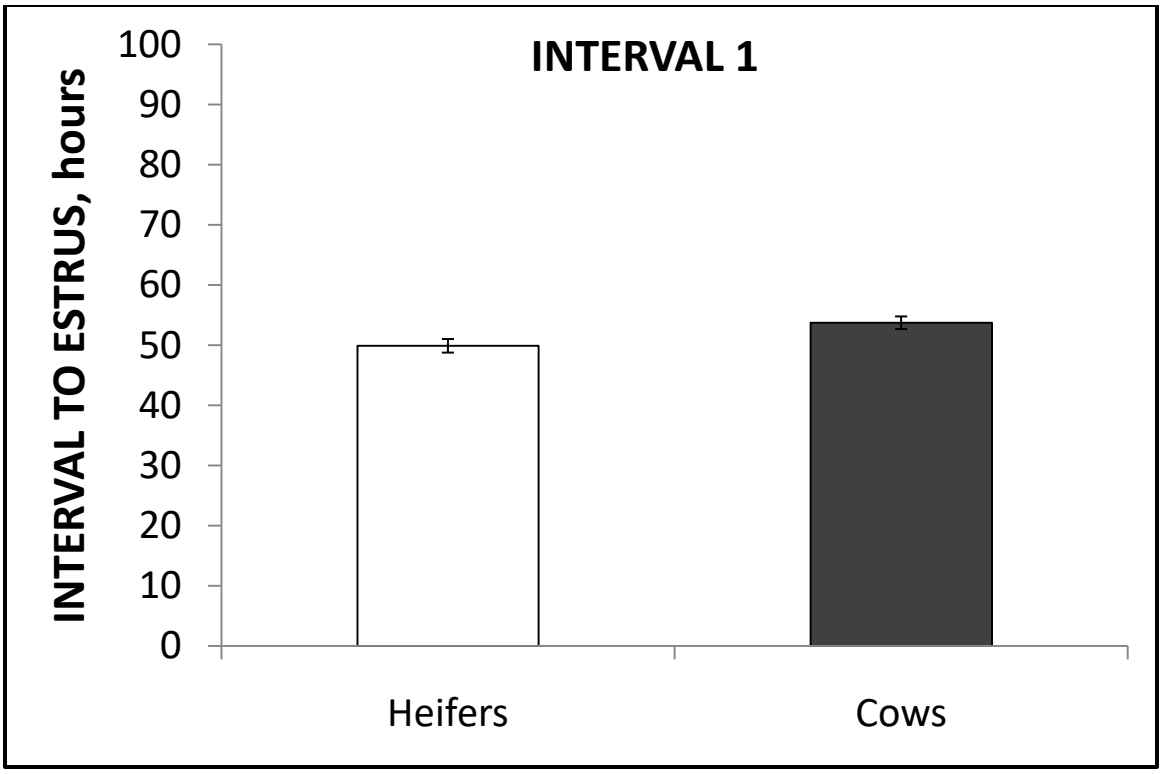

Figure 4. Interval to estrus (h) following CIDR removal for heifers and cows that exhibited estrus ($P < 0.0001$).

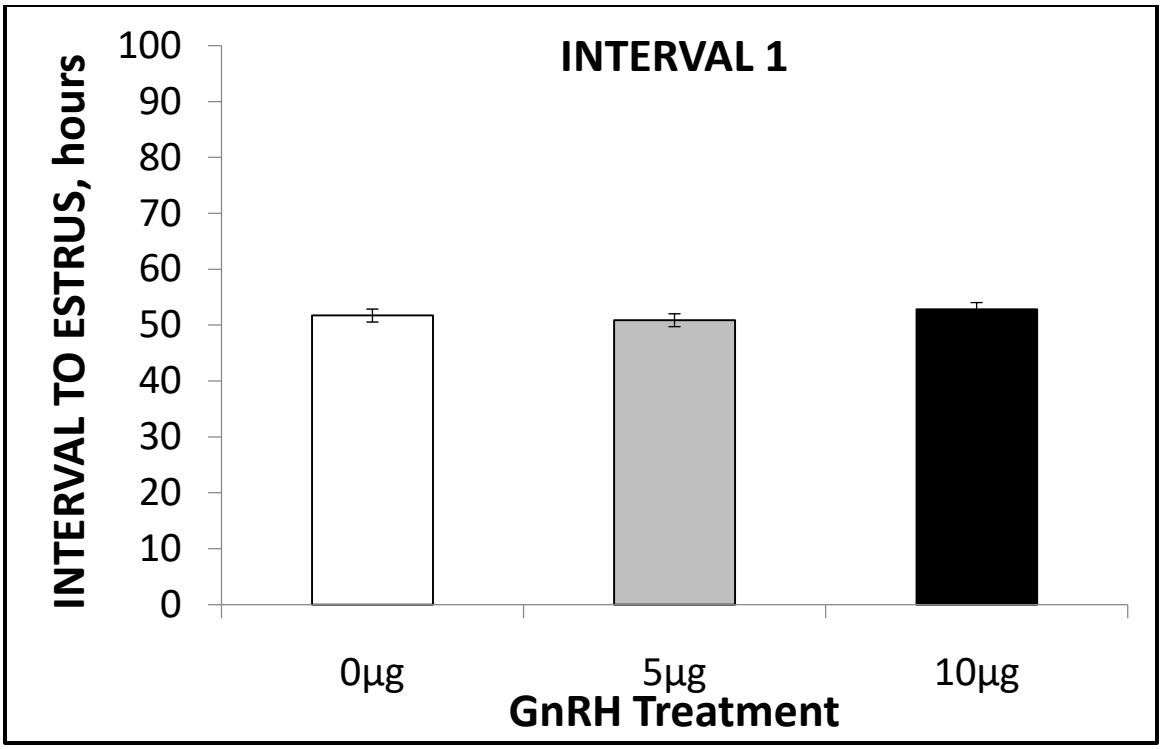

Figure 5. Interval to estrus (h) following CIDR removal for 0, 5, and 10 µg GnRH for animals that exhibited estrus (P = 0.11).

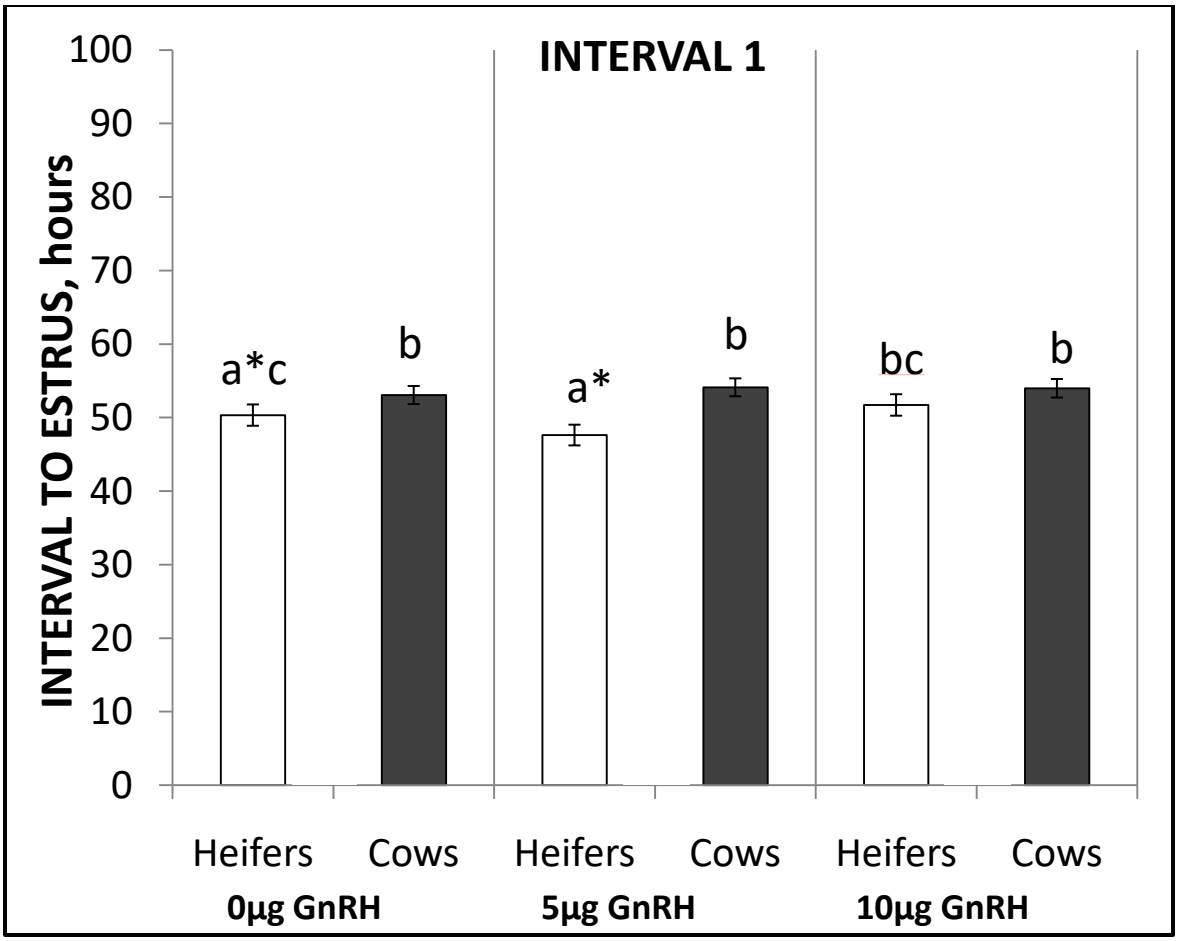

Figure 6. Treatment by age interaction on interval to estrus (h) following CIDR removal for animals that exhibited estrus. [abc]Superscripts differ ($P < 0.05$), similar superscripts with asterisks (*) represent tendencies ($P > 0.05$ but $P \leq 0.10$).

Figure 7. Interval to estrus (h) following CIDR removal for heifers and cows when animals that did not exhibit estrus were included (P = 0.03).

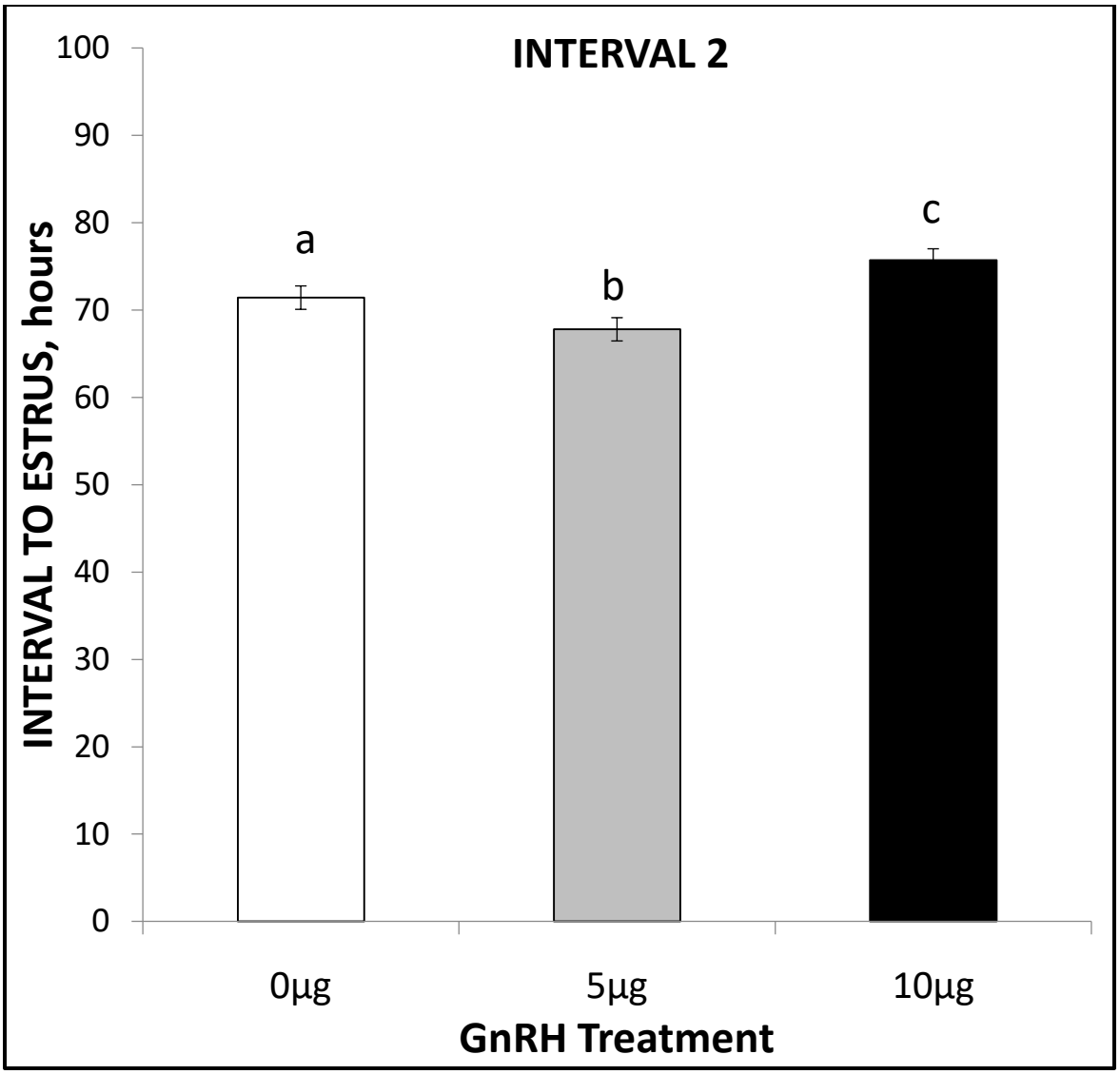

Figure 8. Interval to estrus (h) following CIDR removal for 0, 5, and 10 µg GnRH when animals that did not exhibit estrus were included ($P < 0.0001$), ^{abc}Superscripts differ ($P < 0.05$).

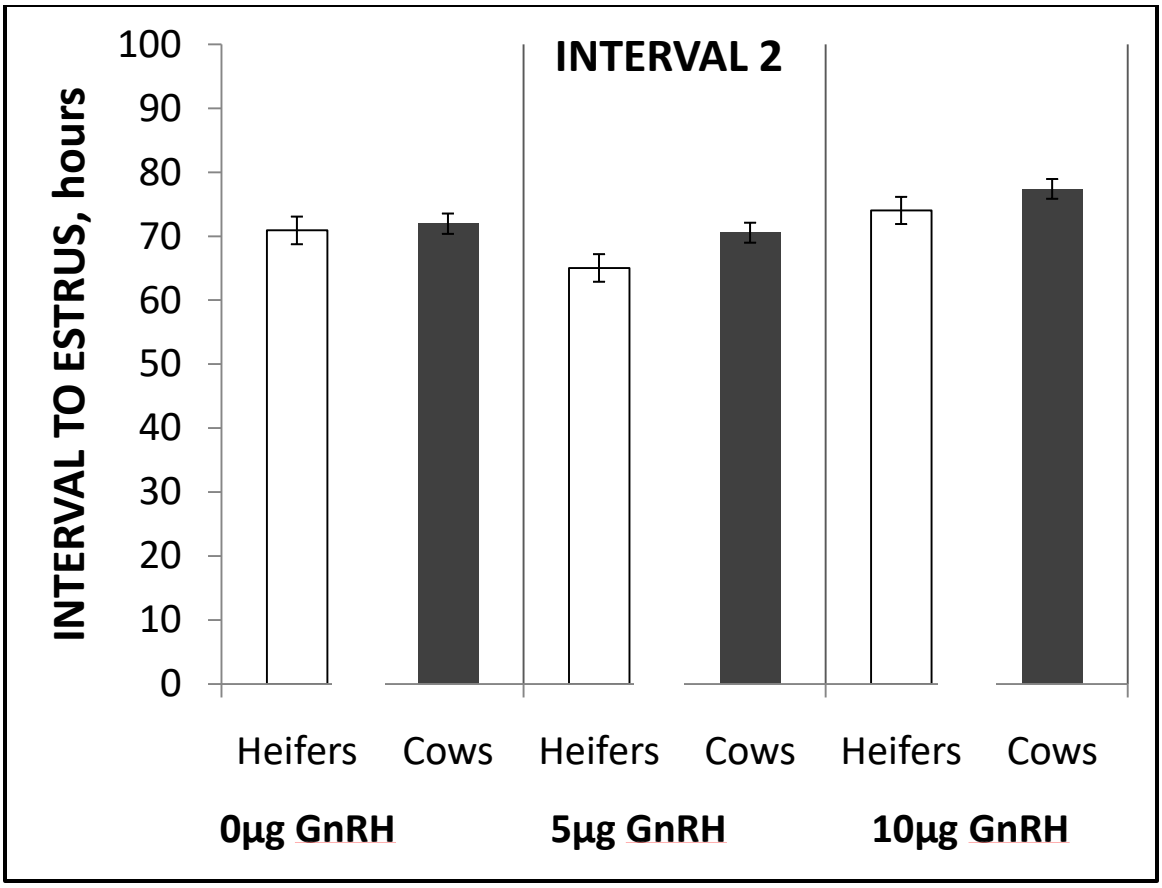

Figure 9. Treatment by age interaction on interval to estrus (h) following CIDR removal when animals that did not exhibit estrus were included (P = 0.50).

