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Understanding the influence of synchronization of ovulation on steroid bioavailability and its association with the presence of *Pseudomonas aeruginosa* within the bovine reproductive tract

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To the Graduate Council:

I am submitting herewith a dissertation written by Sierra Ashley Lockwood entitled "Understanding the influence of synchronization of ovulation on steroid bioavailability and its association with the presence of *Pseudomonas aeruginosa* within the bovine reproductive tract." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

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Dedication

This dissertation is dedicated to my great grandfather, Donald L. Outhouse.

Through his example, I developed a love and passion for agriculture.

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Abstract

Corticosteroid-binding globulin (CBG) transports glucocorticoids and progesterone, but little is known about CBG in the bovine reproductive tract. *Pseudomonas aeruginosa* is an environmental bacteria capable of colonizing the vaginal cavity of other species. *P. aeruginosa* presence within the bovine vaginal cavity is not well characterized, yet evidence suggests that a controlled internal drug release device (CIDR) can alter the bacterial abundance within the cavity. *P. aeruginosa* produces a protease that cleaves the reactive center loop of CBG allowing for the release of the steroid into its active form. Thus, the objective of this study was to examine effects of progesterone released from a CIDR on circulating and vaginal concentrations of CBG, the proportion of free progesterone and cortisol, and presence of *P. aeruginosa* following synchronization of ovulation. Prior to CIDR insertion (d -7) and following removal (d 0), blood and vaginal flush samples were collected from each heifer (n=67). Plasma collected at pregnancy diagnosis (d 38) from pregnant heifers (n=24) had greater (P=0.02) concentrations of progesterone than samples collected on d -7, but were similar to those measured on d 0. Similarly, plasma CBG concentrations measured on d -7 and 0 did not differ, but were greatest (P=0.03) on d 38. The free progesterone index (FPI) calculated from progesterone and CBG concentrations measured on the three sampling days were not different (P=0.16). During the CIDR insertion period, *P. aeruginosa* abundance decreased (P<0.0001), and on d 0, *P. aeruginosa* abundance was related to both CBG concentration (r=-0.25; P=0.05) and FCI (r=0.37; P=0.004). The third study aimed to determine if CBG mRNA is expressed

within the vagina and uterus of abattoir-sourced reproductive tracts (*n*=3) to determine the source of CBG within the vaginal cavity. In the tissues collected, CBG gene was not expressed within the vaginal epithelium, but was expressed in the uterine endometrium. In summary, CIDR insertion did not affect CBG concentrations, but CBG may regulate the free fraction of progesterone in circulation during early gestation. Additionally, *P. aeruginosa* within the vaginal cavity of heifers may alter the free proportion of cortisol as seen by a reduction in CBG concentration.

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Introduction

Infertility poses a major economic detriment to the United States cattle industry. It has been estimated that reproductive inefficiency constitutes a combined loss of approximately $900 million per year within the dairy and beef cattle industries (Bellows et al., 2002). With the world's population expected to grow in excess of 9 billion people by the year 2050 (Godfray et al., 2010), research efforts focused on improving the reproductive efficiency of cattle remains of interest in order to continue the supply of animal-derived protein to our growing population. To bolster reproductive efficiency, the use of estrous synchronization protocols has gained popularity within both cattle industries and relies on the use of coordinated administration of exogenous hormones. Through this management technique, producers can easily identify cows and heifers that fail to conceive during the breeding season and can make informed culling and retention decisions.

The use of a controlled internal drug release device (CIDR) impregnated with progesterone is common among protocols used to synchronize estrus in cows and heifers to allow for coordinated ovulation and to facilitate subsequent artificial insemination. However, a gap in knowledge exists in regards to the effect of exogenous progesterone release from a CIDR on the local endocrine and bacterial profiles within the bovine vaginal cavity. Recently, research has examined the interactions between hormone production and bacterial community profiles that exist within the bovine vagina, and it has been reported that changes in the diversity and abundance of bacteria occur as hormone profiles vary (Laguardia-Nascimento et al., 2015).

Furthermore, during pregnancy, a physiological state characterized by heightened progesterone production, the bovine vaginal microbiome undergoes a reduction in both bacterial diversity and the number of bacteria inhabiting the cavity (Walther-António et al., 2014; Laguardia-Nascimento et al., 2015). Although research has aimed to characterize the existing bacterial community profiles in the bovine vaginal cavity, the direct effect of exogenous progesterone release from a CIDR on the local bacterial community profile remains nebulous.

In regards to women, *Pseudomonas aeruginosa* is a bacterial pathogen that can colonize the vaginal epithelium (Osset et al., 2001). Additionally, studies characterizing the bovine vaginal bacterial community profiles reported that *Pseudomonadaceae*, the family in which *Pseudomonas aeruginosa* belong, is present within the vaginal cavity (Laguardia-Nascimento et al., 2015), and its abundance is augmented by progesterone (Padula and Macmillan, 2006). Notably, this particular bacterial species has been shown to release LasB, a protease that has the ability to cleave the reactive center loop (RCL) of the steroid transporter, corticosteroid-binding globulin (CBG), a member of the serine protease inhibitor super family (Simard et al., 2014; Hammond, 2016). After cleavage of the RCL, steroid hormones such as progesterone and cortisol dissociate from CBG and are then considered free or biologically available, and can serve their physiological functions (Siiteri et al., 1981). Furthermore, it has been reported that under conditions of elevated progesterone production, cortisol is displaced from CBG, and in turn, CBG will primarily bind progesterone (Rosenthal et al., 1969; Hammond,

2016). Therefore, the presence of proteases and local hormone production may both play roles in determining the bioavailability of these steroid hormones.

Thus far, the presence of CBG within the uterine horns of swine and its interaction with cortisol and progesterone concentration has been examined in our lab (Klemcke et al., 1998), but to the best of our knowledge, CBG presence and possible involvement in steroid hormone availability and associated bacterial interaction within the bovine vaginal cavity has not been examined. In the study conducted by Klemcke et al. (1998), it was postulated that CBG presence in the uterine horns of gilts served to shuttle cortisol and progesterone within cavity, but the exact mechanism and source of CBG was not known. It is possible that CBG acts to impede steroid catabolism to ensure a constant steroid reserve (Benassayag et al., 2001). Furthermore, the expression of CBG messenger ribonucleic acid (mRNA) has been reported within various regions of the female reproductive tract in other species (Seralini et al., 1990; Misao et al., 1994), but has not been characterized in cattle. To gain a better understanding of the direct impact of CIDRs used to facilitate coordinated breeding, the main goal of the research included in this dissertation is to investigate the vaginal steroid hormone profile and associated bacterial interactions in beef heifers undergoing synchronization of ovulation. Whereas, the second project in this dissertation aimed to characterize CBG synthesis sites in the female bovine reproductive tract and examine changes in CBG mRNA expression during different stages of the estrous cycle and gestation. Through the completion of these experiments, the expected outcome was to gain a better understanding of bovine CBG and its influence on the reproductive axis,

but to also gain insight as to the local production of CBG within the bovine reproductive tract. Furthermore, investigating the impact of CBG on the bound and biologically available fractions of progesterone within the reproductive tract during synchronization may prove beneficial for further understanding the ability of heifers and cows to become pregnant following estrous synchronization and artificial insemination.

Chapter I

Literature Review

Controlled internal drug release device (CIDR)

Estrous synchronization relies on coordinated administration of exogenous reproductive hormones and many of the protocols developed and approved for use in the United States rely on the use of a controlled internal drug release device (CIDR; Eazi-Breed CIDR, Zoetis, Madison, NJ). In contrast to other exogenous hormones that are administered by injection, CIDRs used to synchronize estrus in cattle are plastic devices that are infused with progesterone (1.38 g; Eazi-Breed CIDR, Zoetis, Madison, NJ, or 1.9 g; Eazi-Breed CIDR, São Paulo, Brazil) and after intravaginal insertion, progesterone is absorbed through the vaginal epithelium and migrates to the vascular system. Within an hour post-insertion, circulating concentrations of progesterone reach a maximum peak and are sustained throughout a 7 d insertion period (Kesler, 2002). Similar to the rapid rise in systemic progesterone concentration after insertion, systemic progesterone rapidly decreases after the CIDR is removed and returns to baseline 8 hr post-removal (Kesler, 2002).

Progesterone released by the CIDR has the capability to promote synchrony among heifers and cows through two mechanisms. During a normal estrous cycle, regression of the corpus luteum (CL) must occur to allow for the onset of estrus. However, at the onset of the estrous synchronization process, cows and heifers may be at various stages of the estrous cycle that may prevent successful luteal regression (Kesler, 2002). Immature CL lack the ability to respond to prostaglandin F2α ($PGF_{2\alpha}$) and luteal regression does not occur until the CL acquires luteolytic capacity at approximately d 6 of the estrous cycle (Tsai and Wiltbank, 1998; Diaz et al., 2000).

Insertion of CIDRs for a period of 7 d prior to $PGF_{2\alpha}$ administration ensures the CL has reached maturity and can undergo luteolysis following $PGF_{2\alpha}$ administration (Lucy et al., 2001; Kesler, 2002).

Another way in which CIDRs are used to synchronize a herd of cattle is by inducing cyclicity in pre-pubertal heifers and anestrous cows (Rhodes, 1998; Lucy et al., 2001; Kesler, 2002). Specifically, Lucy et al. (2001) performed a study to evaluate synchronization rates among pre-pubertal heifers and postpartum cows and reported that heifers and cows administered a CIDR followed by an injection of $PGF_{2\alpha}$ displayed estrus earlier in a 31-d breeding season. Additionally, greater pregnancy rates were observed in heifers and cows that received a CIDR followed by $PGF_{2\alpha}$ when compared to control animals with no hormone therapy and animals that received only an injection of $PGF_{2\alpha}$. During the CIDR insertion period, the low dose release of progesterone prevents follicular wave turn-over and promotes the development of a persistent dominant follicle by increasing luteinizing hormone (LH) pulse frequency (Savio et al., 1993; Kojima et al., 2003). As the persistent follicle continues to develop, estradiol production and release subsequently increases (Kojima et al., 2003). Through positive feedback on the hypothalamus, estradiol promotes the release of GnRH and subsequent LH production and release from the anterior pituitary (Kesner et al., 1981), and in pre-pubertal heifers and anestrous cows and this mechanism allows for the resumption of cyclicity (Anderson et al., 1996; Imwalle et al., 1998). Additionally, the removal of the CIDR and subsequent administration of $PGF_{2\alpha}$ and GnRH, ovulation of the dominant follicle can occur to achieve synchrony and allow for coordinated

insemination. Although the physiological mechanisms behind the use of a CIDR are well characterized, a gap in knowledge exists in regards to the effect of exogenous progesterone release from a CIDR on the local bacterial and endocrine profiles within the bovine vaginal cavity.

Interaction between progesterone and bacterial community profiles

Within the last few years, researchers have started to examine the interactions between hormone production and bacterial community profiles that exist within the bovine vagina, and it has been reported that changes in the diversity and abundance of bacteria occur as hormone profiles vary (Laguardia-Nascimento et al., 2015). Furthermore, during pregnancy, a physiological state characterized by heightened progesterone production, the bovine vaginal microbiome undergoes a reduction in both bacterial diversity and abundance of bacteria inhabiting the cavity (Walther-António et al., 2014; Laguardia-Nascimento et al., 2015). Although research has aimed to characterize the existing bacterial community profiles in the bovine vaginal cavity, the direct effect of exogenous progesterone release from a CIDR on the local bacterial community remains nebulous.

Presence of Pseudomonas aeruginosa in the vaginal cavity

In regards to women, *Pseudomonas aeruginosa* is an aerobic opportunistic bacterial pathogen that has the capability to colonize the vaginal epithelium (Osset et al., 2001; Hammond, 2016). Studies that have characterized the bovine vaginal bacterial community profiles reported that *Pseudomonadaceae*, the family in which

Pseudomonas aeruginosa belong, is present within the vaginal cavity (Laguardia-Nascimento et al., 2015), but the prevalence of this particular bacterial is not well characterized in bovine. Padula and Macmillan (2006) performed an experiment to examine the effect of CIDR (1.9 g progesterone) insertion on bacterial isolates found within the vagina of postpartum Holstein cows. The study was designed such that three treatments were administered (control: no CIDR; CIDR-P4: two intravaginal devices containing progesterone were administered for 14 d; CIDR-Blank: two intravaginal devices without progesterone were administered for 14 d) and vaginal swabs were performed on d 0 (day of CIDR insertion), 7, and 14 to examine culturable bacterial profiles (Padula and Macmillan, 2006). Additionally, CIDRs were swabbed immediately after removal on d 14 to determine bacterial species present on the device. For both CIDR treatment groups, the number of samples positive for *Pseudomonas aeruginosa* increased over the sampling period. Likewise, among the samples collected on d 7 and 14, the presence of *Pseudomonas aeruginosa* was greater in the CIDR-P4 treated cows when compared to the other two treatment groups (Padula and Macmillan, 2006). *Pseudomonas aeruginosa* isolates were also present within the cultures obtained from swabbing the CIDR directly after removal. Padula and Macmillan (2006) indicated there was a potential for cross-contamination between animals, but regardless of contamination, the presence of *Pseudomonas aeruginosa* increased as a result of CIDR insertion.

Although it appears that progesterone released from a CIDR may impact the population of *Pseudomonas aeruginosa* within the vaginal cavity, further investigation is

warranted to dismiss the possibility of contamination (Padula and Macmillan, 2006). Furthermore, the exposure of the vaginal cavity to air during the duration of CIDR insertion may have contributed to the shift in aerobic bacterial populations (Padula and Macmillan, 2006). We suggest that subsequent studies should aim to examine the effect of one CIDR on the vaginal bacterial profiles as only one insert is used in traditional estrous synchronization protocols. Recognizing that only CIDRs containing 1.38 g of progesterone are approved for use in the United States, further investigation is justified to determine if CIDRs impregnated with 1.38 g of progesterone elicit a similar pattern to those observed by Padula and Macmillan (2006).

Influence of Pseudomonas aeruginosa on steroid hormone bioavailability

Pseudomonas aeruginosa has the capability to synthesize and release LasB (*Pseudomonas aeruginosa* elastase), a protease that can cleave the reactive center loop (RCL) of the steroid hormone transporter, corticosteroid-binding globulin (CBG) (Simard et al., 2014; Hammond, 2016). When Simard et al. (2014) incubated human CBG in a variety of bacterial cultures (*Pseudomonas aeruginosa*, *Eschericha coli, Staphylococcus aureus, Burkholderia cenocepacia, Micrococcus luteus, Enterococcus faecalis, Acinetobacter baumannii,* and *Mycobacterium smegmatis*) they observed a 90 % reduction in the cortisol-binding capacity and a 5-10 kDa loss when CBG was incubated in *Pseudomonas aeruginosa* medium. No other bacterial cultures affected the cortisol-binding capacity of CBG. When human CBG was incubated with protease fractions extracted from *Pseudomonas aeruginosa*, only LasB reduced the cortisol-

binding capacity of CBG and proved to have the ability to interfere with the ability of CBG to bind steroids within the RCL (Simard et al., 2014). Based on the study performed by Simard et al. (2014) and the presence of *Pseudomonas aeruginosa* in the bovine vaginal cavity as reported by Padula and Macmillan (2006), further investigation is warranted to determine if this particular bacteria has the ability to influence CBG and free progesterone concentrations during the estrous synchronization process.

Overview of corticosteroid-binding globulin (CBG) in circulation

Corticosteroid-binding globulin is a glycoprotein that is primarily synthesized within hepatocytes (Hammond et al., 1991; Heo et al., 2003a) and functions as a transporter for a small subset of steroid hormones (i.e. glucocorticoids and progesterone) in which it binds with high affinity, but low capacity (Slaunwhite and Sandberg, 1959; Seal et al., 1966; Westphal, 1986a; Heo et al., 2003b). Corticosteroid-binding globulin is a member of the serine protease inhibitor superfamily (Hammond et al., 1987; Law et al., 2006) and acts as a target for proteases (Lin et al., 2010) such as neutrophil elastase (Hammond et al., 1990), chymotrypsin (Lewis and Elder, 2014), and LasB, a protease produced and released by the bacterial species, *Pseudomonas aeruginosa* (Simard et al., 2014). In the presence of CBG, the aforementioned proteases cleave specific sites within the RCL of CBG and compromise the steroid binding capacity (Lewis and Elder, 2017). After cleavage, the steroid dissociates from CBG and can be sequestered and loosely bound by albumin, or remain free within circulation, but in both instances the steroid is considered biologically available (Simard

et al., 2014). Additionally, a consequence of RCL cleavage includes a 5 kDa reduction in molecular weight and conformational change that prevents the ability of CBG to bind an additional steroid molecule (Hammond et al., 1990; Potempa et al., 1994; Silverman et al., 2001).

Free hormone hypothesis and steroid hormone distribution

The free hormone hypothesis suggests that steroid hormones in the unbound or free-state are considered biologically available and are able to passively diffuse from the vasculature to specific effector tissues to serve their respective physiological functions (Siiteri et al., 1981; Mendel, 1989; Hammond, 2016). A limitation that exists within the current literature is that many experiments with the aim to examine fluctuations in cortisol or progesterone, as a result of an imposed treatment, often do not take into consideration CBG concentration. Consequently, this approach only provides insight into the production of the steroid, not alterations in the biologically available, or active fraction of the steroid. The free cortisol index (FCI; nmol/mg) is a calculation that was derived to quantify biologically available cortisol concentrations and is determined by examining the ratio of circulating cortisol concentration (nmol/L) in relation to CBG concentration (mg/L) (Le Roux et al., 2002; Le Roux et al., 2003). The use of this calculation allows for the estimation of cortisol that is biologically active in systemic circulation and is a better depiction of hypothalamic-pituitary-adrenal axis stimulation (Le Roux et al., 2003).

In humans, approximately 80 to 90 % of cortisol is bound to CBG, 5 to 10 % is loosely bound to albumin, and 5 % exists as free or completely unbound in circulation (Siiteri et al., 1982; Hammond et al., 1991; Estrada-Y-Martin and Orlander, 2011). Aside from humans, the distribution of cortisol has been documented in other species (Breuner and Orchinik, 2002; Malisch and Breuner, 2010), but little has been reported for domestic livestock species. Our lab has previously examined the distribution in swine, and similar to humans, CBG is the primary protein transport for cortisol in swine, but it is estimated that approximately 65 % is bound to CBG, 23 % bound to albumin, and 12 % free in systemic circulation (Kattesh et al., 1990; Kattesh et al., 1997). Among species, the RCL amino acid sequence is poorly conserved as compared to the overall amino acid sequence of the glycoprotein (Simard et al., 2014), and may contribute to the variability in the distribution of steroids observed among species. Nonetheless, CBG acts as the primary transporter of cortisol in circulation.

