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Investigating extra hepatic steroid and eicosanoid metabolizing enzymes in cattle

By

Megan Pauline Theresa Owen

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Agricultural Science
in the College of Agriculture and Life Sciences

Mississippi State, Mississippi

December 2017

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Investigating extra hepatic steroid and eicosanoid metabolizing enzymes in cattle

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Steroid and eicosanoid metabolism occurs in two phases and primarily within hepatic tissues, but localized metabolism has been examined in several extra-hepatic tissues in humans and rodents. Phase I of metabolism is performed by Cytochrome P450s (CYP) that add hydroxyl groups to the carbon ring structure which is further metabolized by phase II UDP-glucuronosyltransferase (UGT). The overall objectives of the following experiments were to: 1) determine the amount of extra-hepatic steroid metabolism within reproductive tissues of cattle across the estrous cycle; 2) determine the amount of extra-hepatic steroid metabolism and an oxylipin profile within reproductive tissues of cattle based on pregnancy status; and 3) determine the amount of endometrial blood perfusion in cattle using a novel laser Doppler technique. Activity of CYP1A was found within corpora lutea (CL) tissues of both pregnant and non-pregnant cattle, but not within endometrial tissues. Endometrial perfusion, measured using a novel laser Doppler technique, was also validated by measuring angiogenic factors in close proximity to the location of perfusion. A positive correlation (r = 0.28; $P = 0.04$) was observed between endometrial perfusion and nitrite concentration, an angiogenic factor. Endometrial blood

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perfusion was affected by the proximity to the CL, but not by the proximity of the dominant follicle. In addition, UGT was categorized across the estrous cycle and the activity was dependent upon the proximity of the CL. Oxylipins, including eicosanoids, were also profiled in CL of cattle that were non-pregnant and pregnant with 5 out of 39 oxylipins differentially expressed. The activity and oxylipin products of steroid and eicosanoid enzymes were not correlated with serum or luteal progesterone. Through these experiments, we have verified that there is localized metabolism of steroids and eicosanoids within reproductive tissues of cattle as well as fetal tissues. Also, we have achieved a full oxylipin profile of non-pregnant and pregnant cattle CL with five oxylipins contained in various amounts between pregnancy status.

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CHAPTER I

REVIEW OF LITERATURE

The Estrous Cycle

The estrous cycle consists of two primary phases, the follicular phase and luteal phase. The follicular phase, corresponds to a decrease in progesterone (P_4) from the regressing corpus luteum (CL) and an increase in estrogens, especially estradiol-17β (E2) from the developing follicle (Fortune et al., 1988). The follicular phase ends with the ovulation of a dominant follicle. The ovulated follicle will then transition from a corpus hemorraghicum to a CL, which initiates the start of the luteal phase. If a successful pregnancy is not established by a competent conceptus, the CL will regress and the cycle will repeat every 21 days, on average, in cattle, pigs, and horses, while in sheep the cycle will repeat every 17 days (Henricks et al., 1972; Nett et al., 1976; Baird, 1978; Ginther et al., 1989). One important concept during normal reproductive cyclicity is the cross talk between ovarian and uterine secretions during the follicular and luteal phases of the estrous cycle (Kimmins and Maclaren, 2001). Transplanting ovaries from adjacent to the uterus to the neck of cyclic ewes determined that the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secreted from the uterus affected the ovarian structure, the CL, to cause luteolysis (McCracken et al., 1999). This insured that there were substances coming from the uterus to act upon the ovaries. During the follicular phase, increased secretion of E2 from the dominant follicle on the ovary is transferred in the blood and acts upon several tissues including the uterus.

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Estrogen exposure to the uterus results in increased blood flow, increased smooth muscle motility, and growth and development of uterine glands of the endometrium (Ford, 1982).

Similarly, during the luteal phase, increased secretion of P_4 from the CL on the ovary is transferred in the blood and acts upon several tissues including the uterus. Exposure of P_4 to the uterus results in a blockage of smooth muscle contractions of the uterus and stimulates nutritive secretions from the uterine glands of the endometrium (Nephew et al., 1994). The luteal phase ends with uterine secretion of the hormone PGF2α, which regresses the functional CL, allowing for emergence of the next follicular phase (Shemesh and Hansel, 1975). Uterine secretion of PGF2α is partially initiated by down-regulation of uterine P_4 receptors followed by up-regulation of uterine estrogen receptors (ER) and oxytocin receptors (OTR) as well as a major enzyme involved in prostaglandin synthesis, cyclooxygenase. Irrespective of the exact mechanisms, ovarian steroid secretion, and uterine exposure contributes to normal reproductive cyclicity in cattle. Moreover, these same pathways are also involved in establishing a successful pregnancy.

Pregnancy

Maternal Recognition of Pregnancy

Maternal recognition of pregnancy occurs between d 15 and 17 in cattle, which prolongs the lifespan of the CL. Within ruminant animals, the conceptus secretes interferon-tau (IFNτ), which suppresses the expression of the OTR, stimulating the synthesis of COX-2 within the endometrium. Production of IFNτ reaches its peak at approximately d 15 in sheep (Ashworth and Bazer, 1989) and d 17 in cattle (Demmers et al., 2001). This disallows the pulsatile release of PGF2α within the endometrium, and

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secretes pro-embryonic proteins, which results in a hospitable uterine environment (Bazer, 1992; Cheng et al., 2007). Tight junctions are reduced within the uteri of sheep on day 12 of the estrous cycle, which could allow for small molecules to permeate the endometrium and facilitate the transport of IFNτ. The trophectoderm cells of the fetus secrete IFNτ, which signals the CL to not undergo luteolysis (Roberts et al., 1990; Spencer et al., 1996; Imakawa et al., 2009; Bazer et al., 2010). In cattle, at d 16 of their estrous cycle, the lack of a conceptus allows for expression of the OTR, production of oxytocin (OT) by the CL, inducing COX-2 to increase the synthesis of prostaglandin intermediates, and stimulates the activity of prostaglandin F synthase, which is a vital enzyme of $PGF_{2\alpha}$ synthesis (Kim et al., 2003) within the endometrium and results in an embryo-toxic uterine environment (Bazer, 1992; Cheng et al., 2007; Figure 1).

Placenta

The placentas of livestock species are categorized into two different morphological groups. Cotyledonary placentas are within ruminant species and have synepitheliochorial microscopic anatomy. Diffuse placentas are within horses and pigs and has a the microscopic anatomy is epitheliochorial placenta (Moll and Kunzel, 1973; Nakaya and Miyazawa, 2015). Cotyledonary placentas only attach at portions of the uterine wall that protrude called caruncles and the fetal attachment portions that are complementary to these caruncles are called cotyledons (Moll and Kunzel, 1973; Lemley et al., 2012; Nakaya and Miyazawa, 2015). Similar to gestation length, birth weight, and estrous cycle length, placental morphometric measurements are highly dependent on breed within a certain species and are highly dependent on maternal breed and parity (Bienson et al., 1999; Ford et al., 2002).

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Placentation is the period of maternal and fetal portions of the placenta attaching cand growing together and developing (Wilson, 2002). In ewes, placental attachment begins at approximately d 15 of gestation (Flint et al., 1979). By d 20 of pregnancy in ewes, the conceptus is adhered to the uterine wall and placentation occurs through about d 45 of pregnancy. Placental length increases to about 30 cm between d 25 and 140 of gestation, whereas, total placental weight increases between d 25 and 80 of gestation and remains rather constant thereafter in sheep (Bazer et al., 2011). Placental attachment in cattle, however, does not begin until around d 22 of gestation in cattle (Amoroso, 1959). During d 40 to 70, organ differentiation begins, bone ossification, and formation and completion of orientation and stomach compartments occurs (Caleb O. Lemley et al., 2014). By d 150 of gestation caruncular and cotyledonary vascularization is complete and growth of the placenta increases 2-fold from d 150 to 250 of gestation, while fetal weight significantly increased by 15-fold from d 150 to 250 of gestation (Reynolds and Redmer, 1995).

