5-1-2020

Effects of estradiol benzoate on reproductive characteristics in beef cattle

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The effects of estradiol benzoate on female reproductive characteristics in beef cattle

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A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Agriculture
in the Department of Animal and Dairy Sciences

Mississippi State, Mississippi

May 2020

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Date of Degree: May 1, 2020

Institution: Mississippi State University

Major Field: Agriculture

Committee Chair: Jamie Larson

Title of Study: The effects of estradiol benzoate on female reproductive characteristics in beef cattle

Pages in Study: 35

Candidate for Degree of Master of Science

Assisted reproductive technologies such as estrus synchronization and embryo transfer can aid producers in meeting their goals of improving genetics, calve one calf per cow per year, and wean heavy calves. Most estrus synchronization protocols follow a GnRH-PGF-GnRH sequence. Ovulation prompted by exogenous gonadotropin releasing hormone (GnRH) may cause the release of sub-mature oocytes and lead to decreased pregnancy rates. Inclusion of estradiol benzoate (EB) improved pregnancy rates in some studies. The objective of this study was to determine if EB affects blood perfusion of follicles, corpus luteum (CL), or uterus, concentrations of estradiol and progesterone, or incidence of standing estrus.

DEDICATION

This thesis is dedicated to my family. Without your support and guidance, I would not have made it this far.

ACKNOWLEDGEMENTS

There are numerous people that deserve to be thanked. I am grateful for the guidance, help, and support of my committee that consisted of Dr. Larson, Dr. Lemley, and Dr. Smith. Dr. Larson, thank you for allowing me the opportunity to be your graduate student, to palpate and ultrasound cows as much as my arm could handle, and for teaching skills that can be applied outside of the classroom. Dr. Smith, thank you for your patience and kindness when reviewing statistics. It's because of you I have a better understanding of math. Dr. Lemley, thank you for teaching us to question everything and not take everything at face value. A special thanks to Zully and Riley for your hours spent at the farm, in the cold, and terrible weather. Additionally, a huge thank you to Cody, Corey, Bryan, Sarah, Justin, and Colton. Thanks for always being there even when you did not want to be! I'm eternally grateful.

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CHAPTER I

LITERATURE REVIEW

Introduction

It is the goal to optimize reproductive efficiency among cow-calf producers. There are many ways to improve reproductive efficiency. However, assisted reproductive technologies such as estrus synchronization is a way to improve reproductive efficiency. Most protocols utilize exogenous GnRH to induce ovulation. Research has indicated that exogenous GnRH may cause the release of sub-mature follicles, thus hindering pregnancy rates. Furthermore, research is discovering the crucial role of pre-ovulatory estradiol is becoming known. The following literature review discusses current knowledge about beef cattle reproduction specifically estrus synchronization and preovulatory estradiol as well as future implications of the role of synchronization and estradiol on female reproductive characteristics.

Female reproductive anatomy

The female reproductive system is composed of the vulva, vagina, cervix, uterus, uterine horns, oviduct, and ovaries. All components play a role in cyclicity, pregnancy, and parturition. External genitalia of the female includes the vulva. The vulva functions as the external opening to the reproductive tract and is the first “barrier” to keeping foreign bacteria, feces, and other unwanted items from entering the reproductive tract. The vulva opens into the vagina. The vagina is the site of copulation and semen deposition during natural service in beef cattle. The vaginal environment changes depending upon phases of the estrous cycle. For example, the

vaginal microbiome is altered depending on concentrations of hormones and pregnancy status (Nascimento et al., 2015; Moreno et al., 2016). The vagina leads to the cervix. The cervix in beef cattle is a hard, non-compliant organ, composed of, on average, three cartilaginous rings. The cervix acts as a barrier to keep foreign bacteria and objects from entering the uterus due to is structure. During estrus, privileged pathways are formed by sialomucins in the cervix to aid in sperm transport during natural mating. The uterus is a muscular organ that plays a role in sperm transport, parturition and housing the growing fetus in some species. The oviduct is comprised of three parts: infundibulum, ampulla, and isthmus. Fertilization occurs at the ampulla isthmus junction. The ovary possesses oocytes, follicles, corpora lutea (CL), corpora hemorragica, and corpora albicans. Reproductive organs and ovarian structures play a crucial role in regulating the estrous cycle.

Estrous cycle:

Cattle have a 21-day estrous cycle (Armstrong and Hansel, 1958). The estrous cycle is marked by the beginning of estrus to the succeeding estrus and is comprised of two phases, the follicular phase and luteal phase, which can be further divided into stages (Rodriguez-Martinez et al., 1986). Estrus can be defined as standing heat. The stages that comprise the follicular phase are estrus and proestrus, marked by antral follicles on the ovary and increased concentrations of estradiol. During proestrus the CL is regressing, progesterone is decreasing, and the preovulatory follicle is undergoing the final growth phase. Estrus is observed as standing heat and ovulation occurs during this phase. The length of proestrus contributes to pregnancy establishment (Pohler et al., 2012). Cattle that experience longer proestrus have improved pregnancy rates, embryo development, and luteal function (Burke et al., 2001; Mussard et al., 2003, 2007; Bridges et al.,

2012, Pohler et al., 2012). Mussard et al., (2007) compared pregnancy rates between cattle with a long proestrus period (2.25 d) and a short proestrus period (1.25 d). Cattle with a longer proestrus period had greater pregnancy rates compared to those with a shorter proestrus period (50.0% v 2.6%, respectively) (Mussard et al., 2007). The luteal phase is comprised of the stages metestrus and diestrus, which is characterized by a CL and increased concentrations of progesterone, as it is the CL that synthesizes progesterone.

Throughout the estrous cycle there are other key hormones that help to regulate the cycle, such as GnRH, luteinizing hormone (LH), follicle stimulating hormone (FSH) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Gonadotropin releasing hormone is secreted from the hypothalamus to stimulate the secretion of LH and FSH from the anterior pituitary (Hansel and Snook., 1970). Follicle stimulating hormone (FSH) and LH act to develop and mature the follicle to obtain ovulatory capacity (Hansel and Snook., 1970). As follicles mature, they will increase in size and synthesize more estradiol. Estradiol initiates the preovulatory surge of LH needed for ovulation to occur (Chenault et al., 1974).

Through understanding the estrous cycle and how hormones interact, the estrous cycle can be manipulated in many ways to induce or prohibit cyclicity to enhance producer management of females.