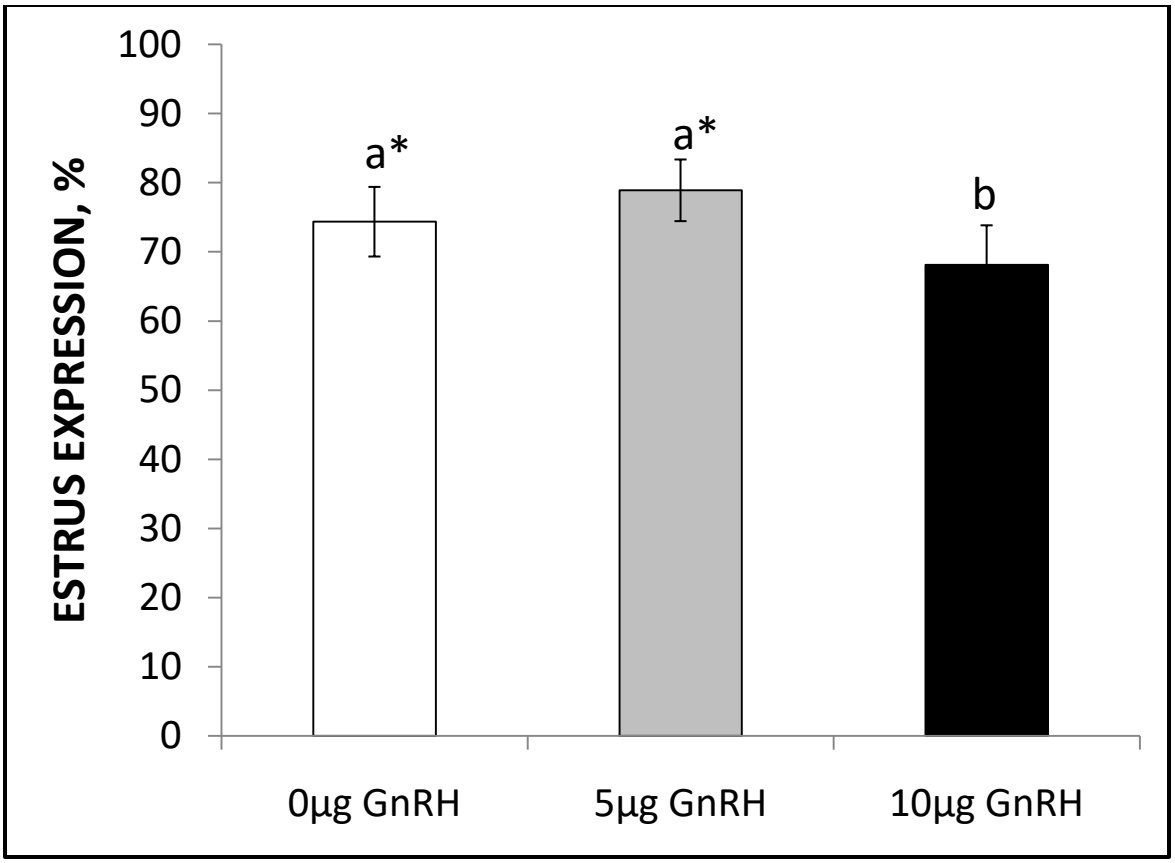

Figure 10. Expression of estrus of animals treated with 0, 5, and 10 µg GnRH ($P < 0.001$). There was no effect of age ($P = 0.96$) or treatment by age ($P = 0.74$) on expression of estrus when all animals were included. [ab]Superscripts differ ($P < 0.05$), similar superscripts with asterisks (*) represent tendencies ($P > 0.05$ but $P \leq 0.10$).

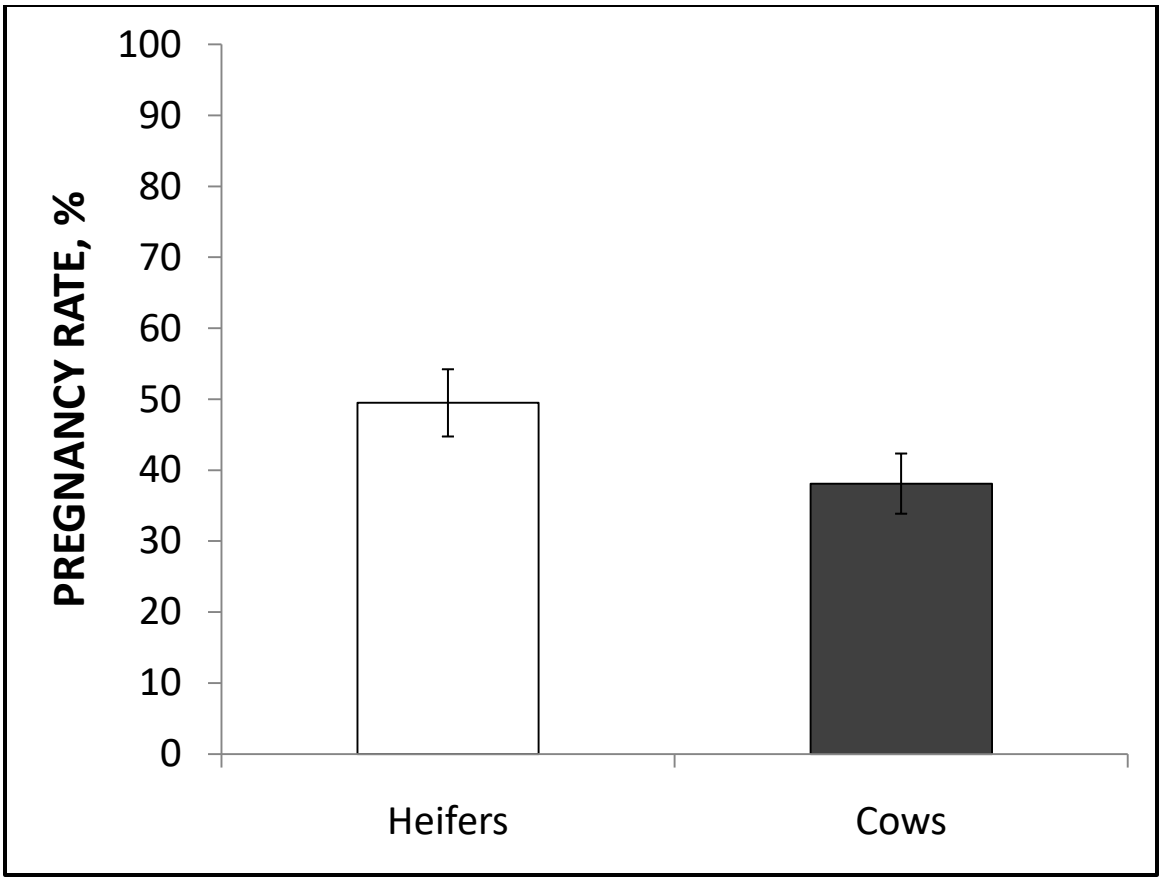

Figure 11. Pregnancy success among heifers and cows when all animals were included ($P < 0.01$).

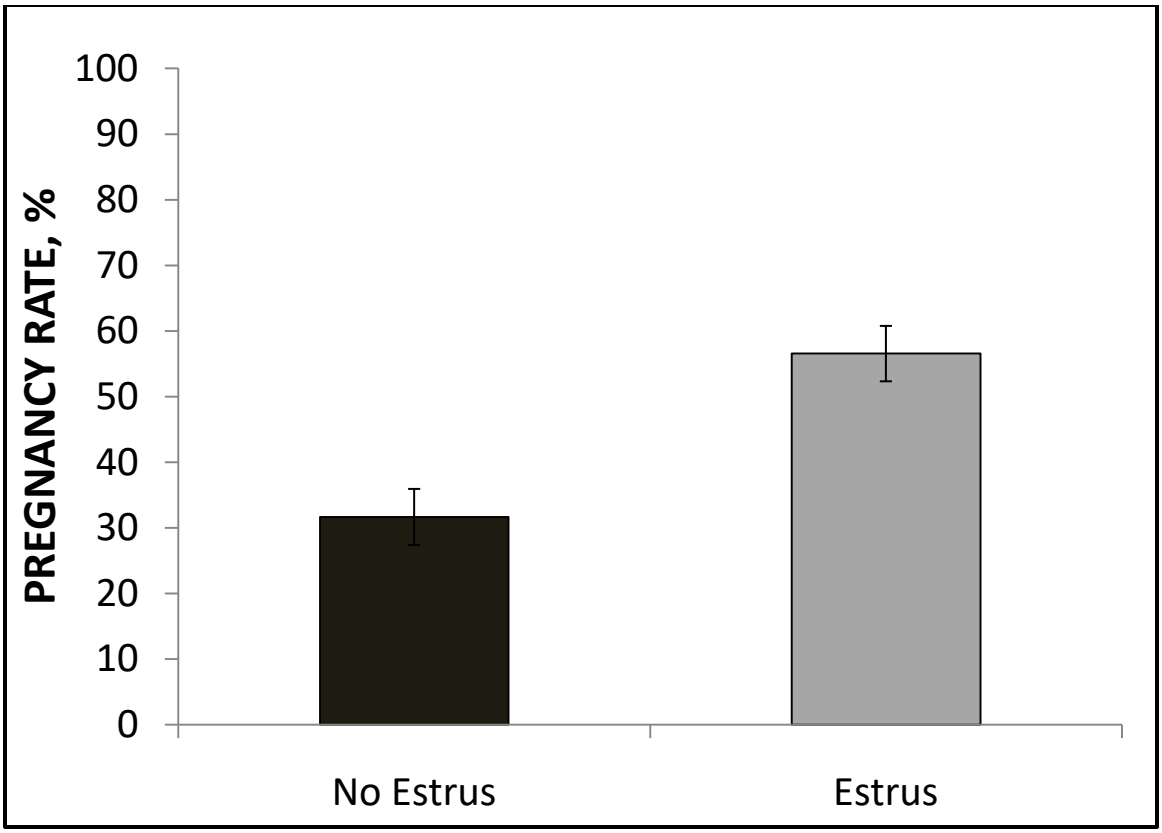

Figure 12. Pregnancy success of animals that did and did not exhibit estrus ($P < 0.0001$). There was no estrus by age interaction ($P = 0.51$) for pregnancy success.

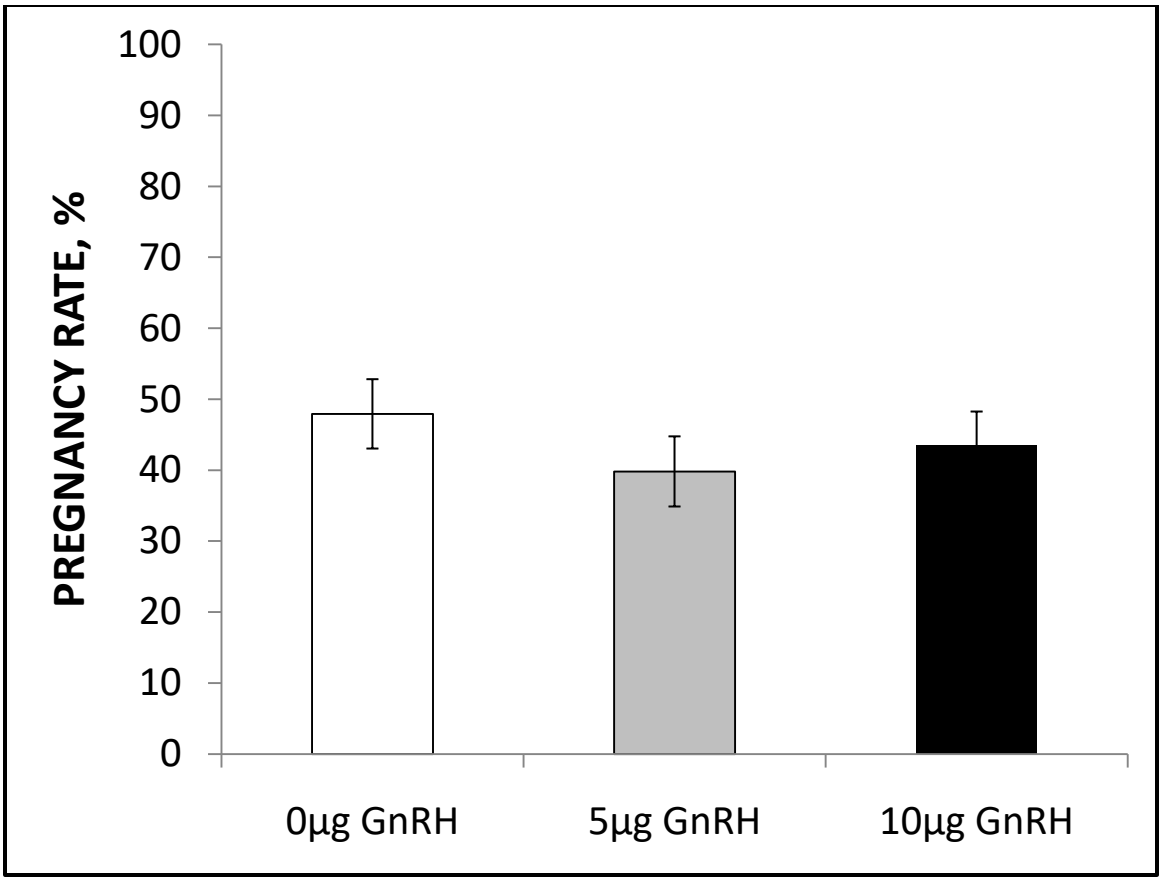

Figure 13. Pregnancy success of the three treatment groups when all animals were included (0, 5, and 10 µg GnRH) (P = 0.22). There was no treatment by age interaction (P = 0.40) for pregnancy success.

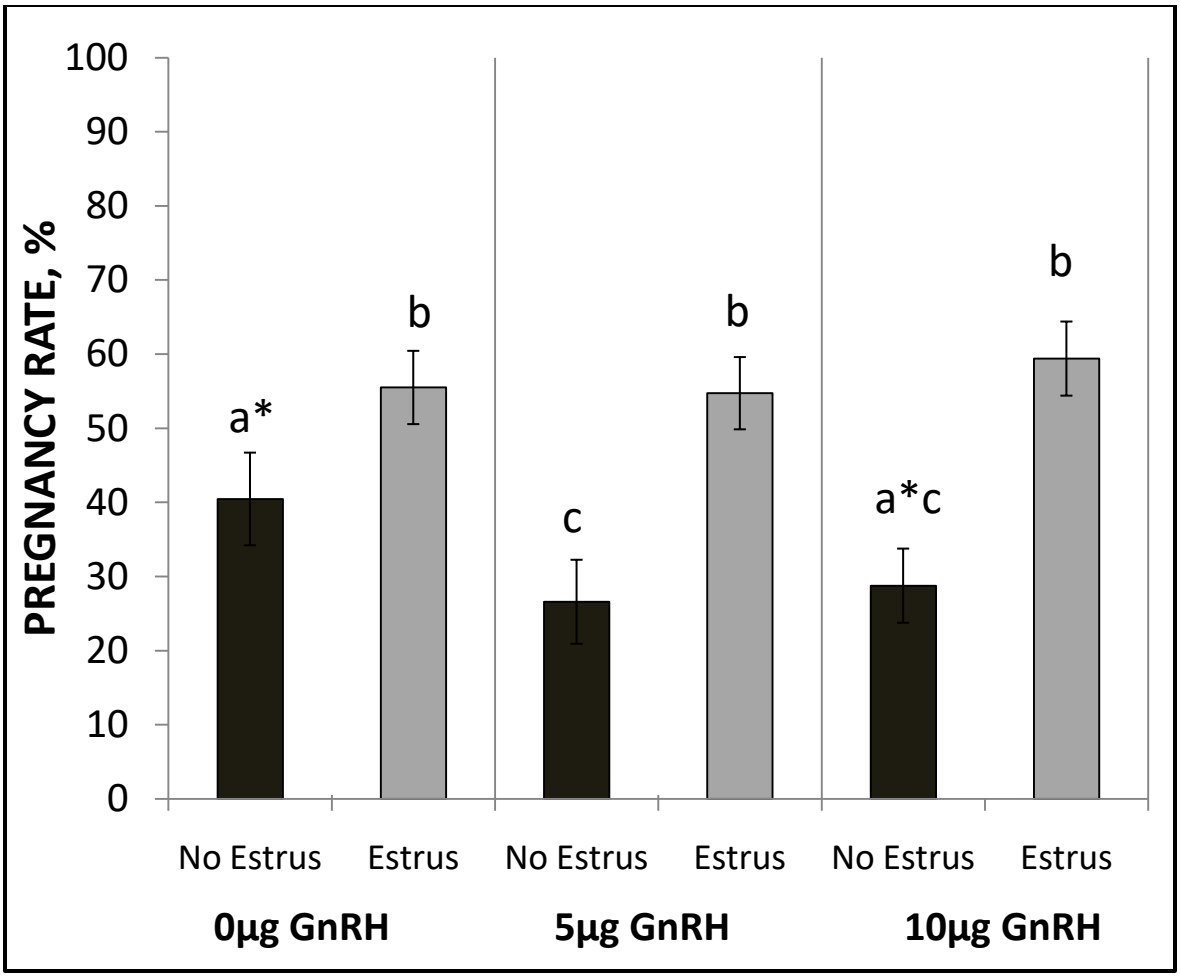

Figure 14. Treatment by estrus interaction on pregnancy success of heifers and cows ($P < 0.08$), [abc]Superscripts differ ($P < 0.05$), similar superscripts with asterisks (*) represent tendencies ($P > 0.05$ but $P \leq 0.10$).

DISCUSSION

Animals that exhibited estrus prior to fixed time AI had increased conception rates versus animals that did not exhibit estrus (Richardson et al., 2016), and variations in expression of estrus, interval to estrus, and/or pregnancy success have been hindrances to the successful use of protocols to synchronize estrus (Roche and Boland, 1991; Bo et al., 1994). In the current study, heifers had a shorter interval to estrus compared to cows. When utilizing the 7 day CO-Synch + CIDR protocol, at time of CIDR removal and prostaglandin $F_{2\alpha}$ injection, follicles are in the growing phase (Lussier et al., 1987; Ginther et al., 1996). Furthermore, heifers have shorter follicular waves compared to cows, so at the time of prostaglandin $F_{2\alpha}$ injection follicles of heifers are in a more advanced stage of growth resulting in a shorter interval to expression of estrus and ovulation compared to cows (Adams, 1998). Among heifers that exhibited estrus, those treated with 5 μg of GnRH at CIDR removal tended to have a shorter interval to estrus compared to those treated with 0 μg and did have a shorter interval to estrus compared to those treated with 10 μg. There were no differences in interval to estrus observed among the cows that exhibited estrus. Estradiol is the primary signal for the initiation of standing estrus (Allrich, 1994), and administration of physiological doses (5 μg) increased circulating concentrations of estradiol (Larimore et al., 2016).