Steroid Hormones

Introduction and Synthesis

Steroid hormones are highly lipophilic and have a 4-ring nucleus that comes from the base structure of cholesterol. Synthesis of steroid hormones can be facilitated by almost every cell type within the body. This process begins with the movement of cholesterol, into the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR). This cyclic-AMP-dependent phosphoprotein (Kohen et al., 2003) is thought to be the rate limiting step of steroid synthesis and has a half-life of about 4 min. (Juengel et al., 2000). Once the cholesterol is within the inner mitochondrial membrane

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cytochrome P450 (CYP) side-chain cleavage (CYP11A1) cleaves the aliphatic side chain between carbon 20 and 22, then oxidizes the 20 carbon to yield a ketone group at the 17 carbon (Payne and Hales, 2004). This newly formed molecule, termed pregnenalone, has 21 carbons and can be further metabolized into various steroid hormones. For the synthesis of P_4, pregnenalone is moved into the smooth endoplasmic reticulum where 3 beta-hydroxysteroid dehydrogenase (3β-HSD) converts it into P_4 (Payne and Hales, 2004). This conversion of pregnenalone to P_4 is highly dependent upon product inhibition (Caffrey et al., 1979). Greater concentrations of P_4 means that there will be less P_4 produced. Progesterone can be further converted to 17α-hydroxyprogesterone, a fellow progestagen, via 17α-hydroxylase (CYP17) within the smooth endoplasmic reticulum (Payne and Hales, 2004). 17α-hydroxyprogesterone will then lose 2 carbons and become a part of the androgen class, specifically androstenedione, by 17, 20 lyase (Morato et al., 1961; Akhtar et al., 2011). Androstenedione can be further converted into other androgens via 17 beta-hydroxysteroid dehydrogenase to yield testosterone and further converted into dihydrotestosterone by 5α-reductase (Payne and Hales, 2004). However, androstenedione and testosterone can also be converted into various types of estrogens via aromatase to yield estrone and estradiol, respectively (Weisz and Gibbs, 1974; Sherman and Korenman, 1975).

Corpus Luteum

The LH surge immediately prior to ovulation induces the luteinization of granulosal and thecal cells (Juengel et al., 1995; Niswender et al., 2000). This luteinization process alters the steroidogenic pathway which helps ensure that P_4 is the primary steroid hormone produced (Juengel et al., 1995; Niswender et al., 2000; Scully et

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al., 2015). However, several species including humans, pigs, and rats, maintain the ability to produce a large amount of E2 by the CL (Niswender et al., 2000). As the CL progresses through maturation, from early to mid to mid-late luteal phase, the large luteal cells undergo hypertrophy, increase in cellular size, whereas the small luteal cells will undergo hyperplasia, increase in cell numbers (Meidan et al., 1990). While the small luteal cells produce a smaller amount of P_4, the large luteal cells produce the greater quantity of P_4 to be secreted into circulation (Rodgers and O'Shea, 1982).

The developing CL has a large amount of angiogenesis and has one of the largest blood flows per gram of tissue (Herzog et al., 2010; Shirasuna, 2010). Blood flow in CL from beef heifers was not different between d 7 and 14 comparing non-pregnant and pregnant CL; however, d 14 to 18, non-pregnant CL decreased in blood flow, whereas pregnant CL maintained a constant amount of blood flow (Scully et al., 2015). This decrease in blood flow of the CL is due to the anti-luteotrophic effects of PGF2α that is secreted at d 15 of the estrous cycle and begins the functional and structural regression of the CL (Scully et al., 2015). Concentrations of mRNA encoding for StAR decreased rapidly which affected downstream steroidogenic enzymes, 3β-HSD, and CYP11A1, 9 hr after administration of $PGF_{2\alpha}$ within CL tissues of ewes (Juengel et al., 2000). The maintenance of blood flow after d 15 in pregnant animals was due to the secretion of IFNτ by the trophectoderm cells, which is luteotrophic and maintains the physiological and structural integrity of the CL in order to maintain the pregnancy (Roberts et al., 1990; Spencer et al., 2008; Scully et al., 2015). As outlined in Figure 1, IFNτ induces the production of COX-2 resulting in the secretion of PGE_2, which increases the amount of cyclic AMP and increases angiogenesis of the endometrium (Cheng et al., 2007).

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Progesterone

Progesterone is a steroid hormone with 21 carbons, and has been noted to create a type of quiescence of the uterus which minimizes the myometrial contractions and affects the movement of blood through capillary beds within the endometrium (Grazzini et al., 1998; Soloff et al., 2011). The primary source of P_4 is the transient organ, the CL, which is present on an ovary post-ovulation. However, the adrenal gland was observed to secrete as much P_4 in stressed rats as compared to the amount of P_4 secreted from the ovary while the rat was in the metestrus phase (Fajer et al., 1971). Once ovulation occurs the cells within the ovulation site begin to differentiate where the granulosal cells become large luteal cells and the theca interna cells become small luteal cells (Milvae et al., 1996). The stimulation for P_4 production is due to the binding of luteinizing hormone (LH) from the gonadotropin cells of the anterior pituitary (Dehejia et al., 1982; Milvae et al., 1996) to the LH receptors. These LH receptors can be induced to degrade and internalize at a slower rate with the supplementation of LH-like substances, such as human chorionic gonadotropin (hCG). Mock and Niswender (1983) observed that internalization and degradation by luteal cells are greatly extended in sheep with radiolabeled hCG, 22.8 ± 2.3 hr, when compared to natural sheep LH, 0.4 ± 0.2 hr. The increased half-life of hCG is due to the increased glycosylation which prevents proteases from degrading the molecule (Kessler et al., 1979).

Cyclooxygenase (COX)

The cyclooxygenase (COX) pathway includes the generation of prostaglandins, resolvins, and protectins (Calder, 2010). Cyclooxygenase acts through two different isoforms cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), which can be

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activated ubiquitously (Inskeep, 1973; Funk, 2001). Actions of COX-1 include renal electrolyte and water homeostasis, cytoprotection of gastric cells, and activation of platelet aggregation and clot formation. While COX-2 is primarily responsible for the metabolism of arachidonic acid into various prostaglandin species (Funk and Powell, 1983; Funk, 2001; Arosh et al., 2004; Shishodia et al., 2004). This pathway begins with arachidonic acid which is then converted to PGG_2 by COX-2, which is then converted to PGH_2 by prostaglandin endoperoxidase H_2 synthase, where it can be converted into various prostaglandins with their respective synthase enzymes.

The amount of arachidonic acid can vary within an animal based on their nutritional availabilities. Certain fatty acids are more readily converted into arachidonic acid and therefore converted into prostaglandins (Herrera-Camacho et al., 2011). Expression of COX-2 has primarily been found in the luminal and superficial glandular epithelial cells (Kim et al., 2003), which coincides with the secretion of $PGF_{2\alpha}$ into the uterine lumen. These prostaglandins include prostaglandin E_2 (PGE_2) which is angiogenic, as well as $PGF_{2\alpha}$ which is known to be embryo-toxic (Canby-Hagino and Thompson, 2005; Figure 2).