Assisted reproductive technologies in beef cattle

Reproductive efficiency is an important economic trait in beef cattle operations. The goal of cow-calf producers is to calve early in the calving season, wean the most pounds of calf, and improve efficiency (Odde, 1990; Rodgers et al., 2012) Assisted reproductive technologies (ART) may help producers achieve these goals and increase reproductive efficiency. Forms of ART

include estrus synchronization, artificial insemination (AI), and embryo transfer (ET). The benefits of utilizing ART include faster genetic progress through selecting for important traits, females becoming pregnant earlier in the breeding season which leads to calving earlier in the calving season and thus, heavier calves at weaning. Beef cattle producers have been slow to adopt ART, such as estrous synchronization, due to the initial cost, labor, and lack of facilities (Lamb et al., 2016). In the United States, currently 8 to 10% of beef cattle producers incorporate estrus synchronization and artificial insemination. In 2008, 4 to 6% of the beef calf crop resulted from AI (Pohler et al., 2012; NAHMS, 2008). An improvement in pregnancy rates when using ART may lead to an increase in usage of ART, allowing for the benefits to be realized.

Estrus synchronization:

Many estrus synchronization protocols are comprised of GnRH, $PGF_{2\alpha}$, and progesterone to mimic the female's natural estrous cycle in a condensed period of time. Estrus synchronization protocols are designed to bring a group of females to the same stage of the estrous cycle. The advantages of ES include synchrony of estrus and ovulation, allowing for timed AI (TAI) and ET. The need for estrus detection is eliminated in TAI protocols. Most synchronization protocols follow a GnRH-PGF-GnRH sequence (Pohler et al., 2012). The 7-day Co-Synch + CIDR protocol is commonly used among beef cow calf producers (Geary et al., 2001). The protocol begins with an injection of GnRH and a controlled internal drug release (CIDR) inserted intravaginally. Seven days later, the CIDR is removed and an injection of PGF is administered. Sixty to sixty-six hours after the injection of PGF, TAI is performed, and the second injection of GnRH is administered. The rate of pregnancy per AI ranges from 40 to 60% in beef cattle (Meneghetti et al., 2009). However, the rate of fertilization, or fertilized oocyte per ovulation, is

90 to 95% (Diskin et al., 2011). The majority of this difference can be attributed to early embryonic loss which occurs primarily in the first three weeks of gestation (Couto et al., 2019). There is an association between insufficient progesterone production from the CLand early embryonic loss (Mann et al., 2006). Research has indicated that induced ovulation from exogenous GnRH administration is a contributing factor to decreased pregnancy rates as a sub-mature follicle may ovulate, decreasing oocyte competence (Arlotto et al., 1996; Perry et al., 2005), decrease in preovulatory concentrations of estradiol (Jinks et al., 2013; Dickenson et al., 2016), and postovulatory progesterone concentrations (Atkins et al., 2010; Perry et al., 2005)

GnRH induced ovulation:

Current ES protocols utilize GnRH to induce ovulation. The injection of GnRH ovulates follicles within a range of sizes (Perry et al., 2005). This range may result in the ovulation of sub-mature follicles. Ovulating follicles that have not reached full ovulatory capacity negatively affects pregnancy rates and embryonic survival (Perry et al., 2015). Follicles induced to ovulate that were smaller than 11.3 mm in diameter after a GnRH-induced ovulation led to decreased pregnancy rates (Lamb et al., 2001; Perry et al., 2005; Perry et al., 2007). Additionally, induced ovulation of small follicles increased late embryonic loss or fetal mortality. Perry et al. (2005), found induced ovulation of follicles <11 mm had a pregnancy rate of approximately 43% and 39% of pregnant females experienced late embryonic loss or fetal mortality. Cows with follicles that were 12.8 mm in diameter had a pregnancy rate of 68.0 ± 4.9% (Perry et al., 2007). Heifers that had follicles that were <10.7 mm or > 15.7 mm had lesser pregnancy rates compared to those that had follicles of 12.8 mm (Perry et al., 2007). Furthermore, heifers that exhibited standing estrus had increased follicle diameter, concentrations of estradiol and pregnancy rates

compared to those that did not (63% compared to 20% pregnancy rates, respectively; Perry et al., 2007). Increased follicle size is correlated to increased oocyte competence (Arlotto et al., 1996). The induction of ovulation of immature follicles results in shorter estrous cycles, reduced embryo survivability, and decreased luteal function (Santos et al., 2004; Perry et al., 2007).

There is a correlation among pregnancy rates, size of follicles, and size of CL. Perry et al. (2007) described a curvilinear association between follicle size and pregnancy rates. The bovine follicle achieves ovulatory capacity at a diameter of 10 mm (Martinez et al., 1999; Sartori et al., 2001; Perry et al., 2007). Smaller follicles (<11.5 mm) have smaller CL; therefore, progesterone secretion is decreased (Vasconcelos et al., 2001; Perry et al., 2007). Concentrations of progesterone must be secreted in adequate amounts from the CL to support pregnancy (McNatty et al., 1979). The number of large luteal cells in the corpus luteum is positively correlated to progesterone being secreted (McNatty et al., 1979). This was established through in vitro studies that followed ablation and replacement technique to establish the steroidogenic capacity of cells. As the number of large luteal cells increases, progesterone will also increase. Luteal cells originate from the theca interna and externa cells of the dominant follicle; which is where steroidogenesis of progesterone and estradiol occur. Large follicles will luteinize into large CL which will secrete more progesterone (Vancocelos et al., 2001). Follicles and CL affect cyclicity and ovarian dynamics.

Ovary dynamics

Ovarian structures change throughout the estrous cycle. In beef cattle, the right ovary houses the ovulatory follicle approximately 60% of the time (Ginther, 2016; Karamishabankareh et al., 2015). Additionally, the right ovary of beef females has a greater response to superovulation and embryos generated from the right ovary have increased cleavage and

blastocyst rates (Ginther, 2019; Karamishabankareh et al., 2015). In humans, at the fetal stage, the right ovary is more developed than the left as indicated by increased weight and protein and DNA content (Mittwoch and Kirk, 1975). With the right ovary biologically favored for ovulation in cattle, a positive intraovarian relationship forms between the preovulatory follicle and CL (Ginther, 2019).

The positive intraovarian relationship between follicles and CL include angio-coupling resulting in more efficient intraovarian blood perfusion (Ginther, 2019). This vascular relationship is positive during the luteal phase for both the CL and preovulatory follicle. However, a negative effect can be found on the largest pre deviated follicle during luteolysis (Ginther, 2019). The relationship between adjacent CL and dominant follicles produced during the first and second follicular wave can have an angio-coupling mechanism, in turn affecting blood perfusion to ovarian structures (Pugliesi et al., 2019; Ginter et al., 2016; Domingues et al., 2018). The idea of functional angio-coupling, where a dominant follicle or CL signals for either an increase or decrease in blood flow, affects either structure (Ginther et al., 2016). During the luteal phase, the CL and adjacent dominant follicle experience a two-way coupling positively effecting both due to an increase in blood flow demonstrated by an increased percentage of color doppler signals of blood flow (Ginther et al., 2016). However, during luteolysis the regressing CL negatively impacts the adjacent dominant follicle due to vasoconstriction (Ginther et al., 2016).