Thus far, progesterone distribution has not been as well characterized in the literature as compared to cortisol. However, research aiming to evaluate the proportion of free and bound progesterone fractions have been investigated in regards to pregnancy. In a study conducted by Rosenthal et al. (1969) where free and bound fractions of progesterone are measured during the trimesters of human pregnancy, it appears that CBG acts a buffer to limit the amount of free progesterone throughout the course of pregnancy. More specifically, the authors noted that CBG-bound progesterone increases from 37 to 43 %, whereas free progesterone is maintained at approximately 2 % throughout pregnancy (Rosenthal et al., 1969). The rise in CBG-

bound progesterone was concurrent with the overall rise in circulating progesterone concentrations throughout the three trimesters and it appears that the proportion of CBG-bound progesterone is concentration dependent (Rosenthal et al., 1969). Additionally, by the second trimester CBG became the primary transporter for progesterone (Rosenthal et al., 1969), is believed to function as a buffer to regulate the amount of free progesterone in circulation, extend the half-life, and to create a reserve of the steroid (Siiteri et al., 1982; Bright, 1995; Klieber et al., 2007).

Reproductive functional roles of CBG

Glucocorticoids and progesterone compete for the one binding site CBG harbors within its RCL, and the concentration of cortisol or progesterone can alter the ratio of steroids bound within the RCL (Westphal, 1986a). For example, under periods of high progesterone production during human pregnancy, progesterone displaces cortisol from the RCL to occupy the binding site (Rosenthal et al., 1969); whereas, during periods of elevated glucocorticoid production, cortisol displaces progesterone from the RCL (Westphal, 1986a). This interaction signifies the main function of CBG which includes serving as a buffer and reservoir for glucocorticoids and progesterone (Rosner, 1991; Sivukhina and Jirikowski, 2014).

Regulatory role of CBG during pregnancy

The role of CBG in reproductive function still remains unclear, but previous research conducted in the field of human reproduction has revealed that CBG likely assumes the role of regulating the bioavailability of steroids at the maternal-fetal

interface throughout the course of pregnancy (Benassayag et al., 2001; Hammond, 2016). In support of this claim, Benassayag et al. (2001) examined concentrations of cortisol, progesterone, CBG, and albumin within maternal circulation, intervillous blood, the umbilical vein, and umbilical arteries at time of delivery in women with normal, full-term pregnancies (Benassayag et al., 2001). The authors noted no differences in albumin concentration among the 4 vascular sampling locations, and circulating albumin concentrations in pregnant women were similar to that of non-pregnant controls (Benassayag et al., 2001). However, concentrations of CBG were different among the sampling locations. Maternal peripheral samples contained the greatest concentration of CBG, and CBG concentration in the intervillous blood space was greater than those measured within the umbilical vein and arteries (Benassayag et al., 2001). Peripheral concentrations of CBG were approximately three times greater in full-term pregnant women when compared to the non-pregnant controls (Benassayag et al., 2001). Interestingly, concentrations of progesterone and cortisol also varied greatly among the 4 sampling locations. Progesterone was the greatest in the intervillous space and lowest in peripheral circulation, but the opposite was observed for cortisol (Benassayag et al., 2001). It is likely that the differences in CBG concentration between peripheral and intervillous circulation are a reflection of the steroid load in those particular sites. Additionally, the authors hypothesized that CBG within the intervillous space functions as a transporter of progesterone from the maternal vasculature to the fetal placenta (Benassayag et al., 2001). Because of greater concentrations of CBG within the intervillous space when compared to the umbilical vein and arteries, and distinct varying

isoforms between maternal and fetal CBG, it appears that maternal-derived CBG is the primary source of CBG at the maternal-fetal interface (Benassayag et al., 2001). Furthermore, it is suggested that the elevated CBG concentration in maternal circulation act to buffer the greater cortisol concentrations associated with pregnancy, and to prevent the transfer of this steroid to fetal circulation (Murphy, 1979; Westphal, 1986b; Benassayag et al., 2001), but also to prevent the maternal development of hypercorticism (Sandberg et al., 1966).

Role of CBG in transport of progesterone

In addition to steroid buffering, CBG may also play a role in transporting progesterone from the corpus luteum (CL) to the uterus (Graham and Clarke, 1997), but, the exact mechanism of action is not well understood. The presence of CBG mRNA in steroidogenic tissues, and specifically within both the human corpus luteum and endometrium supports the theory that CBG plays a role in progesterone transport and steroidogenic function (Misao et al., 1994; Misao et al., 1997; Misao et al., 1999). Misao et al. (1997) examined the expression of CBG mRNA in human CL tissue following hysterectomy procedures and reported a greater expression of CBG mRNA during the mid-luteal phase of the menstrual cycle when compared to the early and late-luteal phases when concentrations of progesterone are lower than that of the mid-luteal phase (Misao et al., 1997; Misao et al., 1999). Similarly, CBG mRNA levels are higher during the secretory phase of the menstrual cycle, a phase characterized by greater progesterone concentrations when compared to the proliferative phase (Misao et al.,

1994). Therefore, it is speculated that CBG may play a role in the localized transport of progesterone within the reproductive tract of humans, but to the best of our knowledge, no other mechanisms have been proposed, and this relationship has not been examined in regards to the bovine estrous cycle.

Regulatory role of CBG during parturition

In addition to endometrial and luteal production, CBG can be found in other tissues with glucocorticoid or progesterone demands. However, similar to other steroid transporter proteins such as albumin and sex hormone-binding globulin, CBG is predominantly synthesized within hepatocytes (Khan et al., 1984), and has been reported in many species including humans (Khan et al., 1984), swine (Heo et al., 2003a), rats (Weiser et al., 1979), guinea pigs (Perrot-Applanat et al., 1981), and rabbits (Seralini et al., 1990). Previous work conducted in our lab indicated that CBG biosynthesis changes throughout the course of an individual's life and is related to physiological events that occur during that particular phase of development (Heo et al., 2003b). For example, fetal hepatic CBG mRNA levels decrease toward the end of gestation and was reflected by an overall reduction in plasma CBG concentrations, but the reverse is observed following birth through the first 40 d of the post-natal period when CBG mRNA levels and plasma CBG concentration were greater than those observed at the time of birth (Heo et al., 2003b). In humans, the heightened production and release of fetal glucocorticoids (i.e. cortisol) near the end of gestation plays a vital role in preparing the fetus and dam for birth by aiding in fetal lung maturation (Smith,

2007), and upregulating estradiol and pro-inflammatory prostaglandin production to stimulate uterine myometrial contractions (Whittle et al., 2001). Therefore, it is likely Heo et al. (2003b) observed a reduction in hepatic CBG production at the time of birth to allow for an increase in free, or biologically active cortisol associated with the parturition process. Furthermore, it is hypothesized that the overall rise in circulating CBG concentration toward the end of gestation limits negative feedback on the hypothalamus and anterior pituitary to allow for continued hypothalamic-pituitary-adrenal (HPA) axis activation necessary for parturition (Ballard et al., 1982; Challis and Brooks, 1989; Berdusco et al., 1995).

Factors that influence CBG biosynthesis

Glucocorticoids

Glucocorticoids, interleukin-6 (IL-6), and estradiol are three main proposed factors that are capable of regulating CBG biosynthesis. In many species, periods in which an individual experiences stress and is under heightened glucocorticoid (i.e. cortisol) influence are often associated with an overall reduction in circulating CBG concentration (Kattesh et al., 1980; Heo et al., 2005; Ho et al., 2006). Previously it has been reported that the glucocorticoid receptor (GR), in conjunction with its ligand, regulates the transcription of the CBG promotor in mice and rats (Smith and Hammond, 1992; Cole et al., 1999), but until recently the full mechanism in which glucocorticoids regulate CBG biosynthesis was not understood. Verhoog et al. (2014) were the first to describe the mechanism for which glucocorticoids suppress CBG biosynthesis though a

series of *in vivo* and *in vitro* experiments. First, male mice (*n* = 40) were subjected to four treatments for the duration of 10 d that included the following: no treatment (control), voluntary exercise, involuntary swimming (1 hr/d), and restraint in a small cage (1 hr/d). After the 10 d period, mice were sacrificed and livers were harvested and used to evaluate CBG mRNA expression. When compared to the control mice, the mice in the stress-related treatment groups (involuntary swimming and restraint) had a 27 and 55 % reduction in CBG mRNA expression, respectively (Verhoog et al., 2014). Next, the authors exposed human (HepG2) and mouse (BWTG3) hepatoma cells to dexamethasone (1 nM), a synthetic glucocorticoid, and reported a reduction in both CBG mRNA expression and CBG protein levels within both cell types which indicated that glucocorticoids are capable of hindering CBG biosynthesis at the transcriptional level (Verhoog et al., 2014).

To verify that dexamethasone suppression of CBG biosynthesis acts through the GR, Verhoog et al. (2014) then examined the effect of dexamethasone on CBG promoter reporter constructs transfected within mouse hepatoma cells and reported a dose-dependent reduction in promotor activity within the cells. Additionally, they reported a heightened suppressive effect on promoter activity occurred when the hepatoma cells were co-transfected with GRα and incubated with dexamethasone (Verhoog et al., 2014). Similar to results reported by Cole et al. (1999), in which dexamethasone suppression act by way of the GR, when hepatoma cells were co-transfected with RU486, an antagonists of the GR, the suppressive effect of dexamethasone was nullified, indicating that glucocorticoids inhibit CBG biosynthesis

though interactions with the GR (Verhoog et al., 2014). More specifically, the authors reported that glucocorticoids cause a migration of C/EBPβ, a transcription factor, to the proximal promoter of the CBG gene (SerpinA6) to repress CBG transcription (Verhoog et al., 2014).

Previously, Underhill and Hammond (1995) showed that C/EBPβ has the capability to bind to the promotor region on the CBG gene. To further support this claim, Verhoog et al. (2014) performed an experiment in which C/EBPβ was knocked-down in mouse hepatoma cells and reported a reduction in the ability of dexamethasone to repress CBG mRNA expression when C/EBPβ protein expression was reduced. Similarly, the migration of the GR to the proximal promotor region of the CBG gene was reduced (Verhoog et al., 2014). All together, these results indicate that CBG biosynthesis is controlled in part by glucocorticoids and their receptor and it appears that the mechanism controlling CBG biosynthesis lies at the transcriptional level where the glucocorticoid activated GR, in conjunction with C/EBPβ, bind to the promotor of the CBG gene and represses CBG transcription (Verhoog et al., 2014).

Interleukin-6

Unlike other acute phase cytokines, IL-6 has the ability to regulate hepatic CBG biosynthesis (Bartalena et al., 1993; Emptoz-Bonneton et al., 1997). Interleukin-6, a pro-inflammatory acute phase cytokine produced by leukocytes, has the ability to suppress hepatic CBG biosynthesis in a dose-dependent manner, and the mechanism as to which it is able to regulate CBG biosynthesis is similar to that of glucocorticoids

(Emptoz-Bonneton et al., 1997). In HepG2 cells, IL-6 caused a 71 and 61 % reduction in the secretion of the glycoprotein and the expression of CBG mRNA, respectively which indicates that IL-6 is a transcriptional regulator of the CBG gene (Emptoz-Bonneton et al., 1997; Perogamvros et al., 2012). In support of this claim, Underhill and Hammond (1995) previously reported that IL-6 suppressed the transcriptional process of the rat CBG gene by interacting with C/EBPβ, the transcription factor involved in glucocorticoid-induced transcriptional inhibition process mentioned above. As a result of IL-6 and glucocorticoids sharing the same mechanism for inhibiting hepatic CBG biosynthesis, there is an accumulative suppressive effect when HepG2 cells are cultured in the presence of both IL-6 and dexamethasone (Emptoz-Bonneton et al., 1997).

An *in vivo* study performed in men elicited a similar response to the *in vitro* study mentioned above. A single administration of IL-6 at a concentration of 3.0 µg/kg caused a decline in circulating CBG concentrations and one week post-treatment was required before baseline concentrations of CBG were observed (Tsigos et al., 1998). It is noteworthy to mention that the authors chose a dose of 3.0 µg/kg to ensure HPA axis activation to mimic the response that would be observed during periods of septic shock or trauma which have also been associated with reduced CBG concentrations (Tsigos et al., 1998; Ho et al., 2006). The inverse relationship observed between CBG biosynthesis and IL-6 supports the idea that CBG acts as a negative acute phase protein to increase the bioavailable fraction of cortisol in circulation as a way to mediate the inflammatory response (Tsigos et al., 1998; Perogamvros et al., 2012).

The role of CBG as a negative acute phase protein is important for its role in the mitigation of the inflammatory response (Hammond et al., 1990; Perogamvros et al., 2012). As mentioned earlier, in the presence of neutrophils, a particular group of leukocytes that respond during the innate immune response to inflammation, CBG undergoes a 5 kDa reduction in molecular weight and a loss to its steroid-binding capacity (Hammond et al., 1990). The reduction in binding capacity of CBG allows for targeted delivery of glucocorticoids to sites of inflammation and localized immune cells (Hammond et al., 1990; Perogamvros et al., 2012). Neutrophils are one of the first responders to sites of inflammation and are responsible for targeting invading pathogens and for the release of reactive oxygen species, but under periods of chronic inflammation, these actions can be harmful and lead to tissue destruction (Nathan, 2006; Mantovani et al., 2011). Glucocorticoids such as cortisol serve as immune regulators by suppressing the inflammatory response through a direct interaction with neutrophils at the site of inflammation (Hammond et al., 1990). Once cortisol is cleaved from the RCL of CBG, dissociates from the transport protein, and is taken up by neutrophils, the steroid is able to alter gene expression within the immune cell and to reduce the release of reactive oxygen species (Burton et al., 2005; Buckham Sporer et al., 2007). Therefore, in regards to periods of inflammation, it is evident that IL-6 induced cortisol release occurs to prevent the negative effects of prolonged inflammation and to regulate the localized immune response. Furthermore, CBG can be classified as a negative acute phase protein because hepatic CBG expression is

reduced during inflammation to allow for greater levels of bioavailable glucocorticoids (Pugeat et al., 1989).

Estrogen

Unlike glucocorticoids and IL-6, estrogen increase hepatic CBG biosynthesis and elevations in CBG concentration are associated with the administration of oral contraceptives, but to the best of our knowledge the mechanism of action is not presently understood. Previously, Doe et al. (1967) conducted an experiment to examine the effect of oral administration of diethylstilbestrol, a synthetic estrogenic compound, on fluctuations in CBG concentration in a test male patient undergoing treatment for prostate cancer. Diethylstilbestrol was administered daily (1 mg/d) for 4 weeks followed by a period of 4 weeks in which no treatment was administered. The dose was then increased by 1 mg/d and the treatment schedule repeated until a maximum dose of 5 mg/d was achieved. Over the treatment period, circulating concentrations of CBG increased in a dose-dependent manner, but following the treatment period, a withdrawal period of 4 weeks was required to return CBG concentrations back to baseline levels (Doe et al., 1967). Similarly, other studies have reported an increase in circulating CBG concentrations following administration of diethylstilbestrol and ethinylestradiol in women (Sandberg and Slaunwhite, 1959), and a two-fold increase in hepatic production rate of CBG following estradiol administration in male rats (Feldman et al., 1979). Therefore, it appears that estrogenic compounds are

capable of increasing circulating CBG concentrations by directly stimulating hepatic CBG biosynthesis.

More recently, Ågren et al. (2011) administered combined oral contraceptives to women to investigate the impact of estrogen and progestin-based contraceptive drugs on circulating levels of CBG. The experimental subjects ranged from 18 to 50 years of age and were stratified into two treatment groups that received one of the following treatments for six 28-d cycles: nomegestrol acetate/17β-estradiol (2.5 mg/1.5 mg; NOMAC/E2) or levonorgestrel/ethinylestradiol (150 µg/30 µg; LNG/EE) (Ågren et al., 2011). Blood samples were collected prior to the start of the study and during cycles 3 and 6 to examine changes to CBG concentration. Both treatment groups exhibited greater CBG concentrations at the end of the study when compared to the baseline sample collected. Women within the NOMAC/E2 treatment group experienced a 27 % (910 vs. 1116 nmol/L) increase in CBG concentration, but this rise was far less than that observed in the LNG/EE treatment group which had an overall increase of 118 % (932 vs. 1980 nmol/L) (Ågren et al., 2011). Differences in the overall rise in CBG concentration observed at the end of the experiment are attributed to the different estrogenic compounds used in the two treatments (Zeun et al., 2009; Ågren et al., 2011). Nonetheless, circulating CBG concentrations rise as a result of heightened hepatic biosynthesis during periods of estrogen influence, but are not affected by the progestin compound in the combined oral contraceptive drug (Hammond et al., 1984).

Conclusion

Although the role of CBG in regards to the regulation of glucocorticoids at various stages of life have been well documented in a variety of species, an overall lack in knowledge remains in regards to the interaction between CBG and the steroid hormones it transports in the bovine specifically. Additionally, the role of CBG in reproductive-specific events and the transport of progesterone remains unclear and further investigation is warranted. Recognizing that the use of estrous synchronization has become a common cattle management practice and the majority of protocols rely on the use of a CIDR, an opportunity exists to explore the effect of exogenous progesterone administration on local vaginal and systemic concentrations of CBG. Likewise, delving into this response will help us to better understand the role of CBG as a transporter and buffer for progesterone within the bovine reproductive tract.

Furthermore, the opportunity exists to examine CBG synthesis sites within the bovine reproductive tract. As mentioned previously, CBG mRNA is expressed in reproductive tissues of a variety of species, but has not been examined in cattle. In other species, the presence of CBG within in the uterus has been documented (Milgrom and Baulieu, 1970; Klemcke et al., 1998), but to the best of our knowledge, concentrations within the vaginal cavity have not been measured. Evaluating CBG mRNA expression within the vagina, uterus, oviduct, and ovary in conjunction with directly measuring concentrations of CBG within the reproductive tract will indicate if the glycoprotein is locally produced or shuttled to the reproductive tract from the vascular system or via migration from other regions within the tract.