Expression of COX isoforms are dependent upon the pregnancy status of the host animal. Cycling cows and ewes yielded an increase in COX-2 mRNA in endometrial tissue from the mid- to late-luteal portion of the estrous cycle (Kim et al., 2003; Arosh et al., 2004), and expression of COX-2 in bovine endometrium is over 4 fold greater than COX-1 (Emond et al., 2004). Staining intensity for COX-2 increased in cattle epithelial stroma tissue, ipsilateral to the CL as compared to contralateral to the CL, at day 16 when the uterus is infused with IFNτ (Emond et al., 2004). Pregnant ewes resulted in greater

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COX-2 mRNA than cycling ewes beginning on d 12 through d 16 (Kim et al., 2003), and fetal tissues at d 18 of pregnancy expressed COX-2 mRNA and protein (Kim et al., 2003).

Lipoxygenase (LOX)

The lipoxygenase (LOX) pathway primarily produces leukotrienes, resolvins, and protectins (Calder, 2010). Lipoxygenases are iron dioxygenases that specifically insert oxygen into polyunsaturated fatty acids, resulting in the formation of hydroxy-eicosatetraenoic acid (HETE) molecules (Funk, 2001). Leukotrienes are made predominately by inflammatory cells in response to activation of immune complexes. Resolvins and protectins have been noted to inhibit migration of neutrophils which in turn minimizes the inflammatory response elements (tumor necrosis factor α - TNFα, interleukins, and necrosis factor- κB - NF-κB) and thereby act as anti-inflammatory (Calder, 2010). Administration of PGF2α increased T cell proliferation in regressing luteal cells (Davis and Pate, 2007). Leukocytes are recruited to the CL throughout its lifespan to either encourage chemokine mediators, during luteolysis, and recruitment of antiapoptotic molecules to potentiate survival (Pate et al., 2010). Products of enzymatic activity of LOX, including several HETE, are greater in amniotic fluid of women that were in labor as compared to women that were not (Romero et al., 1987). However, immediately prior to labor, leukotrienes were in greater amounts as compared to HETE (Mitchell and Grzyboski, 1987).

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Oxylipins

Oxylipins are a family of lipids that result from biosynthesizing polyunsaturated fatty acids (PUFA) which result in products such as eicosanoids, epoxides, diols, thromboxanes, and leukotrienes (Calder, 2010; Gouveia-Figueira et al., 2015; Mavangira and Sordillo, 2017). Eicosanoids are derived from arachidonic acid by the COX-2 pathway as described above and outlined in Figure 2. These eicosanoids cannot be stored, which indicates that they are to be formed de novo when cells are mechanically or chemotoxically challenged by any cell within the body (Funk, 2001). Metabolism of omega fatty acids into oxylipins occurs via CYP, COX, and LOX enzymes and further metabolism by UGT to inactivate these oxylipins (Heemskerk et al., 2014). Metabolites of CYP are classified as dihydroxyoctadecenoic acid (HOME) and dihydroxyeicosatrienoic acid (HODE), metabolites of COX enzymes are classified as prostaglandins (PG) and thromboxanes (TX), and metabolites of LOX enzymes are classified as HETE and octadecadienoic acids (ODE; Calder, 2010; Chang et al., 2012; Bruins et al., 2013; Gouveia-Figueira and Nording, 2015a).

Some eicosanoids are important for the regression of the CL, such as PGF2α (Rekawiecki et al., 2008), which allows for the non-pregnant female to begin another follicular phase of the estrous cycle, but other eicosanoids are important for the maintenance of the CL, such as PGE2, to prolong during gestation and produce P_4 (Weems et al., 2011). Production of these oxylipins have been linked to inflammatory responses (Calder, 2010; Zivkovic et al., 2012; Mavangira and Sordillo, 2017) and increased intake of omega fatty acids (White et al., 2012; Gouveia-Figueira and Nording, 2015b; Mavangira and Sordillo, 2017). The ODE are specifically α-linoleic acid, linoleic,

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and oleic acid via LOX enzymes and have been identified in human serum and plasma, hepatic, and intestinal microsomes (Little et al., 2004; Zivkovic et al., 2012; Gouveia-Figueira et al., 2015; Berkecz et al., 2017). Supplementation of omega fatty acids have decreased the inflammatory oxylipins (Zivkovic et al., 2012; Bruins et al., 2013), increased density of omega 3 fatty acids in the blood (White et al., 2012), and increase in ODE concentration which have been noted to be involved with cellular proliferation (Reynolds et al., 2006). Several oxylipins are expressed in a sexually dimorphic manner. In pigs, supplementation with arachidonic acid in the diet, increased 12,13-DiHOME in females, but decreased 14,15-DiHETrE in females (Bruins et al., 2013).

Expression of epoxide hydrolase mRNA was greatest in macaque CL tissue between mid-late and late luteal phase (Irusta et al., 2007). Epoxide hydrolase metabolites, such as 9,10-DiHOME and 19,20-DiHDPE, have been differentially expressed in the cow brain, heart, and liver, with the liver containing the greatest quantity and the brain with the lesser (Gouveia-Figueira and Nording, 2015b). Supplementation of human breast cancer cells with 19,20-DiHDPE decreased angiogenic factors and tumor growth. Supplementation also decreased metastasis of lung cancer in rat lungs (Bruins et al., 2013). These metabolites are potent vasodilators as compared to other categories of oxylipins (Ulu et al., 2014). Production of these oxylipins has been linked to both pro- and anti-inflammatory responses (Calder, 2010; Zivkovic et al., 2012; Mavangira and Sordillo, 2017) and increased intake of omega fatty acids (White et al., 2012; Gouveia-Figueira and Nording, 2015b; Mavangira and Sordillo, 2017). Increased omega fatty acids within the diet has increased these omega fatty acids within CL tissues and concurrently increasing production of P_4 in sheep (Abayasekara and Wathes, 1999).

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Metabolism of these omega fatty acids into oxylipins occurs via CYP enzymes and further metabolism by UGT to inactivate these oxylipins (Heemskerk et al., 2014). Specific CYP and UGT isoforms for these pathways are discussed in further detail below.

Phase I Steroid and Eicosanoid Metabolism

Cytochrome P450

Cytochrome P450 1A (CYP1A)

Cytochrome P450 1A (CYP1A) is involved in metabolizing and activating polycyclic aromatic hydrocarbons, which includes steroid hormones, eicosanoids, fatty acids, and xenobiotics (Chun et al., 1999). Expression of CYP1A has been observed in the human lung, placenta, lymphocytes, and in low amounts in the liver. Oxidation of estrone and estradiol to 2-hydroxyestradiol is performed by CYP1A in the human (Figure 3; Khan and Khan, 2013). The metabolite of CYP1A, 2-hydroxyestradiol, has been observed to reduce angiogenesis, and the methylated metabolite of 2-hydroxyestradiol produced by catechol-o-methyltransferase (2-methoxyestradiol) has exhibited pro-inflammatory and anti-angiogenic properties (Khan and Khan, 2013). Bovine fertilized oocytes exposed to 2-methoxyestradiol decreased the development of d 2 and 4 early morula in a dose dependent fashion; however, treatment with 2-methoxyestradiol on d 6 post-fertilization did not affect cellular replication and progression from the blastocyst stage (Lattanzi et al., 2003).

Janele et al., (2006) observed a dose dependent increase in secretion of TNFα when human male whole blood samples were stimulated with cortisol and E2, and when they were stimulated with 2-hydroxyestradiol and 2-hydroxyestrone (CYP mediated metabolites of estradiol and estrone, respectively). Our laboratory was the first to

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discover CYP activity in cultured bovine endometrial cells. Moreover, the activity of CYP enzymes in these cells was decreased by treatment of IFNτ (Gilfeather and Lemley, 2016). Intra-liver activity of CYP1A was not affected throughout gestation of beef cows, but activity of CYP1A was significantly decreased in those beef cattle that had been nutrient restricted (60% of NRC requirements) compared to non-nutrient restricted cattle beginning on d 30 of gestation until d 140 of gestation (Hart et al., 2014). In vivo uterine expression and/or activity of CYP1A has not been examined to date.