Estradiol

Anabolic steroids like estradiol 17β are excreted from ruminants by changing estradiol into estrone and then into estradiol 17α, which possesses lower estrogenic capabilities than

estradiol 17β (Rico, 1981). Estradiol 17β is secreted by granulosa cells of a follicle (Moon et al., 1978) and estradiol 17α can be found in bile (Rico, 1981). Endogenous estradiol will peak 36 hours before ovulation (Chenault et al., 1975). Estrogens, such as estradiol 17β are metabolized by the liver and has a half-life of 30 minutes (Rico, 1981).

Estradiol causes sexual behavior (Ford and Oochio, 1988), secretions in the female reproductive tract, uterine tone, and increased blood flow (Rodriguez- Martinez et al., 1986). Estradiol plays a role in the transport of spermatozoa to the site of fertilization by decreasing uterine pH. Decreasing uterine pH slows motility of sperm and extends the viable life span of sperm in the reproductive tract (Jones et al., 2000). Additionally, estradiol plays a role in sperm capacitation. Sperm incubated in oviduct specific – estradiol associated glycoproteins had higher rates of fertilization compared to sperm incubated in oviduct specific glycoproteins (King et al., 1994). Estradiol is also instrumental in glycoprotein secretion (Dickinson et al., 2016; Buhi et al., 2002), embryo development (Spencer and Bazar, 2004), and secretion of progesterone from the corpus luteum (Dickinson et al., 2016; Atkins et al., 2013). Peters et al. (1997) compared conception and pregnancy rates of cattle that received prostaglandin and estradiol benzoate to those that only received prostaglandin. They concluded estradiol benzoate did not significantly change pregnancy rates (Peters et al., 1997). However recent research has shown that incorporating estradiol into estrus synchronization protocols increases pregnancy rates as preovulatory estradiol is important for pregnancy establishment (Jinks et al., 2012; Dickinson et al., 2016; Martinez et al., 1998).

Preovulatory estradiol:

The growing follicle synthesizes increasing amounts of estradiol as it increases in size. Synthesis of estradiol by the follicle can be described by the two cell – two gonadotropin theory described by Fortune and Quirk (1988). It is imperative that the dominant follicle attains final maturation as maturation of the follicle is related to maturation of the oocyte (Pohler et al., 2012). Preovulatory secretion of estradiol affects the uterine and oviduct environment through inducing uterine receptors and uterine proteins (Bartol et al., 1981; Pohler et al., 2012). Estradiol is integral in the formation of gap junctions between granulosa cells, increased LH receptors, increased cellular proliferation, increased stimulatory action of FSH on aromatase activity, and enhanced stimulation of progestin synthesis after gonadotropin stimulation (Perry et al., 2005). There is a strong relationship between serum estradiol concentration, follicle size and pregnancy rates (Atkins et al., 2010). The exact mechanism of how preovulatory estradiol effects pregnancy establishment remains unknown (Pohler et al., 2011).

Industry usage of estradiol

There are synthetic variations of estradiol that have been approved for use in cattle production in the United States and South America. Estradiol Cypionate (ECP) was approved for use to treat anestrous females and ovarian cysts. Estradiol cypionate causes an increase in circulating estradiol 4 to 8 hours after injection (Madsen et al., 2015). The dairy industry utilized ECP in the synchronization protocol Heat synch (Stevenson, 2004). Estradiol valerate is a longer acting form of estradiol. Estradiol benzoate (EB) is the synthetic analog to estradiol 17β with a similar half-life. Estradiol benzoate induces ovulation 20.5 hours after injection (Stevenson, 2004). After the injection of EB a two to three-fold increase in circulating estradiol can be observed compared to ECP (Madsen et al., 2015). This rise in circulating estradiol can be

measured until ovulation (Madsen et al., 2015). In South America, estradiol is commonly used in estrus synchronization protocols. Additionally, Jinks et al. (2012), found that recipient cattle with increased amounts of circulating estradiol had increased pregnancy rates from embryo transfer.

Estradiol benzoate can also be used as a growth promoter in feedlot cattle. Growth implants such as Synovex- C and Synovex B use a combination of estradiol benzoate and trenbolene acetate to improve performance in feedlot heifers (Herschler et al., 1995). Estradiol cypionate and estradiol valerate were once approved for use in the United States but due to a limited market pharmaceutical companies ceased production of these products. Therefore, the FDA withdrew its approval of estradiol for use in synchronization protocols (Lauderdale, 2015).

Progesterone

Large follicles will luteinize into large CL which will secrete more progesterone (Vansconcelos et al., 2001). Vasconcelos et al (2001) reported follicles larger than 15mm secreted 3.06 ng/mL compared to follicles smaller than 14 mm secreted 2.48 ng/mL. Progesterone is needed in adequate concentrations to support and maintain pregnancy (McNatty et al., 1979). Pugliesi et al. (2015) evaluated the effects of long-acting progesterone and size of the preovulatory follicle on fertility in suckled beef cows. Four experiments were performed to evaluate ovarian dynamics and pregnancy rates. In all experiments, cattle received an ES protocol and transrectal ultrasonography was used to determine size of the preovulatory follicle, size of the CL, and vascularization of the CL. Cattle were categorized based on the size of the preovulatory follicle as small (< 10 mm) or large (> 10 mm). Cattle were then assigned to a control group or a long-acting progesterone group. In this study, a smaller, less vascularized preovulatory follicle decreased ovulation rate, pregnancy per artificial insemination, and led to CL with decreased function. Cattle with small follicles subjected to progesterone

supplementation compared to cattle with large follicles subjected to progesterone supplementation were statistically similar when comparing pregnancy rates (Pugliesi et al., 2015).

In conclusion, large CL's will secrete increased concentrations of progesterone compared to small CL's. Progesterone is needed for maintenance of pregnancy. Presence of vascularization of the pre ovulatory follicle effects CL function positively.

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CHAPTER II

EFFECTS OF ESTRADIOL BENZOATE ON FEMALE REPRODUCTIVE CHARACTERISRICS IN BEEF CATTLE

Introduction

Reproductive efficiency is an important economic trait in beef cattle operations. Reproductive failure is estimated to cost the beef industry $500 million annually (Bellows et al., 2002). Goals of cow-calf producers include having females calve early in the calving season and weaning heavy calves. Assisted reproductive technologies (ART) may help producers achieve these goals and increase reproductive efficiency. Beef cattle producers have been slow to adopt ART, such as estrus synchronization (ES), due to the initial cost, labor, and lack of facilities (Lamb et al., 2016). However, improving pregnancy rates when using ART could increase usage in the beef industry.

Current ES protocols follow a gonadotropin releasing hormone (GnRH) – prostaglandin (PGF) – GnRH sequence to synchronize estrus (Pohler et al., 2012). In most protocols, GnRH is administered to induce ovulation – but this could include ovulation of sub-mature follicles which may have a negative effect on pregnancy rates (Perry et al., 2005). Incorporating estradiol in an ES protocol may enhance pregnancy rates as preovulatory concentrations of estradiol are important for establishment of pregnancy (Jinks et al., 2012; Dickinson et al., 2016; Martinez et al., 1998). However, we lack knowledge on implications of estradiol on follicles and subsequent CL.