Realizing that *Pseudomonas aeruginosa* is a common environmental bacterial pathogen and its colonization within the bovine vaginal cavity appears to be influenced by exogenous progesterone release from a CIDR (Padula and Macmillan, 2006), further investigation is warranted to determine if the presence of this particular bacteria within the vaginal cavity has an influence on the level of free, or biologically available progesterone during the estrous synchronization process, or pregnancy success following fixed-time artificial insemination. Therefore, the goal of the present dissertation was to investigate the vaginal endocrine response and associated bacterial interactions in beef heifers undergoing synchronization of ovulation to gain a better understanding of the direct impact of CIDRs used to facilitate synchrony. Additionally, this dissertation aimed to characterize CBG synthesis in the female bovine reproductive tract and examine changes in CBG mRNA expression during different stages of the estrous cycle and gestation. Through the completion of these experiments, the expected outcome was to gain a better understanding of bovine CBG and its influence on the reproductive axis, but to also gain insight as to the local production of CBG within the bovine reproductive tract.

Chapter II

Influence of synchronization of ovulation and early pregnancy on peripheral and vaginal concentrations of corticosteroid-binding globulin and the distribution of progesterone and cortisol in beef heifers

Abstract

Corticosteroid-binding globulin (CBG) is a transporter for glucocorticoids (i.e. cortisol) and progesterone and functions to regulate the amount of free steroid in circulation, but little is known about CBG and its role in bovine reproductive function. The objective of this study was to examine the effect of exogenous progesterone release from a controlled internal drug release device (CIDR) on circulating and vaginal concentrations of CBG and the proportion of free progesterone and cortisol following synchronization of ovulation using the 7 d CO-Synch + CIDR protocol. Consigned beef heifers (*n* = 67) were enrolled in the study and on d -7, immediately prior to CIDR insertion, blood and vaginal flush samples were collected. Following CIDR removal on d 0, the same samples were collected again. Blood samples were collected for a third time at the time of pregnancy diagnosis on d 38. Steroid concentrations were determined using a commercially available RIA and CBG was quantified by an in-house ELISA. Steroid hormone concentration (nmol/L) was then divided by CBG concentration (mg/L) to calculate a free hormone index (nmol/mg) for both progesterone (FPI) and cortisol (FCI). A mixed model analysis of variance with repeated measures was performed. Plasma samples collected at pregnancy diagnosis on d 38 from heifers with successful pregnancies (*n* = 24) following synchronization and AI had greater (*P* = 0.02) concentrations of progesterone than samples collected d -7, but were not different than those measured on d 0. Similarly, plasma CBG concentrations measured on d -7 and 0 did not differ, but were greatest (*P* = 0.03; *n* = 24) on d 38. The FPI calculated from progesterone and CBG concentrations measured on the three sampling days were

not different (P = 0.16). Similar to plasma progesterone concentrations, circulating concentrations of cortisol were consistent during synchronization of ovulation (d -7 and 0), but were lower (P = 0.01; n = 24) at pregnancy diagnosis (d 38). Likewise, FCI did not change during synchronization of ovulation, but was lowest (P < 0.0001; n = 24) at pregnancy diagnosis on d 38. Among heifers that became pregnant following AI, progesterone concentrations contained within vaginal flush samples collected immediately after CIDR removal on d 0 were approximately four times greater (P < 0.0001) than concentrations measured immediately prior to CIDR insertion on d -7, but CBG concentrations measured on d -7 were not different (P = 0.21) from those measured on d 0, and thus, the FPI on d 0 was greater (P < 0.0001) than on d -7. Vaginal cortisol concentrations measured immediately following CIDR removal were approximately two times greater (P < 0.0001) than those measured before the CIDR was inserted. Similarly, vaginal FCI was greater (P < 0.0001) at CIDR removal than at CIDR insertion. In the current study, CIDR insertion did not affect circulating CBG concentrations, but it appears that CBG may play a role in regulating the free fraction of progesterone during early gestation.

Introduction

Estrous synchronization relies on coordinated administration of exogenous reproductive hormones and many of the protocols developed and approved for use in the United States rely on the use of a controlled internal drug release device (CIDR). In contrast to other exogenous hormones that are administered by injection, CIDRs are

plastic devices that are infused with progesterone (1.38 g) and after intravaginal insertion, progesterone is absorbed through the vaginal epithelium and migrates to the vascular system. Within 1 h post-insertion, circulating concentrations of progesterone reach a maximum peak (approximately 4.5 ng/mL) and are sustained throughout a 7 d insertion period (Kesler, 2002). Similar to the rapid rise in systemic progesterone concentration after insertion, systemic progesterone rapidly decreases after the CIDR is removed and returns to baseline concentrations 8 h post-removal (Kesler, 2002).

Insertion of a CIDR for a period of 7 d ensures the corpus luteum (CL) has reached maturity and can undergo luteolysis following $PGF_{2\alpha}$ administration (Lucy et al., 2001; Kesler, 2002). Likewise, during the CIDR insertion period, the low dose release of progesterone prevents follicular wave turn-over and promotes the development of a persistent dominant follicle by increasing luteinizing hormone (LH) pulse frequency (Savio et al., 1993; Kojima et al., 2003). As the persistent follicle continues to develop, estradiol production and release subsequently increases (Kojima et al., 2003). Through positive feedback on the hypothalamus, estradiol promotes the release of gonadotropin-releasing hormone (GnRH) and subsequent LH production and release from the anterior pituitary gland (Kesner et al., 1981), and in pre-pubertal heifers and anestrous cows this mechanism allows for initiation or resumption of cyclicity (Anderson et al., 1996; Imwalle et al., 1998).

Corticosteroid-binding globulin (CBG) is a glycoprotein that is predominantly synthesized within hepatocytes (Hammond et al., 1991; Heo et al., 2003a) and functions as a transporter for a small subset of steroid hormones (i.e. glucocorticoids and

progesterone) in which it binds with high affinity, but low capacity (Slaunwhite and Sandberg, 1959; Seal et al., 1966; Westphal, 1986a; Heo et al., 2003b). Corticosteroid-binding globulin is a member of the serine protease inhibitor superfamily (Hammond et al., 1987; Law et al., 2006), and acts as a target for proteases (Lin et al., 2010) such as neutrophil elastase, a protease specifically synthesized and released by neutrophils (Hammond et al., 1990). More recently chymotrypsin, and LasB, an elastase produced and released by the bacterial species, *Pseudomonas aeruginosa* have also been reported as proteases capable of disrupting the reactive center loop (RCL) (Lewis and Elder, 2014; Simard et al., 2014). In the presence of CBG, the aforementioned proteases cleave specific sites within the RCL of CBG and compromise the steroid binding capacity (Lewis and Elder, 2017). After cleavage, the steroid dissociates from CBG and can be sequestered and loosely bound by albumin, or remain free within circulation, but in both instances the steroid is considered biologically available (Simard et al., 2014).

In humans, approximately 80 to 90 % of cortisol is bound to CBG, 5 to 10 % is loosely bound to albumin, and 5 % exists as free or completely unbound in circulation (Siiteri et al., 1982; Hammond et al., 1991; Estrada-Y-Martin and Orlander, 2011). Aside from humans, the distribution and characterization of cortisol has been documented in other species (i.e. wild birds, chimpanzees, gorillas, rats, chickens, etc.) (Gayrard et al., 1996; Breuner and Orchinik, 2002; Malisch and Breuner, 2010), but little has been reported for domestic livestock species. Our lab has previously examined the distribution in swine, and we estimated that approximately 65 % is bound to CBG, 23 %

bound to albumin, and 12 % free in systemic circulation (Kattesh et al., 1990; Kattesh et al., 1997).

Thus far, progesterone distribution has not been as well characterized in the literature as compared to cortisol. However, research aiming to evaluate the proportion of free and bound progesterone fractions have been investigated in regards to human pregnancy. In a study conducted by Rosenthal et al. (1969) where free and bound fractions of progesterone are measured during the trimesters of human pregnancy, it appears that CBG acts a buffer to limit the amount of free progesterone throughout the course of pregnancy. More specifically, the authors noted that CBG-bound progesterone increases from 37 to 43 %, whereas free progesterone is maintained at approximately 2 % throughout pregnancy (Rosenthal et al., 1969). The rise in CBG-bound progesterone was concurrent with the overall rise in circulating progesterone concentrations throughout the three trimesters and it appears that the proportion of CBG-bound progesterone is concentration dependent (Rosenthal et al., 1969). Additionally, by the second trimester CBG became the primary transporter for progesterone (Rosenthal et al., 1969), is believed to function as a buffer to regulate the amount of free progesterone in circulation, extend the half-life, and to create a reserve of the steroid (Siiteri et al., 1982; Bright, 1995; Klieber et al., 2007).

The free hormone hypothesis suggests that steroid hormones in the unbound or free-state are considered biologically available and are able to passively diffuse from the vasculature to specific effector tissues to serve their respective physiological functions (Siiteri et al., 1981; Mendel, 1989; Hammond, 2016). The free cortisol index

(FCI; nmol/mg) is a calculation that was derived to quantify biologically available cortisol concentrations and is determined by examining the ratio of circulating cortisol concentration (nmol/L) in relation to CBG concentration (mg/L) (Le Roux et al., 2002; Le Roux et al., 2003). The use of this calculation allows for the estimation of cortisol that is biologically active and is a better indicator of hypothalamic-pituitary-adrenal axis activation (Le Roux et al., 2003). For this manuscript, we also adapted this calculation to estimate the proportion of biologically available progesterone.

Recognizing that the use of estrous synchronization has become a common cattle management practice and the majority of protocols rely on the use of a CIDR, an opportunity exists to explore the effect of exogenous progesterone administration on the local vaginal and systemic endocrine response. Therefore, the goal of the present study was to investigate vaginal steroid hormone profiles and systemic endocrine response in beef heifers undergoing synchronization of ovulation using the 7 d CO-Synch + CIDR protocol to gain a better understanding of the direct impact of CIDRs used to facilitate synchrony on CBG concentrations. We hypothesized that CBG concentrations would rise during synchronization of ovulation to regulate the proportion of free progesterone in the vaginal cavity and in circulation.

Materials and Methods

Animals

Prior to animal manipulation, procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee (protocol number 2398-1115).

Heifers (*n* = 67) enrolled in the study were consigned to the University of Tennessee Beef Heifer Development Program in Lewisburg, TN on October 3, 2016 and remained in the program until September, 2017. At arrival, heifer age and body weight ranged from 182 to 383 d of age and 198.7 to 390.1 kg, respectively. Breed composition included the following: Angus (*n* = 28), Angus x Hereford (*n* = 4), Angus x Shorthorn (*n* = 2), Angus-cross (*n* = 9), Charolais (*n* = 7), Gelbvieh (*n* = 4), Hereford (*n* = 4), Simangus (*n* = 4), and Simmental (*n* = 5). All heifers were housed on pasture and received the same feed ration over the course of the program. Ad libitum access to water and supplemented hay was also provided.

Tissue collection and synchronization of ovulation

Synchronization of ovulation began on March of 2017 when heifers were 416 ± 34 d of age and had an average body weight of 415 ± 36 kg. Heifers enrolled in the study included those that had not undergone prior synchronization of ovulation procedures at the testing center. On d -7, immediately prior to the administration of GnRH (100 ug, Factrel; Zoetis, Madison, NJ) and CIDR (1.38 g progesterone; Eazi-Breed CIDR, Zoetis, Madison, NJ) insertion as part of the 7 d CO-Synch + CIDR protocol, blood (6 mL) was collected in sodium heparin vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) via coccygeal venipuncture. Blood samples were centrifuged (Sero-Fuge Centrifuge, Clay Adams, Inc., Parsippany, NJ) for 10 min and plasma was harvested, aliquoted into three 2 mL microcentrifuge tubes, and then stored at -20°C until analyzed for total cortisol, progesterone, and CBG concentrations.

Following blood collection, the vulva was cleaned with 70 % ethanol and vaginal cytological samples were collected by inserting a cytobrush (Cooper Surgical, Inc., Trumbull, CT) into the vaginal lumen and rotating the brush clockwise against the vaginal epithelium (Stratman et al., 2016). Cytobrushes were rolled onto microscope slides and slides were allowed to air dry prior to being stained with a modified Wright-Giemsa stain (Protocol hema 3 stain set, Fisher Scientific Co, Pittsburgh, PA) (Stratman et al., 2016). Under 100X magnification, approximately 200 cells were counted to determine the proportion of polymorphonuclear neutrophils (PMN) to total cells present on the slide (Ghasemi et al., 2012; Stratman et al., 2016).

Vaginal flushes were also performed on d -7, immediately prior to CIDR insertion. Briefly, a rubber catheter attached to a 60 mL syringe was inserted into the vagina and 60 mL of 0.9 % sodium chloride sterile saline was infused into the vagina and recovered via vaginal lavage and caught in a plastic cup, then transferred to a 50 mL conical tube and immediately stored on ice. Vaginal flush samples were centrifuged at 2,000 rpm (821 x *g*; Beckman Coulter Allegra X-14R Centrifuge, Brea, CA) for 5 min at 4°C, aliquoted into three 13 mL culture tubes, and then stored at -20°C until analyzed for total cortisol, progesterone, and CBG concentrations as well as bacterial analyses.

The same procedures mentioned above to collect blood, cytological samples, and perform vaginal swabs and flushes were again performed on d 0 when CIDRs were removed and $PGF_{2\alpha}$ was administered. On d 2, heifers received another injection of GnRH immediately prior to AI. An additional blood sample for progesterone, cortisol,

and CBG analyses was obtained at time of pregnancy diagnosis (d 38) as determined by transrectal ultrasonography.

Plasma steroid hormone analyses

Total plasma cortisol and progesterone concentrations (ng/mL) were measured in duplicate by following the procedures of commercially available RIA kits (MP Biomedicals, Solon, OH; cat # 07-221102 and 07-170102, respectively) (Hulbert et al., 2013; Pohler et al., 2016). Cortisol standards ranged from 5 to 1,000 ng/mL with a cross-reactivity less than 12.3 % for related endogenous steroids. Intra- and inter-assay CV were 17.3 % and 25.9 % for low (2.27 ng/mL) and, 12.2 % and 15.0 % for high (16.2 ng/mL) cortisol controls. Progesterone standards ranged from 0.2 to 50 ng/mL with a cross-reactivity less than 5.4 % for related endogenous steroids. Intra- and inter-assay CV were 6.7 % and 6.0 % for low (0.89 ng/mL) and, 11.3 % and 15.1 % for high (5.0 ng/mL) progesterone controls.

Vaginal flush steroid hormone analyses

Total progesterone concentrations within the vaginal flush samples were determined by following the same RIA procedures as described for the progesterone measured in plasma samples, but an initial validation was performed to ensure concentrations of progesterone in the flush samples were within the limits of the standard curve of the assay. Briefly, vaginal flush samples collected on d -7 (immediately prior to CIDR insertion) and d 0 (immediately post-CIDR removal) from four random heifers were used for the validation. Undiluted samples from d -7 and 0

were analyzed for each heifer. Additionally, dilutions of 300 uL sample:100 uL saline, 200 uL sample:200 uL saline, and 100 uL sample:300 uL saline were performed to determine if dilutions were necessary to ensure sample concentrations fit within the standard curve of the assay. Based on the results of the validation (Table 1), concentrations of the undiluted d -7 samples and the diluted (100 uL sample: 300 uL saline) d 0 samples fit within the standard curve of the progesterone RIA. Samples were measured in duplicate and intra- and inter-assay CV were 3.6 % and 3.3 % for low (5.93 ng/mL) and, 3.4 % and 5.7 % for high (51.0 ng/mL) progesterone controls.

A two-step validation process was performed for measuring cortisol within the vaginal flush samples. Concentrations within the samples were too low to be measured using the same RIA kit that was used for plasma cortisol concentrations. Therefore, we tested the efficacy of the High Sensitivity Salivary Cortisol Enzyme Immunoassay (EIA) Kit (Salimetrics, Inc., State College, PA) in regards to measuring cortisol within the flush samples. The first step of the validation included a recovery assay to determine the recovery capability of Sep-Pak Classic C18 Cartridges (Waters Corporation, Milford, MA). We followed the procedures reported by Shackleton and Whitney (1980) for concentrating urine samples for cortisol determination with slight modification. Instead of concentrating urinary samples, we concentrated 1 mL of ^{125}I Cortisol Tracer Solution (MP Biomedicals, Solon, OH; cat # 06B256617), reconstituted in 1 mL of 0.9 % sodium chloride sterile saline, and measured gamma emissions (counts per minute; cpm) from the Sep-Pak recovered sample in comparison to 1 mL of straight ^{125}I Cortisol Tracer Solution. The straight sample and Sep-Pak recovered sample had gamma emissions of

47,101.7 cpm and 46,914.7 cpm, respectively, with an overall recovery of 99.6 %. Due to the high recovery capabilities of the Sep-Pak Classic C18 Cartridges, we continued with the High Sensitivity Salivary Cortisol EIA validation to determine if concentrations of cortisol obtained following the use of the cartridges fall within the standard curve of the assay.

First, we concentrated pre- and post-CIDR flush samples at varying volumes (1, 2, 5, and 10 mL) using Sep-Pak Classic C18 Cartridges as described previously by Shackleton and Whitney (1980), and then reconstituted in 100 uL 0.9 % sodium chloride sterile saline. Concentrated samples were then measured for total cortisol concentration following the manufacturer instructions provided within the Salimetrics High Sensitivity Salivary Cortisol EIA kit. Based on concentrations obtained after performing the assay (Table 2), concentrating samples with a volume of 10 mL achieves concentrations that are within the middle portion of the standard curve (0.12, 0.37, 1.10, 3.30, 10.0, and 30.0 ng/mL). All subsequent vaginal flush cortisol analyses were performed by concentrating 10 mL of flush sample using the Sep-Pak Classic C18 Cartridges and were quantified in duplicate via the Salimetrics High Sensitivity Salivary Cortisol EIA with intra- and inter-assay CV of 5.0 and 7.5 % for low (1.17 ng/mL) and 3.3 and 7.8 % for high (9.62 ng/mL) cortisol standards. Within the results section, concentrations of cortisol within the vaginal flush samples were adjusted for the 10 mL of flush used to concentrate the samples and are reported as nmol/L.