Cytochrome P450 2C (CYP2C)

Metabolism and oxidation are primarily the role of CYP2C and focuses on metabolizing P_4, as outlined in Figure 3. Various tissues in monkeys were tested for the expression of mRNA for CYP2C within the testis, small intestines, adrenal glands, kidneys, uterus, and brain, with minimal amounts found; however, there was no presence in the heart and lung, but there was a greater presence within the liver (Uno et al., 2006). Cattle receiving a high fiber diet compared to cattle receiving a high corn starch diet were also observed to have a significant increase in CYP2C activity in hepatic tissue, and yielded a significant decrease in insulin in peripheral blood circulation (Lemley et al., 2010). A recently discovered CYP2C (CYP2C44) in mice was found to convert arachidonic acid into epoxyeicosatrienoic acids (EET; DeLozier et al., 2004). These EET influence vasorelaxation as well as anti-inflammatory and pro-fibrinolytic effects in the cardiovascular system (Roman, 2002). Also, DeLozier et al. (2004) found that CYP2C was expressed more in female adrenal and kidney tissues compared with male kidney and adrenal tissues. Intra-liver activity of CYP2C was not affected throughout gestation of beef cows (Hart et al., 2014). Also, CYP2C activity was significantly decreased in those

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beef cattle that had been nutrient restricted (60% of NRC requirements) compared to non-nutrient restricted cattle beginning on d 30 of gestation until d 140 of gestation (Hart et al., 2014). Expression of several isoforms of CYP2C were observed in CL tissue of rhesus macaques and were more greatly expressed during the mid to late luteal phase (Irusta et al., 2007). Uterine expression and/or activity of CYP2C has not been examined to date. Finally, there were significant amounts of staining within the hepatocyte cells of the mouse liver for CYP2C (DeLozier et al., 2004). Metabolism and epoxygenation of PUFA are produced primarily by the isoforms of CYP2C to produce oxylipins (Mavangira and Sordillo, 2017).

Cytochrome P450 3A (CYP3A)

Metabolism and oxidation are primarily the roles of CYP3A and focuses on metabolizing P_4 and E2, as outlined in Figure 3. Holstein cows yielded a greater rate of P_4 clearance, indicated by the significant decrease in P_4 half-life, in cattle receiving a high fiber diet compared to cattle receiving a high corn starch diet (Lemley et al., 2010). These Holstein cattle were also observed to tend to have an increase in CYP3A activity in hepatic tissue (Lemley et al., 2010). Holstein cows, during late-lactation, treated with 1,000 IU of hCG did not increase activity of CYP3A in hepatic tissues even though blood perfusion score was significantly greater in the CL between d 10 and 17 of the estrous cycle of those treated cattle compared to the control cattle (Voelz et al., 2015). Hepatic activity of CYP3A was significantly less on d 254 of gestation when compared to d 85 or 140 in beef cows (Hart et al., 2014). Also, CYP3A was not different between cattle that had been nutrient restricted (60% of NRC requirements) compared to non-nutrient

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restricted cattle beginning on d 30 of gestation until d 140 of gestation (Hart et al., 2014). In vivo uterine expression and/or activity of CYP3A has not been examined to date.

Phase II Steroid and Eicosanoid Metabolism

Introduction

Uridine diphosphate-glucuronosyltransferase (UGT) is a membrane-bound enzyme that is part of the second phase of P_4 metabolism which conjugates hydroxyprogesterone metabolites with glucuronic acid. This reaction is initiated by transferring a glucuronosyl group from the uridine 5'-diphospho-glucuronic acid (UDPGA) resulting in a more polar substance which is easier to move within the blood to the kidneys where they are filtered out and finally excreted via urination (Radominska-Pandya et al., 1998). In addition to steroids and eicosanoids, UGT enzymes are well established catalyzers of several drugs and are often referred to as a "drug metabolizing enzyme" or DME. The UGTs exhibit 117 different members within mammalian species with the genetic homology being at least 40% between families and upwards of 60% between isoforms (Jancova et al., 2010). Multiple isoforms are found throughout the human body including the kidney, adipose tissue, lung, skin, intestines, and brain; however, the primary location is the liver, similar to the phase I enzymes (Collier et al., 2002).

UDP-glucuronosyltransferase 1 (UGT1)

Interestingly, mice that utilized in-vitro fertilization and intra-cytoplasmic sperm injection to achieve pregnancy, yielded 61-fold and 106-fold greater mRNA, respectively, for UGT1A1 within the placenta when compared to pregnancies achieved

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through a natural mating (Collier et al., 2012). Healthy post-menopausal women have yielded expression of UGT1A1 mRNA in endometrial tissues. Human endometrial cancer cell lines exhibited greater activity of UGT1A1 when treated with the substrate 2-hydroxyestradiol compared to the substrate 4-hydroxyestradiol (Duguay et al., 2004). Mouse placental UGT1A1 and UGT1A2 mRNA was negatively correlated with tissue lysate levels of P_4 ($r = -0.52$, $P = 0.007$) and cholesterol ($r = -0.45$, $P = 0.018$), respectively (Collier et al., 2012). However, variants of the UGT1A subfamily were not found using Western blotting, qPCR, and immunohistochemistry in human term placental tissues (Collier et al., 2002). Estrogen receptor alpha transfected cells, HepG2, that were stimulated with estradiol expressed a significantly greater UGT1A4 expression compared to those cells that did not exhibit estrogen receptor alpha (Chen et al., 2009). In humans, UGT1A8 is primarily found extrahepatically, which indicates that it may have a role in the regulation of peripheral concentrations of hormones (Collier et al., 2002).

UDP-glucuronosyltransferase 2 (UGT2)

There are two separate subfamilies of UGT2, UGT2A that is found within nasal tissue, and UGT2B has been identified in human liver cells as well as extra hepatic tissues, kidney, prostate, mammary gland, and ovary (Strassburg et al., 1999; Albert et al., 2000; Juengel et al., 2000). In addition, UGT2B is highly involved in androgen clearance and is more greatly active in patients with prostate cancer (Nadeau et al., 2011). Human term placentas showed the presence of UGT2B4 and 2B7 using both Northern and Western blotting techniques, however the band intensities were different when comparing Western blot procedures (Collier et al., 2002).

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Uterine Blood Perfusion

Blood flows through the body via arteries to get to tissues, where it is then perfused through capillary beds for nutrient and waste exchange (Gannon et al., 1997). Ford and Chenault (1981) determined a negative correlation ($r = -0.25$, $P < 0.05$) between P_4 and uterine blood flow. Conversely, E2 increases blood flow to the uterus (Ford and Chenault, 1981; Ford, 1982) and endometrium (Gannon et al., 1997) of cattle and swine. This increased blood flow and perfusion in humans has been indicative of endometrial receptiveness and poor uterine perfusion is one cause of failure of implantation of embryos (Gannon et al., 1997; Yang et al., 1999; Sarnik et al., 2007; Barad et al., 2014). Measurement of endometrial perfusion via laser Doppler fluxmetry is a non-invasive procedure that has been used in human medicine and research for years (Gannon et al., 1997; Barad et al., 2014). In humans, an increase in uterine perfusion exists during the early follicular phase, during increased production of E2, as compared to the menses or ovulation phase of the menstrual cycle (Gannon et al., 1997). Conscious human subjects exhibited a varied amount of endometrial blood perfusion, whereas anaesthetized patients did not exhibit variable perfusion across the regions of the uterus and endometrium (Gannon et al., 1997).