Therefore, the objectives of this study were to determine if exogenous estradiol affects blood perfusion to follicles and CL in the ovary and to determine if exogenous estradiol alters uterine blood perfusion. Our hypothesis was that exogenous estradiol would increase blood perfusion to dominant follicles, CL, and the uterus.

Materials and methods

All procedures in this study were approved by the Institutional Animal Care and Use Committee of Mississippi State University and followed the FASS Guide for the Care and Use of Agricultural Animals in Research and Teaching.

Animals and treatment

A total of 47 suckled beef cows (range of 2 to 9 years, mean of 4 years of age; mean of 45 d post-partum) at the H. H. Leveck Animal Research Center – Beef Unit (Mississippi State, MS) were initially enrolled in the experiment. Animals were randomly divided into 2 replicates and started the experiment one day apart which allowed time for the same technician to ultrasound all appropriate animals on the same day relative to treatment. All cows received an injection of GnRH (2 mL i.m.; Factrel, Zoetis, Kalamazoo, MI, USA) and a vaginal insert containing 1.38 g of progesterone (Eazi-Breed CIDR, Zoetis) on day -9 (d 0 = expected day of estrus). Seven days later, an injection of $PGF_{2\alpha}$ (5 mL i.m.; Lutalyse, Zoetis) was administered with CIDR removal along with application of an Estrotect patch (Estrotect Breeding Indicator, Estrotect Rockway Inc, Spring Valley, WI, USA; day -2). On day -1, cows were randomly assigned to one of two treatments: either no additional treatment (n = 23; Control) or an injection of estradiol benzoate (1 mg/2 mL i.m.; n = 24; E2). Two hundred and fifty milligrams of

estradiol benzoate (Sigma Aldrich E8515) was mixed with 50 mL benzyl alcohol to dissolve and was then added to 450 mL sesame oil. After mixed, solution was stored in an amber glass container. Final solution contained 0.5 mg estradiol benzoate per 1 mL of solution. Cattle were observed for signs of estrus twice per day (AM/PM) and Estrotect patchs were observed and scored on a scale of 1 to 4, based on amount of color present (1 = 25% color, 2 = 50% color, 3 = 75% color, and 4 = 100% color showing). If females had a score of 3 or greater, she was recorded as displaying standing estrus. Cattle that displayed primary signs of standing estrus between day 0 and 3 received an embryo on day 7.

Blood samples and analysis

Blood samples were obtained by venipuncture of the coccygeal vein or artery in a 10 mL serum tube (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA) on days -1 and 0 for all cows and days 6, 14, 21 and 28 only for cows that received an embryo. Blood samples were immediately placed on ice. Once returned to laboratory, samples were centrifuged for 20 min at 2,000 × *g* at 4°C. Serum was removed and frozen at -20°C for later analysis. Circulating concentrations of estradiol-17β were analyzed in serum samples from days -1 and 0 in a single assay by RIA using methodology described by Perry and Perry (2008). The intra-assay CV was 3.19%. Assay sensitivity was 0.4 pg/mL. Circulating concentrations of progesterone were analyzed in serum samples from days -1, 0, 6, 14, 21, and 28 and were determined via RIA in the same laboratory. The intra-assay CV was 3.87%.

Ultrasonography

Transrectal ultrasonography (10.0- to 5.0-MHz linear-array transducer, MicroMaxx, Sonosite Inc., Bothell, WA, USA) was used to identify blood perfusion to dominant follicles and CL. Using the Doppler function of the ultrasound, three images were saved, analyzed, and averaged for the amount of perfusion to the tissue. Perfusion was measured using imageJ (version 1.47; U.S. National Institutes of Health, Bethesda, MD, USA) to count the number of pixels of perfusion to the dominant follicle or CL.

Laser Doppler perfusion was used, according to Owen et al., 2018, to determine blood perfusion to the uterine body at day -1, 0, and 6. An artificial insemination (AI) rod with a sheath was passed through the cervix into the uterine body. Once the rod placed, the rod portion was removed leaving the sheath in place. The laser was inserted and passed through the AI sheath. Perfusion was recorded for 10 s while the animal was still. Mean perfusion units were analyzed.

Embryo transfer

Embryos were transferred into the 29 cows which exhibited standing estrus. Frozen grade 1 and 2 embryos, from previous collections, were thawed at 23.9° C for 20 s. Embryos at differing stages of development were matched to the recipient based on timing of estrus. Embryos were transferred on d 7 according to guidelines of the International Embryo Transfer Society (Savoy, IL, USA) and were placed in the uterine horn ipsilateral to the CL.

Statistical analysis

The mixed model procedure of SAS (SAS software version 9.4; SAS Inst. Inc., Cary, NC, USA) was used for all statistical analyses. The final model for blood perfusion of the dominant

follicle included cow (random variable), treatment, replicate and size of the follicle. The final models for blood perfusion of the uterus on d -1 and 0 included cow (random variable) treatment, replicate and concentration of estradiol. The final models for blood perfusion of the CL on d 6, 14, and 21 included cow (random variable), treatment, replicate, d 0 follicle perfusion, and d 0 follicle size. The final model for size of the follicle included cow (random variable), treatment, replicate, and blood perfusion. The final model for concentration of estradiol included treatment, replicate, size of the follicle, and perfusion of the follicle. The final model for concentration of progesterone included treatment, replicate, size of the CL, and perfusion of the CL. All 2-way interactions were initially included and they, along with other variables initially tested, were removed in a reverse step-wise fashion when *P*-values were greater than 0.10.

A chi-square analysis (frequency procedure of SAS) was used to analyze standing estrus by treatment group.

Repeated measure analysis (mixed procedure of SAS) was used to analyze the effect of day on uterine blood perfusion (d -1, 0, 6) and CL size (d 6, 14, and 21). Several covariance structures were tested and those with the lowest AIC and BIC was used for analysis. The variance component covariance structure provided the lowest AIC and BIC for the models. All 2-way interactions were initially included and they, along with other variables initially tested, were removed in a reverse step-wise fashion when *P*-values were greater than 0.10.

When statistically significant, means were separated using the PDIFF option of the LSMEANS statement; least squares means and SEM are reported. Statistical significance was declared at $P \leq 0.05$.

Results and discussion

After treatment with EB on d -1, concentrations of estradiol in systemic circulation on day 0 were greater ($P = 0.003$) in cows in the EB group, compared to cows in the control group (21.35 ± 4.49 vs 9.45 ± 4.61 pg/mL, respectively) (Figure 1.2). This indicates treatment with EB on d -1 increased circulating concentrations of the hormone. Total, 61% of all cows exhibited standing estrus. More ($P < 0.001$) cows that received EB exhibited standing estrus compared to cows in the control group (21 of 24 [87.5%] vs 8 of 23 [29.6%], respectively) (Figure 1.1). Cook et al (1986) reported that when 500 μg estradiol benzoate and 10 cc of saline solution were administered, 80% of cattle exhibited estrus. In comparison, when 200 μg estradiol benzoate and 500 μg of GnRH were administered, only 60% of cattle exhibited estrus. The interval from administration of EB to the onset of standing estrus was, on average, 15.5 h (Cook et al., 1986). The duration of estrus was not altered by the injection of EB (Cook et al., 1986).