Previous research has also reported that the distribution of expression of estrus following CIDR removal when implementing the 7 day CO-Synch + CIDR protocol ranged from 36 to 120 hours (Busch et al., 2008; Wilson et al., 2010). It is unclear why some growing follicles produce more estradiol than others (Jinks et al., 2013; Perry et al., 2014). When all animals were included in the analysis, 5 μg of GnRH at CIDR removal shortened the interval to estrus compared to animals that did not receive GnRH (0 μg), and both the 5 μg and 0 μg treatments had shorter interval to estrus compared to the 10 μg treatment. Therefore, the additional LH pulse stimulated by the 5 μg dose of GnRH following CIDR removal likely positively contributed to estradiol production, allowing animals to reach concentrations of estradiol sufficient enough to initiate standing estrus sooner than their counterparts.

Increased LH pulsatility increased estradiol production (Fortune et al., 2001), and 5 μg of GnRH administered systemically elicited an LH pulse similar to a physiological pulse which positively contributed to LH pulsatility (Ginther et al., 1996). Thus in the current study, the additional physiological pulse of LH likely resulted in the shorter interval to estrus and increased expression of estrus, seen in animals treated with 5 μg of GnRH. Cattle continuously infused with GnRH (2 μg, i.v.) were reported to have blocked LH pulsatile secretion as a result of pituitary desensitization and GnRH receptor down regulation (Vizcarra et al., 1997). Thus the difference in the response observed between the 5 μg treatment and the 10 μg treatment, might be overstimulation with the increased dose of GnRH.

In this study, animals that exhibited estrus had increased pregnancy success compared to animals that did not exhibit estrus, which agrees with previous research by

Richardson et al. (2016) who reported that cows exhibiting estrus prior to fixed-time AI had increased pregnancy success compared to cows that did not exhibit estrus. Further, heifers had increased pregnancy success compared to cows. Small doses of GnRH following CIDR removal did not impact pregnancy success, however, among animals that did not exhibit estrus the 0 μg treatment had increased pregnancy success compared to 5 μg and 10 μg, which did not differ. In this instance, the extra LH pulse as a result of the supplemental GnRH dose was likely inducing animals that would have exhibited estrus to have a shorter interval to estrus and increased expression of estrus, leaving those animals that would not respond, explaining why pregnancy success was decreased in the GnRH treatment groups compared to control (0 μg).

In conclusion, animals that exhibited estrus had greater pregnancy success compared to animals that did not. Supplementation of a small dose (5 μg) of GnRH at CIDR removal during the 7-day CO-Synch + CIDR protocol shortened the interval to estrus and increased expression of estrus. Care must be taken not to administer too large of a dose (10 μg) as overstimulation may occur and have negative effects on estrus and pregnancy success.

CHAPTER 4

INFLUENCE OF SMALL DOSES OF GnRH FOLLOWING CIDR REMOVAL ON REPRODUCTIVE PARAMETERS IN BEEF CATTLE SUBJECTED TO A FIXED-TIME AI PROTOCOL

ABSTRACT

Animals that exhibit estrus prior to fixed-time artificial insemination have increased circulating concentrations of estradiol and greater conception rates compared to those that do not. Thus, when using a fixed-time AI protocol it is necessary to maximize expression of estrus in order to achieve maximum conception rates. Previous research has reported multiple small doses of systemically administered GnRH after CIDR removal increased estradiol concentrations. Therefore, our objective was to determine if small doses of GnRH following CIDR removal would impact estradiol concentrations, expression of estrus, or pregnancy success in beef cattle subjected to a fixed-time AI protocol. Beef cows and heifers (n = 247) were synchronized using the 7-day CO-Synch + CIDR protocol and were evenly assigned to one of three treatment groups [0 µg GnRH at CIDR removal (0 µg); 10 µg GnRH at CIDR removal (10 µg), or 5 µg at CIDR removal plus 5 µg 12 h later (5 + 5 µg)]. Animals were visually observed for estrus and artificially inseminated 55 (heifers) or 60 (cows) hours following CIDR removal. Blood samples were collected beginning at CIDR removal and every 12 hours until time of AI. Data were analyzed using the GLIMMIX procedure of SAS with estrus, treatment, and age as independent variables for pregnancy success, and treatment and age as independent variables for expression of estrus. Estradiol concentrations were analyzed using analysis of repeated measures in the MIXED procedure of SAS. Concentrations of

estradiol were not influenced by treatment ($P = 0.66$) or a treatment by time interaction ($P = 0.87$), but were influenced by time ($P < 0.0001$). Estradiol concentrations increased from CIDR removal to 48 hours after CIDR removal and then were decreased at the time of AI. Expression of estrus was not influenced by age ($P = 0.31$), or treatment ($P = 0.60$); however, there was a treatment by age interaction ($P < 0.01$) for expression of estrus. For animals administered the 5 + 5 μg treatment, expression of estrus was increased ($P < 0.01$) among heifers compared to cows (84 ± 3% vs 70 ± 3%, respectively). Pregnancy success was influenced by estrus ($P < 0.01$), treatment ($P = 0.02$), and a treatment by estrus ($P < 0.01$) interaction, but was not influenced by age ($P = 0.91$), treatment by age ($P = 0.83$), or treatment by estrus by age ($P = 0.94$). Animals that exhibited estrus had increased pregnancy success compared to animals that did not exhibit estrus (67 ± 2% vs 41 ± 7% respectively). Animals administered the 5 + 5 μg treatment had increased pregnancy success ($P \leq 0.05$; 69 ± 6%) compared to animals administered the 0 μg and 10 μg treatments which did not differ (53 ± 6% vs 41 ± 8%, respectively). Pregnancy success was similar ($P \geq 0.27$) between animals that did and did not exhibit estrus for animals administered the 0 μg and 5 + 5 μg treatments. For animals administered the 10 μg treatment, conception rates were decreased ($P < 0.01$) among animals that did not exhibit estrus compared to animals that exhibited estrus. In conclusion, the 5 + 5 μg treatment did not impact concentrations of estradiol or expression of estrus, but did improve pregnancy success of beef cattle subjected to a fixed-time AI protocol.

INTRODUCTION

Artificial insemination allows producers to introduce new genetics into their herd and, when coupled with synchronization protocols it allows condensing of their calving season and production of a more uniform calf crop. In order to have increased adoption of AI in the beef industry, synchronization protocols need to be easy to administer, effectively induce expression of estrus and ovulation, and lastly contribute positively to pregnancy success rates. Fixed-time AI protocols are designed to induce ovulation; however, in order for these protocols to be adopted they must produce pregnancy rates similar to those synchronization protocols focused on estrus detection.

Cattle that exhibit estrus prior to fixed-time AI have increased circulating concentrations of estradiol (Perry and Perry, 2008) and increased conception rates compared to those that do not (Richardson et al., 2016). Therefore in order to maximize conception rates to fixed-time AI it is necessary to improve expression of estrus. Cattle can exhibit estrus up to 5 days (120 h) following CIDR removal (Stevenson et al., 2000; Busch et al., 2008; Wilson et al., 2010; Patterson et al., 2013), however many fixed time artificial insemination protocols recommend inseminating heifers and cows between 54 and 72 hours following CIDR removal. Thus, variations in interval to estrus, expression of estrus, and pregnancy success have been hindrances to the successful implementation of FTAI synchronization protocols (Roche and Boland, 1991; Bo et al., 1994) and prove to be an area where more research is needed.

Gonadotropin releasing hormone injections (100 μg, i.m.) administered during synchronization protocols can induce ovulation of small dominant follicles that have not

produced sufficient concentrations of estradiol to induce estrus (Jinks et al. 2013; Perry et al., 2014). Supplementation of estradiol cypionate during the preovulatory period increased pregnancy rates in cows induced to ovulate smaller dominant follicles, but had no influence when large follicles ovulated (Jinks et al., 2013). Since estradiol cannot be used in production settings to increase estradiol concentrations and expression of estrus, investigation in methods to manipulate the estrous cycle and enhance estradiol production and expression of estrus are imperative areas of research.

Gonadotropin releasing hormone has been reported to favorably impact multiple factors contributing to reproduction, either directly or indirectly through gonadotropin secretion and subsequent estradiol production. A 5 μg dose of GnRH was reported to stimulate an LH pulse similar to that of a physiological pulse (Ginther et al., 1996). Furthermore, LH pulsatility stimulated estradiol production by ovarian follicles, and increased LH pulsatility further increased estradiol production (Fortune et al., 2001). Cows and heifers administered small doses of GnRH following CIDR removal were reported to have increased estradiol concentrations (Larimore et al., 2016), and increased preovulatory estradiol was reported to positively impact uterine pH (Perry and Perry, 2008), endometrial gene expression favorable to implantation (Bauersachs et al 2005), and sperm transport (Bauersachs et al., 2005; Perry and Perry, 2008; Larimore et al., 2015). Thus indicating small doses of GnRH administered systemically may be a useful tool in increasing preovulatory estradiol and its associated favorable effects on reproduction. Therefore, the objective of this study was to determine if small doses of GnRH administered systemically following CIDR removal would impact estradiol

concentrations, expression of estrus, or pregnancy success in beef cattle subjected to a fixed-time AI protocol.

MATERIALS AND METHODS

All procedures were approved by the South Dakota State University Institutional Animal Care and Use Committee.

Experimental Design

Beef cows and heifers (n = 247) at South Dakota State University beef breeding unit and cow calf unit (two breeding seasons) were assigned evenly to one of three treatment groups [0 µg GnRH as Factrel (0 µg), 10 µg GnRH as Factrel (10 µg), or 5 µg GnRH as Factrel at CIDR removal (d 0), plus 5 µg 12 hours later (5 + 5 µg)] based on breed, age, AI sire, and calving interval. The animals were synchronized using the 7-day CO-Synch + CIDR protocol. Animals were administered GnRH (100 µg as 2 cc of Factrel i.m.; Pfizer Animal Health, Madison, NJ) on day -7, and a CIDR insert (Pfizer Animal Health, Madison, NJ) was inserted intravaginally. On day 0, $PGF_{2\alpha}$ was adminstered ($PGF_{2\alpha}$; 25 mg as 5 cc of Lutalyse i.m.; Pfizer Animal Health, Madison, NJ), and CIDRs were removed, at this time animals were administered one of the three GnRH treatments (0 µg, 10 µg, or 5 + 5 µg) and EstroTect patches (Western Point, Inc., Apple Valley, MN) were applied to assist with detection of estrus. Cows and heifers at the SDSU cow calf unit were artificially inseminated 54 ± 2 (heifers) or 60-66 (cows) hours following CIDR removal (FTAI) and administered GnRH (100 µg as 2 cc Factrel i.m.; Pfizer Animal Health, Madison, NJ). One inseminator artificially inseminated all animals

with semen from one of eight bulls, which were evenly distributed among treatments. Cows and heifers were maintained separate from bulls for 30 days. Animals with greater than half of their patch rubbed off were documented as having exhibited standing estrus. All cows and heifers at their respective units were maintained as single groups throughout the experiment.

Ultrasonography

Pregnancy diagnoses were conducted approximately 30 and 90 days following artificial insemination via transrectal ultrasonography using an Aloka 500V ultrasound with a 7.5 MHz transrectal linear probe (Aloka, Wallingford, CT). This study was conducted over two breeding seasons.

Blood Sampling and Radioimmunoassay

Blood samples were collected by jugular venipuncture into 10 mL vacutainer tubes (Fisher Scientific, Pittsburgh, PA), but blood collections were conducted differently between years. In year 1, blood samples were collected at CIDR removal (day 0) and every 12 hours up until time of AI or 60 h. In year 2, blood samples were collected at CIDR removal (day 0), and at time of AI, 54 ± 2 hours (heifers) and 60-66 hours (cows) after CIDR removal.

Blood was allowed to coagulate at room temperature, stored at 4˚C for 24 hours, and centrifuged at 1,200 x *g* for 30 minutes at 4˚C. Serum was collected and stored at -20˚C until radioimmunoassays (RIA) were performed. Radioimmunoassays were

performed to determine estradiol-17β concentrations using methods described by Perry and Perry (2008).

Statistical Analysis

Circulating concentrations of estradiol were analyzed by analysis of repeated measures using the MIXED procedure of SAS as described by Littell and coworkers (1998). All covariance structures were modeled in the initial analysis. The indicated best fit covariance structure, compound symmetry, was used for the final analysis. The model included the independent variables of treatment, time, and treatment by time. The effect of treatment was analyzed using animal within treatment as the error term, and effects of time and any interaction were analyzed using the residual as the error term. Since expression of estrus and pregnancy status are binomial distributions, the proportion of animals in each classification were analyzed as binomial distributions in the GLIMMIX procedure of SAS. The statistical model for expression of estrus included treatment, age (heifer or cow), and the treatment by age interaction. The statistical model for pregnancy included treatment, estrus and age and all 2- and 3-way interactions. Differences were considered to be significant when $P \leq 0.05$ and a tendency when $P > 0.05$ but $P \leq 0.10$.

RESULTS

Estradiol Concentration

Concentrations of estradiol were not influenced by treatment ($P = 0.66$; Figure 15) or a treatment by time interaction ($P \geq 0.68$; Figures 16-18), but were influenced by

time ($P < 0.0001$; Figure 19). Estradiol concentrations increased from CIDR removal to 48 hours after CIDR removal (2.44 ± 0.18 to 6.0 ± 0.34 pg/mL from 0 h to 48 h) and then were decreased at the time of AI (3.44 ± 0.18 pg/mL). When animals that exhibited estrus and those that did not were analyzed separately, concentrations of estradiol were, again, not influenced by treatment ($P \geq 0.74$) or a treatment by time interaction ($P \geq 0.69$), but were influenced by time ($P < 0.01$). For animals that exhibited estrus, estradiol concentrations increased from CIDR removal to 48 hours after CIDR removal (2.49 ± 0.20 to 7.07 ± 0.43 pg/mL from 0 h to 48 h; Figure 20) and then were decreased at time of AI (3.50 ± 0.20 pg/mL). The same pattern was not observed for the animals that did not exhibit estrus, concentrations of estradiol were lowest at time of CIDR removal (2.19 ± 0.32; $P < 0.03$) compared to all other time points, however, there were no significant differences ($P \geq 0.44$) in concentrations of estradiol from 12 h to time of AI (Figure 21).

Expression of Estrus

Expression of estrus was not influenced by treatment ($P = 0.60$; Figure 22) or age ($P = 0.31$; Figure 23); however, it was influenced by a treatment by age interaction ($P < 0.01$; Figure 24). Expression of estrus was similar ($P \geq 0.24$) between cows and heifers administered the 0 and 10 μg treatments. However, for animals administered the 5 + 5 μg treatment, expression of estrus was increased ($P < 0.01$) among heifers compared to cows.

Pregnancy Success

Pregnancy success was influenced by estrus ($P < 0.01$; Figure 25), treatment ($P = 0.02$; Figure 26), and a treatment by estrus ($P < 0.01$; Figure 27) interaction, but was not influenced by age ($P = 0.91$; Figure 28), treatment by age ($P = 0.83$; Figure 29), or treatment by estrus by age ($P = 0.94$). Animals that exhibited estrus had increased pregnancy success compared to animals that did not exhibit estrus (67 ± 2% vs 41 ± 7%, respectively). Animals administered the 5 + 5 µg treatment had increased pregnancy success ($P \leq 0.05$; 69 ± 6%) compared to animals administered the 0 µg and 10 µg treatments which did not differ (53 ± 6% vs 41 ± 8%, respectively). Pregnancy success was similar ($P \geq 0.27$) between animals that did and did not exhibit estrus for animals administered the 0 µg and 5 + 5 µg treatments. However, for animals administered the 10 µg treatment, conception rates were decreased ($P < 0.01$) among animals that did not exhibit estrus compared to animals that exhibited estrus (Figure 27).

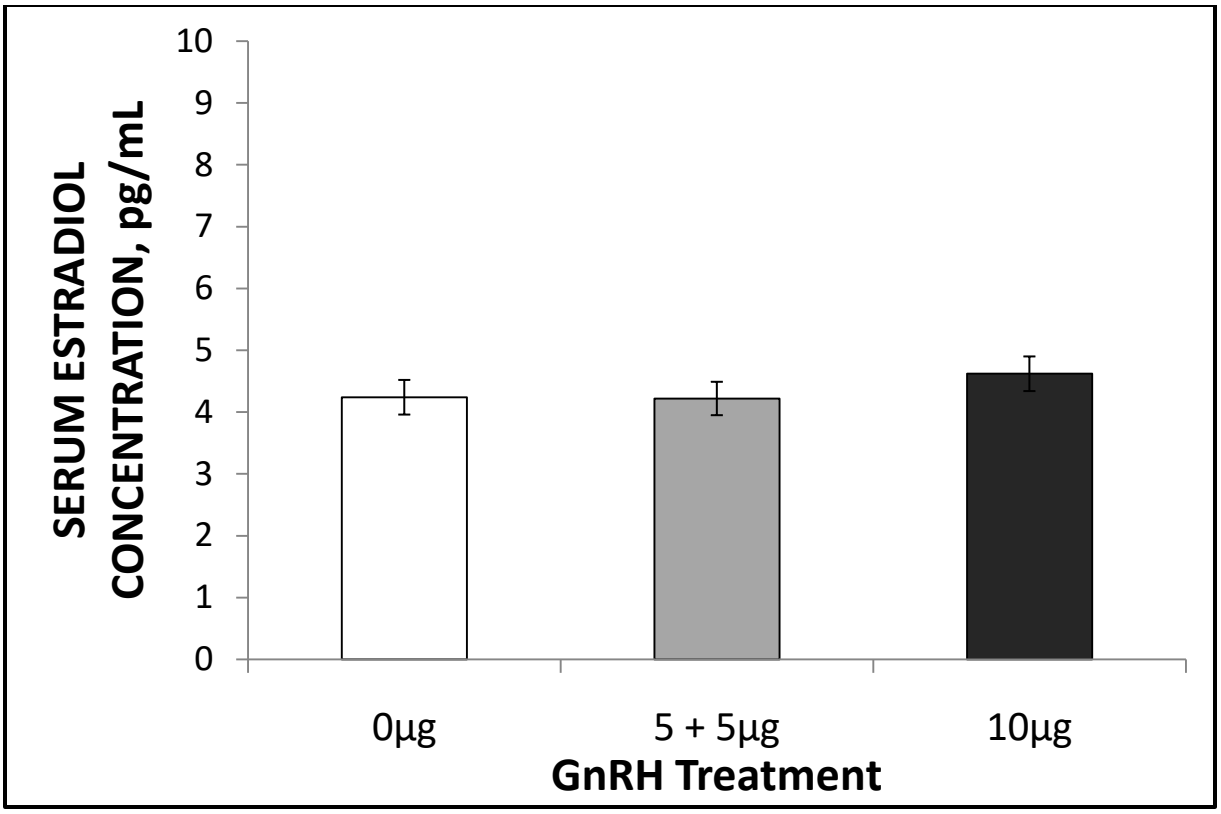

Figure 15. Serum concentrations of estradiol (pg/mL) for all animals treated with 0, 5 + 5, or 10 µg of GnRH at CIDR removal (P = 0.66).