Several researchers have utilized various techniques to measure uterine blood flow using color Doppler ultrasonography (Lemley et al., 2012; Brockus et al., 2016) or surgically implanted transducers (Ford and Chenault, 1981). Infusion of radioactive microspheres have been utilized to measure endometrial perfusion, but this technique is terminal and cannot be used during extended repeated samplings (Rosenfeld et al., 1973). In sheep, blood flow to the uterus and mammary gland ultimately increases to result in

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18% of total cardiac output, which is the amount of blood pumped by the heart per minute by multiplying heart rate and stroke volume (Rosenfeld, 1977; Clapp, 1978). In cattle, uterine artery blood flow increases by 250% and heart rate by 118% from days 180 to 262 days of gestation (Brockus et al., 2016b).

Angiogenic factors

Vascularization is greatly dependent upon vasculogenesis and angiogenesis (Bruno et al., 2009) with several key factors assisting with one or both and are vital to the development of the placenta.

Vascular Endothelial Growth Factor

A key angiogenic protein is vascular endothelial growth factor (VEGF). Actions of VEGF include stimulation of endothelial cell migration, increased in capillary permeability, and it acting like a survival factor for endothelial cells (Bruno et al., 2009). It is variably expressed in intercaruncle tissue across the estrous cycle of cattle, with the greatest expression accompanying the early stages of diestrus during CL formation (Tasaki et al., 2010). Vascular endothelial growth factor positively affects primordial follicle survival, mitogenic effects on granulosa cells, and assists in the transitions between primary to secondary follicles (Bruno et al., 2009). Supplementation of VEGF to cattle bovine pulmonary artery endothelial cells significantly decreases the amount of basal tension and increases the diameter within the pulmonary artery (Jacobs et al., 2006).

Nitric Oxide

Nitric oxide synthases are cytochrome enzymes that require three co-substrates (L-arginine, NADPH, and oxygen) and several other cofactors and prosthetic groups to

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synthesize nitric oxide (NO; Nathan and Xie, 1994). Nitric Oxide Synthase 3 is also known as eNOS or endothelial, constitutive nitric oxide synthase. Bovine endometrial stromal cells exhibited a lack of stimulation by supplementation of TNFα or oxytocin to increase expression of NOS3, which typically increases the expression of NOS3 in luteal cells (Woclawek-Potocka et al., 2004; Skarzynski et al., 2009). Nitric oxide assists with angiogenesis (Nathan and Xie, 1994) and mediates vasodilatory responses in muscular arteries (Ignarro et al., 1987). Supplementation of 17β-estradiol significantly increases uterine blood flow within 2 hr of administration and is interrelated with the concentration of nitrites, a metabolite of nitric oxide, in the system (Van Buren et al., 1992). Supplementation of increasing increments of metabolizable protein also increased serum nitrites in pregnant ewes, but also tended to decrease uterine blood flow (Lekatz et al., 2015). Metabolites of NO, nitrites, increases in maternal serum as gestation progresses from d 180 through d 240 of gestation in Holstein heifers (Brockus et al., 2016b).

Melatonin

Melatonin (*N*-acetyl-5-methoxytryptamine) is a neurohormone secreted primarily by the pineal gland during scotophase and acts as a powerful antioxidant (Reiter et al., 2009). This hormone is widely known to regulate sleep wake cycles and reproductive cyclicity in seasonal breeding animals, and is involved in pigmentation in the dermal layer of some species, protects DNA within the nucleus and mitochondria, and influences pubertal development (Reiter et al., 2006; Pandi-Perumal et al., 2008; Reiter et al., 2009). Melatonin acts as a powerful antioxidant (Reiter et al., 2009) that reduces oxidative stress on reproductive tissues through direct and indirect action. Directly, melatonin binds to its receptors that influences either vasoconstriction or vasodilation (Dubocovich and

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Markowska, 2005), and indirectly via the Nitric Oxide pathway (Pandi-Perumal et al., 2008) which reduces the number of reactive oxigen species.

Melatonin Receptors

Melatonin is facilitated by two G-protein coupled receptors labelled MT_1 and MT_2 (Morgan et al., 1994; Dubocovich and Markowska, 2005; Pandi-Perumal et al., 2008). Receptor MT_1 has a greater affinity for melatonin as compared to MT_2, and is primarily found in the brain, small intestine, ovary, lung, cardiac tissues, and myometrium (Dubocovich and Markowska, 2005; Pandi-Perumal et al., 2008). Melatonin elicits the dilation of cardiac vessels through the MT_2 receptor, but constriction of cardiac vessels through the MT_1 receptor. Recently, a third melatonin receptor (MT_3) has been found but belongs to the family quinone reductases, which regulate transcription factors that code for apoptosis (Witt-Enderby et al., 2003). This MT_3 receptor is known to elicit metabolic and antioxidative effects.

Therapeutic Administration to Livestock

Previous research in our laboratory demonstrated similar birth weights in calves from cattle which were orally supplemented with melatonin. However, calves from the melatonin-supplemented dams increased in BW and heart girth at 9 wk of age compared to calves from non-supplemented dams (Brockus et al., 2016a). Supplementation of melatonin to semen extender has been noted to improve freeze-thaw survivability in bulls and buffalo (Cruz et al., 2014). The addition of 500 mg of melatonin to beef tallow administered to multiparous cattle increased the interval between calving to first estrus and ovulation (Sharpe et al., 1986). Dietary enhancement of melatonin (20 mg per day)

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throughout the last third of gestation increased uterine blood flow, heart rate, and total antioxidant capacity, but it decreased maternal serum estradiol-17β and P_4 concentrations (Brockus et al., 2016b). Intrauterine infusion and dietary supplementation of melatonin to sheep, increases umbilical artery blood flow, fetal descending aorta, abdominal girth, kidney size, and lamb weights one month after weaning (Forcada et al., 1995; Lemley et al., 2012; Lemley et al., 2013b; Eifert et al., 2015). However, melatonin supplementation decreases caruncle and cotyledon weights (Lemley et al., 2013b; Eifert et al., 2015). Umbilical blood flow increases within 10 days of initiation of supplementation in ewes along with a 58% increase in the occurrence of twins and reduced the proportion of non-pregnant ewes (Williams et al., 1992; Lemley et al., 2012).

Implications and Conclusions

There are over 92 million cattle in the U.S. that contains almost 50 million cows and heifers with an annual pregnancy rate of 75% (Minton et al., 1992; National Cattlemen's Beef Association, 2015; Jones, 2017). This loss in pregnancy rates or infertility and embryonic mortality costs the beef industry over $1.63 billion annually (Minton et al., 1992; National Cattlemen's Beef Association, 2015; Jones, 2017). A typical estrous cycle in cattle lasts approximately 21 days and is divided into two primary phases: the follicular and the luteal phase, which are named for the primary structure in the ovary at the time. The conceptus within the uterus around d 15 of the estrous cycle, during the late luteal phase, begins to secrete IFNτ which has significant impacts on the CL and uterus as outlined above under maternal recognition of pregnancy. One primary impact is the production of hormones including steroids and oxylipins, which aids in the angiogenesis and blood flow to the CL and uterus. This localization of oxylipins that are

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differentially expressed in CL of pregnant as compared to non-pregnant females could accommodate future research to determine pregnancy earlier than current methods. Also, characterization of these localized oxylipins within the CL could allow for identification of dysfunctional CL.

An increase in vascularity and endometrial perfusion has been linked to increased reproductive receptivity in humans (Gannon et al., 1997; Yang et al., 1999); however, utilization of this technique has not been utilized in livestock to date. Measurement of endometrial perfusion may provide an additional breeding soundness evaluation for cows and heifers. Metabolism of steroids and eicosanoids occurs in two phases, CYP and UGT, with a paucity of information on these enzymes in extra hepatic tissues within livestock animals. Hydroxyestrogens have been noted to have anti-embryonic properties, which led us to evaluate the CYP1A and 3A activity, without success, in cyclic and early pregnant endometrium. Production of EET by CYP2C has exhibited anti-inflammatory effects and analysis of CL tissues for activity of CYP2C failed. However, localization of UGT within endometrial tissues was a success and could provide evidence for localized clearance of steroid metabolites and oxylipin via glucuronidation.