Treatment with EB did not affect blood perfusion of the uterus, size of the dominant follicle, or blood perfusion of the dominant follicle on d 0, as values were similar among cows in the EB group versus control group (Table 1.1 and Table 1.2, respectively). This was somewhat unexpected because estrogen increases blood flow to the female reproductive tract through diminishing activity of periarterial alpha adrenergic receptors (Miller and Duckles, 2008). Blood perfusion is dependent upon blood flow (Carr, 2012). We would expect that when estradiol increases blood flow, blood perfusion would also increase. There was an effect of day on uterine blood perfusion ($P = 0.001$) on d -1, 0, and 6. Uterine blood perfusion was lower on d 6 compared to d -1 and d 0 ($P = 0.001$ and $P = 0.0002$, respectively) which were similar ($P = 0.6996$) (Figure 1.4) . However, while using laser Doppler, Owen et al. (2018) found a negative

correlation between the concentration of estradiol and blood perfusion to the uterine horn ipsilateral of a dominant follicle in cattle.

In cows that exhibited standing estrus and thus were further included in data collection, treatment with EB did not affect size of the CL on d 6 or 14 (Table 1.3). However, EB did (P = 0.0211) cause a smaller CL on d 21 compared to cows in the control group, although it should be noted a small number of total animals had a CL on d 21 (Table 1.3). On all day's post-estrus (d 6, 14, and 21), blood perfusion of the CL was similar between cows in the EB and control groups (Table 1.3). There were no treatment effects and perfusion stayed relatively similar in the CL from d 6 to d 14 and d 21 (5.9, 6.5, and 6.0 area of perfusion, respectively) (Table 1.3). This could be due to normal concentrations of estradiol decreasing in metestrus and diestrus and concentrations of progesterone increasing in metestrus and diestrus. Progesterone has vasoconstrictive properties (Cook et al., 1986) and may influence blood perfusion of the CL in diestrus.

However, blood perfusion of the CL on d 6 was different (P = 0.0069) among cows in the two replicates. Cows in replicate 1 averaged 1.1588 ± 0.7336 area of perfusion and cows in replicate 2 averaged 9.4888 ± 0.5686 area of perfusion. This difference was driven by 3 cows in replicate 2 that had unusually elevated blood perfusion relative to the others while cows in replicate 1 were more consistent. This Effect continued (P = 0.0399) on d 14 with cows in replicate 1 and 2 averaging 4.6610 ± 0.7295 and 7.2686 ± 0.7431 area of perfusion, respectively. However, on d 21 values became similar (P = 0.5492) with cows in replicate 1 and 2 averaging 6.12 ± 0.66 and 6.35 ± 1.07 area of perfusion, respectively.

Size of the dominant follicle on d 0 also affected (P = 0.0173) blood perfusion of the CL on d 6 with large follicles leading to more perfusion compared to smaller follicles. Large follicles

resulted in a CL with 9.40 ± 1.3812 area of perfusion compared to small follicles which resulted in a CL with 4.66 ± 0.9977 area of perfusion. This effect did not continue to d 14 and 21. As expected, day affected CL size ($P < 0.0001$). On d 14 CL size was larger compared to d 6 and 21 ($P = 0.0005$ and $P < 0.0001$, respectively), which were similar ($P = 0.1768$) (Figure 1.4). The relationship between adjacent CL and dominant follicles produced during the first and second follicular wave can have an angio-coupling mechanism, in turn affecting blood perfusion to ovarian structures (Pugliesi et al.,2019; Ginther et al., 2016; Domingues et al., 2018). The idea of functional angiocoupling, where a dominant follicle or CL signals for either an increase or decrease in blood flow, affects either structure (Ginther et al., 2016). During the luteal phase, the CL and adjacent dominant follicle experience a two-way coupling positively affecting both due to an increase in blood flow demonstrated by an increased percentage if color doppler signals of blood flow (Ginther et al., 2016). However, during luteolysis the regressing CL negatively impacts the adjacent dominant follicle due to vasoconstriction (Ginther et al., 2016). During this experiment, record of adjacent structures was not recorded. Therefore, adjacent structures or lack thereof may contribute to the differences in blood perfusion.

On d 6, concentration of progesterone was influenced by replicate ($P = 0.0130$), size of the CL on d 6 ($P = 0.0421$) and tended ($P = 0.0913$) to be influenced by treatment of EB. Blood perfusion of the CL did not influence ($P > 0.05$) concentrations of progesterone on d 6, 14, or 21. Concentration of progesterone on d 0 were increased ($P = 0.0265$) by treatment, with treated animals having 1.1543 ± 0.1034 and control animals having 0.8177 ± 0.1024 ng/mL. However, 13 cows did not respond appropriately to the ES protocol and had a CL present on d 0 and did not exhibit standing estrus thus, were removed from further analysis. Size of the dominant follicle on day 0 did not influence concentrations of progesterone on day 0, nor did we detect any

influence of blood perfusion of the follicle on concentrations of progesterone. Similarly, Perry et al. (2005) reported that follicle size did not affect concentrations of progesterone of cows that ovulated spontaneously. Size of the dominant follicle can affect the size of the CL, although we did not see that effect in the current study. When ovulation is induced through administering exogenous hormones, the resulting CL and production of progesterone may be affected. Interestingly, Herzog et al. (2010) found a positive correlation between luteal volume and concentrations of progesterone. Although we did not detect an effect of blood perfusion to the CL on concentrations of progesterone, Pugliesi et al. (2019) concluded that increased blood perfusion to luteal tissue increased circulating concentrations of progesterone and incidence of pregnancy in bos indicus-influenced cattle. However, Pugliesi et al. (2019) measured blood perfusion via visual observation rather than quantitatively. In dairy cattle, Voelz et al. (2015) did not find a correlation between blood perfusion of the CL and concentration of progesterone.

Concentrations of progesterone were different between cows declared pregnant and or non-pregnant females on d 28, on d 14, 21 and 28 $P = 0.0573$, 0.0021, and <0.001, respectively (Figure 1.5). Concentrations of progesterone were different among females that received estradiol to those that did not on d 21 (P =0.0294) (Figure 1.5).