CBG concentration determination

Concentrations of bovine CBG (mg/L) were quantified in triplicate by an indirect competitive ELISA developed previously in our laboratory (Kattesh et al., 2014). Isolation and purification of CBG from bovine plasma (bCBG) and development and validation of an ELISA for its quantification followed the procedures outlined by Roberts et al. (2003) for porcine CBG. Briefly, bovine plasma was collected (~400 ml) and denuded of bound cortisol by incubation with Amberlite XAD-2 beads (Sigma-Aldrich, St. Louis, MO), dialyzed against 50 mM Tris, 0.5M NaCl, pH 7.6 (column buffer), and applied to a Sepharose affinity column containing the ligand 11 β-hydroxy-3-one-pregn-4-one-17β carboxylic acid (HCAC) (Roberts et al., 2003). The column was washed and eluted sequentially with column buffer containing 200 ng/mL of cortisol. Fractions containing CBG were dialyzed again using the same buffer and passed over a 10 mL Concanavalin A-Sepharose 4B (C9017; Sigma-Aldrich, St. Louis, MO) column (Roberts et al., 2003). The column was washed and eluted with 10 % and warmed 20 % methyl α-D-mannopyranoside (Sigma-Aldrich, St. Louis, MO) (Roberts et al., 2003). The eluted fraction was dialyzed against 10 mM Tris, pH 7.6 and applied to a HPLC-DEAE anion exchange column and eluted with a linear gradient of 0 to 0.3 M NaCl (Roberts et al., 2003). The resulting purified bCBG was dialyzed against 10 mM Tris, pH of 7.6, and cortisol was added for storage (Roberts et al., 2003). Location of bCBG in column eluates was monitored by an optical density at 280 nm, SDS-PAGE, and ^{3}H-cortisol binding (Schiller and Pétra, 1976).

To generate a population of polyclonal antibodies specific for bCBG, 600 ug of purified bCBG obtained from the above procedures was sent to Pacific Immunology Corp. (Ramona, CA). Two rabbits were immunized with 120 ug of bCBG and were provided three subsequent boosters of 60 ug of bCBG that occurred 22, 43, and 71 d after the first immunization. Rabbit anti-bCBG serum was harvested via exsanguination and retained for the bCBG ELISA described below.

Bovine CBG assay development was performed by following the procedures of Signorella and Hymer (1984) and Roberts et al. (2003). First, 96-well plates were coated with purified bCBG (20 ng/100 uL/well) in coating buffer (20 mM Tris, 100 mM NaCl, pH 8.4), sealed, and incubated overnight at 4° C (Roberts et al., 2003). A separate set of 96-well plates were blocked with 200 uL glassware treatment solution consisting of 0.1 % BSA and 1X PBS, pH 7.4, sealed, and incubated at 4° C overnight (Roberts et al., 2003). Both coated and blocked plates were washed for 4 cycles with 300 uL of 1X TBS (150 mM NaCl, 20 mM Tris, 3.1 mM sodium azide, pH 7.4), tapped dry, and stored at -20 ºC with desiccant (Roberts et al., 2003). In glass tubes, plasma samples (40 uL) were combined with 160 uL diluent (10 % BSA, 10X PBS, and 10 % Tween 20) and 200 uL rabbit anti-bCBG serum at a dilution of 1/80,000, vortexed, and stored overnight at 4° C. Similarly, vaginal flush samples (25 uL) were combined with 175 uL diluent and 200 uL rabbit anti-bCBG serum at a dilution of 1/80,000, vortexed, and stored overnight at 4° C. For each plate, standards ranging from 0.3 to 160 ng/100 uL/well were prepared and combined with rabbit anti-bCBG at a dilution of 1/80,000 and stored overnight at 4° C. Tubes containing either standard-rabbit anti-bCBG or sample-

rabbit anti-bCBG mixtures were vortexed and 120 uL of the mixture was transferred to a blocked plate, sealed, and allowed to incubate overnight at 4° C. An aliquot of 100 uL from each well of blocked plate well was transferred to the corresponding well on a bCBG coated well and incubated at 37° C for 2 hr in a humid chamber. Following the incubation period, the bCBG coated plates containing sample-rabbit anti-bCBG mixtures were washed for 4 cycles with 300 uL of tris buffered saline with Tween-20 (TBST; 150 mM NaCl, 0.05 % Tween 20, 20 mM Tris, 3.81 mM sodium azide, pH 7.4) and tapped dry (Roberts et al., 2003). Then, 100 uL of a 1/16,000 dilution solution of goat anti-rabbit IgG conjugate to alkaline phosphatase (A3687; Sigma-Adrich) was added to each well and allowed to incubate at 37° C for 2 hr in a humid chamber. Plates were washed again for 4 cycles with TBST, tapped dry, and 100 uL of phosphatase substrate, para-nitro-phenyl-phosphate in diethanolamine buffer (SeraCare Life Sciences, Inc., Milford, MA), was added to each well followed by an incubation period of 1.5 hr at 37° C in a humid chamber (Roberts et al., 2003). Following incubation, 100 uL of stop solution (0.05 % Na2EDTA) was added to each well and absorbance was read at a 405 nm wavelength by an ELX808 (BioTek Instruments, Inc., Winooski, VT). Data were quantified using Gen5 software version 2.03 (BioTek Instruments, Inc., Winooski, VT). For plasma samples, intra- and inter-assay CV were 13.0 % and 20.0 % for internal control A (3.78 mg/L), and 6.5 % and 24.3 % for internal control B (4.62 mg/L). For vaginal flush samples, intra- and inter-assay CV were 15.5 % and 19.2 % for internal control A (2.02 mg/L), and 15.8 % and 18.7 % for internal control B (3.14 mg/L).

Free hormone index

Progesterone and cortisol concentrations were converted from ng/mL to nmol/L by a multiplicative conversion factor of 3.18 and 2.76, respectively (Graham et al., 2011). Steroid hormone concentration (nmol/L) was then divided by CBG concentration (mg/L) to calculate a free hormone index (nmol/mg) for both progesterone (FPI) and cortisol (FCI) (Le Roux et al., 2003).

Statistical analysis

All heifers enrolled in the study (n = 67) were used to examine the relationship between the steroid hormones and CBG profiles and the ability to conceive following synchronization of ovulation and AI. In addition, all heifers that were confirmed pregnant following timed AI (n = 24) were used as a sub-population to examine the influence of progesterone during synchronization of ovulation and early gestation on circulating and vaginal concentrations of cortisol, progesterone, and CBG.

All statistical analyses were performed in SAS 9.3 (SAS Institute, Cary, NC, USA). A mixed model analysis of variance with heifer as the experimental unit was performed to assess the effect of synchronization of ovulation and early gestation on measured dependent variables. Sampling day was used in the model as a repeated measure, and pen and heifer were included as random blocking effects. Fisher's LSD test was used to separate means ($P \leq 0.05$).

Results

Systemic endocrine response to synchronization of ovulation and pregnancy

All tables and figures are included in the appendix at the end of this chapter. Plasma progesterone, cortisol, and CBG concentrations measured prior to CIDR insertion and immediately following CIDR removal were similar ($P > 0.10$) among heifers that became pregnant following AI and those that remained open (Table 3). Likewise, plasma FPI and FCI calculated from progesterone, cortisol, and CBG concentrations measured prior to and immediately following CIDR insertion and removal were not different ($P > 0.40$) among heifers that became pregnant following AI and those that remained open (Table 3). However, plasma samples collected at pregnancy diagnosis on d 38 from heifers with successful pregnancies following synchronization and AI had significantly greater ($P = 0.02$) concentrations of progesterone than samples collected on d -7 (Figure 1A), but were not different than those measured on d 0. Similarly, plasma CBG concentrations measured on d -7 and 0 did not differ, but were greatest ($P = 0.03$) on d 38 at pregnancy diagnosis (Figure 1B). The FPI calculated from progesterone and CBG concentrations measured on the three sampling days were not different ($P = 0.16$; Figure 1C). Similar to plasma progesterone concentrations, circulating concentrations of cortisol were consistent during synchronization of ovulation (d -7 and 0), but were lower ($P = 0.01$) at pregnancy diagnosis (d 38; Figure 1D). Likewise, FCI did not change during synchronization of ovulation, but was lowest ($P < 0.0001$) at pregnancy diagnosis on d 38 (Figure 1E).

Vaginal polymorphonuclear neutrophil (PMN) percentage

The insertion of a CIDR for a period of 7 d resulted in an increase ($P < 0.0001$) of approximately 42 % in the percentage of PMN within the vaginal cavity. At the time of CIDR insertion (d -7), PMN accounted for 7.28 ± 2.83 % of the total cells on the cytological slides whereas PMN accounted for 48.85 ± 2.81 % of the total cells when the CIDR was removed (d 0). Although day differences existed, no differences in PMN were observed between heifers that became pregnant or remained open following synchronization of ovulation and artificial insemination (Table 4).

Vaginal steroid hormone profiles in response to synchronization of ovulation

Vaginal flush progesterone, cortisol, and CBG concentrations measured prior to CIDR insertion and immediately following CIDR removal were not different ($P > 0.30$) among heifers that became pregnant following AI and those that remained open (Table 4). Likewise, vaginal flush FPI and FCI calculated from progesterone, cortisol, and CBG concentrations measured in the recovered flush solution prior to and immediately following CIDR insertion and removal were not different ($P > 0.70$) among heifers that became pregnant following AI and those that remained open (Table 4). However, among heifers that became pregnant following AI, progesterone concentrations contained within vaginal flush samples collected immediately after CIDR removal on d 0 were approximately four times greater ($P < 0.0001$) than concentrations measured immediately prior to CIDR insertion on d -7 (Figure 2A). Although there was a rise in progesterone concentration within vaginal flush samples as a result of CIDR insertion,

CBG concentrations measured on d -7 were not different ($P = 0.21$) from those measured on d 0, and thus, the FPI on d 0 was greater ($P < 0.0001$) than on d -7 (Figure 2B and C). Vaginal cortisol concentrations measured immediately following CIDR removal were approximately two times greater ($P < 0.0001$) than those measured before the CIDR was inserted (Figure 2D). Similarly, vaginal FCI was greater ($P < 0.0001$) at CIDR removal than at CIDR insertion (Figure 2E).

Discussion

In the present study, the pregnancy rate of heifers synchronized following the 7 d CO-Synch + CIDR protocol and TAI was 35.8 %. The overall low pregnancy rate observed in our study in comparison to a typical pregnancy rate of 53 % in heifers undergoing TAI may be a result of the synchronization protocol used or the imposed research sample collection methods (Hall et al., 2005). In previous years at the University of Tennessee Beef Heifer Development Program, synchronization of ovulation was performed using the 14-d CIDR-PG protocol, but heifers in our study underwent the 7 d CO-Synch + CIDR protocol. In pre-pubertal heifers, CIDRs are used to induce cyclicity among a cohort of individuals at various stages of pubertal development (Rhodes, 1998; Lucy et al., 2001; Kesler, 2002). During the CIDR insertion period, the low dose release of progesterone prevents follicular wave turn-over and promotes the development of a persistent dominant follicle by increasing luteinizing hormone (LH) pulse frequency (Savio et al., 1993; Kojima et al., 2003). As the persistent follicle continues to develop, estradiol production and release subsequently increases (Kojima et al., 2003). Through positive feedback on the hypothalamus,

estradiol promotes the release of GnRH and subsequent LH production and release from the anterior pituitary gland (Kesner et al., 1981), and in pre-pubertal heifers this mechanism allows for the initiation of cyclicity (Anderson et al., 1996; Imwalle et al., 1998). Therefore, the reduction in pregnancy rates observed in our heifers may be a refection in the inability of some heifers to achieve cyclicity or synchrony at the time of synchronization and breeding (Busch et al., 2007). However, we cannot be certain because estrus detection patches or observation of standing heat were not components of this study.

In addition to the synchronization protocol used, we cannot rule out the possibility that the method used to collect vaginal flush samples did not affect pregnancy success in the heifers. Vaginal flush collections are a common method to assess reproductive status in other species (Caligioni, 2009), and to our knowledge, no adverse effects following sample collection have been reported. However, although we used sterile biological saline to infuse into the vaginal cavity and collection was performed in an aseptic manner, it is possible that the collection method may have had an adverse effect on the ability of heifers to become pregnant following the synchronization period. Thus, precaution is advised to ensure external contaminants are not introduced into the reproductive tract and all infused saline is recovered to minimize the impact of the collection method on the reproductive tract.

In the present study, CIDR insertion did not significantly alter circulating concentrations of progesterone, cortisol, or CBG. Recognizing that FPI and FCI are directly proportional to progesterone, cortisol, and CBG concentration, it is not

surprising that FPI and FCI were not affected by the insertion period. However, the numeric rise in circulating progesterone concentration at the time of CIDR removal indicates that the CIDRs increased plasma progesterone concentrations and were consistent with concentrations previously reported for beef cattle (Kesler, 2002). At the time of CIDR insertion, mean progesterone concentrations were 7.15 nmol/L (2.25 ng/mL) for heifers that failed to conceive following TAI, and 8.15 nmol/L (2.56 ng/mL) for those that became pregnant. Considering heifers were not pre-synchronized and were at various stages of the estrous cycle at the time of CIDR insertion, we cannot rule out the possibility that some heifers had not achieved puberty by the time the study started. Puberty in beef cattle can be assessed by collecting consecutive weekly samples in which circulating progesterone concentrations exceed 1 ng/mL in two of the three samples collected. In the current study, 13 heifers had plasma progesterone concentrations less than 1 ng/mL at the start of our study, but because only one blood sample was taken before the CIDR was inserted, we cannot conclude if the low progesterone concentrations observed in those 13 heifers was a result of anestrus or the stage of the estrous cycle in which the samples were collected.

Although CIDR insertion did not appear to affect plasma concentrations of CBG, CBG was greatest at the time of pregnancy diagnosis on d 38 among heifers that became pregnant following TAI. During this time, plasma progesterone concentrations were also greater than those measured during the synchronization period. Although CBG concentrations have not been examined in regards to progesterone and pregnancy in bovine, the response observed in the present study is consistent with

those reported for human pregnancy. Rosenthal et al. (1969) conducted a study to observe the free and bound fractions of progesterone during the trimesters of human pregnancy, and it was postulated that CBG acts a buffer to limit the amount of free progesterone throughout the course of pregnancy. The authors observed an increase in CBG-bound progesterone from 37 to 43 %, whereas free progesterone was maintained at approximately 2 % throughout pregnancy, and the rise in CBG-bound progesterone was concurrent with the overall rise in circulating progesterone concentrations throughout the three trimesters (Rosenthal et al., 1969). Additionally, by the second trimester CBG became the primary transporter for progesterone (Rosenthal et al., 1969), and is believed to function as a buffer to regulate the amount of free progesterone in circulation, extend the half-life, and to create a reserve of the steroid (Siiteri et al., 1982; Bright, 1995; Klieber et al., 2007). Plasma progesterone concentrations measured here in pregnant heifers at the time of pregnancy diagnosis were greater than those measured during synchronization, and were concurrent with the rise in plasma CBG levels observed on d 38. Also in agreement with Rosenthal et al. (1969), we found no differences in the amount of free progesterone in circulation at the time of pregnancy diagnosis. This response suggests that similar to humans, CBG may act to regulate the proportion of free progesterone in circulation especially during periods of high progesterone influence such as pregnancy. However, we must also consider the relationship between CBG and cortisol, another steroid in which it binds with a high affinity (Klieber et al., 2007).

During periods of stress or during the inflammatory response, CBG acts as a negative acute phase protein as shown by a reduction in circulating CBG concentrations (Pugeat et al., 1989; Tsigos et al., 1998). During the inflammatory response, there is an increase in neutrophils which release neutrophil elastase that can cleave the RCL of CBG (Hammond et al., 1990). Once the RCL has been cleaved, CBG can no longer bind the steroid in which it was transporting, thus allowing the steroid to be in its biologically available state (Simard et al., 2014; Lewis and Elder, 2017). Therefore, when cortisol concentrations are elevated, a reduction in circulating CBG concentration occurs to allow for an increase in bioavailable cortisol. At the time of pregnancy diagnosis in pregnant heifers in the present study, plasma cortisol concentrations were lowest during the time in which CBG concentrations were the highest. Although this response is consistent with CBG's role as a negative acute phase protein, plasma cortisol concentrations on d 38 were 21.0 nmol/L (7.62 ng/mL) lower than those measured on d 0 and were less than baseline cortisol concentrations previously reported in beef heifers (Edwards et al., 1987). Therefore, we speculate that the increase in CBG concentrations during early gestation was not a result of reduced cortisol concentration, but a reflection in the sustained increase in progesterone concentration that occurs with pregnancy. However, to support the previous statement, a subsequent study would have to be performed in which multiple blood samples were collected throughout early gestation to examine the fluctuations in plasma progesterone, cortisol, and CBG concentration, and to evaluate the binding distribution of CBG.

Although CIDR insertion did not appear to influence the systemic endocrine response in heifers undergoing synchronization of ovulation, CIDRs directly altered the local vaginal endocrine response, but these changes were not related to the ability of the heifers to conceive following TAI. Additionally, CIDRs induced a localized inflammatory response within the vaginal cavity as indicated by a 42 % increase in PMN migration within the vaginal lumen at the time of CIDR removal which is consistent with other studies conducted that have reported an increased infiltration of neutrophils within the reproductive tract of cattle undergoing synchronization (Ahmadi et al., 2007; Çevik et al., 2010; Fischer-Tenhagen et al., 2012). We did not examine the percentage of neutrophils in circulation, but because plasma cortisol concentration did not differ between d -7 and 0, it does not appear that CIDR insertion induced a systemic inflammatory response which would be represented by a rise in cortisol concentration (Chrousos, 1995; Steensberg et al., 2003).

CIDR insertion resulted in an increase in progesterone concentration measured within the vaginal lumen, but did not alter the amount of CBG within the vagina. Therefore, there was also a rise in the amount of free progesterone within the vaginal cavity. These data are not surprising because we know that the mechanism as to which CIDRs work to synchronize cattle relies on the absorption of exogenous progesterone through the vaginal epithelium to cause a rise in systemic progesterone concentrations. Surprisingly, vaginal cortisol concentrations also increased during the CIDR insertion period, but because cortisol acts as a regulatory agent during the inflammatory response (Hammond et al., 1990), we believe this response occurred as a result of

neutrophil migration that occurred during CIDR insertion. The presence of a CIDR has been linked to a numeric increase in the proportion of neutrophils within the vaginal cavity and an overall reduction in circulating neutrophils (Ahmadi et al., 2007; Walsh et al., 2008). These patterns were noted for both progesterone impregnated and non-impregnated devices and thus, the response appears to be a result of the presence of the device within the vaginal cavity (Ahmadi et al., 2007; Walsh et al., 2008). In addition to mechanical induction of the immune response, hormones have the capability to impact the activity and presence of neutrophils within an individual. Specifically, Lasarte et al. (2016) reported that progesterone administration induced a Cxcl1 chemokine gradient that promoted neutrophil infiltration into the vaginal cavity of mice. Others have reported that progesterone can also impact the survivability of immune cells, such that neutrophil apoptosis was reduced in vitro when the immune cells were subjected to progesterone, and neutrophils harvested from women showed a greater reduction in apoptosis when compared to those harvested from men (Molloy et al., 2003). Thus, it appears that CIDRs impregnated with progesterone have the potential to impose a multifaceted effect on the local immune response within the vagina.