Research into therapeutic substances for improvement of reproductive success is critical for improved profitability. Our lab has previously done research with the supplementation of melatonin to cattle. Supplementation with melatonin has yielded an increase in enzyme activity of CYP within the liver, uterine blood flow, and umbilical blood flow (Lemley et al., 2012; Lemley et al., 2013a; Brockus et al., 2016b). Increasing the activity of the CYP via supplementation of melatonin could provide an increased

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production of oxylipins, either pro- or anti-inflammatory and embryonic, which could provide a greater chance of maintaining a pregnancy.

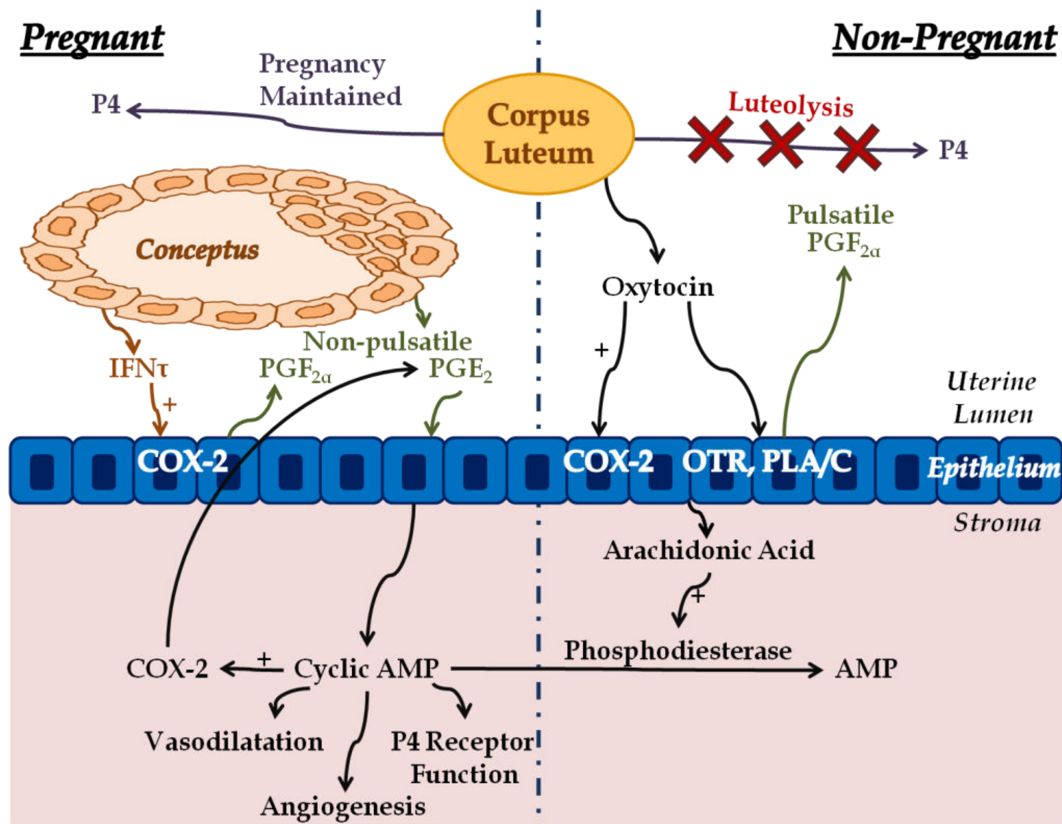

Figure 1 Proposed interactions between luteotrophic and luteolytic pathways in ruminants

Proposed interactions between luteotrophic and luteolytic pathways in the choice between pregnancy and a return to cyclicity in polyestrus ruminants, in which IFNτ is the maternal recognition of pregnancy signal and there is no decidualization. (Redrawn from Cheng et al., 2007)

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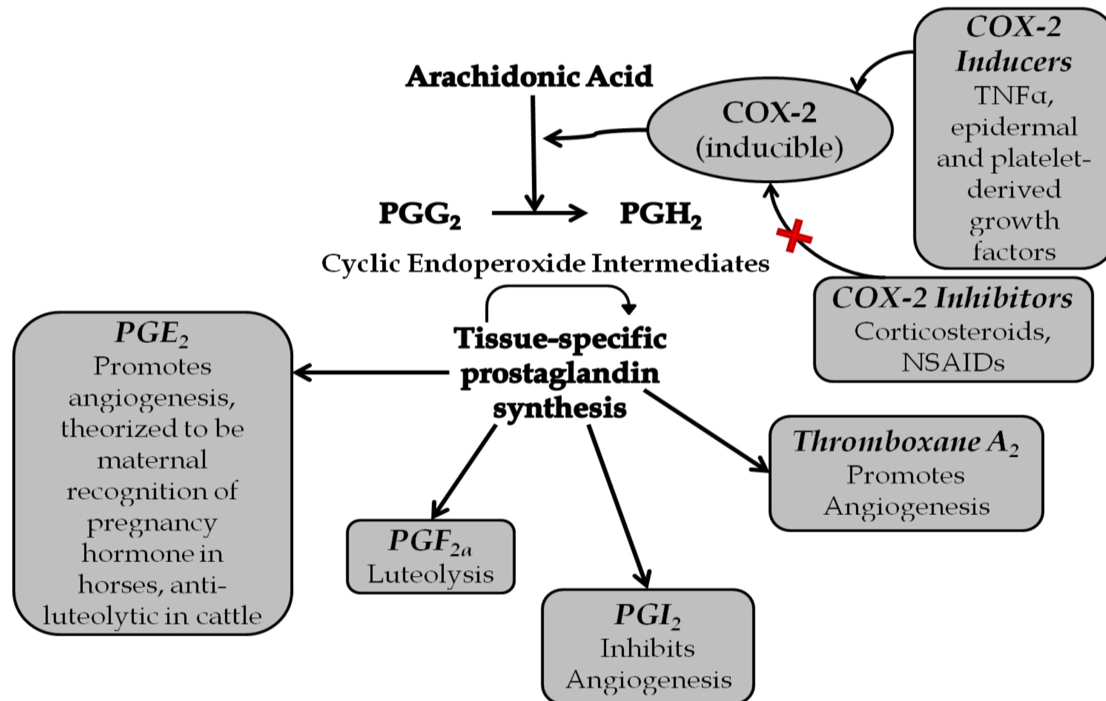

Figure 2 Adaptation of prostaglandin pathway

(Redrawn from Canby-Hagino and Thompson, 2005)