Conclusion

In conclusion, estradiol causes vasodilation and positively affects the female reproductive tract in preparation for pregnancy. It was hypothesized that administration of EB on day -1 would increase blood perfusion to the dominant follicle, CL, and uterus. In conclusion, administration of EB on day -1 increases the number of females that display estrus and increases circulating concentrations of estradiol compared to females that do not receive estradiol. Females

treated with estradiol did not have an increase in uterine blood perfusion. Additionally, females treated with estradiol did not differ in blood perfusion or size of dominant follicle when compared to those that did not receive treatment. Differences in size of CL between females treated with estradiol and those that did not receive treatment were found on d 21. Interestingly, there was an effect of replicate on day 6 and 14 on CL blood perfusion. More research on the effect of preovulatory estradiol on CL function needs to be conducted to gain understanding of why concentrations of preovulatory estradiol influence pregnancy rates.

Table 2.1 LSmeans ± standard errors for blood perfusion of the uterus on days -1, 0, and 6 relative to d of expected standing estrus.

	Control n = 23	Estradiol n = 24	*P*-value
Blood perfusion of uterus, d -1, perfusion units	453.40 ± 88.46	403.83 ± 89.09	0.9575
Blood perfusion of uterus, d 0, perfusion units	274.76 ± 89.85	407.18 ± 87.43	0.8545
Blood perfusion of uterus, d 6, perfusion units	125.12 ± 26.37	120.42 ± 15.76	0.8826

[+]Cows were treated with estradiol benzoate (1ng/2mL) on day -1
Uterine blood perfusion is presented as perfusion units on day -1, 0, and 6. Standard errors for each mean are presented. Statistical significance was determined at $P \leq 0.05$.

Table 2.2 LSmeans ± standard errors for size of the dominant follicle and blood perfusion of the dominant follicle on d -1 (day of treatment with estradiol benzoate) and 0

	Treatment		
	Control n = 23	Estradiol n = 24	*P*- Value
Size of dominant follicle, d -1, mm	13.15 ± 1.02	12.55 ± 1.02	0.1241
Size of dominant follicle, d 0, mm	13.09 ± 1.60	15.87 ± 1.57	0.7937
Blood perfusion of dominant follicle, d -1, perfusion area	7.14 ± 0.71	9.98 ± 0.66	0.0646
Blood perfusion of dominant follicle, d 0, perfusion area	7.30 ± 0.77	7.13 ± 0.76	0.8734

Table 2.3 LSmeans ± standard errors for size and blood perfusion of the CL on d 6, 14, and 21 relative to treatment with estradiol benzoate on d -1.

	Treatment		
	Control n = 8[a]	Estradiol n = 21[a]	*P*-value
Size of CL, d 6, mm	18.79 ± 1.13	19.72 ± 0.66	0.4910
Size of CL, d 14, mm	21.35 ± 1.53	21.45 ± 0.87	0.9558
Size of CL, d 21, mm	19.96 ± 0.90	17.31 ± 0.49	0.0211
Blood perfusion of CL, d 6, perfusion area	10.44 ± 1.43	10.91 ± 1.33	0.8131
Blood perfusion of CL, d 14, perfusion area	7.08 ± 1.34	7.28 ± 0.75	0.8981
Blood perfusion of CL, d 21, perfusion area	8.06 ± 1.07	7.13 ± 0.57	0.6222

[a] includes animals that were observed in standing estrus and received an embryo on d 7.

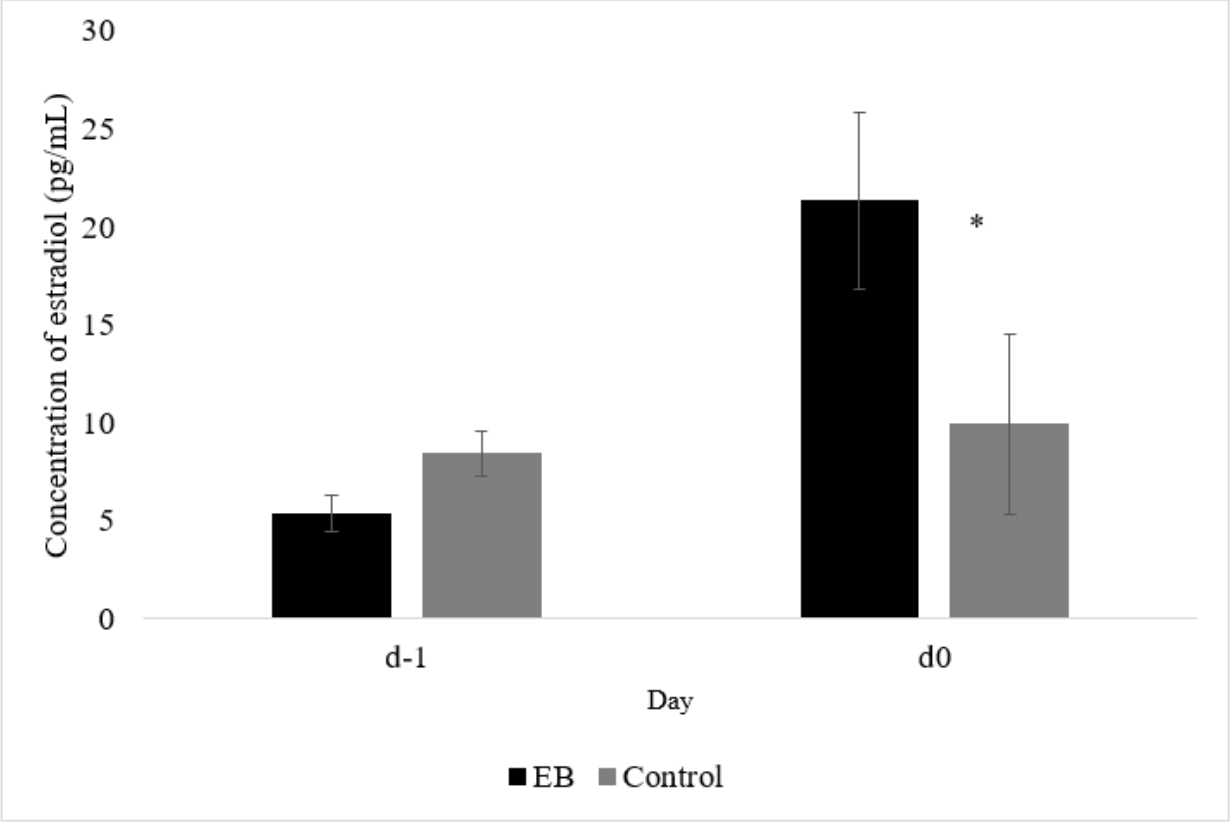

Figure 2.1 LSmeans ± standard errors for concentrations of estradiol on days -1 and day 0.