We did not evaluate the cortisol and progesterone binding properties of bovine CBG as part of this study. In regards to human CBG, the glycoprotein binds cortisol with a higher affinity than progesterone, but both with a much higher affinity than that of albumin (Mickelson et al., 1981; Hammond, 2016). We were able to detect fluctuations in the amount of CBG measured within plasma as a result of early gestation in cattle that we believe is a reflection of heightened progesterone influence. Future work should

be aimed at evaluating the binding affinity of CBG for both progesterone and cortisol via Scatchard analysis to determine if bovine CBG binding affinities are similar to those reported for human CBG.

Conclusion

To the best of our knowledge, this study is the first to examine the effect of exogenous progesterone release from a CIDR on local and systemic concentration of CBG and the corresponding FPI and FCI in heifers undergoing synchronization of ovulation. Progesterone release from a CIDR inserted for a 7 d period as part of the 7 d CO-Synch + CIDR protocol did not affect systemic or vaginal concentrations of CBG in heifers enrolled in the current study. Likewise, the endocrine profiles measured did not affect the ability of the heifers to become pregnant following synchronization and TAI. At the time of the CIDR insertion on d -7, heifers were at various stages of the estrous cycle. To determine if in fact CIDR insertion does not alter circulating concentrations of CBG during the 7 d period, a subsequent study should be conducted such that heifers are pre-synchronized and are at the same stage of the estrous cycle at the time of the CIDR insertion. In regards to early pregnancy, it appeared that CBG concentrations increased in response to the heightened output of progesterone resulting in stabilization of the amount of free progesterone in circulation. Altogether, based on the preliminary data reported in this study, there is evidence to suggest that bovine CBG functions in a similar manner to human CBG during early gestation.

Appendix

Table 1. Validation of MP Biomedicals Progesterone Double Antibody RIA kit for determination of progesterone concentration within vaginal flush samples collected on d 0 and 7 of synchronization of ovulation.

Heifer[1]	Day	Dilution	[P4] (ng/mL)	Corrected [P4] (ng/mL)
A	-7	undiluted	4.52	-
	0	undiluted	113.32	-
		300 uL sample : 100 uL saline	69.03	91.81
		200 uL sample : 200 uL saline	48.00	96.00
		100 uL sample : 300 uL saline	21.44	85.77
B	-7	undiluted	10.83	-
	0	undiluted	38.38	-
		300 uL sample : 100 uL saline	33.20	44.15
		200 uL sample : 200 uL saline	25.04	50.07
		100 uL sample : 300 uL saline	11.31	45.24
C	-7	undiluted	6.27	-
	0	undiluted	99.49	-
		300 uL sample : 100 uL saline	74.75	99.42
		200 uL sample : 200 uL saline	51.32	102.64
		100 uL sample : 300 uL saline	26.26	105.02
D	-7	undiluted	BDL[2]	-
	0	undiluted	60.74	-
		300 uL sample : 100 uL saline	45.48	60.49
		200 uL sample : 200 uL saline	29.86	59.72
		100 uL sample : 300 uL saline	14.22	56.87

[1]Four heifers were selected at random for the validation.
[2]Below detectable limits (BDL).

Table 2. Validation of vaginal flush cortisol concentrations (ng per volume concentrated) obtained following Sep-Pak Classic C18 Cartridge extraction and Salimetrics High Sensitivity Salivary Cortisol EIA quantification.

	Day of vaginal flush collection[1]	
Sample preparation[2]	**Pre-CIDR (d -7)**	**Post-CIDR (d 0)**
1 mL concentrated	0.02	0.19
2 mL concentrated	0.02	0.24
5 mL concentrated	0.11	0.42
10 mL concentrated	0.61	1.81

[1]Vaginal flush samples were collected immediately prior to CIDR insertion on d -7 and immediately following CIDR removal on d 0.
[2]Samples (pre-CIDR and post-CIDR) from one heifer were chosen at random for this validation. Sample aliquots at varying volumes (1, 2, 5, and 10 mL) were concentrated using the Sep-Pak C18 cartridges and reconstituted in 100 uL of 0.9 % sodium chloride sterile saline. Concentrations are represented as total ng of cortisol contained within each volume (1, 2, 5, and 10 mL) concentrated.

Table 3. Comparison of measured plasma variables (mean ± SEM) on d -7 and 0 between heifers that became pregnant (*n* = 24) following synchronization of ovulation followed by artificial insemination and those that remained open (*n* = 43).

		Pregnancy status on d 38		
Plasma variable[1]	**Day**	**Open**	**Pregnant**	**P-Value**
Progesterone (nmol/L)	-7	7.15 ± 1.52	8.15 ± 2.03	0.15
	0	11.46 ± 1.94	10.69 ± 2.28	
Cortisol (nmol/L)	-7	54.06 ± 5.25	51.58 ± 7.08	0.55
	0	76.89 ± 5.28	61.39 ± 7.02	
CBG (mg/L)	-7	4.11 ± 0.31	5.22 ± 0.52	0.15
	0	4.37 ± 0.33	4.58 ± 0.46	
FPI[2] (nmol/mg)	-7	1.68 ± 0.27	1.55 ± 0.36	0.43
	0	2.42 ± 0.34	2.25 ± 0.42	
FCI[2] (nmol/mg)	-7	9.75 ± 1.61	7.91 ± 1.77	0.47
	0	14.42 ± 2.42	11.42 ± 2.52	

[1]Plasma samples were collected from each heifer prior to CIDR insertion on d -7 and immediately following CIDR removal on d 0.
[2]Steroid hormone concentration (nmol/L) was then divided by CBG concentration (mg/L) to calculate a free hormone index (nmol/mg) for both progesterone (FPI) and cortisol (FCI).

Table 4. Comparison of variables measured in flush vaginal flush samples (mean ± SEM) on d -7 and 0 between heifers that became pregnant (*n* = 24) following synchronization of ovulation followed by artificial insemination and those that remained open (*n* = 43).

		Pregnancy status on d 38		
Vaginal variable[1]	**Day**	**Open**	**Pregnant**	**P-Value**
Progesterone (nmol/L)	-7	33.63 ± 15.62	34.08 ± 17.18	0.95
	0	139.87 ± 31.75	136.34 ± 34.69	
Cortisol (nmol/L)	-7	0.038 ± 0.006	0.038 ± 0.006	0.66
	0	0.078 ± 0.012	0.073 ± 0.013	
CBG (mg/L)	-7	2.60 ± 0.18	2.32 ± 0.21	0.35
	0	2.66 ± 0.18	2.66 ± 0.21	
FPI[2] (nmol/mg)	-7	14.38 ± 7.84	14.92 ± 8.54	0.95
	0	52.73 ±15.04	52.92 ± 16.20	
FCI[2] (nmol/mg)	-7	0.016 ± 0.003	0.018 ± 0.004	0.75
	0	0.030 ± 0.007	0.029 ± 0.007	
PMN[3] (%)	-7	7.81 ± 3.22	6.75 ± 3.87	0.92
	0	47.98 ± 3.20	49.72 ± 3.81	

[1]Vaginal flush samples were collected from each heifer by infusing 60 mL of sterile saline into the vaginal cavity prior to CIDR insertion on d -7 and immediately following CIDR removal on d 0. Concentrations are represented as whole concentrations contained within the flush sample.

[2] Steroid hormone concentration (nmol/L) was then divided by CBG concentration (mg/L) to calculate a free hormone index (nmol/mg) for both progesterone (FPI) and cortisol (FCI).

[3] Polymorphonuclear neutrophils (PMN) within the vaginal cavity were assessed prior to CIDR insertion on d -7 and immediately following CIDR removal on d 0.

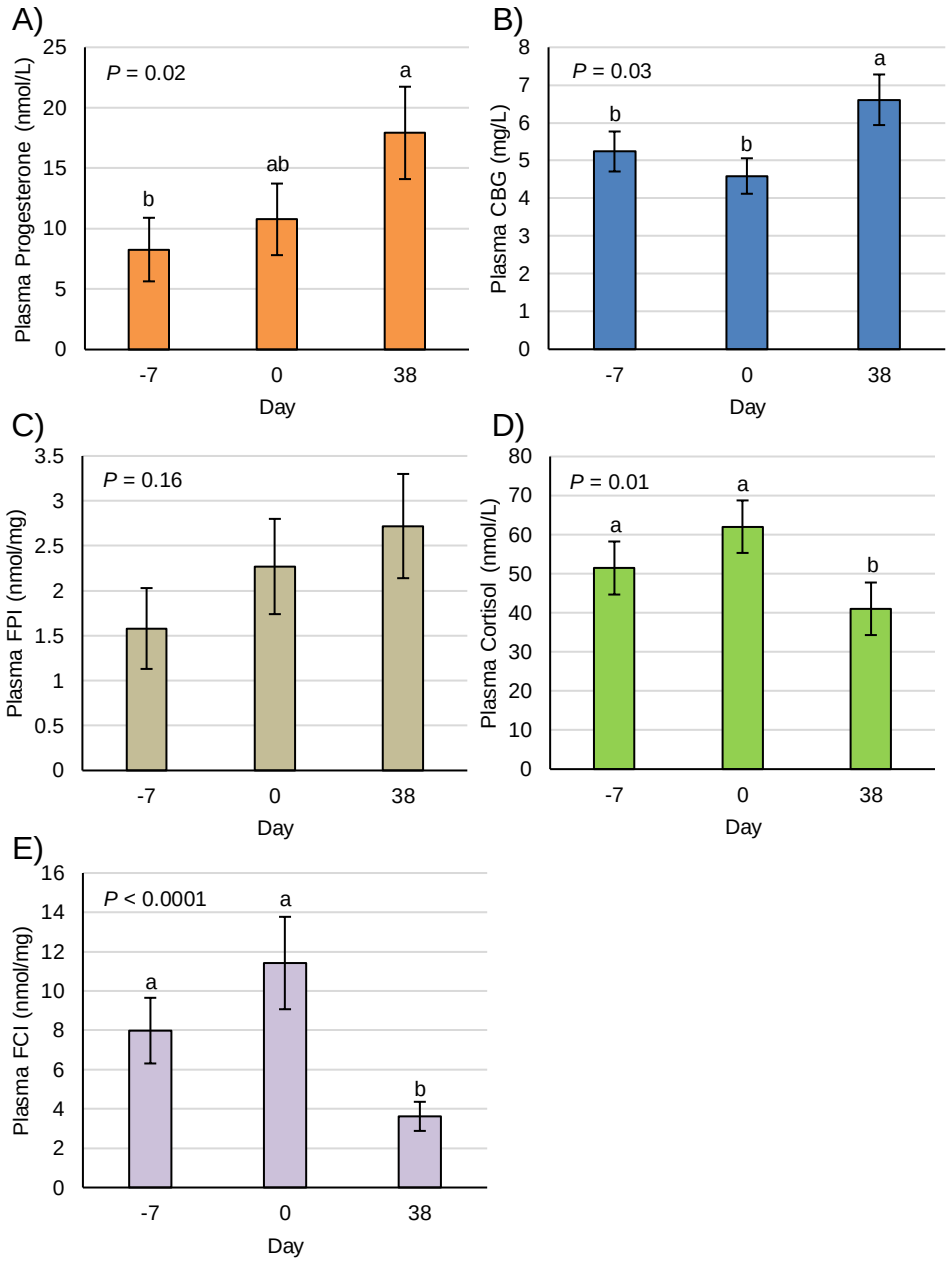

Figure 1. Mean ± SEM plasma constituents measured during synchronization of ovulation and early gestation in heifers that became pregnant following synchronization and AI (*n* = 24). Progesterone impregnated CIDRs were inserted on d -7 and removed on d 0. Pregnancy diagnosis via transrectal ultrasonography occurred on d 38. Steroid hormone concentration (nmol/L) was divided by CBG concentration (mg/L) to calculate a free hormone index (nmol/mg) for progesterone (FPI; Figure 1C) and cortisol (FCI; Figure 1E). [a,b]Means with different superscripts differ.

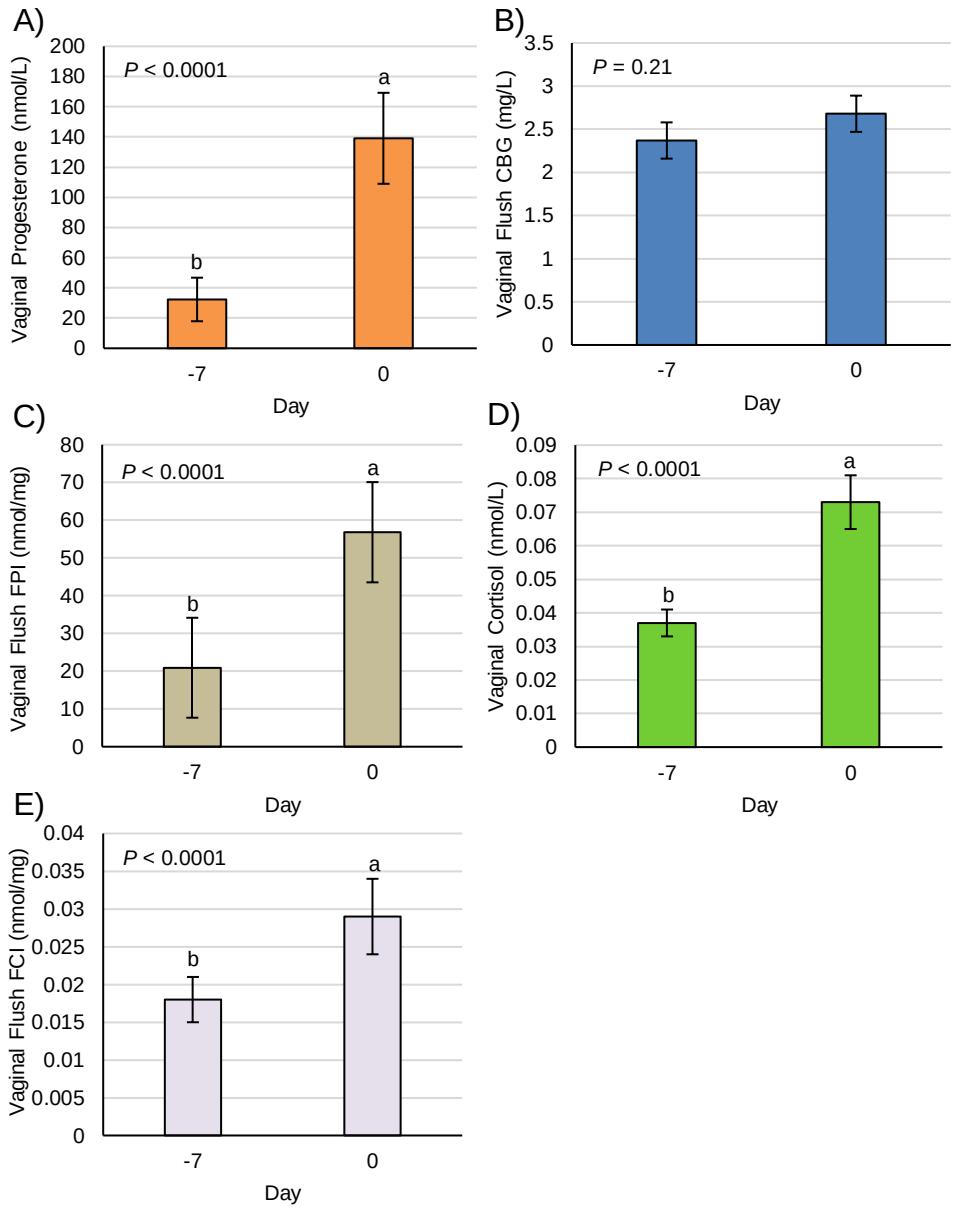

Figure 2**.** Mean ± SEM progesterone, CBG, cortisol, FPI, and FCI measured in vaginal flush samples collected during synchronization of ovulation in heifers that became pregnant following synchronization and AI (*n* = 24). Progesterone impregnated CIDRs were inserted on d -7 and removed on d 0. Steroid hormone concentration (nmol/L) was divided by CBG concentration (mg/L) to calculate a free hormone index (nmol/mg) for progesterone (FPI; Figure 2C) and cortisol (FCI; Figure 2E). [a,b]Means with different superscripts differ.

Chapter III

Presence of *Pseudomonas aeruginosa* within the vaginal cavity of heifers undergoing synchronization of ovulation and the influence on biologically available steroid hormones

Abstract

Pseudomonas aeruginosa is a gram negative aerobic bacteria that is commonly found in the environment and is capable of colonizing the vaginal cavity of a variety of species. The presence of *Pseudomonas aeruginosa* within the bovine vaginal cavity has not been well characterized, but there is evidence to suggest that the presence of a controlled internal drug release device (CIDR) can alter the abundance of the bacteria inhabiting the cavity. *Pseudomonas aeruginosa* produces and releases LasB, a protease that is capable of cleaving the reactive center loop (RCL) of corticosteroid-binding globulin (CBG), a transporter for cortisol and progesterone. When cleavage occurs, the steroid dissociates from CBG and is considered biologically available. The main objective of the present study was to examine the effect of exogenous progesterone released from a CIDR on the presence of *Pseudomonas aeruginosa* within the vaginal cavity of heifers undergoing synchronization of ovulation using the 7 d CO-Synch + CIDR protocol. Additionally, we examined the relationship between *Pseudomonas aeruginosa* and CBG concentration in the vaginal cavity as it relates to the biologically available fractions of progesterone and cortisol. Beef heifers (n = 67) were enrolled in the study and immediately prior to CIDR insertion on d -7, and immediately following CIDR removal on d 0, vaginal flush samples were collected. The abundance of *Pseudomonas aeruginosa* was quantified using quantitative PCR. Steroid concentrations were determined using a commercially available RIA and CBG was quantified by an in-house ELISA. Steroid hormone concentration (nmol/L) was then divided by CBG concentration (mg/L) to calculate a free hormone index (nmol/mg)

for both progesterone (FPI) and cortisol (FCI). A mixed model ANOVA with repeated measures was performed, and Spearman correlations were used to examine the relationship between the presence of *Pseudomonas aeruginosa* and the endocrine variables measured. During the 7 d CIDR insertion period, the abundance of *Pseudomonas aeruginosa* within the vaginal flush samples decreased ($P < 0.0001$), but presence of *Pseudomonas aeruginosa* within the vaginal cavity was not related ($P = 0.96$) to pregnancy status following synchronization of ovulation and AI. *Pseudomonas aeruginosa* was unrelated ($P > 0.10$) to progesterone concentration, cortisol concentration, and the FPI measured within the vaginal flush samples. In contrast, *Pseudomonas aeruginosa* abundance at the time of CIDR insertion on d -7 was negatively related ($r = -0.32$; $P = 0.04$) to the percentage of PMN within the vaginal cavity, but no relationship ($P = 0.68$) between the two variables was observed on d 0 when CIDRs were removed. On d 0, *Pseudomonas aeruginosa* abundance was related to both CBG concentration ($r = -0.25$; $P = 0.05$) and FCI ($r = 0.37$; $P = 0.004$). In summary, the presence of *Pseudomonas aeruginosa* decreased as a result of CIDR insertion, but was unrelated to vaginal progesterone concentration. However, under the current methods, it appears that *Pseudomonas aeruginosa* within the vaginal cavity of heifers may have to potential to alter the free proportion of cortisol as seen by a reduction in CBG concentration.