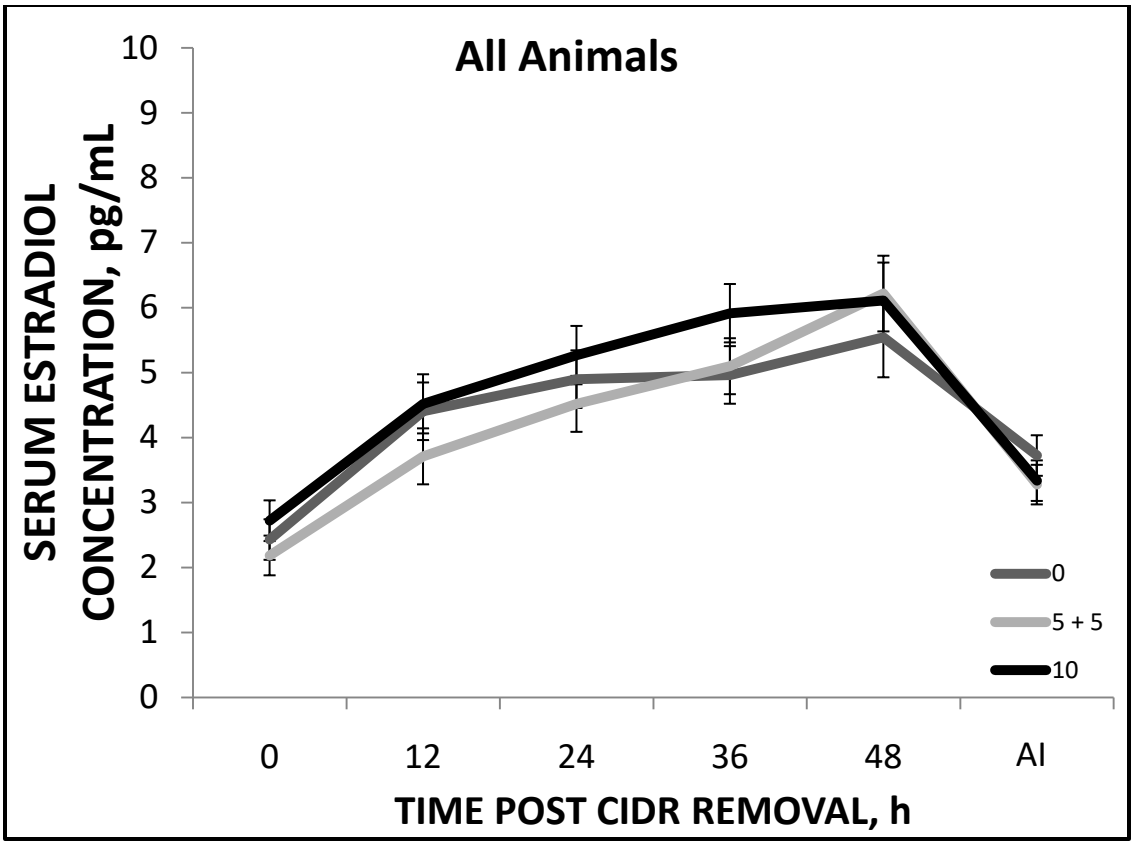

Figure 16. Treatment by time interaction on serum estradiol concentrations (pg/mL) for all animals (P = 0.86).

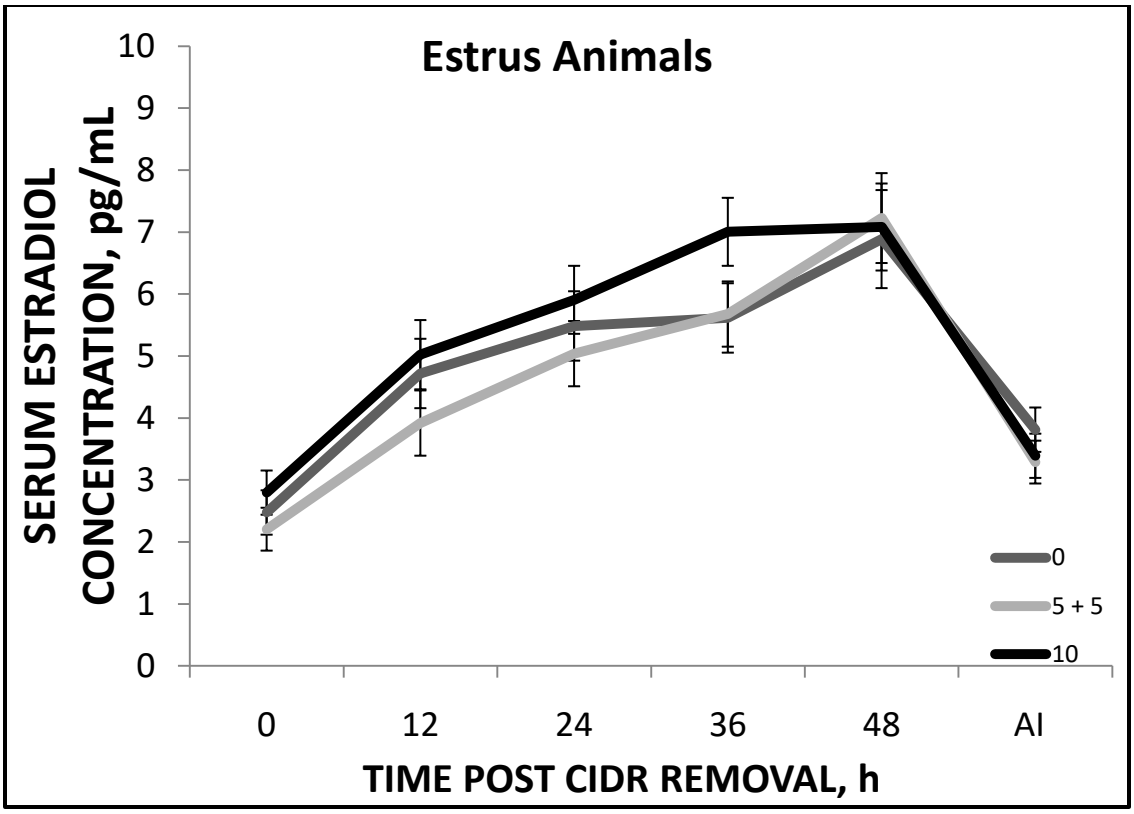

Figure 17. Treatment by time interaction on serum estradiol concentrations (pg/mL) for animals that exhibited estrus (P = 0.68).

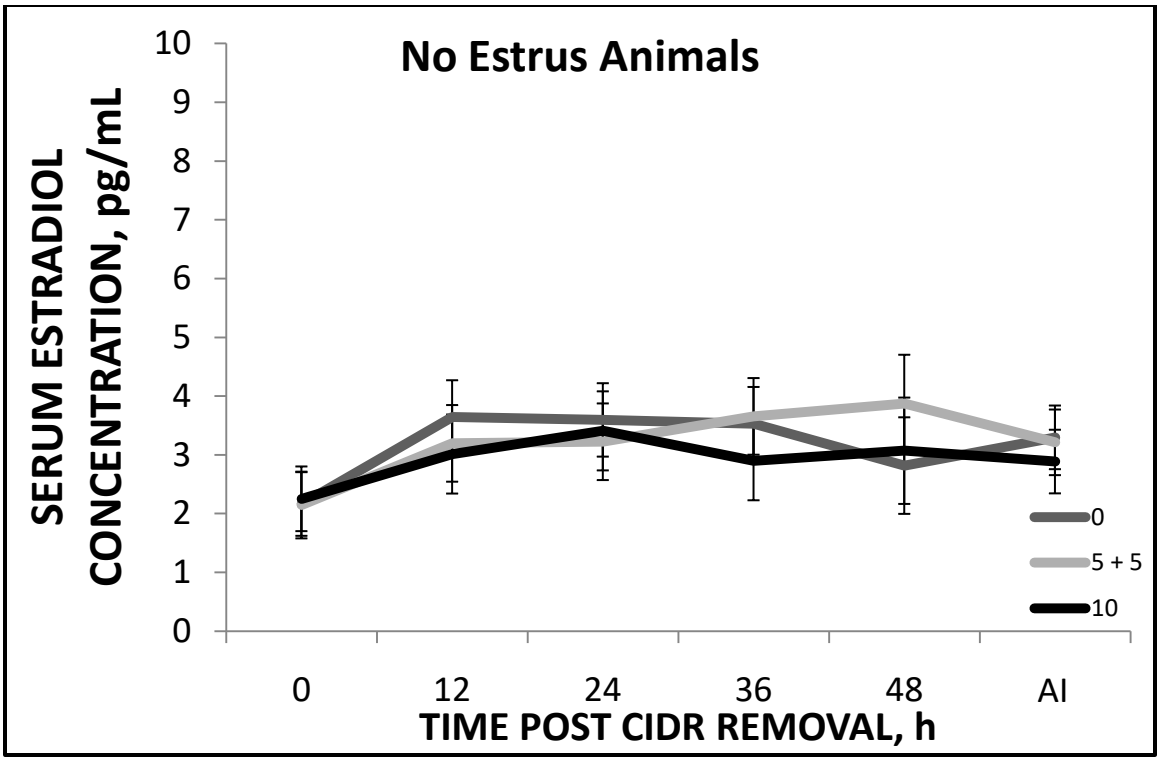

Figure 18. Treatment by time interaction on serum estradiol concentrations (pg/mL) for animals that did not exhibit estrus (P = 0.95).

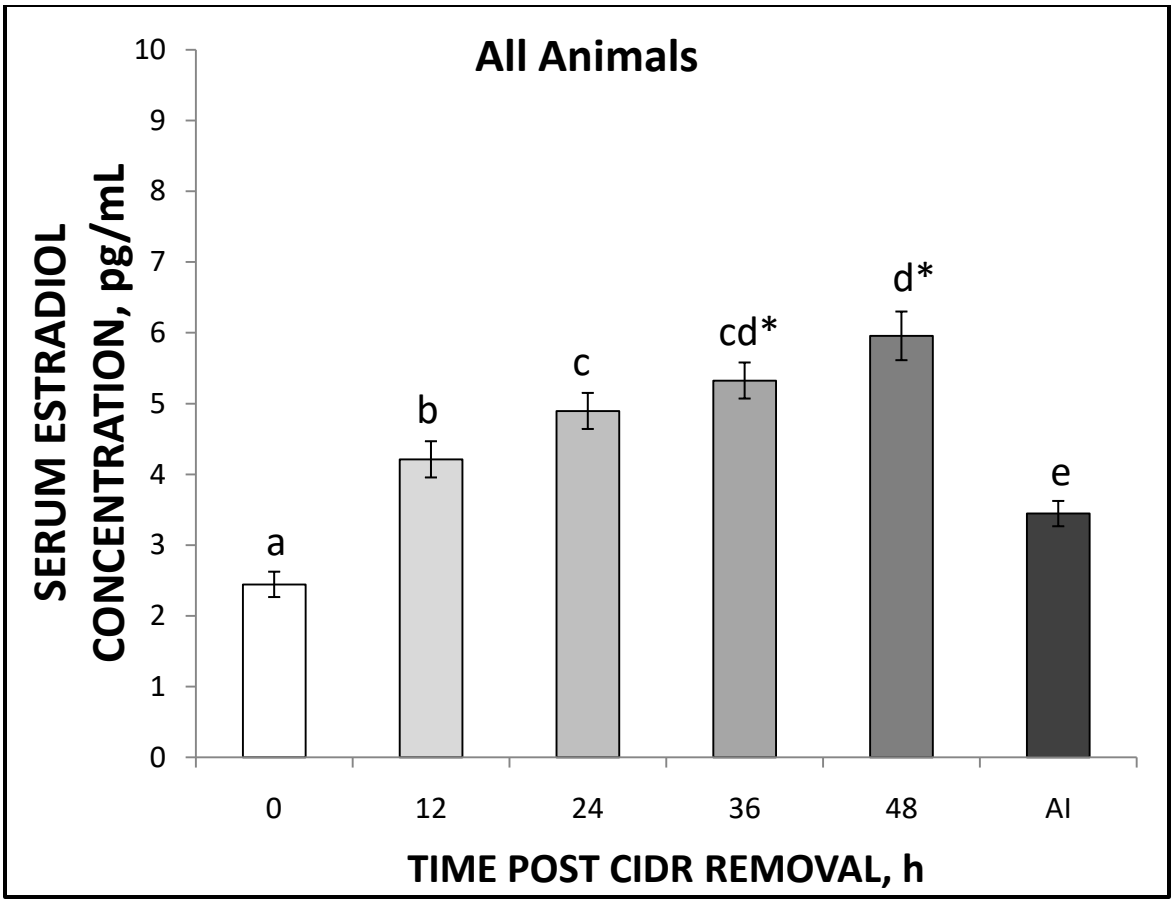

Figure 19. Serum concentrations of estradiol for all animals (pg/mL) starting at 0 hours post CIDR removal to time of AI ($P < 0.0001$). [abcde]Supercripts differ ($P \leq 0.05$), similar superscripts with asterisks (*) represent tendencies ($P > 0.05$ but $P \leq 0.10$).

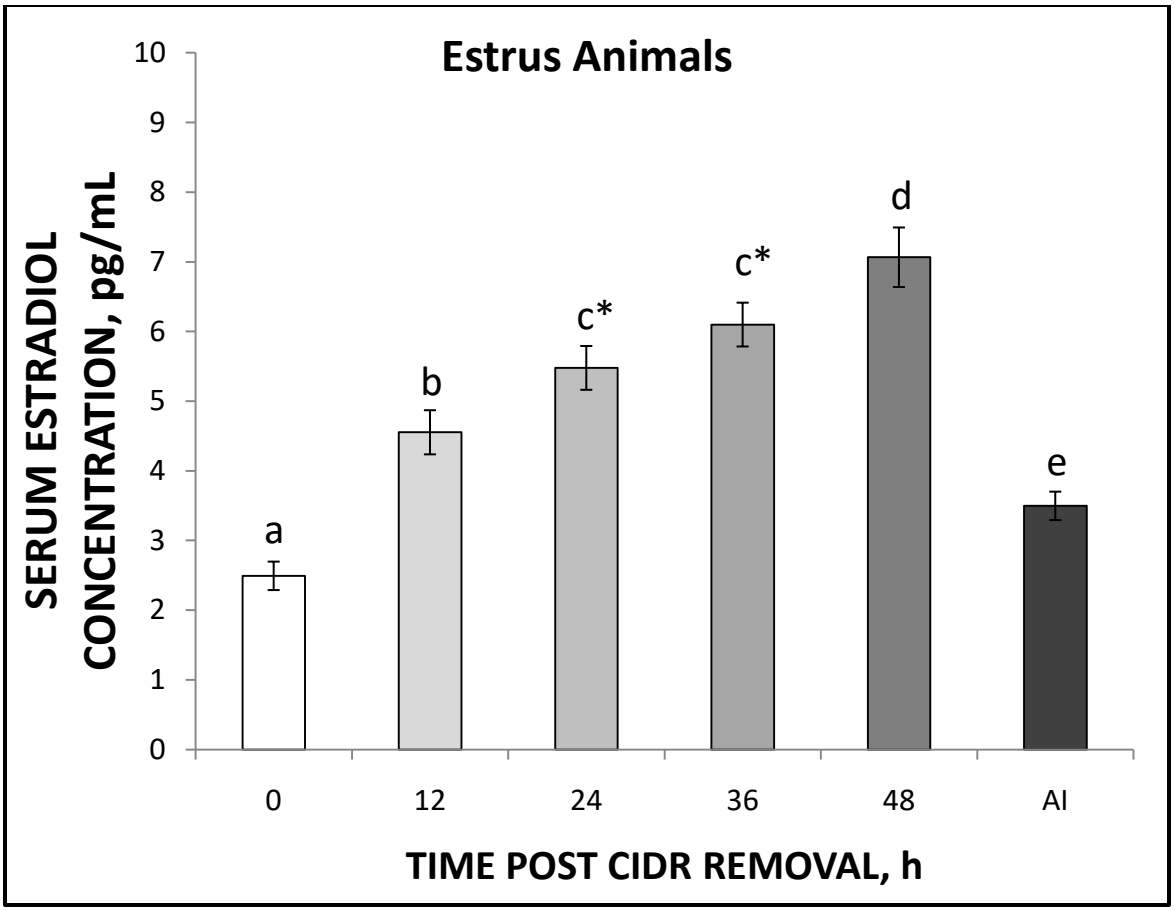

Figure 20. Serum concentrations of estradiol for estrus animals (pg/mL) starting at 0 hours post CIDR removal to time of AI ($P < 0.0001$). ^{abcde}Supercripts differ ($P \leq 0.05$), similar superscripts with asterisks (*) represent tendencies ($P > 0.05$ but $P \leq 0.10$).