* ($P < 0.0003$)

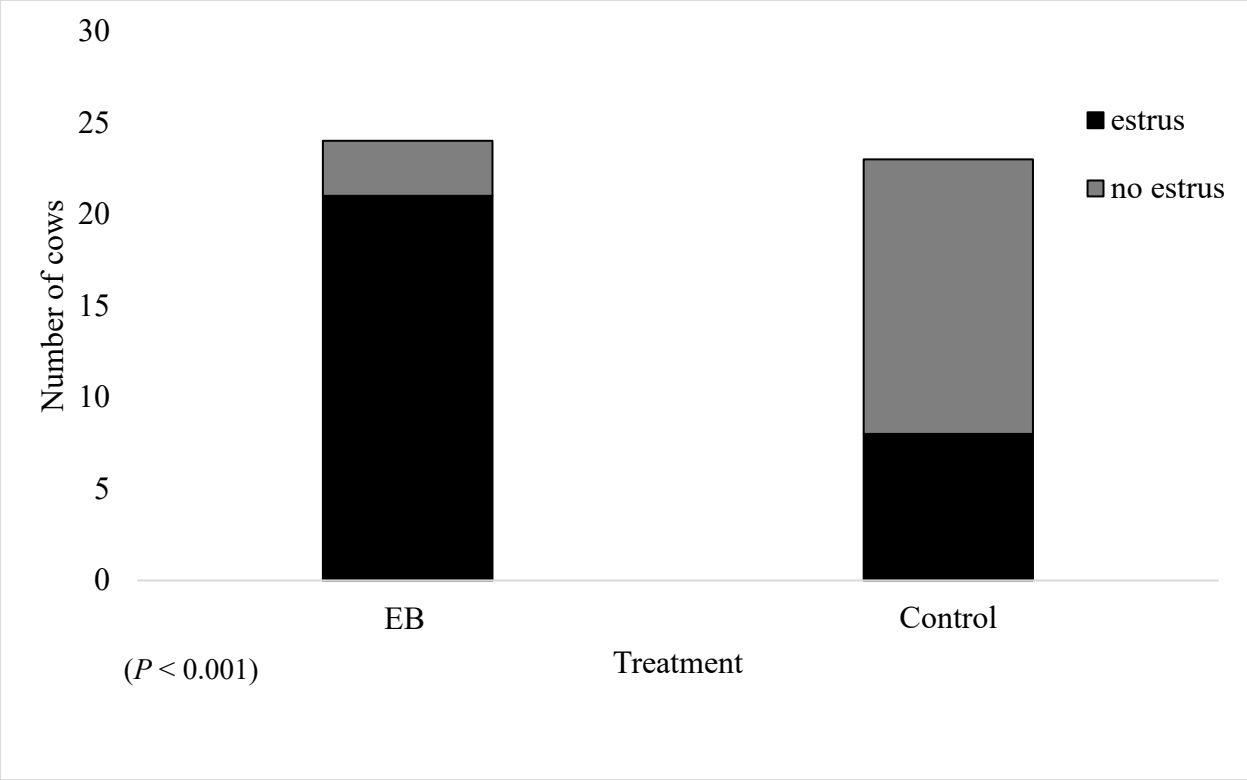

Figure 2.2 Incidence of standing estrus comparing the treatment of estradiol benzoate (1ng/2mL) versus control group.

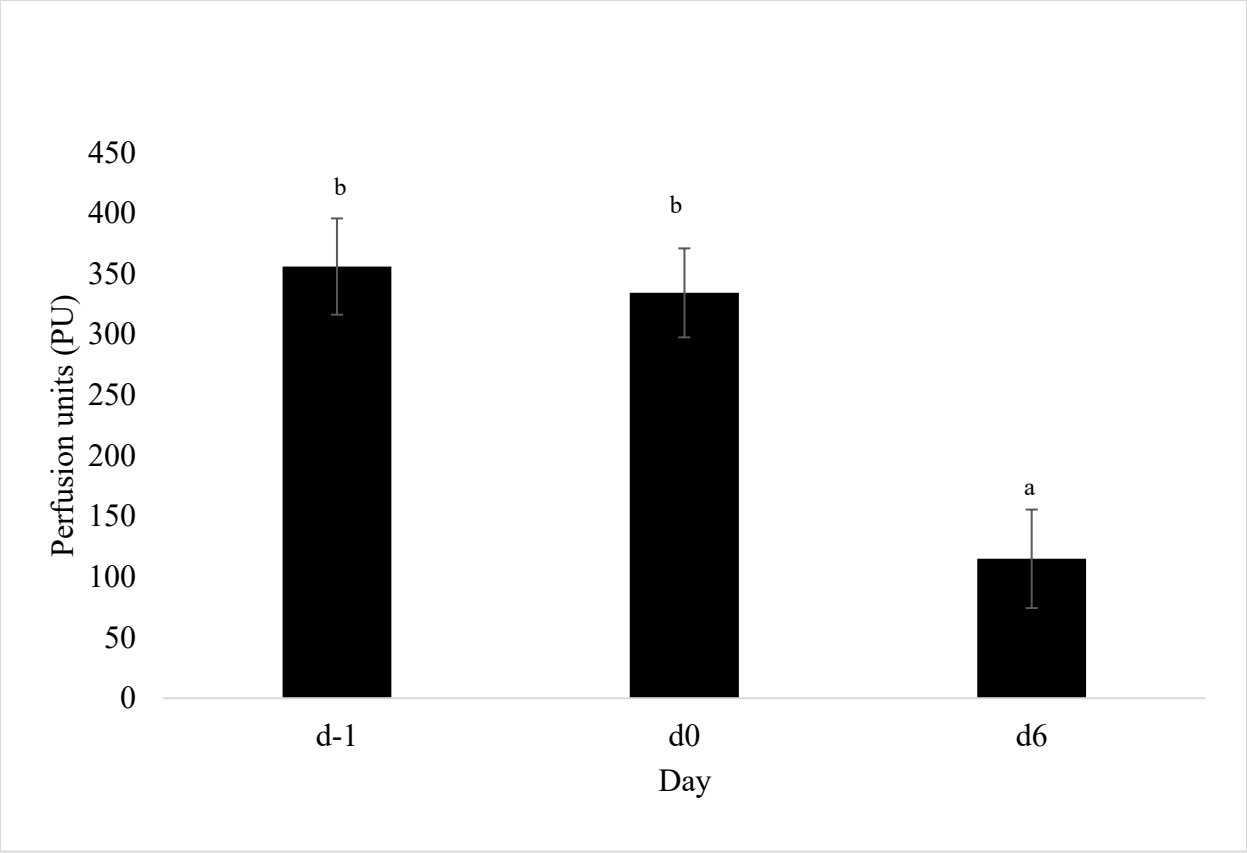

Figure 2.3 LSmeans ± standard errors for uterine blood perfusion in perfusion units on d -1, 0, and 6.

Uterine blood perfusion on d 6 was decreased compared to uterine blood perfusion on d -1 and 0.