Introduction

Within the last few years, researchers have started to examine the interactions between hormone production and bacterial community profiles that exist within the bovine vagina, and it has been reported that changes in the diversity and abundance of bacteria occur as hormone profiles vary (Laguardia-Nascimento et al., 2015). Furthermore, during pregnancy, a physiological state characterized by heightened progesterone production, the bovine vaginal microbiome undergoes a reduction in both bacterial diversity and abundance of bacteria inhabiting the cavity (Walther-António et al., 2014; Laguardia-Nascimento et al., 2015). Although research has aimed to characterize the existing bacterial community profiles in the bovine vaginal cavity, the direct effect of exogenous progesterone release from a controlled internal drug release device (CIDR) on the population of local bacterial communities in cattle undergoing estrous synchronization remains unclear.

In women, *Pseudomonas aeruginosa* is an aerobic opportunistic bacterial pathogen that can colonize the vaginal epithelium and lead to infection (Osset et al., 2001; Hammond, 2016). Studies that have characterized the bovine vaginal bacterial community profiles reported that *Pseudomonadaceae* are present within the vaginal cavity (Laguardia-Nascimento et al., 2015), but the prevalence of *P. aeruginosa* is not well documented in cattle. Previously, Padula and Macmillan (2006) examined the effect of CIDR (1.9 g progesterone) insertion on cultured bacterial isolates found within the vagina of postpartum Holstein cows. The study was designed such that three treatments were administered (control: no CIDR; CIDR-P4: two intravaginal devices

containing progesterone were administered for 14 d; CIDR-Blank: two intravaginal devices without progesterone were administered for 14 d) and vaginal swabs were performed on d 0 (day of CIDR insertion), 7, and 14 to examine culturable bacterial profiles (Padula and Macmillan, 2006). They reported that the number of samples positive for *P. aeruginosa* increased over the sampling period in cows that received a CIDR. Likewise, the presence of *P. aeruginosa* was greater in the CIDR-P4 treated cows when compared to the other two treatment groups (Padula and Macmillan, 2006). Thus, it appears that the presence of the CIDR and progesterone are capable of altering the abundance of *P. aeruginosa* within in the bovine vaginal cavity.

P. aeruginosa has the capability to synthesize and release LasB (*P. aeruginosa* elastase), a protease that can cleave the reactive center loop (RCL) of the steroid hormone transporter, corticosteroid-binding globulin (CBG) (Simard et al., 2014; Hammond, 2016). When Simard et al. (2014) incubated human CBG in a culture of *P. aeruginosa*, they observed a 90 % reduction in the cortisol-binding capacity of CBG and a 5-10 kDa loss. Furthermore, when human CBG was incubated with protease fractions extracted from *P. aeruginosa*, only LasB reduced the cortisol-binding capacity of CBG and proved to have the ability to interfere with the ability of CBG to bind steroids within the RCL (Simard et al., 2014). Based on the study performed by Simard et al. (2014) and the presence of *P. aeruginosa* in the bovine vaginal cavity as reported by Padula and Macmillan (2006), further investigation is warranted to determine if this particular bacteria has the ability to influence CBG and free progesterone concentrations during the estrous synchronization process.

The objective of the present study was to examine the effect of exogenous progesterone released from a CIDR on the presence of *P. aeruginosa* within the vaginal cavity of heifers undergoing synchronization of ovulation using the 7 d CO-Synch + CIDR protocol. Additionally, we aimed to examine the relationships between *P. aeruginosa* and CBG concentration in the vaginal cavity as it relates to the free fractions of progesterone and cortisol. Based on the literature presented above, we hypothesized that CIDR insertion would increase the abundance of *P. aeruginosa* within the vaginal lumen, and that heifers with a greater abundance of *P. aeruginosa* would have lower CBG concentrations and greater proportion of free steroid within the vaginal lumen.

Materials and Methods

Animals

Prior to animal manipulation, procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee. Heifers (*n* = 67) enrolled in the study were consigned to the University of Tennessee Beef Heifer Development Program in Lewisburg, TN on October 3, 2016 and remained in the program until September, 2017. At arrival, heifer age and body weight ranged from 182 to 383 d of age and 198.67 to 390.09 kg, respectively. Breed composition included the following: Angus (*n* = 28), Angus x Hereford (*n* = 4), Angus x Shorthorn (*n* = 2), Angus-cross (*n* = 9), Charolais (*n* = 7), Gelbvieh (*n* = 4), Hereford (*n* = 4), Simangus (*n* = 4), and Simmental (*n* = 5). All heifers were housed on pasture and received the same feed

ration over the course of the program. Ad libitum access to water and supplemented hay was also provided.

Tissue collection and synchronization of ovulation

Synchronization of ovulating began on March 2017 when heifers were 416 ± 33.73 d of age and had an average body weight of 415 ± 36 kg. On d -7, immediately prior to the administration of GnRH (100 ug, Factrel; Zoetis, Madison, NJ) and CIDR (1.38 g progesterone; Eazi-Breed CIDR, Zoetis, Madison, NJ) insertion as part of the 7 d CO-Synch + CIDR protocol, blood (6 mL) was collected in sodium heparin vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) via coccygeal venipuncture. Blood samples were centrifuged (Sero-Fuge Centrifuge, Clay Adams, Inc., Parsippany, NJ) for 10 min and plasma was harvested, aliquoted into three 2 mL microcentrifuge tubes, then stored at -20°C until later cortisol, progesterone, and CBG analyses. Following blood collection, the vulva was cleaned with 70 % ethanol, and vaginal cytological samples were collected by inserting a cytobrush (Cooper Surgical, Inc., Trumbull, CT) into the vaginal lumen and rotating the brush clockwise against the vaginal epithelium (Stratman et al., 2016). Cytobrushes were rolled onto microscope slides and slides were allowed to air dry prior to being stained with a modified Wright-Giemsa stain (Protocol hema 3 stain set, Fisher Scientific Co, Pittsburgh, PA) (Stratman et al., 2016). Under 100X magnification, approximately 200 cells were counted to determine the proportion of polymorphonuclear neutrophils (PMN) to total cells present on the slide (Ghasemi et al., 2012; Stratman et al., 2016). Vaginal flushes were then performed by

inserting a rubber catheter attached to a 60 mL syringe into the vagina and 60 mL of 0.9 % sodium chloride sterile saline was infused into the vagina and recovered via vaginal lavage and caught in a plastic cup, then transferred to a 50 mL conical tube. Following collection, vaginal flush samples were centrifuged at 2,000 rpm (821 x *g*; Beckman Coulter Allegra X-14R Centrifuge, Brea, CA) for 5 min at 4°C, aliquoted into three 13 mL culture tubes, and stored at -20°C until later cortisol, progesterone, and CBG determination.

The same procedures mentioned above to collect blood and vaginal flush samples were performed again on d 0 when CIDRs were removed and $PGF_{2\alpha}$ was administered. On d 2, heifers received another injection of GnRH immediately prior to AI.

Pseudomonas aeruginosa quantification within vaginal swabs

P. aeruginosa ATCC 27853 was grown overnight in 5 mL of Tryptic Soy Broth (Becton, Dickinson, and Company, Sparks, MD) with shaking at 37º C. Serial dilutions were performed to obtain sub-samples ranging from 10^{-1} to 10^{-9}. Then, 100 uL from 10^{-3} to 10^{-7} samples were plated in triplicate on Luria-Bertani agar plates (Becton, Dickinson, and Company, Sparks, MD), incubated overnight at 37º C, and colony forming units (CFU) were counted on each plate. Following DNA isolation procedures described above, DNA was extracted from 1 mL of the sub-samples ranging from 10^{0} to 10^{-9}, and were used to generate a standard curve on each PCR 96-well plate that ranged from 7.0×10^{2} to 7.0×10^{8} $CFU \cdot mL^{-1}$.

Vaginal flush samples were thawed, vortexed, and centrifuged at 3,280 x *g* for 20 min, then the resulting supernatant was discarded and pellets were re-suspended in 180 uL of Bufffer ATL provided within the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany)(Clemmons et al., 2017). Subsequent bacterial DNA extraction was performed as per the manufacturer instructions within the DNeasy Blood & Tissue Kit protocol for gram negative bacteria (Clemmons et al., 2017). Following extraction, DNA concentration within each sample was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) (Lee et al., 2011).

Forward (722F: 5'-GGCGTGGGTGTGGAAGTC-3') and reverse (788R: 5'-TGGTGAAGCAGAGCAGGTTCT-3') primers were designed to target a *gyrB* gene specific sequence of *P. aeruginosa* (Lavenir et al., 2007; Lee et al., 2011). Each PCR reaction contained a total volume of 40 uL which consisted of 20 uL PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Life Technologies, Carlsbad, CA) and 2 uL of each the forward and reverse primers (500 nM) (Lee et al., 2011). The remaining 16 uL consisted of varying volumes of nuclease-free water and DNA template to achieve a final mass of 150 ng of DNA template per PCR reaction. A negative control was constructed following the PCR reaction recipe above, but 16 uL of nuclease-free water was used in place of the of DNA template. Amplification of DNA was performed in triplicate using the QuantStudio™ 3 Real-Time PCR System with the following thermal cycling settings. A preliminary hold stage at 50º C for 2 min followed by 95º C for 2 min was performed, and 40 cycles of denaturation at 95º C for 15 sec, annealing at 58.5º C for 1 min, and extension at 72º C for 1 min was performed.

Plasma steroid hormone analyses

Total plasma cortisol and progesterone concentration (ng/mL) were determined by following the procedures of commercially available radioimmunoassay kits (MP Biomedicals, Solon, OH; cat # 07-221102 and 07-170102, respectively) with a sensitivity of 5 ng/mL and 0.2 ng/mL, respectively (Hulbert et al., 2013; Pohler et al., 2016). Cortisol standards ranged from 5 to 1,000 ng/mL with a cross-reactivity less than 12.3 % for related endogenous steroids. Intra- and inter-assay coefficients of variation (CV) were 17.3 % and 25.9 % for low (2.27 ng/mL) and, 12.2 % and 15.0 % for high (16.2 ng/mL) cortisol controls. Progesterone standards ranged from 0.2 to 50 ng/mL with a cross-reactivity less than 5.4 % for related endogenous steroids. Intra- and inter-assay coefficients of variation (CV) were 6.7 % and 6.0 % for low (0.89 ng/mL) and, 11.3 % and 15.1 % for high (5.0 ng/mL) progesterone controls.

Vaginal flush steroid hormone analyses

Total progesterone and cortisol concentrations within the vaginal flush samples were determined by following the same RIA procedures as described in Chapter II using the MP Biomedical Progesterone Double Antibody RIA kit and Salimetrics High Sensitivity Salivary Cortisol EIA. Intra- and inter-assay CV were 3.6 % and 3.3 % for low (5.93 ng/mL) and, 3.4 % and 5.7 % for high (51.0 ng/mL) progesterone controls. Cortisol EIA with intra- and inter-assay CV of 5.0 and 7.5 % for low (1.17 ng/mL) and 3.3 and 7.8 % for high (9.62 ng/mL) cortisol standards.

CBG concentration determination

Concentrations of bovine CBG (mg/L) were quantified by an indirect competitive ELISA developed in our laboratory as described in Chapter II (Roberts et al., 2003; Kattesh et al., 2014). For plasma samples, intra- and inter-assay CV were 13.0 % and 20.0 % for internal control A (3.78 mg/L), and 6.5 % and 24.3 % for internal control B (4.62 mg/L). For vaginal flush samples, intra- and inter-assay CV were 15.5 % and 19.2 % for internal control A (2.02 mg/L), and 15.8 % and 18.7 % for internal control B (3.14 mg/L).

Free hormone index

To calculate the free fraction of cortisol and progesterone, concentrations of cortisol and progesterone were converted from ng/mL to nmol/L by a multiplicative conversion factor of 2.76 and 3.18, respectively (Graham et al., 2011). Steroid hormone concentration (nmol/L) was then divided by CBG concentration (mg/L) to calculate a free hormone index (nmol/mg) for both cortisol (FCI) and progesterone (FPI) (Le Roux et al., 2003).

Statistical analysis

All heifers enrolled in the study (n = 67) were used to examine the relationship between the bacterial, steroid hormones, and CBG profiles following synchronization of ovulation and AI. In addition, heifers that were confirmed pregnant following timed artificial insemination (n = 24) were used as a sub-population to examine the influence

of progesterone during synchronization of ovulation and early gestation on vaginal bacterial presence and concentrations of cortisol, progesterone, and CBG.

All statistical analyses were performed in SAS 9.3 (SAS Institute, Cary, NC, USA). A mixed model analysis of variance with heifer as the experimental unit was performed to assess the effect of synchronization of ovulation on measured dependent variables. Sampling day was used in the model as a repeated measure, and pen and heifer were included as random blocking effects. Fisher's LSD test was used to separate means ($P \leq 0.05$). Spearman correlations were also performed to examine relationships between the abundance of *P. aeruginosa* and the measured endocrine-related variables.

Results

All tables are included in the appendix at the end of this chapter. All vaginal flush samples were positive for *P. aeruginosa* as detected by qPCR analysis. During the 7 d CIDR insertion period of the 7 d CO-Synch + CIDR protocol, the abundance of *P. aeruginosa* within the vaginal flush samples exhibited a 3.5-fold decrease ($P < 0.0001$) from 9,818.10 ± 2,034.94 CFU·mL^{-1} on d -7 to 2,797.69 ± 527.62 CFU·mL^{-1} on d 0. However, no differences ($P = 0.96$) in *P. aeruginosa* abundance were observed on d -7 and 0 among heifers that became pregnant ($n = 24$) and those that remained open ($n = 43$) following synchronization of ovulation and timed AI (Table 5).

Overall means for progesterone, cortisol, CBG, FPI, FCI, and PMN measured on d -7 and 0 are reported in Table 6. Spearman correlations were performed to assess

the relationship between *P. aeruginosa* abundance and the other dependent variables measured in the vaginal flush samples obtained on d -7 and 0. *P. aeruginosa* was unrelated ($P > 0.10$) to progesterone concentration, cortisol concentration, and the FPI measured within the vaginal flush samples collected on d -7 and 0 (Table 7). In contrast, *P. aeruginosa* abundance within the vaginal cavity on d -7 was inversely related ($P = 0.04$) to the percentage of PMN within the vaginal cavity such that heifers with a greater abundance of PMN had fewer *P. aeruginosa* inhabiting the vaginal cavity, but no relationship ($P = 0.68$) between the two variables was observed on d 0 when CIDRs were removed (Table 7). On d 0, *P. aeruginosa* abundance was negatively related to both CBG concentration ($P = 0.05$) and FCI ($P = 0.004$) measured in the vaginal flush such that heifers with a greater abundance of *P. aeruginosa* had lower CBG concentrations, but a greater proportion of free cortisol within the vaginal cavity (Table 7). Likewise, FCI was directly related ($r = 0.30$; $P = 0.02$) to the percentage of PMN within the vaginal cavity on d 0, such that heifers with a greater proportion of PMN had a higher FCI.

Discussion

In addition to vaginal flush samples, vaginal swab samples were collected on d -7 and 0 with the intent to determine *P. aeruginosa* presence using a cotton swab technique instead of vaginal flush collection method. Recognizing that *P. aeruginosa* is a gram negative bacteria that is capable of adhering to host cells (Woods et al., 1980; Holden et al., 1999), we hypothesized that vaginal bacterial swabs would serve as a

better collection method than the vaginal flush method. Following DNA extraction, swab samples contained an average of 38.7 ng/uL of purified DNA on d -7 and 53.1 ng/uL on d 0, but the qPCR methods described above were unable to detect *P. aeruginosa* within the swab samples. In contrast, vaginal flush samples contained an average of 17.3 ng/uL of purified DNA on d -7 and 88.0 ng/uL on d 0 and *P. aeruginosa* was detectable in samples collected using the vaginal flush method. We suspect that the bacterial swab collection method was not adequate for obtaining a sufficient representative sample of bacteria within the vaginal cavity of heifers. It is also possible that the swab method collected primarily host cells from the vaginal epithelial instead of bacterial cells within the lumen. Instead, vaginal flush samples are capable of flushing the vaginal lumen by making contact with a greater surface area, and may be a more representative sample of the bacteria communities inhabiting the vaginal lumen (Ball et al., 1988).

Heifers enrolled in our study displayed a 3.5 fold reduction in the abundance of *P. aeruginosa* following the 7 d CIDR insertion period. However, this was contrary to our hypothesis and data reported by Padula and Macmillan (2006) who observed an increase in the abundance of *P. aeruginosa* within the vaginal cavity as a result of CIDR insertion. Although both studies were conducted in beef heifers consisting of *Bos taurus* lineage, the experiment performed by Padula and Macmillan (2006) was conducted in the Southern region of Australia during autumn which is characterized by warmer seasonal temperatures and more rainfall than the spring climate in which our experiment occurred. *P. aeruginosa* is an opportunistic environmental pathogen (Stover

et al., 2000), and within the United States, its prevalence is affected by season such that *P. aeruginosa*-induced respiratory infections in humans are observed more commonly during the summer and autumn months when compared to winter and spring months (Psoter et al., 2013). Thus, it is possible that environmental conditions may have contributed to the varying results in our study and those reported by (Padula and Macmillan, 2006). Likewise, all cows in their study were within the luteal phase of the estrous cycle at the time of CIDR insertion. Recognizing the impact of progesterone on the vaginal microbiome (Walther-António et al., 2014; Laguardia-Nascimento et al., 2015), and that heifers in our study were at different stages of the estrous cycle, it is also possible that our results conflicted to those reported by Padula and Macmillan (2006) due to both the physiological hormone state of the animals at CIDR insertion and the type of CIDR used.