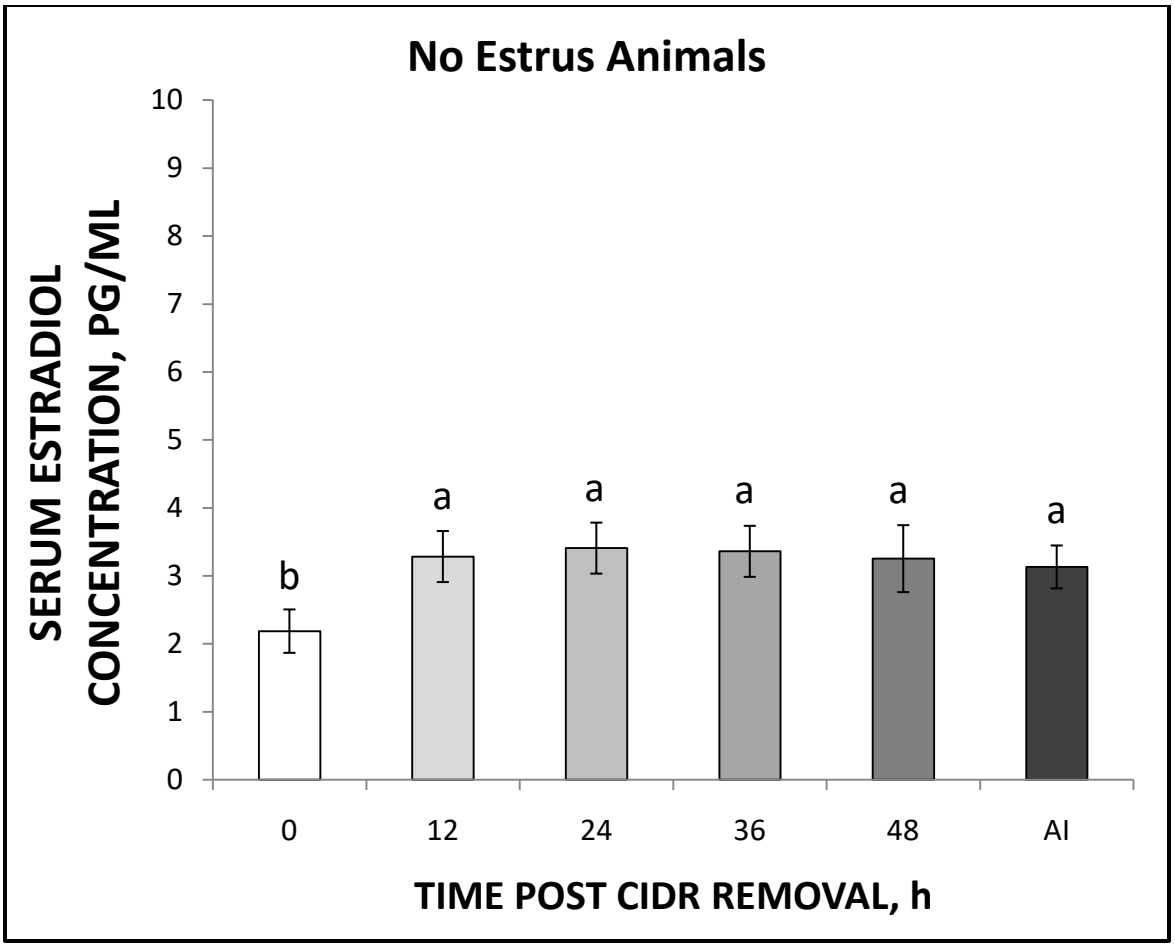

Figure 21. Serum concentrations of estradiol for no estrus animals (pg/mL) starting at 0 hours post CIDR removal to time of AI ($P < 0.0001$). [ab]Supercripts differ ($P < 0.05$).

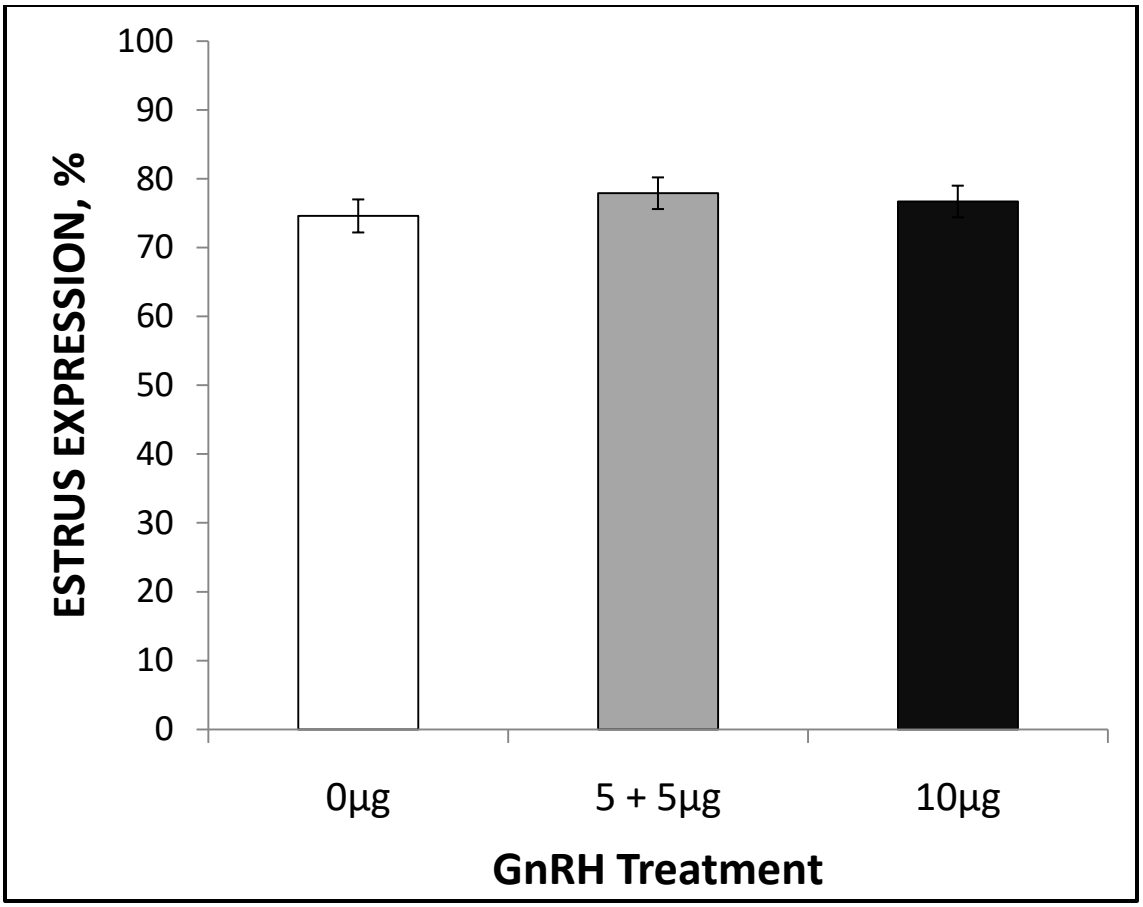

Figure 22. Expression of estrus of animals treated with 0, 5 + 5, and 10 µg of GnRH at CIDR removal (P = 0.60).

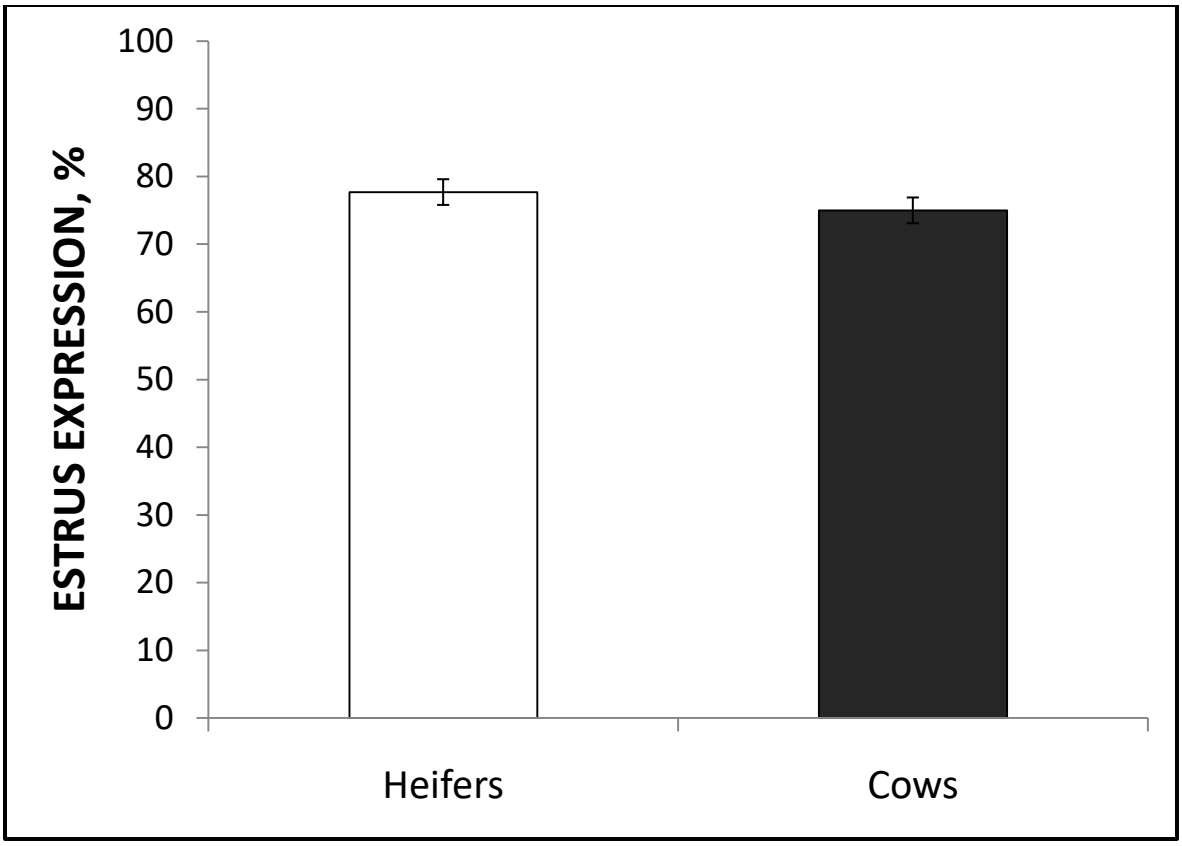

Figure 23. Expression of estrus among heifers and cows (P = 0.31).

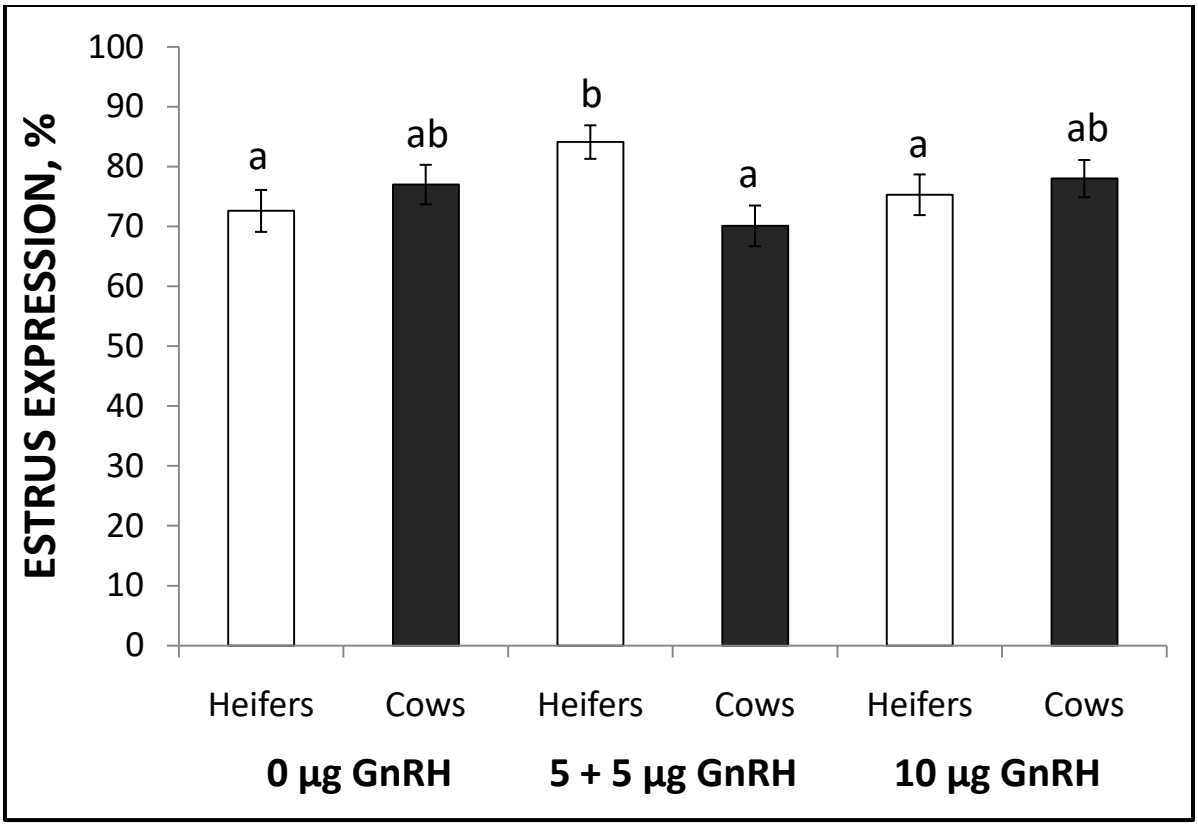

Figure 24. Treatment by age interaction on expression of estrus ($P < 0.01$). [ab]Superscripts differ ($P \leq 0.05$).

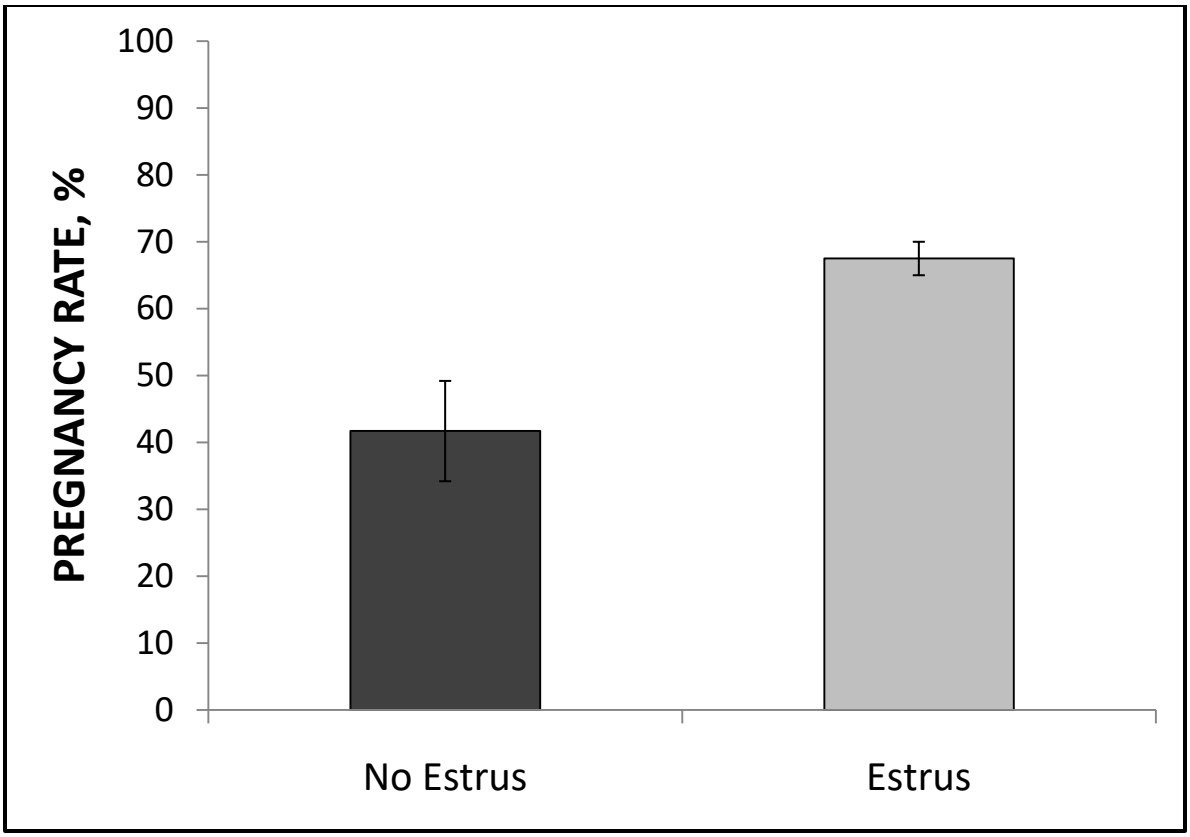

Figure 25. Pregnancy success among animals that did and did not exhibit estrus ($P < 0.01$).