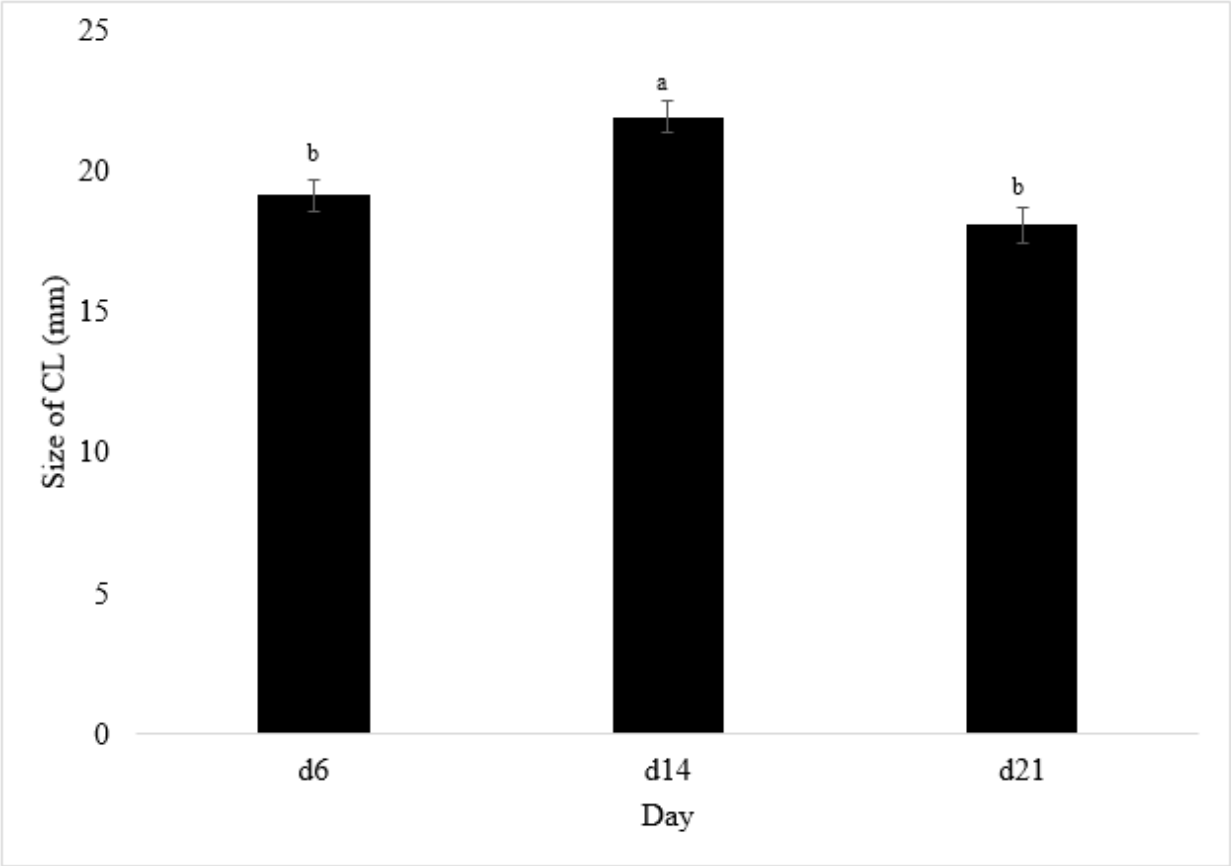

Figure 2.4 LSmeans ± standard errors for CL size on d 6,14, and 21 in recipients that received an embryo on d 7, in both the treatment and control group.

Day influenced ($P < 0.0001$) size of CL. Day 14 had increased CL size compared to d 6 and 21.

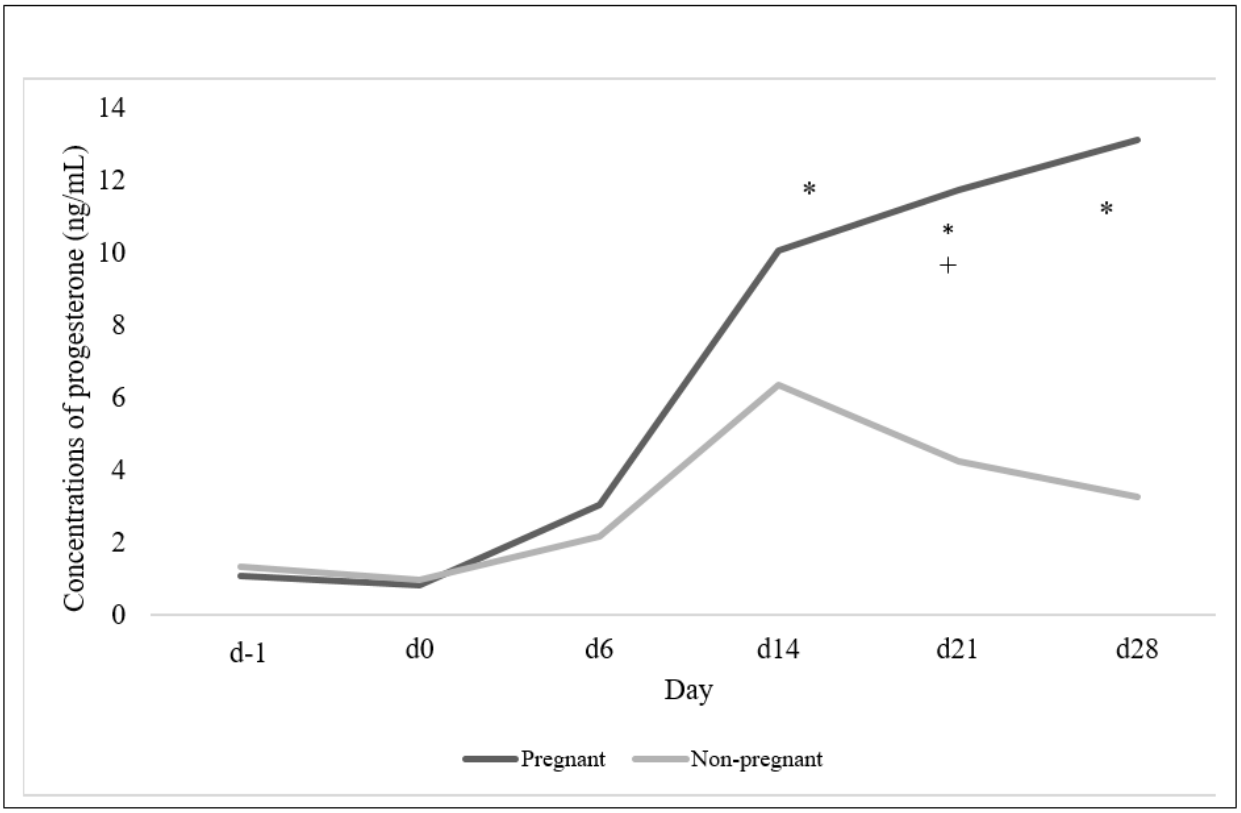

Figure 2.5 Concentrations of progesterone throughout the estrous cycle comparing pregnant and non-pregnant females.

Concentrations of progesterone were different between pregnant and non-pregnant females, as determined on d 28, on d 14, 21 and 28 P = 0.0573, 0.0021, and <0.001, respectively. Concentrations of progesterone were different among females that received estradiol to those that did not on day 21 (P =0.0294).

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