Additionally, Padula and Macmillan (2006) recognized the chance of sampling contamination among the cows enrolled in their study (Padula and Macmillan, 2006). Therefore, it is possible that their observed response was a result of cow to cow contamination and not necessarily a direct effect of CIDR insertion. However, among the current literature it appears that there are disagreements in regards to the effect of progesterone on bacterial communities within the bovine reproductive tract such that Laguardia-Nascimento et al. (2015) observed a reduction in both the diversity and abundance of bacteria within the vaginal cavity during pregnancy, a state characterized by sustained progesterone influence, which is consistent with our results.

In the present study, CIDR were inserted and the plastic tail attached to the device was left intact. Previous research conducted by Fischer-Tenhagen et al. (2012) suggested that the presence of the plastic tail is capable of altering the bacterial communities within the vagina and reported an increase in the occurrence of purulent discharge which was also observed in our samples. More specifically, the authors noted an increase *Escherichia coli*, a pathogenic bacteria, within the vagina of heifers who received a CIDR with intact tail (Fischer-Tenhagen et al., 2012). They also reported that heifers who received CIDRs with intact tails were more likely to have their CIDRs removed by pen-mates before the end of the synchronization period (Fischer-Tenhagen et al., 2012). Taken together, it appears that CIDR insertion may provide a route for pathogenic bacteria to enter the vaginal cavity either by transmission from the host individual into the vaginal cavity or through curious behaviors of pen-mates (Fischer-Tenhagen et al., 2012). Likewise, it is possible that CIDRs allow for an influx of air into the vaginal cavity that may alter bacterial community profiles.

In regards to the physiological measurements collected as part of this study, a relationship existed between the presence of *P. aeruginosa* and the amount of PMN, CBG, and the FCI within the vaginal cavity. More specifically, at the time of CIDR insertion, heifers with a higher percentage of PMN had a lower abundance of *P. aeruginosa* within the cavity. Notoriously, PMN (i.e. neutrophils) are one of the first defense mechanisms during the initiation of the innate immune response to inflammation or tissue damage, and function in this manner by killing pathogenic cells or by destroying host tissue through phagocytosis (Henson and Johnston, 1987; Weiss,

1989; Kobayashi and DeLeo, 2009). Therefore, we speculate that among heifers with a greater population of neutrophils within the vagina, the phagocytic properties of the immune cell may have led to the observed reduction in *P. aeruginosa* as part of the host's innate immune response.

In support of our hypothesis, we observed a negative correlation between the presence of *P. aeruginosa* and concentrations of CBG measured within vaginal flush samples on d 0, such that heifers with a greater abundance of the bacteria had lower concentrations of CBG. *P. aeruginosa* produces and releases a protease (LasB) that is capable of cleaving multiple sites within the amino acid sequence of the RCL (Simard et al., 2014; Hammond, 2016; Lewis and Elder, 2017). Thus, it is possible that among heifers with a higher abundance of the bacteria within the vaginal cavity had less CBG present due to the proteolytic properties of the bacteria. Also during this time, there was a positive correlation between *P. aeruginosa* and the free proportion of cortisol (FCI) within the vaginal flush sample. When proteolytic cleavage of the RCL occurs, the steroid in which is bound to CBG dissociates and is considered biologically available which increases the free proportion of the steroid (Siiteri et al., 1981). We also observed a large increase in the percentage of PMN within the vaginal cavity at the time of CIDR removal which was directly related to FCI such that heifers with more PMN had a greater proportion of free cortisol. Therefore, it appears that the amount of free cortisol may be related to *P. aeruginosa* and PMN within the cavity, both of which are capable of causing a reduction in CBG concentration which would result in an increase in FCI.

Conclusion

To our knowledge, this is the first study to examine the relationship between CIDR insertion and the presence of *P. aeruginosa* inhabiting the vaginal cavity of heifers using qPCR technologies. Similarly, no other studies have been performed to examine the relationship between *P. aeruginosa* abundance and CBG concentration within the reproductive tract of cattle. As a result of CIDR insertion, we observed a 3.5 fold reduction in the number of *P. aeruginosa* inhabiting the vaginal lumen, but we do not know what induced this response, but we speculate environment and the greater ability of air to enter the cavity may have altered the bacterial communities. Based on the results presented in our study, a positive relationship exists between the abundance of *P. aeruginosa* and the proportion of free cortisol in the vaginal cavity. Thus, further investigation is warranted to determine the ability of the *P. aeruginosa* and PMN to disrupt steroid binding within the RCL of bovine CBG and if it has the ability to impact reproductive function in regards to steroid bioavailability in addition to its pathogenic properties.

Appendix

Table 5. Comparison of the abundance (mean CFU·mL^{-1} ± SEM) of *Pseudomonas aeruginosa* in vaginal flush samples collected on d -7 and 0 between heifers that became pregnant (*n* = 24) following synchronization of ovulation followed by artificial insemination and those that remained open (*n* = 43).

	Pregnancy Status on d 38		P-Value	
Day[1]	**Open**	**Pregnant**	**Day*Preg Status**	**Day**
-7	10504.23^{a} ± 2455.81	8779.79^{a} ± 2577.22	0.96	<0.0001
0	2980.70^{b} ± 619.24	2550.48^{b} ± 639.47		

[1]Vaginal flush samples were collected from each heifer immediately prior to CIDR insertion on d -7 and immediately following CIDR removal on d 0.
^{ab}Means within a row or column with different superscripts differ.

Table 6. Mean ± SEM concentrations of endocrine-related variables measured within the vaginal flush samples collected on d -7 and 0 from heifers undergoing synchronization of ovulation.

	Day		
Vaginal flush variable[1]	**-7**	**0**	**P-Value**
Progesterone (nmol/L)	33.43[b] ± 13.64	136.47[a] ± 27.57	<0.0001
Cortisol (nmol/L)	0.038[b] ± 0.006	0.076[a] ± 0.0012	<0.0001
CBG (mg/L)	2.49 ± 0.15	2.66 ± 0.15	0.25
FPI[2] (nmol/mg)	14.58[b] ± 7.45	52.81[a] ± 14.20	<0.0001
FCI[2] (nmol/mg)	0.016[b] ± 0.004	0.030[a] ± 0.006	<0.0001
PMN (%)	7.44[b] ± 2.70	48.61[a] ± 2.68	<0.0001

[1]Vaginal flush samples were collected from each heifer immediately prior to CIDR insertion on d -7 and immediately following CIDR removal on d 0.
[2]Steroid hormone concentration (nmol/L) was divided by CBG concentration (mg/L) to calculate a free hormone index (nmol/mg) for both progesterone (FPI) and cortisol (FCI).
[ab]Means with different superscripts differ.

Table 7. Spearman correlation analysis between *Pseudomonas aeruginosa* abundance and endocrine variables measured in vaginal flush samples collected on d -7 and 0 from heifers undergoing synchronization of ovulation.

Day[1]	Variable 1	Variable 2	Correlation coefficient (*r*)	P-Value
-7	*P. aeruginosa* abundance	Vaginal [CBG]	-0.002	0.99
0	*P. aeruginosa* abundance	Vaginal [CBG]	-0.25	0.05
-7	*P. aeruginosa* abundance	% PMN	-0.32	0.04
0	*P. aeruginosa* abundance	% PMN	0.05	0.68
-7	*P. aeruginosa* abundance	Vaginal [Progesterone]	-0.11	0.48
0	*P. aeruginosa* abundance	Vaginal [Progesterone]	-0.19	0.14
-7	*P. aeruginosa* abundance	Vaginal [Cortisol]	0.16	0.29
0	*P. aeruginosa* abundance	Vaginal [Cortisol]	0.19	0.14
-7	*P. aeruginosa* abundance	Vaginal FPI[2]	-0.11	0.46
0	*P. aeruginosa* abundance	Vaginal FPI	-0.06	0.67
-7	*P. aeruginosa* abundance	Vaginal FCI[2]	0.16	0.31
0	*P. aeruginosa* abundance	Vaginal FCI	0.34	0.004

[1] Vaginal flush samples were collected from each heifer immediately prior to CIDR insertion on d -7 and immediately following CIDR removal on d 0.
[2]Steroid hormone concentration (nmol/L) was divided by CBG concentration (mg/L) to calculate a free hormone index (nmol/mg) for both progesterone (FPI) and cortisol (FCI).

Chapter IV

Characterization of CBG mRNA expression within bovine vaginal epithelium and uterine endometrium: a pilot study

Abstract

Corticosteroid-binding globulin (CBG) is a glycoprotein that serves as a transporter for glucocorticoids and progesterone. Thus far, CBG has been measured within the uterus and vaginal lumen in swine and cattle, respectively, but the source of this glycoprotein remains ill-defined. Therefore, the objective of this pilot study was to determine if CBG mRNA is expressed locally within the bovine reproductive tract. Reproductive tracts (n = 3) were collected at the time of evisceration from cattle within the luteal phase of the estrous cycle. Vaginal epithelial tissue was harvest 2.5 cm caudal to the cervix, and endometrial tissue from each uterine horn was collected 2.5 cm cranial to the bifurcation and 2.5 cm caudal to the oviduct. Total RNA was extracted from each tissue sample and cDNA was synthesized via reverse transcription PCR. CBG gene expression was quantified relative to β-actin via quantitative PCR. A mixed model analysis of variance was performed to determine if CBG expression levels varied among the sample collection sites. Within vaginal epithelial tissue samples, the CBG gene was not expressed. However, expression of the gene was present in the endometrium, but expression levels did not differ (P = 0.95) among the collection sites. In summary, it appears that CBG biosynthesis may occur within the uterine endometrium, but not the vaginal epithelium.

Introduction

Corticosteroid-binding globulin (CBG) is a glycoprotein that is predominantly synthesized within hepatocytes (Hammond et al., 1991; Heo et al., 2003a) and functions

as a transporter for glucocorticoids and progesterone in which it binds with high affinity, but low capacity (Slaunwhite and Sandberg, 1959; Seal et al., 1966; Westphal, 1986a; Heo et al., 2003b). Corticosteroid-binding globulin is a member of the serine protease inhibitor superfamily (SERPIN A6) (Hammond et al., 1987; Law et al., 2006). As a member of this superfamily, CBG acts as a target for proteases (Lin et al., 2010) such as neutrophil elastase, a protease specifically synthesized and released by neutrophils (Hammond et al., 1990). More recently chymotrypsin, and LasB, an elastase produced and released by *Pseudomonas aeruginosa* have also been reported as proteases capable of disrupting the RCL (Lewis and Elder, 2014; Simard et al., 2014). In the presence of CBG, these proteases cleave specific sites within the reactive center loop (RCL) of CBG and compromise the steroid binding capacity (Lewis and Elder, 2017). After cleavage, the steroid dissociates from CBG and can be sequestered and loosely bound by albumin, or remain free within circulation, but in both instances the steroid is considered biologically available (Simard et al., 2014). Additionally, a consequence of RCL cleavage includes a 5 kDa reduction in molecular weight and conformational change that prevents the ability of CBG to bind an additional steroid molecule (Hammond et al., 1990; Potempa et al., 1994; Silverman et al., 2001).

Glucocorticoids and progesterone compete for the one binding site within the RCL of CBG, and the concentration of cortisol or progesterone can alter the ratio of steroids bound within the RCL of CBG (Westphal, 1986a). For example, under periods of high progesterone production during human pregnancy, progesterone displaces cortisol from the RCL to occupy the binding site (Rosenthal et al., 1969); whereas,

during periods of elevated glucocorticoid production, cortisol displaces progesterone from the RCL (Westphal, 1986a). This interaction signifies the main function of CBG which includes serving as a buffer and reservoir for glucocorticoids and progesterone (Rosner, 1991; Sivukhina and Jirikowski, 2014).

In addition to steroid buffering, CBG may also play a role in transporting progesterone from the corpus luteum (CL) to the uterus (Graham and Clarke, 1997), but, the exact mechanism of action is not well understood. The presence of CBG mRNA in steroidogenic tissues, and specifically within both the human corpus luteum and endometrium supports the theory that CBG plays a role in progesterone transport and steroidogenic function (Misao et al., 1994; Misao et al., 1997; Misao et al., 1999). Misao et al. (1997) examined the expression of CBG mRNA in human CL tissue following hysterectomy procedures and reported a greater expression of CBG mRNA during the mid-luteal phase of the menstrual cycle when compared to the early and late-luteal phases when concentrations of progesterone are lower than that of the mid-luteal phase (Misao et al., 1997; Misao et al., 1999). Similarly, CBG mRNA expression within endometrial tissue is greater during the secretory phase of the menstrual cycle, a phase characterized by greater progesterone concentrations when compared to the proliferative phase (Misao et al., 1994). Therefore, it is speculated that CBG may play a role in the localized transport of progesterone within the reproductive tract of humans, but to the best of our knowledge the expression of CBG mRNA within the reproductive tract has not been studied in regards to cattle.

So far, our lab has shown the ability to quantify CBG within the uterine lumen of swine (Klemcke et al., 1998), and within the vaginal cavity of cattle as described in Chapter II of this dissertation. Based on the previous studies described above, CBG mRNA is expressed locally in the endometrium and luteal tissue in other species, but to our knowledge synthesis of CBG within the vaginal epithelium has not been explored and uncertainty remains regarding the source of CBG measured in our experiments. Thus, the objective of this experiment is to determine if CBG mRNA is expressed locally in vaginal epithelial and uterine tissue of cattle to allow us to better understand the source of CBG within the bovine vaginal cavity.

Materials and Methods

Animals

Female reproductive tracts (n = 3) were harvested from cull cattle at a local abattoir at the time of evisceration which occurred approximately 45 min post-exsanguination. Exact breed composition is unknown, but two tracts were from cattle of dairy breed phenotype and one tract was from that of a cow of beef breed phenotype. Only non-gravid tracts and those without visible signs of infection were collected. The stage of the estrous cycle was determined at the time of collection by assessing visible characteristics of the corpus luteum (CL) as described by Ireland et al. (1980). For this study, only tracts classified as stage 2 (d 5 to 10 of estrous cycle; red or brown apex with a yellow body) or 3 (d 11 to 17 of estrous cycle; whole CL is orange or yellow)

according to the methods of Ireland et al. (1980) were used to determine if bovine CBG mRNA is expressed within the reproductive tract.

Tissue collection

Using a scalpel, sections of vaginal epithelial and uterine endometrial tissue samples were cut from the inside of each reproductive tract at locations designated in Figure 8 of the appendix (Bauersachs et al., 2006; Okumu et al., 2010). Immediately after collection, tissue sections were placed in a 15 mL conical tube, submerged in RNAlater (Ambion, Austin, TX), and stored on ice until samples were transported back to the laboratory (Forde et al., 2009; Okumu et al., 2010). Once back at the laboratory, tubes containing the tissue sample and RNAlater solution were stored at -80 ºC until later RNA extraction.

RNA extraction

Prior to total RNA extraction, the sections of tissue were frozen in liquid nitrogen, pulverized using a BioPulverizer (BioSpec Products Inc., Bartlesville, OK), and further ground to a powder with a mortar and pestle. Ground uterine epithelium (30 mg) and vaginal endometrium (70 mg) were transferred to culture tubes containing 1 mL Buffer RTL provided within the Qiagen RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Tissues were further homogenized for 30 s using a homogenizer (Laboratory Homogenizer, Model 125, Thermo Fisher Scientific Co. Pittsburgh, PA) and were then passed through a 20 gauge needle and syringe to further lyse the tissue. Total RNA extraction procedures were performed to the specifications of protocol provided within

the Qiagen RNeasy Plus Mini Kit (Okumu et al., 2010). All samples were eluted in 50 uL of RNase-free water and RNA concentrations were quantified using an Ultrospec 3100 Pro UV/Visible Spectrophotometer (Amersham Bioscience Corp., Buckinghamshire, UK). Eluted samples were stored at -80 º C until cDNA synthesis was performed.

cDNA synthesis

Total RNA (500 ng) was converted to cDNA through reverse transcription PCR (RT-PCR) by following the instructions provided within the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Pittsburgh, PA). Each RT-PCR reaction contained a total volume of 20 uL which consisted of 10 uL of RT-PCR Master Mix. The remaining 10 uL consisted of varying volumes of RNA template and RNase-free water to achieve a final mass of 500 ng of template RNA per RT-PCR reaction tube. Thermal cycling conditions were performed using a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA) as per the instructions provided within the High Capacity cDNA Reverse Transcription Kit (25 ºC for 10 min, 37 ºC for 120 min, 85 ºC for 5 min, and 4 ºC for ∞) and cDNA samples were stored at -20 ºC until later quantitative PCR (qPCR) analyses.

Quantitative PCR analysis

Forward (5'-CGCCATGGATGATGATATTGC-3') and reverse (5'-AAGCCGGCCTTGCACAT-3') primers for β-actin and concentrations (900/300 nM) used for our experiment were previously validated in bovine endometrial tissue and were used as a housekeeping gene (Forde et al., 2009; Okumu et al., 2010). Using the

transcript of SERPINA6 (ENSBTAG00000039808) in Ensembl (www.ensembl.org) and Primer3Plus (www.bioinformatics.nl/primer3plus), forward (5'-AGCTCAGCCACACTCAT CCT-3') and reverse (5'-TTCTCTGGTGCTTTCCAGGT-3') primers (500 nM) were designed to span an exon-intron-exon junction within the bovine CBG gene.

Within each qPCR reaction, 500 ng of template cDNA harvested from uterine endometrial or vaginal epithelial tissue was used (Okumu et al., 2010). For β-actin, a total reaction volume of 20 uL was used and consisted of 10 uL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Life Technologies, Carlsbad, CA), 1.8 uL of forward and 0.6 uL of reverse primers, 6.6 uL RNase-free water, and 1 uL of template cDNA. Thermal cycling settings included a hold stage of 50 ºC for 2 min then 95 ºC for 2 min followed by 40 cycles of denaturation at 95º C for 15 sec, annealing at 53º C for 1 min, and extension at 72º C for 1 min. For quantification of CBG gene expression, a total reaction volume of 20 uL was used and consisted of 10 uL of PowerUp™ SYBR™ Green Master Mix, 1 uL of forward and 1 uL of reverse primers, 7 uL RNase-free water, and 1 uL of template cDNA. Thermal cycling settings included a hold stage of 50 ºC for 2 min then 95º C for 2 min followed by 40 cycles of denaturation at 95º C for 15 sec, annealing at 54º C for 1 min, and extension at 72º C for 1 min.