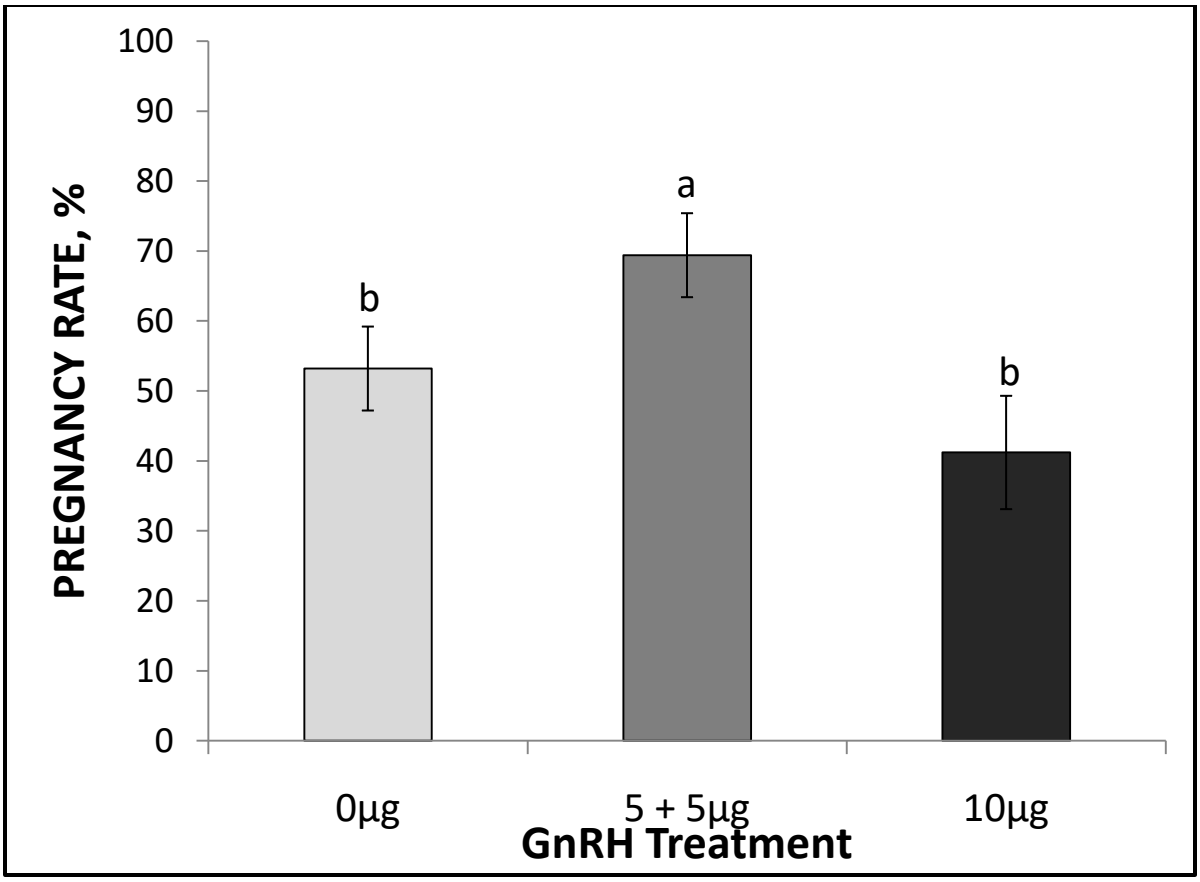

Figure 26. Pregnancy success among animals treated with 0, 5 + 5, and 10 µg of GnRH following CIDR removal ($P < 0.02$). [ab]Supercripts differ ($P \leq 0.05$).

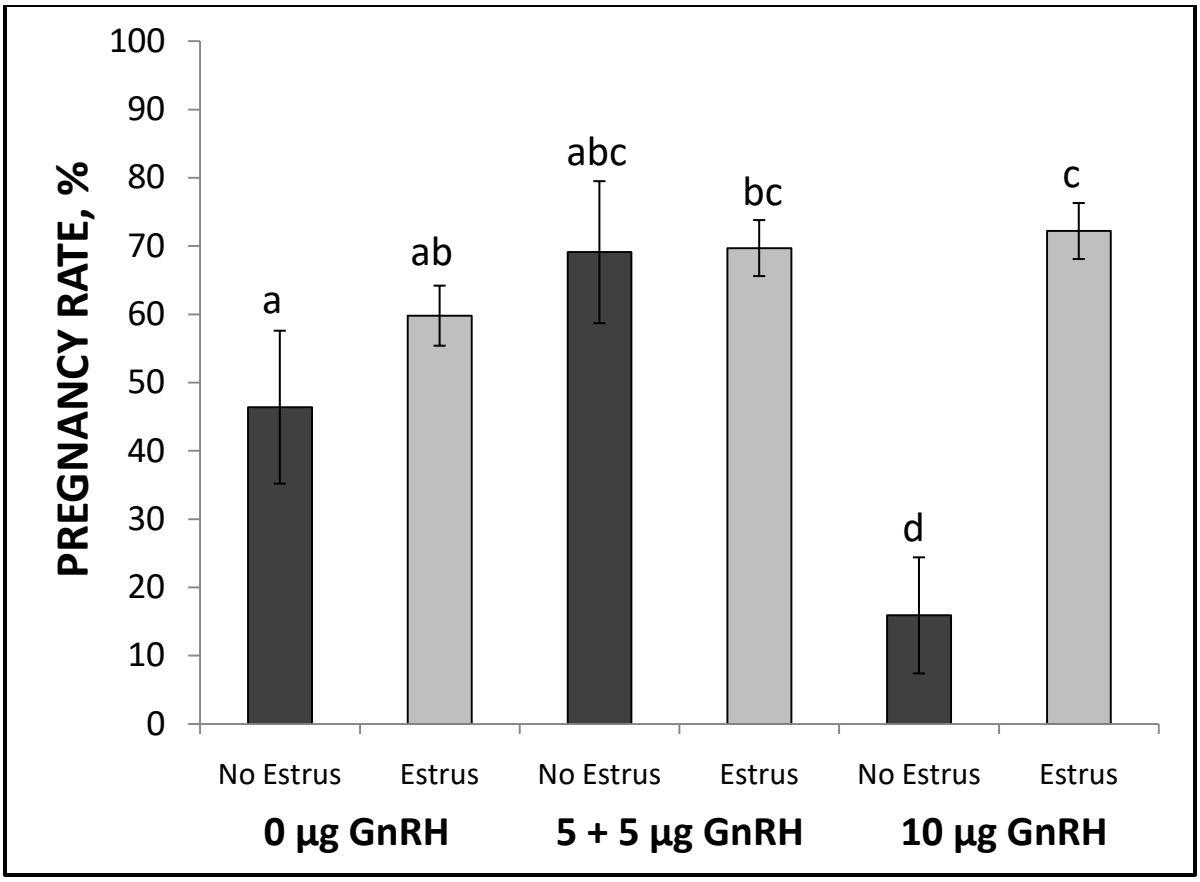

Figure 27. Treatment by estrus interaction for pregnancy success ($P < 0.01$).
[abcd]Superscripts differ ($P \leq 0.05$).

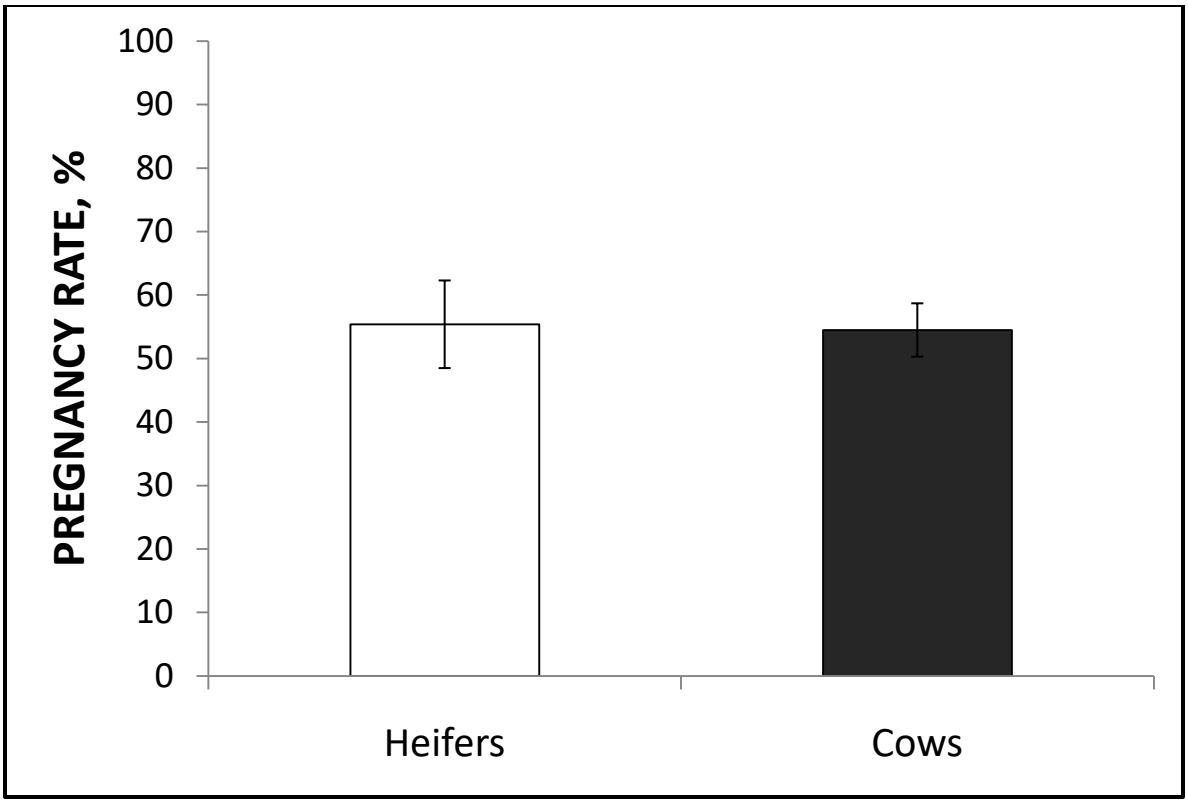

Figure 28. Pregnancy success among heifers and cows (P = 0.91).

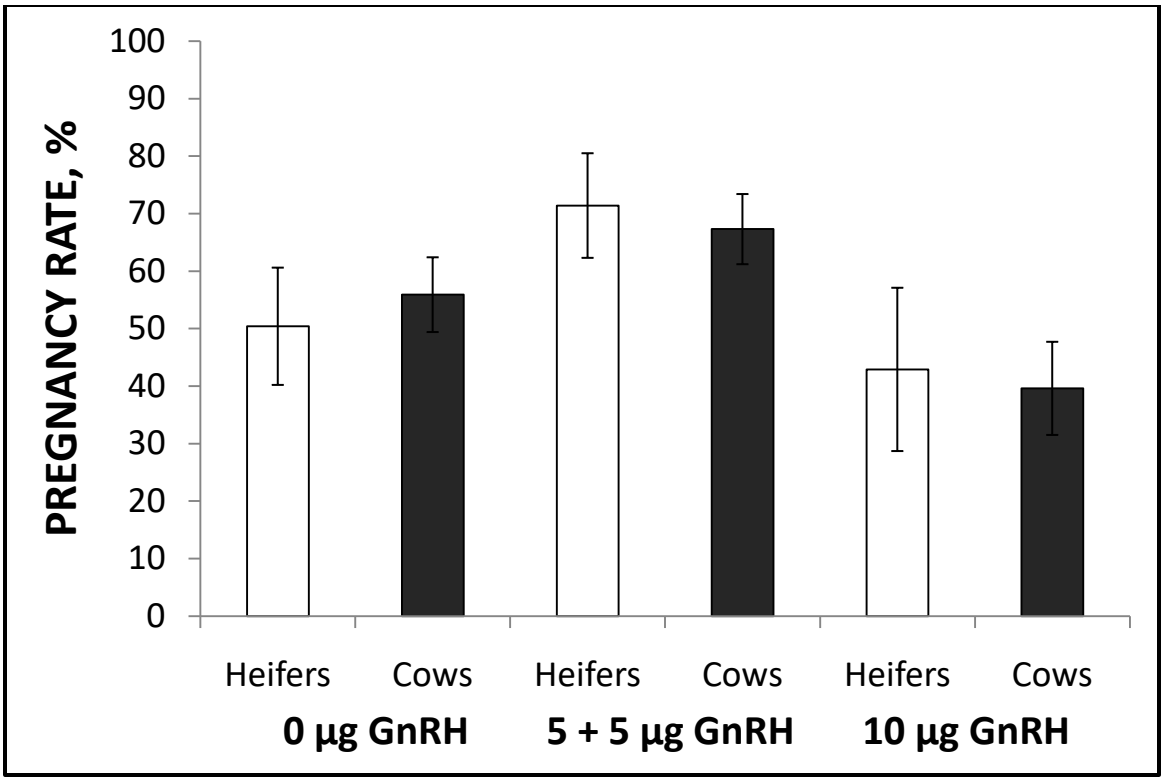

Figure 29. Treatment by age interaction for pregnancy success (P = 0.83).

DISCUSSION

Estradiol plays a critical role in the initiation of estrus and the ovulatory cascade (Allrich, 1994), sperm transport (Larimore et al., 2015), and regulation of uterine environment and embryo survival (Perry and Perry, 2008; Jinks et al., 2013). Cows and heifers that express estrus prior to FTAI have increased estradiol concentrations (Perry and Perry, 2008) and increased pregnancy success compared to those that do not exhibit estrus (Richardson et al., 2016).

Estradiol production is believed to occur via the two-cell two-gonadotropin theory (Fortune and Quirk, 1988), and increased estradiol production is stimulated by increased LH pulsatility (Fortune et al., 2001). A 5 μg dose of GnRH administered systemically has been reported to elicit an LH pulse similar to a physiologic pulse and stimulated an increase in estradiol similar to what is observed with a naturally occurring pulse (Ginther et al., 1996). Furthermore, in a study by Larimore et al. (2016) multiple small doses (5 μg) of GnRH stimulated increased estradiol concentrations, specifically via increased LH pulsatility. In this study supplementing 5 + 5 μg or 10 μg of GnRH at CIDR removal did not impact circulating estradiol concentrations. However, estradiol concentrations did increase from CIDR removal (0 h) to 48 hours and then were decreased at time of AI. At time of AI all animals were administered GnRH (100 μg) which would result in an LH surge, following an LH surge, aromatase activity is down regulated resulting in decreased circulatory and follicular estradiol concentrations (Komar et al., 2001).

When circulating concentrations of estradiol were analyzed separately for animals that exhibited estrus and those that did not exhibit estrus, the supplemental GnRH

treatment (5 + 5 μg or 10 μg at CIDR removal), again, did not produce significant differences. When animals that exhibited estrus were analyzed separately, circulating estradiol concentrations peaked at a higher concentration over time, this is supported by previous research which reported estrus animals had increased circulating concentrations of estradiol compared to animals that do not exhibit estrus (Perry and Perry, 2008). For animals that did not exhibit estrus there was a different pattern observed in circulating estradiol concentrations over time. Estradiol concentrations increased from CIDR removal (0 h) to 12 hours later but did not change significantly after 12 hours.

When it came to expression of estrus there was a treatment by age interaction. Specifically, among animals treated with the 5 + 5 μg dose of GnRH at CIDR removal, heifers responded more favorably compared to cows, with increased expression of estrus. There were no significant differences between treatments among cows. The 5 + 5 μg contributing to more LH pulsatility, whereas 10 μg treatment had decreased expression of estrus. A dose of 10 μg might be over stimulating and potentially negatively impacting estradiol production, similar to what was observed in a previous study by Larimore et al. (2016) where multiple small doses (5 μg) of GnRH were administered and initially positively contributed to estradiol production however after 8 hours estradiol production was reduced to baseline. This over-stimulatory effect was also noted in another study where cattle continuously infused with GnRH (2 μg, i.v.) had LH pulsatile secretion blocked and GnRH receptor down regulation (Vizcarra et al., 1997).

In this study, small doses of GnRH following CIDR removal positively impacted pregnancy success, specifically the 5 + 5 μg dose of GnRH had increased pregnancy success compared to the 0 μg and 10 μg treatments. Overall among animals that

exhibited estrus prior to FTAI, both the 5 + 5 μg and the 10 μg of GnRH following CIDR removal had or tended to have increased pregnancy success compared to estrus animals in the 0 μg group, this could be a result of GnRH stimulating an LH pulse, which could be modulating estradiol concentrations enough to induce favorable conditions, altered uterine pH, increased expression of endometrial genes supportive of implantation, and sperm transport (Bauersachs et al., 2005; Larimore et al. 2015; Perry and Perry, 2008). Among animals that did not exhibit estrus the 5 + 5 μg induced favorable pregnancy success, however the 10 μg treatment lead to decreased pregnancy success.

In addition to stimulating LH pulses GnRH may also be impacting pregnancy success through other mechanisms. Among humans, GnRH and its analogs were reported to increase binding of spermatozoa to the zona pellucida (Morales and Llanos, 1996). Gonadotropin releasing hormone agonists were also reported to increase the cleavage rates of bovine oocytes *in vitro* (Funston and Seidel, 1995). Overall GnRH seems to play a vital role both directly and indirectly in the processes involved with successful fertilization. In conclusion, supplementation with small doses of GnRH following CIDR removal (5 + 5 μg) did not impact circulating concentrations of estradiol or expression of estrus, but favorably impacted conception rates.