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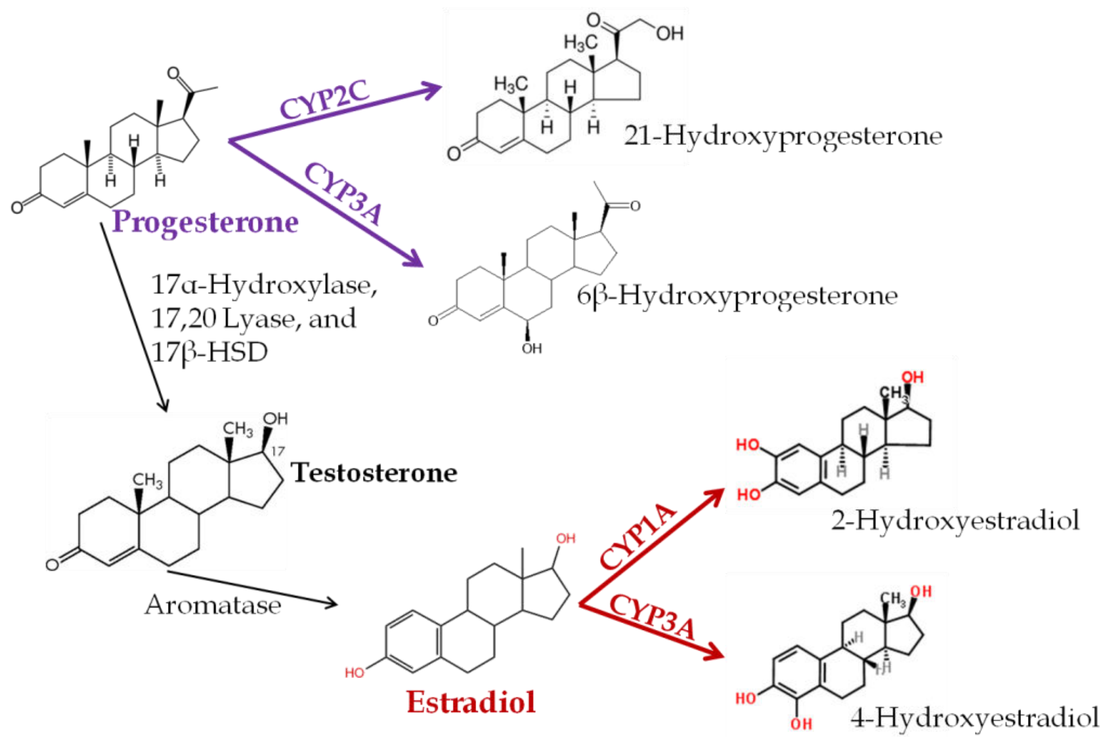

Figure 3 Cytochrome P450 metabolism/hydroxylation of progesterone and estrogen.

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CHAPTER II

EXAMINING ENDOMETRIAL BLOOD PERFUSION USING A NOVEL LASER DOPPLER TECHNIQUE IN ANGUS COWS THROUGHOUT THE ESTROUS CYCLE

Abstract

Previous studies have characterized ovarian steroid synthesis which directly affects uterine environment and blood flow. Clearance of steroids occurs primarily in hepatic tissues, however, we discovered abundant activity of the phase II steroid metabolizing enzyme UDP-glucuronosyltransferase (UGT) in uterine biopsies. No minimally invasive techniques for collecting endometrial perfusion, which is affected by steroids and indicative of reproductive health, have been proposed for livestock. We aimed to characterize UGT activity and endometrial blood perfusion during a normal bovine estrous cycle. We hypothesized increased steroid metabolism during the luteal phase of the estrous cycle and in the uterine horn ipsilateral to the corpus luteum (CL). During the first synchronized estrous cycle, progesterone and UGT activity increased on day 6 compared to day 0 and 3. Endometrial perfusion was increased ipsilateral to the CL versus contralateral on day 12, and decreased ipsilateral to the CL versus contralateral on day 18. Endometrial expression of vascular endothelial growth factor A (VEGFA) was decreased on day 18, whereas expression of NOS3 was least on day 12 and greatest on day 18. Similar to perfusion results, nitric oxide metabolites (nitrites) were greatest in the endometrium ipsilateral to the CL as compared to contralateral. Moreover, a positive

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correlation (r = 0.28; P = 0.04) was observed between endometrial perfusion and nitrite concentration. We conclude activity of UGT within the endometrium is affected by the location of the CL, and collection of endometrial perfusion using a laser Doppler probe could be a viable measurement that is supported by nitrite concentration.

Introduction

Progesterone (P_4) has been noted to elicit a variety of responses (Lau et al., 1993; Spencer et al., 1996; Atkinson et al., 1998) in the cow's body to prepare the reproductive tract for an impending pregnancy. Production of P_4 is primarily performed by the corpus luteum (CL), which is the predominant ovarian structure from days 4 through 16 of the cow's estrous cycle (Walters et al., 1984; Ginther et al., 1989). Metabolism of P_4 occurs in two phases (Giantin et al., 2008), beginning with phase I cytochrome P450 2C (CYP2C) and 3A (CYP3A) converting P_4 into hydroxyprogesterone metabolites (Lemley et al., 2010) which are further conjugated to glucuronic acid by the phase II enzyme uridine 5'-diphospho-glucuronosyltransferase (UGT; Hart et al., 2014). A majority of steroid metabolism occurs in the liver, but others have shown UGT expression and/or activity in several extra-hepatic tissues in cattle, such as kidneys, lungs, small and large intestines, and brains (Iwano et al., 2001; Darwish et al., 2010; Girolami et al., 2016). Our preliminary studies have shown minimal to no activity of phase I enzymes in bovine reproductive tissues (Owen and Lemley, unpublished). Conversely, our preliminary studies have shown abundant activity of UGT enzymes in bovine reproductive tissues, which deserves further investigation. Uterine biopsies have been utilized in the cattle and horse industries as a valuable method of detecting endometrial health and assessing the bacterial load within the uterine environment (Ricketts, 1975; Bonnett et al., 1993). Due

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to the vital importance of P_4 to the uterine and endometrial mechanisms, an understanding of the localized metabolism of P_4 within the uterus needs to be established. Therefore, our first aim was to characterize endometrial UGT activity during the normal bovine estrous cycle. Furthermore, we hypothesized that endometrial UGT activity would be greatest during the luteal phase, and would be greater in the uterine horn ipsilateral to the CL.

Progesterone has also been noted to create a type of quiescence of the uterus which minimized the myometrial contractions and affects the movement of blood through capillary beds within the endometrium (Grazzini et al., 1998; Soloff et al., 2011). Conversely, estrogen increased blood flow to the uterus (Ford and Chenault, 1981; Ford, 1982) and endometrium (Gannon et al., 1997) of cattle and swine. This increased blood flow and perfusion in humans has been indicative of endometrial receptiveness and poor uterine perfusion is one cause of failure of implantation of embryos (Gannon et al., 1997; Yang et al., 1999). Collection of endometrial blood perfusion via laser Doppler fluxmetry is a non-invasive procedure that has been used in human medicine and research for years (Gannon et al., 1997; Barad et al., 2014). In humans, an increase in uterine perfusion exists during the early follicular phase, during estrogen-dominance, as compared to the menses or ovulation phase of the menstrual cycle (Gannon et al., 1997). Several researchers have appled various techniques to measure uterine blood flow using color Doppler ultrasonography (Lemley et al., 2012; Brockus et al., 2016) or surgically implanted transducers (Ford and Chenault, 1981). Infusion of radioactive microspheres have been applied to measure endometrial perfusion, but this technique is terminal and cannot be used during extended repeated samplings (Rosenfeld et al., 1973). There are no

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minimally invasive or non-terminal techniques to acquire endometrial perfusion in livestock species, which led us to our second objective: validating laser Doppler to examine bovine endometrial perfusion in relation to angiogenic factor expression and total endometrial nitrite concentrations during the estrous cycle. We hypothesized that endometrial blood perfusion would be greater ipsilaterally to the dominant follicle. Therefore, our objectives through these hypotheses were to determine endometrial UGT enzyme activity, angiogenic factor expression, and endometrial blood perfusion in relation to the CL and dominant follicle (DF) during a normal bovine estrous cycle.