Statistical analysis

Relative expression of the CBG gene was determined by calculating the ΔC_t for each sample relative to the expression of the β-actin gene within the same sample. A mixed model ANOVA was performed in SAS 9.3 to assess if expression levels varied

among tissue collection sites. Cow was included as a random blocking term to account for individual animal variation and Fisher's LSD test was used to separate means ($P \leq 0.05$).

Results

All figures are included in the appendix at the end of this chapter. Although β-actin was expressed consistently among vaginal epithelial tissue samples ($C_t = 20.45 \pm 1.50$) no amplification of the CBG gene was observed using the qPCR analysis methods described in the present study. However, we did observe expression among uterine endometrial tissue samples (Figure 4). Among all four tissue collection sites, CBG mRNA expression levels were similar ($P = 0.95$) regardless of proximity to the corpus luteum, but an overall low expression profile was observed.

Discussion

Based on previous research conducted in our laboratory in which we reported that CBG concentrations within the uterine lumen rise concurrently with progesterone concentrations during early gestation in gilts (Klemcke et al., 1998), we chose to incorporate reproductive tracts harvested from cows within the luteal phase of the estrous cycle to determine if the CBG gene is expressed locally within the uterine endometrium and vaginal epithelium. Likewise, in Chapter II we were able to quantify CBG concentrations within the vaginal lumen of heifers undergoing synchronization of ovulation. Previous studies conducted by Misao et al. (1994) and Seralini et al. (1990) reported CBG gene expression within the human endometrium and ovarian, uterine,

and placental tissues within rabbits. Therefore, within this pilot study, we aimed to determine if CBG mRNA is expressed within the bovine reproductive tract, and to the best of our knowledge, this is the first study to investigate CBG gene expression in regards to cattle.

Due to complications with tissue collection, we were limited to the use of three reproductive tracts within the luteal phase of the estrous cycle. Nonetheless, we were able to detect the presence of CBG mRNA expression within the endometrial tissue samples collected, but not within the vaginal epithelial tissue. Recognizing the histological difference between the two tissues, it was not surprising that we did not observed CBG gene expression in the vaginal tissue. The lining of the vagina is characterized by the presence of stratified squamous epithelial tissue with two vestibular gland ducts that provide mucus to the vaginal lumen (Bloom and Fawcett, 1975; Blazquez et al., 1987a; Blazquez et al., 1987b). In contrast, the uterine endometrium consists of a layer of simple columnar epithelial cells that cover the underlying stroma layer that is comprised by highly glandular tissue that secrete proteins to the uterine lumen (Dawson, 1959; Bloom and Fawcett, 1975; Gray et al., 2001). In swine, Murray et al. (1972) observed differences in the concentration of total protein that was present within uterine flush samples collected at different stages of the estrous cycle such that protein secretions were greatest under during the luteal phase and were concurrent with elevated progesterone production (Murray et al., 1972), and heightened protein synthesis within the endometrial tissue (Basha et al., 1980; Roberts and Bazer, 1988).

Among the four sampling locations within the uterus, the bovine CBG mRNA was present, but expression of the gene was low. This observation was in agreement with data reported by Seralini et al. (1990) who showed that CBG mRNA is expressed within the uterus of rabbits, but at much lower of an abundance when compared to hepatic tissue. Across the sampling sites, CBG mRNA expression was the same. Recognizing that sample size is a major limitation for this study, a follow-up study with a larger population size should be conducted to determine if this pattern is consistent or if levels of CBG mRNA differ among tissue samples collected at varying proximities to the CL. Within uterine tissue, a progesterone concentration gradient exists such that tissue samples collected closer to the ovary with the CL contain greater concentrations of progesterone when compared to tissues collected further away from the CL (Pope et al., 1982; Weems et al., 1988). Likewise, tissue collected from the contralateral uterine horn relative to the CL have lower concentrations of progesterone than tissue collected from the ipsilateral horn (Pope et al., 1982). Based on these observations and the heightened uterine protein production observed by Murray et al. (1972) during the luteal phase of the estrous cycle, it is worth investigating to determine if changes in CBG mRNA expression occur throughout the estrous cycle and if expression levels vary within samples collected from different regions of the uterus.

Conclusion

To the best of our knowledge, this is the first study to examine bovine CBG gene expression. The CBG gene was expressed within uterine endometrial tissue, but not in

vaginal epithelial tissue. Thus, it is possible that CBG measured within the vaginal cavity of heifers, as described in Chapter II, was originally derived from the endometrium. Recognizing that the majority of CBG in circulation is produced within the liver, and CBG possesses a molecular weight of approximately 50-60 kDa, it is not likely that hepatic-derived CBG alters the concentration within other tissues (Hammond et al., 1991; Hammond, 2016). To support this claim, immunohistochemical techniques should be employed to determine if the glycoprotein is produced locally within endometrial tissue. Nonetheless, it appears that CBG plays a role within the bovine reproductive tract, but further investigation is warranted to determine its specific function as it relates to reproductive function.

Appendix

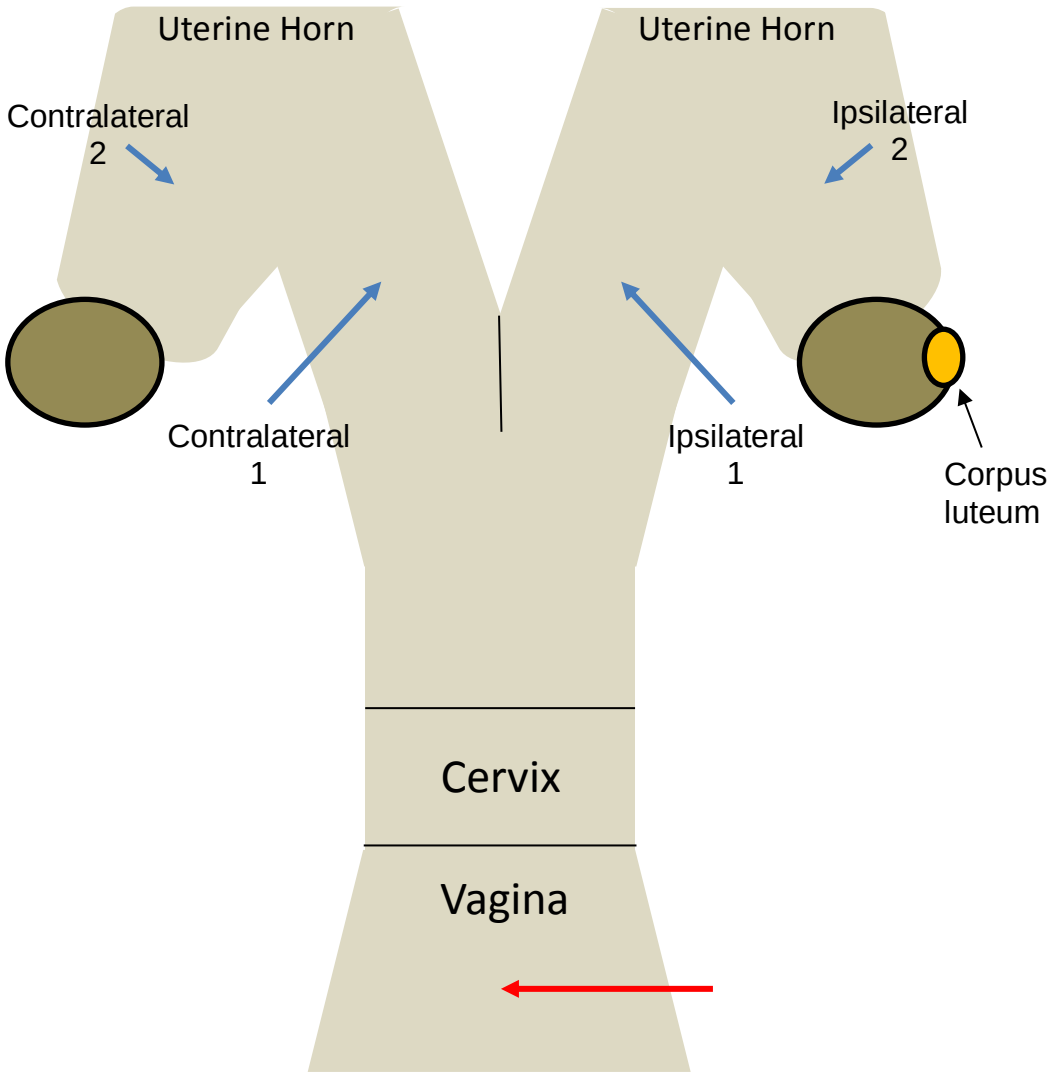

Figure 3. Collection sites for vaginal epithelial (red arrow) and uterine endometrial tissue (blue arrows) harvested from bovine reproductive tracts. Vaginal epithelial tissue was collected approximately 2.5 cm caudal to the cervix. Uterine endometrial tissue was collected approximately 2.5 cm cranial to bifurcation and another sample was collected approximately 2.5 cm caudal to the beginning of the oviduct.

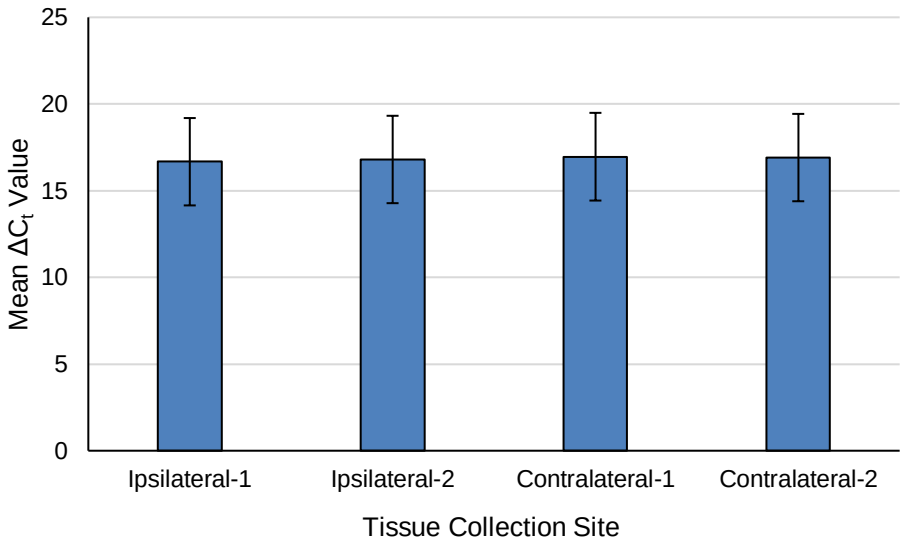

Figure 4. Relative expression of the bovine CBG gene in endometrial tissue collected from four sites within the female reproductive tract. Within the uterine horn ipsilateral to the corpus luteum, endometrial tissue was collected approximately 2.5 cm cranial to bifurcation (Ipsilateral-1) and another sample was collected approximately 2.5 cm caudal to the beginning of the oviduct (Ipsilateral-2). Another set of endometrial samples were collected within the contralateral uterine horn approximately 2.5 cm cranial to bifurcation (Contralateral-1) and 2.5 cm caudal to the beginning of the oviduct (Contralateral-2). Expression of the CBG gene is relative to the expression of β-actin. CBG expression was similar (P = 0.95) among tissue collection sites.

General Research Conclusions

The studies contained within this dissertation are the first to examine the influence of progesterone on concentrations of CBG in circulation and within the reproductive tract of cattle. Likewise, we are the first to explore CBG gene expression within vaginal epithelial and uterine endometrial tissue in cattle. Previously, research conducted in regards to humans has shown that CBG biosynthesis and concentrations of the glycoprotein are altered during heightened progesterone output (Rosenthal et al., 1969; Benassayag et al., 2001). Recognizing the role of CBG in regulating the proportion of biologically available steroid, we were interested in examining the effect of exogenous progesterone administration during synchronization of ovulation and early gestation in heifers on concentrations of CBG within the vaginal cavity and in circulation.

In Chapter II, no alterations in vaginal or systemic CBG concentrations were observed during the CIDR insertion period. However, among heifers confirmed as pregnant on d 38, CBG concentrations in circulation increased during early gestation when compared to those measured during the synchronization period. Additionally, based on our FPI calculation that estimates the proportion of bound progesterone to CBG, it appeared that the overall rise in CBG during early gestation helped to buffer the amount of free progesterone in circulation during that time period. Across a variety of species, including humans and cattle, insufficient progesterone concentrations early in gestation pose a threat to pregnancy sustainability. Based on our preliminary results, there is evidence to believe that CBG plays a role in progesterone bioavailability. Thus, future work should be aimed at examining CBG biosynthesis and the amount of free, or

biologically available progesterone during early gestation to determine if the rate of CBG production within an animal has the potential to impact the maintenance of pregnancy.

Both *P. aeruginosa* and neutrophils synthesize and release proteases that cleave the RCL of CBG to modulate steroid bioavailability by reducing the binding capacity of the transporter (Hammond et al., 1990; Simard et al., 2014). In Chapter III, CIDR insertion caused a localized inflammatory response within the vaginal cavity as denoted by an increase in PMN. Additionally, both PMN and the presence of *P. aeruginosa* were directly related to the amount of free cortisol in circulation, but only *P. aeruginosa* was related to CBG such that heifers with a higher abundance of the bacteria had lower concentrations of CBG. Thus far, this study is the first to examine the relationship between *P. aeruginosa* and the bioavailability of steroids in cattle, but our preliminary data suggest that the presence of the bacteria in the vaginal cavity may be capable of altering the local ratio of free steroid hormone. Although these results were generated by correlation analysis, future work should be aimed to investigate *P. aeruginosa*'s role in disrupting the steroid binding capacity of bovine CBG via *in vitro* analyses. Additionally, the potential for *P. aeruginosa* and PMN to alter the amount of free cortisol within the vaginal cavity suggests that both are capable of direct impact on the local inflammatory response.

Lastly, our pilot study described in Chapter IV provided insight as to the source of CBG measured within the vaginal cavity. Previous research has indicated that although hepatic production of CBG is the main source for CBG in circulation, is not reflective of extrahepatic CBG biosynthesis (Hammond, 2016), thus we wanted to examine CBG

gene expression within the bovine reproductive tract. In our study, CBG mRNA was not expressed within the vaginal tissue, but was expressed at low levels within the uterine endometrium. Therefore, we believe CBG measured in the vaginal cavity in Chapter II was derived from the endometrium. These results are novel in the sense that CBG gene expression has not been characterized in cattle, but these data reinforce the notion that CBG is present within tissues under high steroid influence.

In summary, although our studies were limited in regards to the number of cattle and the number of sampling time points used, our results indicate that CBG acts as a buffer for steroid hormones during early gestation in beef heifers and is active within the bovine reproductive tract. We believe the results from these studies set the foundation for future work in regards to the role of CBG and reproductive function in cattle. Furthermore, previous research in humans have reported polymorphisms associated with the SERPINA6 gene that alter the production and steroid binding properties of CBG (Hammond, 2016). Therefore, further investigation into the variants of the bovine CBG gene may be warranted to determine if the proportion of free steroid differs among cattle with varying genotypes.

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Appendices

Appendix A. Percent recovery assay for evaluating cortisol recovery capability of Sep-Pak Classic C18 Cartridges (Waters Corporation, Milford, MA)

Procedure (adapted from procedures reported by Shackleton and Whitney (1980))

1. Attach the Sep-Pak Classic C18 Cartridge to a 6 or 12 mL syringe.
2. Prime
    a. Add 2 mL of methanol to the barrel of the syringe. Allow the methanol to flow through the cartridge one drop at a time.
    b. Add 5 mL of sterile deionized water to the barrel of the syringe and allow the water to flow through the cartridge one drop at a time.
3. Sample
    a. Add 1 mL of ^{125}I Cortisol Tracer Solution (MP Biomedicals, Solon, OH; cat # 06B256617) to the barrel and allow contents to flow through the cartridge one drop at a time.
4. Wash
    a. Add 5 mL of sterile deionized water to the barrel of the syringe and allow water to flow through the cartridge one drop at a time.
5. Recovery/elute
    a. Add 2 mL of methanol to the barrel of the syringe, allow methanol to flow through the cartridge one drop at a time, and collect methanol in a borosilicate glass test tube.
6. Evaporation and reconstitution

a. Evaporate recovered methanol under air until borosilicate glass tube is completely dry.
b. Reconstitute sample with 1 mL of 0.9 % sodium chloride sterile saline.

7. Recovery determination
    a. In one polypropylene tube, add 1 mL of straight ^{125}I Cortisol Tracer Solution. In a separate tube, transfer all contents of the reconstituted sample in step 6.
    b. Run samples through the gamma counter to evaluate counts per minute (cpm) for each sample.
    c. Calculate percent recovery by using the following equation:

        % recovery = (recovered sample cpm ÷ straight sample cpm) × 100

Appendix B. Protocol for concentrating vaginal flush samples for cortisol EIA using Sep-Pak Classic C18 Cartridges (Waters Corporation, Milford, MA)

Procedure (Shackleton and Whitney, 1980)

1. Attach the Sep-Pak Classic C18 Cartridge to a 6 or 12 mL syringe.
8. Prime
    a. Add 2 mL of methanol to the barrel of the syringe. Allow the methanol to flow through the cartridge one drop at a time.
    b. Add 5 mL of sterile deionized water to the barrel of the syringe and allow the water to flow through the cartridge one drop at a time.
9. Sample
    a. Add 5 mL of vaginal flush sample to the barrel of the cartridge and allow contents to flow through the cartridge one drop at a time.
10. Wash
    a. Add 5 mL of sterile deionized water to the barrel of the syringe and allow water to flow through the cartridge one drop at a time.
11. Recovery/elute
    a. Add 2 mL of methanol to the barrel of the syringe, allow methanol to flow through the cartridge one drop at a time, and collect methanol in a borosilicate glass test tube.
12. Evaporation and reconstitution

a. Evaporate recovered methanol under air until borosilicate glass tube is completely dry.
b. Reconstitute sample with 100 uL of 0.9 % sodium chloride sterile saline.
c. Freeze samples for later analyses.

Vita

Sierra A. Lockwood was born in Syracuse, New York in 1990. A little while after, her family moved back to the family farm in Canandaigua, New York where she was involved in the Ontario County 4-H program while growing up. In 2008, she graduated from Canandaigua Academy and moved to Knoxville, Tennessee to attend The University of Tennessee. She graduated from The University of Tennessee with a Bachelor of Science in Animal Science with a minor in Biology in May of 2012 and a Master of Science in Animal Science with a minor in Statistics in December of 2014. Since then, Sierra has been working toward obtaining her PhD in Animal Science under the guidance of co-mentors Dr. Henry Kattesh and Dr. Justin Rhinehart at The University of Tennessee.