CHAPTER 5

DIFFERENCES IN ABUNDANCE OF GnRH-I AND GnRH-II AMONG BOVINE ANTRAL FOLLICLES

ABSTRACT

Estradiol plays a critical role in fertility among cattle, specifically it has been reported that preovulatory estradiol regulates uterine pH, sperm transport, and uterine secretions that impact embryo development and survival. Both Gonadotropin-releasing hormone (GnRH) and GnRH receptors have been reported in granulosa cells of cattle, and among other species GnRH has been reported to modulate follicular steroidogenesis. Thus, the objective of the current study was to determine changes in the relative abundance of GnRH-I and GnRH-II mRNA within granulosa cells of bovine follicles. Beef cows/heifers were synchronized using the CO-Synch protocol and artificially inseminated. On day 16 after insemination animals were transported to a local abattoir. Following slaughter ovaries were collected and all follicles were classified as small (< 5 mm), medium (5 to 10 mm), or large (> 10 mm). Follicles were aspirated to collect follicular fluid and granulosa cells. Follicles were pooled by size within animal (n = 23, 16, and 18 for small, medium, and large, respectively). Follicular fluid concentrations of estradiol were determined by radioimmunoassay. Total cellular RNA was extracted from the granulosa cells and RT-PCR was performed to determine relative abundance of mRNA for GnRH-I, GnRH-II, and GAPDH. Data were analyzed using the mixed procedure in SAS. Follicle size influenced concentration of estradiol. Large follicles had increased estradiol ($P < 0.0001$) compared to small and medium follicles (18,626 ± 2,650 vs 1,270 ± 2,307 and 8,925 ± 2,763 pg/mL, respectively). There was no difference ($P =$

0.31) in relative abundance of GnRH-I mRNA among small, medium, or large follicles (3.8 ± 0.78, 3.40 ± 0.85, and 1.95 ± 0.94; respectively). However, relative abundance of GnRH-II mRNA was influenced by follicle size ($P < 0.05$), with greater abundance in small follicles (40.97 ± 9.27) compared to medium (6.32 ± 11; $P = 0.02$) or large (7.85 ± 11.11; $P = 0.02$) follicles. However, there was no difference ($P = 0.92$) in relative abundance between medium and large follicles. When follicles were classified by concentration of estradiol, follicles with the lowest 25% of estradiol had decreased (LOWE2; $P < 0.0001$) concentrations of estradiol (434 ± 1,074 pg/mL) compared to the middle 50% (MIDE2; 7,029 ± 961 pg/mL) which was decreased ($P < 0.0001$) compared to the greatest 25% (HIGHE2; 46,423 ± 2,025 pg/mL). LOWE2 had greater ($P < 0.01$; 5.37 ± 0.67) abundance of GnRH-I mRNA compared to MIDE2 and HIGHE2 which did not differ (1.85 ± 0.55 and 1.23 ± 1.65, respectively). Relative abundance of GnRH-II mRNA was greater ($P < 0.01$) in LOWE2 (42.33 ± 9.56) compared to MIDE2 (5.98 ± 8.90). HIGHE2 was similar ($P \geq 0.12$) to the other two groups (8.90 ± 19.12). Thus the decreased abundance of GnRH-I and GnRH-II mRNA may play a role in increased production of estradiol.

INTRODUCTION

Since the discovery of GnRH three distinct forms have been characterized (GnRH-I, GnRH-II, and GnRH-III); however, only GnRH-I and GnRH-II have been found in mammals. Gonadotropin releasing hormone-I is the isoform that is thought to work predominantly through the classical hypothalamo-pituitary-gonadal axis to stimulate gonadotropin release, while GnRH-II is thought to be associated with sexual behavior (Millar, 2005). There have been many reports on extra-pituitary actions of GnRH, those of special interest to reproductive physiologists being actions within the gonads.

Expression of GnRH in the ovary was first characterized in rats (Clayton et al., 1979; Reeves et al., 1980; Jones et al., 1980). Expression was next localized to primary, secondary, and tertiary follicles in the rat (Clayton et al., 1992; Whitelaw et al., 1995). More recently, GnRH-R mRNA expression has been reported in both bovine granulosa cells and luteal tissue, specifically small antral follicles were reported to have increased GnRH receptor mRNA compared to both medium and large follicles (Ramakrishnappa et al., 2003).

A GnRH-like protein was identified in bovine follicles, and this protein suppressed LH-stimulated accumulation of cAMP in a dose dependent manner (Aten et al., 1987). This GnRH-like protein was found to be in greatest concentrations in the granulosa cells (Ireland et al., 1988). Gonadotropin releasing hormone has been reported to have both stimulatory and inhibitory actions on steroidogenesis within the ovary in many species such as the rat, humans, pigs and cows (Sharpe, 1982; Janssens et al., 2000;

Millar, 2005; Ramakrishnappa et al., 2005). Furthermore, when a GnRH agonist was added to cultured granulosa cells at a concentration of 200 ng/mL it stimulated estradiol production, but at concentrations of 500 ng/mL and 1000 ng/mL it decreased estradiol production (Ramakrishnappa et al., 2005). Some large follicles fail to produce elevated concentrations of estradiol while some small follicles do produce elevated concentrations of estradiol (Jinks et al., 2013; Perry et al., 2014), intra-follicular GnRH may be a contributing factor in these variable amounts of estradiol and can directly impact steroidogenesis. Therefore, the objective of this study was to determine the relative abundance of GnRH-I and GnRH-II mRNA among bovine antral follicles when classifying follicles based on size and based on follicular fluid concentrations of estradiol.

MATERIALS AND METHODS

All procedures were approved by the South Dakota State University Institutional Animal Care and Use Committee.

Experimental Design

Beef cows and heifers (n = 37) at the SDSU Beef Breeding Unit were synchronized using a CO-Synch protocol. Gonadotropin releasing hormone was administered (100 µg as 2 cc of Factrel i.m.; Pfizer Animal Health, Madison, NJ) on day -9, followed by $PGF_{2\alpha}$ administration (PG; 25 mg as 5 cc of Lutalyse i.m.; Pfizer Animal Health, Madison, NJ) on day -2. On day 0 animals were administered GnRH (100 µg as 2 cc of Factrel i.m.; Pfizer Animal Health, Madison, NJ) and artificially inseminated.

Follicular dynamics and ovulation were characterized by transrectal ultrasonography using an Aloka 500V ultrasound with a 7.5 MHz linear probe (Aloka, Wallingford, CT) on day -9, 0, and 3. Animals were monitored for estrus behavior daily with the use of EstroTect patches (Western Point, Inc., Apple Valley, MN). Animals with greater than half of their patch rubbed off were documented as having exhibited standing estrus.

Tissue Collection

Animals were sent to a local abattoir on day 16 following insemination, and reproductive tracts were collected immediately following slaughter and kept on ice. Ovaries were collected and all visible surface follicles (tertiary follicles containing a fluid filled antrum, an oocyte surrounded by multiple layers of granulosa cells, and a theca interna and theca externa layer) were counted and classified by size using calipers; small (< 5 mm) n = 23, medium (5 to 10 mm) n = 16, and large (> 10 mm) n = 18. Needle aspirations were conducted to collect the follicular fluid and granulosa cells (n = 57 bovine follicles). Granulosa cells were separated from the follicular fluid by centrifugation, placed in RNase Free tubes (USA Scientific) and snap frozen in liquid nitrogen. Granulosa cell samples were stored in a -80˚C freezer until total RNA was extracted. Follicular fluid samples were diluted to 1:100 and 1:1000 for small and medium follicles, and 1:1000 and 1:10000 for large follicles then stored in a -20˚C freezer until radioimmunoassays were performed.

Radioimmunoassay

Follicular fluid concentrations of Estradiol 17-β were determined by radioimmunoassay (RIA) using the methods described by Perry and Perry (2008). Animals were classified as LOWE2 (lowest 25%), MIDE2 (middle 50%), or HIGHE2 (highest 25%). Inter- and intra-assay CV were 14.9% and 5.06%, respectively and assay sensitivity was 0.4 pg/mL.

RNA Extraction and Real Time RT-PCR

Total cellular RNA was extracted from granulosa cells using a Qiagen RNeasy Plus Mini Kit (Austin, Texas) prior to RNA isolation. Pure RNA was dissolved in nuclease free water, and a spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to determine RNA concentration for each sample. The RNA samples were then stored in a -80˚C freezer. The RNA was diluted to 70 ng/µl and Real Time RT-PCR was performed in duplicate using iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad Laboratories, Inc.) and a Stratagene MX 3000p QPCR machine. Transcript abundance of GnRH-I and GnRH-II was measured using the primers in Table 1, and GAPDH was used as the endogenous reference gene. All of the primers were diluted to a concentration of 10 µM and all products were verified for identity by sequencing (Iowa State Genomics Core). Each plate contained negative controls to ensure no background contamination was occurring. The PCR program was 10 minutes at 50˚C, 1 minute at 95˚C for melting, and 30 seconds at 60˚C for annealing and elongation for 40 cycles. Base pair size of all PCR products was confirmed through gel electrophoresis and the intra-assay CV for all primer pairs was < 28%.

Statistical Analysis

Differences in follicular fluid concentrations of estradiol, relative abundance of GnRH-I, and relative abundance of GnRH-II were analyzed by analysis of variance using the PROC MIXED of SAS (SAS Inst. Inc., Cary, NC). When the F statistic was significant ($P < 0.05$), mean separation was performed using least significant differences (Means ± SEM, Snedecor and Cochran, 1989). Differences were considered to be significant when $P \leq 0.05$ and a tendency when $P > 0.05$ but $P \leq 0.10$.

RESULTS

Follicular Fluid Estradiol Concentration

Estradiol concentrations were influenced by follicle size ($P < 0.0001$), large follicles had increased estradiol (18,626 ± 2,650; Figure 30) compared to medium follicles (8,925 ± 2,763 pg/mL) which had increased estradiol compared to small follicles (1,270 ± 2,307 pg/mL). When animals were classified by follicular fluid concentration of estradiol (Figure 31), follicles with the lowest 25% of estradiol had decreased (LOWE2, $P < 0.01$) concentrations of estradiol (434 ± 1,074 pg/mL) compared to the middle 50% (MIDE2; 7,029 ± 961 pg/mL) which was decreased ($P < 0.01$) compared to the greatest 25% (HIGHE2; 46,423 ± 2,025 pg/mL).

GnRH-I mRNA Abundance

There was no difference ($P = 0.31$; Figure 32) in relative abundance of GnRH-I mRNA among small, medium, or large follicles (3.8 ± 0.78, 3.40 ± 0.85, and 1.95 ± 0.94; respectively). When animals were classified by follicular fluid estradiol concentration, LOWE2 had greater ($P \leq 0.03$; 5.37 ± 0.67) abundance of GnRH-I mRNA compared to MIDE2 and HIGHE2 (1.85 ± 0.55 and 1.23 ± 1.65, respectively; Figure 33).

GnRH-II mRNA Abundance

Relative abundance of GnRH-II mRNA was influenced by follicle size ($P = 0.02$; Figure 34), with greatest abundance in small follicles (40.97 ± 9.27) compared to medium (6.32 ± 11, $P = 0.02$) or large follicles (7.85 ± 11.11, $P = 0.02$). However, there was no difference ($P = 0.92$) in relative abundance between medium and large follicles. When animals were classified by follicular fluid estradiol concentration, relative abundance of GnRH-II mRNA was ($P < 0.01$) greater in LOWE2 (42.33 ± 9.56) compared to MIDE2 (5.98 ± 8.90). HIGHE2 was similar ($P \geq 0.12$) to the other two groups (8.90 ± 19.12; Figure 35).

Gene	Primer	Primer Sequence	Fragment Size	Reference
GnRH I	Forward Reverse	5'-GCAGCTCTGGAAAGTCTGATTG-3' 5'-TCCATGCAACCTGGTGTAAGAA-3'	170	(Ramakrishnappa et al., 2003)
GnRH II	Forward Reverse	5'-TCGGGGGATGATACACCACT-3' 5'-TTCAGCGTGCGGGTTTTCTA-3'	226	(Ramakrishnappa et al., 2003)
GAPDH	Forward Reverse	5'-GATTGTCAGCAATGCCTCCT-3' 5'-GGTCATAAGTCCCTCCACGA-3'	94	(Han et al., 2006)

Table 1. Genes, Primer sequences, and fragment size for genes amplified during Real Time RT-PCR.

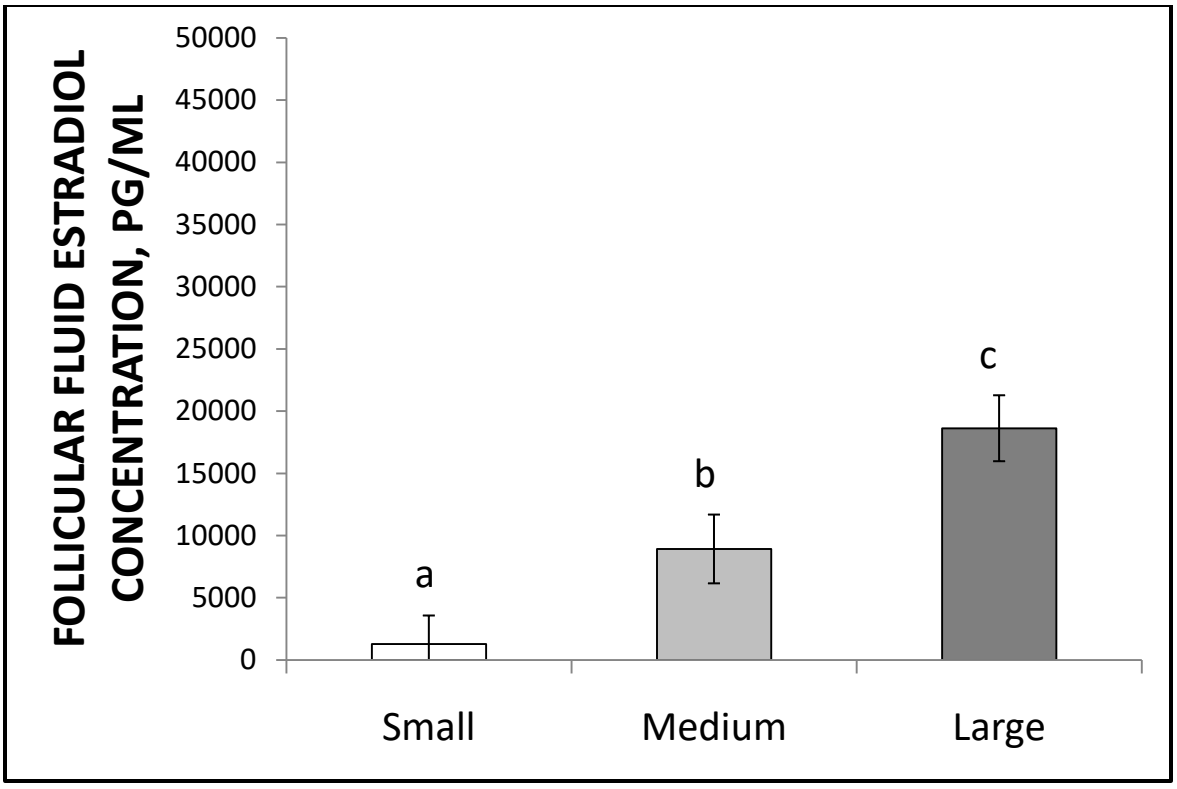

Figure 30. Follicular fluid estradiol concentrations of small, medium, and large follicles. [abc]Supercripts differ ($P < 0.05$).

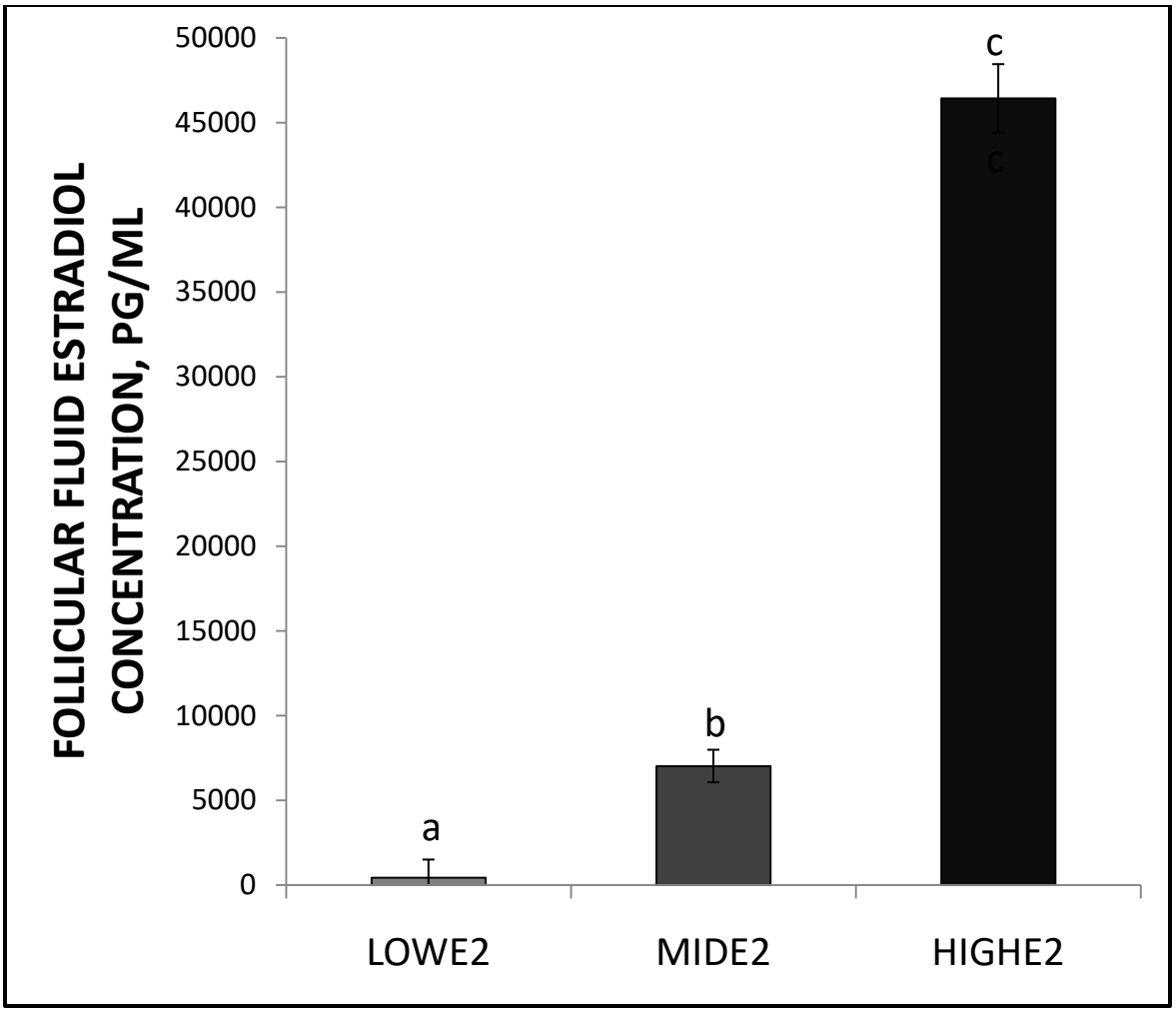

Figure 31. Follicular fluid estradiol concentrations of LOWE2, MIDE2, and HIGHE2 follicles.[abc]Supercripts differ ($P < 0.05$).

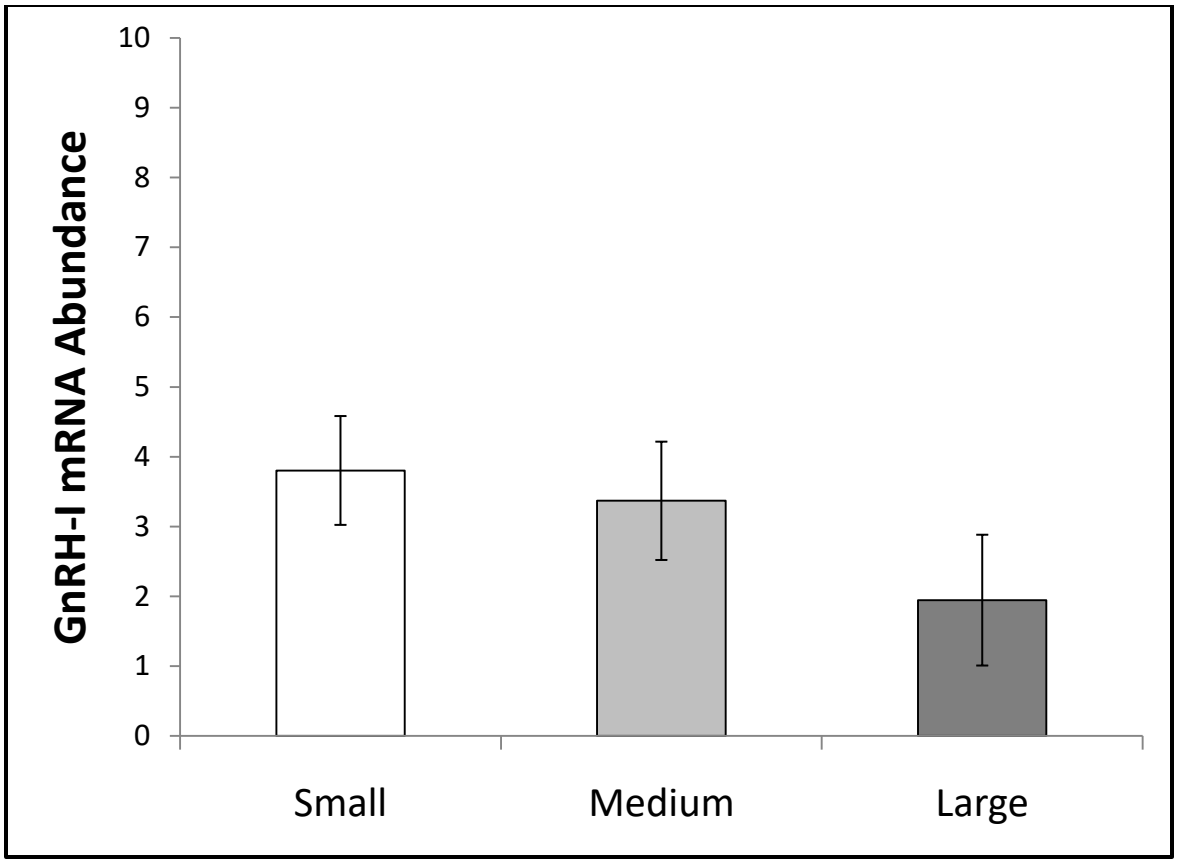

Figure 32. Relative abundance of GnRH-I mRNA in granulosa cells of small, medium, and large follicles ($P = 0.31$).

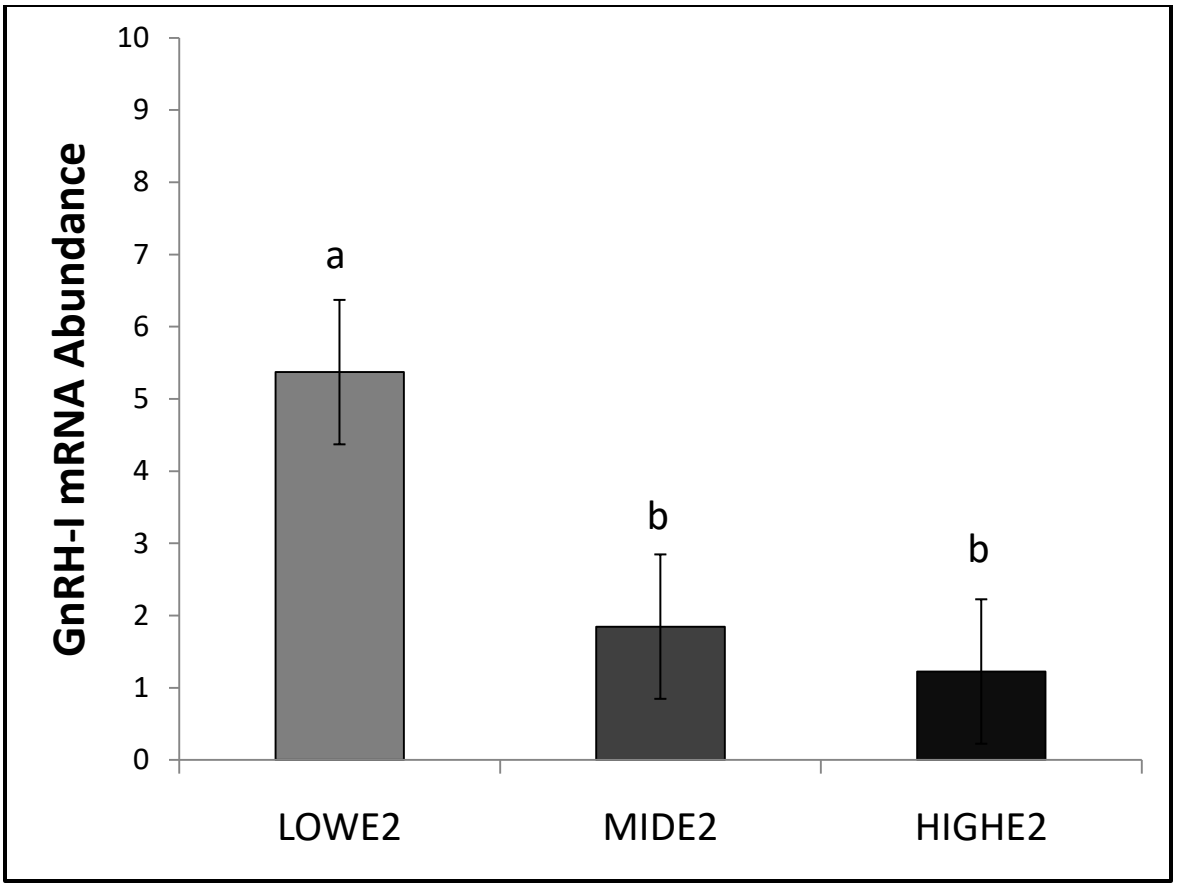

Figure 33. Relative abundance of GnRH-I mRNA in granulosa cells of LOWE2, MIDE2, and HIGHE2 follicles. [ab]Supercripts differ ($P < 0.05$).

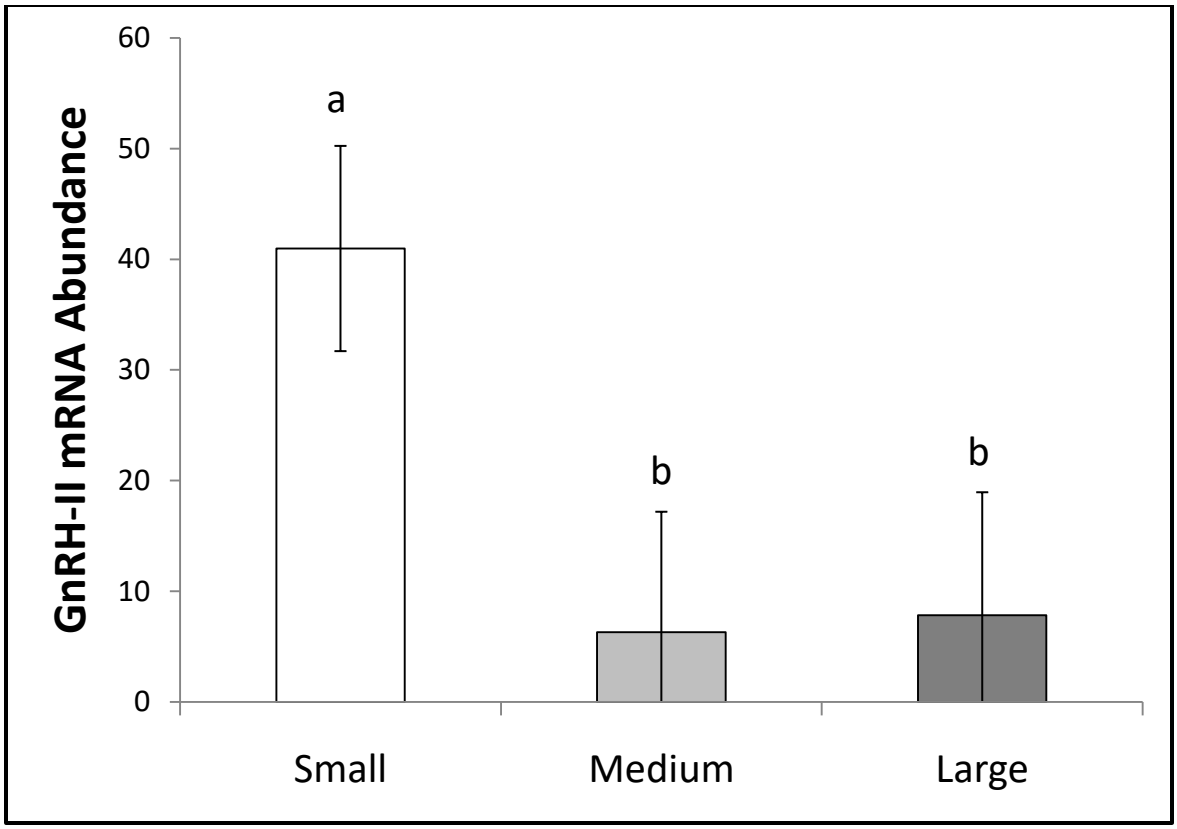

Figure 34. Relative abundance of GnRH-II mRNA in granulosa cells of small, medium, and large follicles. [ab]Supercripts differ ($P < 0.05$).

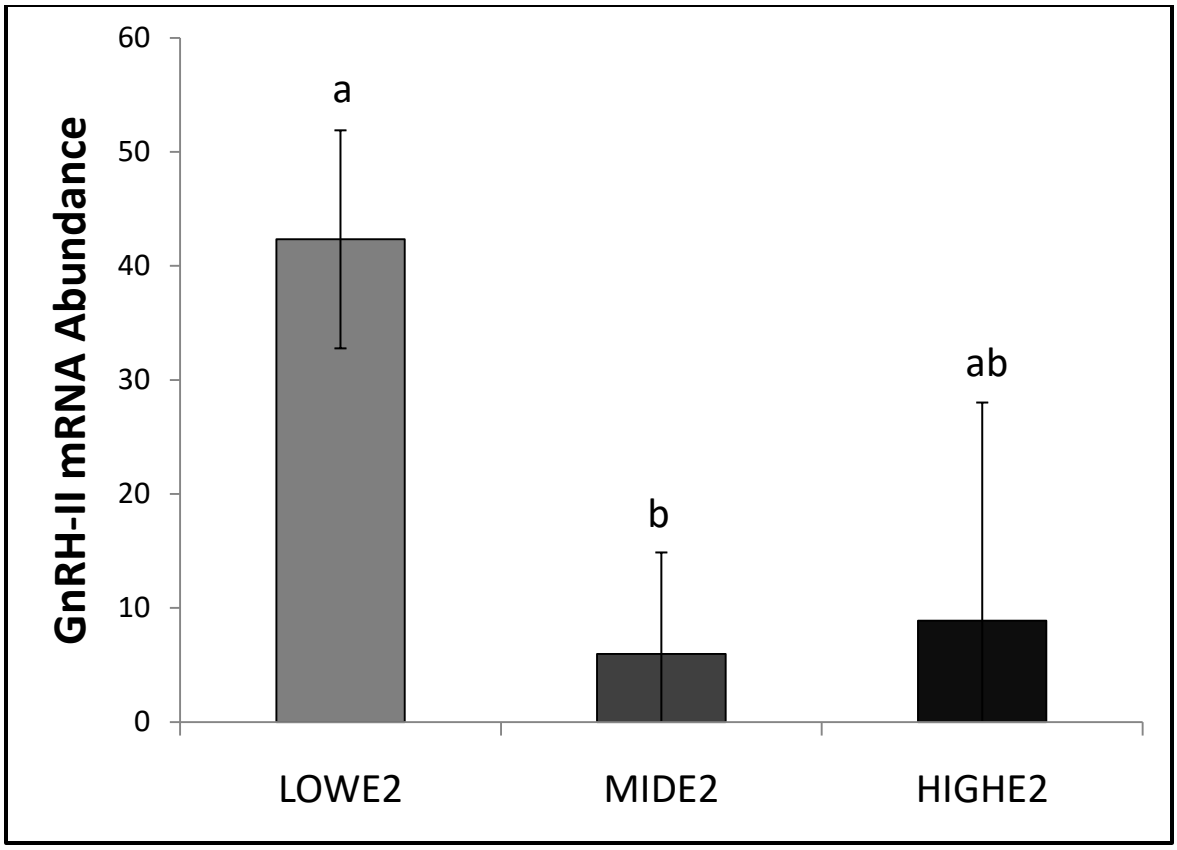

Figure 35. Relative abundance of GnRH-II mRNA in granulosa cells of LOWE2, MIDE2, and HIGHE2 follicles. [ab]Supercripts differ ($P < 0.05$).

DISCUSSION

Estradiol plays a critical role in fertility among cattle, specifically it has been reported that preovulatory estradiol regulates uterine pH (Perry and Perry, 2008), sperm transport, and uterine secretions that impact embryo development and survival (Jinks et al., 2013; Larimore et al., 2015). Estradiol is the primary signal to the hypothalamus to initiate the expression of estrus (Vailes et al., 1992; Diskin and Sreenan, 2000) and is also critical within the ovary as it is associated with growth, divergence, ovulation, termination, and eventual regression/atresia of follicles (Bodensteiner et al., 1996b). In a study by Perry et al. (2014) follicle diameter had a positive relationship with peak concentrations of estradiol, but only among cows that exhibited standing estrus, and estradiol increased earlier in cows that exhibited estrus compared to cows that did not. Furthermore, cows with greater circulating concentrations of estradiol during the preovulatory period had up-regulation of the steroidogenic pathway within the granulosa cells and tended to have increased LH pulse frequency immediately following initiation of luteal regression with $PGF_{2\alpha}$ compared to cows that did not exhibit estrus.

Classically, estradiol from the follicle is believed to be produced via "the two-cell two-gonadotropin theory" (Liu and Hsueh, 1986; Fortune and Quirk, 1988). A 5 μg dose of GnRH stimulates an LH pulse similar to a physiological pulse (Ginther et al., 1996). Further, multiple small doses of GnRH administered systemically following CIDR removal increased circulating concentrations of estradiol (Larimore et al., 2016). Animals that exhibit estrus prior to a fixed time AI have increased concentrations of estradiol (Perry and Perry, 2008) and increased conception rates versus animals that do not exhibit estrus (Richardson et al., 2016). These studies have demonstrated that GnRH

can impact estradiol concentrations secondary to gonadotropin release, but whether or not that GnRH is having any effect directly at the ovary remains unknown.

In the present study, relative abundance of GnRH-I and GnRH-II mRNA was greater in small follicles as well as in LOWE2 follicles, these results are similar to patterns of expression reported by Ramakrishnappa et al. (2003) for GnRH receptor mRNA in bovine antral follicles. Furthermore when a GnRH agonist was added to cultured granulosa cells at a concentration of 200 ng/mL it stimulated estradiol production, but at concentrations of 500 ng/mL and 1000 ng/mL it decreased estradiol production (Ramakrishnappa et al., 2005). Therefore one could infer that the decreased abundance of GnRH-I and II mRNA in medium and large or MIDE2 and HIGHE2 follicles may play a role in increased production of estradiol.

Previous studies have also reported GnRH to induce follicular atresia in rats, porcine, and human follicular cells (Hsueh et al., 1984; Piquette et al., 1991; Billig et al., 1994; Whitelaw et al., 1995; Motomura, 1998; Sridaran et al., 1998; Zhao et al., 2000), specifically GnRH-R mRNA was highly expressed in atretic follicles of rats during the follicular phase (Whitelaw et al., 1995). In the current study, highly variable amounts of GnRH-I and II mRNA were observed in large bovine antral follicles. The stage of follicular development that these follicles were in was not well known, therefore some of those large follicles could have been undergoing atresia. Thus the variable abundance of GnRH-I and II mRNA in large follicles could be from some follicles initiating atresia.

In conclusion, relative abundance of GnRH-I and GnRH-II mRNA in bovine granulosa cells was increased in LOWE2 and some of the HIGHE2 follicles, which we

speculate to have been undergoing atresia. Thus the decreased abundance of GnRH-I and II in follicles with elevated concentrations of estradiol may indicate that GnRH is capable of regulating estradiol production in bovine antral follicles. Further investigations are warranted to determine the specific ways in which GnRH-I and II are impacting estradiol production of growing bovine antral follicles and at what specific stages of follicular growth those actions are having the most impact.

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