Materials and Methods

Experiment 1

Animal care and treatments

Animal care and use were according to protocols approved by the Mississippi State University Institutional Animal Care and Use Committee. Cattle were maintained on winter pasture and allowed ad libitum access to native pasture hay and water. Twenty-one non-pregnant Angus cows had their estrous cycles synchronized using a modified 7-day CO-Synch + CIDR protocol. Briefly, a 2 ml, i.m injection of GnRH (Factrel, Fort Dodge Animal Health, Fort Dodge, IA, USA) was administered, a CIDR (Eazi-Breed™ CIDR® Cattle Insert, Zoetis, Parsippany, NJ, USA) was placed in the vagina of cows for 7 days, upon CIDR removal cattle were administered 5 ml, i.m Lutalyse (Zoetis, Parsippany, NJ, USA), and estrus was detected in 60 ± 6 hrs. Cows were enrolled in the study on their first day of estrus, using observational indications along with Estrotect™ Heat Detection patches (Rockway Inc., Spring Valley, WI, USA), which was considered day 0 of the study. Four cows were removed from the study due to lameness issues (1

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cow) and lack of response to estrous synchronization (3 cows) for the first estrous cycle. Uterine biopsy and blood samples were collected on days 0, 3, 6, and 9 for the first estrous cycle. Cattle were allowed to finish this first synchronized estrous cycle with no further samples collected. A consecutive set of samples, similar to the first estrous cycle, were collected on days 12, 15, 18, and 21 of the second estrous cycle after undergoing estrous synchronization similar to the first estrous cycle. Five cows were removed from the study due to failure to respond to estrous synchronization. Only cattle that exhibited a normal estrous cycle, as determined by peripheral P_4 profiles, were maintained throughout the analysis portion of the study. Due to this criterion, 3 cows were removed from the first estrous cycle and 2 cows were removed from the second estrous cycle analysis. Also, due to this criterion 10 out of 13 cattle showed a decrease in peripheral P_4 profiles on day 9 and therefore day 9 was removed from the analysis.

Sample collection

Cattle were subjected to rectal ultrasonography on day 6 of the first estrous cycle and day 11 and 19 of the second estrous cycle to determine CL and follicular locations and collect ultrasound images. These images were used to determine the diameter and volume of the CL and DF using image J software following previously published methods (Vasconcelos et al., 2001). Also, on each sampling day (days 0, 3, 6, and 9 of the first estrous cycle and days 12, 15, 18, and 21 of the second estrous cycle) blood was collected via venipuncture of the coccygeal vein for hormone analysis. Serum tubes were incubated at room temperature for at least 30 min prior to being centrifuged and plasma tubes were inverted several times and held on ice for transportation. Centrifugation was performed at 4°C for 15 min at 2000 x g. Serum and plasma were harvested and

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subsequently stored at -80°C until assayed. Cattle were subjected to an endometrial biopsy utilizing a mare uterine biopsy punch (Mare Uterine Biopsy Punch 27", Santa Cruz Biotechnology Inc., Dallas, TX, USA) with a sample collected from each horn and snap frozen in liquid nitrogen.

Tissue processing

Approximately 50 mg of tissue was placed into a polypropylene tube with 500 µL of potassium phosphate (KPO_4) buffer (400 mM, pH = 7.4). Samples were then mechanically homogenized using the Tissue Tearor (Biospec Products, Bartlesville, OK, USA) following the manufacturer's protocol. Tissue homogenate samples were placed into microcentrifuge tubes and then centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was stored at −80°C until enzyme assays were conducted. The protein concentration of the supernatant was determined by a Coomassie Plus (Bradford) protein assay following the manufacturer's protocol (Thermo Scientific, Rockford, IL, USA) and diluted to a concentration of 4000 µg/mL.

UGT assay

The UGT assay kit was purchased from Promega Corporation (Madison, WI, USA) and the assay performed according to Hart et al. (2014) with minor adaptations. Briefly, uridine diphosphoglucoronic acid (UDPGA) was added to half the plates to act as reaction wells, and distilled water was added to the other half as control wells. The UGT reaction mixture containing UGT multienzyme substrate was then combined with tissue homogenates (28 µg of tissue protein per well), and the plates were preincubated for 90 min at 37°C. After incubation, detection reagent was added to each of the wells followed

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by an incubation period of 20 min at room temperature while protected from light. The plates were then analyzed using a Promega Multi- Plus plate reader with luminescence detection mode.

Hormone analysis

Serum hormone analysis was determined using a P_4 radioimmunoassay kit performed following the manufacturer's instructions (MP Biomedicals, LLC, Solon, OH, USA) and Cline et al., 2016. Briefly, standards, control samples, and serum samples were allowed to thaw at room temperature and 100 µL of each were pipetted into their respective tubes. The total and non-specific binding tubes that serve as controls utilized clear, non-coated polypropylene tubes whereas the standards and serum samples utilized the ImmuChem Progesterone Coated Tubes that were provided in the kit. After all samples were allocated into their respective tubes, 1.0 mL of iodinated P_4 was added and allowed to incubate in 37°C water bath for 2 hours. The samples were then decanted and placed into a gamma counter (Packard Instrument Company, Meriden, CT, USA). Samples falling below the detectable limit (0.02 ng/mL) were presumed to have 0.02 ng/mL for further statistical analysis. Inter- and intra-assay CV were 6.6 and 8.2% respectively.

Experiment 2

Animal care and treatments

Animal care and use were according to protocols approved by the Mississippi State University Institutional Animal Care and Use Committee. Cattle were maintained on winter pasture and allowed ad libitum access to native pasture hay and water. Twenty

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non-pregnant Angus cows had their estrous cycles synchronized using a modified 7-day CO-Synch + CIDR. Cows were enrolled in the study on their first day of estrus, using observational indications along with Estrotect™ Heat Detection patches, which was considered day 0 of the study. Five cows were removed from the study due to failure to respond to estrous synchronization. A consecutive set of samples, similar to the first experiment, were collected on days 12, 15, 18, and 21 of the second estrous cycle. This second set of samples included the collection of endometrial perfusion using a novel laser Doppler probe (PeriFlux 5000 LDPM, Perimed Inc., Ardmore, PA, USA). Only cattle that exhibited a normal estrous cycle, as determined by P_4 hormone profiles, were maintained throughout the analysis portion of the study. Due to this criterion, 2 cows were removed from the analysis.

Sample collection

Prior to endometrial biopsies, an equine artificial insemination rod was passed through the cervix and into each uterine horn of the cow. Next, the laser Doppler perfusion probe was inserted through the artificial insemination rod until gaining contact with the uterine endometrium. The correct distance for insertion through the lumen of the artificial insemination rod was marked with laboratory tape. The laser Doppler probe collected endometrial blood perfusion and microvascular flow of the tissue surface without traumatizing the endometrium. This probe utilizes a 780-nm wavelength laser, with a measuring depth of approximately 0.5 – 1.0 mm, in order to collect blood perfusion. Optimal perfusion was obtained by selecting 10 sec of relatively constant perfusion for measurement, as measured by arbitrary Perfusion Units (PU) following

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calibration of the middle of each uterine horn. After blood perfusion was collected, cattle were subjected to an endometrial biopsy similar to Experiment 1.

Tissue processing

Total RNA was extracted from individual uterine biopsy samples using the Qiagen miRNeasy kit (Qiagen, Hilden, Germany) according to the manufacturers' protocols. Briefly, approximately 100 mg uterine biopsy sample was mechanically homogenized in 700 µL Quizol. Protein was precipitated via the addition of chloroform, agitation, and the upper aqueous phase was purified through a series of ethanol washes through the column provided in the kit. Finally, 30 µL of nuclease-free water was passed through the column to free the RNA. The RNA was quantified using UV-Vis Spectrophotometer (NanoDrop One, Thermo Scientific, Rockford, IL, USA), and RNA was diluted to a constant concentration (650 ng/µL). The diluted RNA treated with DNase then made into cDNA using the QuantiNova Reverse transcription kit (Qiagen, Hilden, Germany) in the Eppendorf Mastercycler gradient (Applied Biosystems, Foster City, CA, USA). Equal amounts of cDNA from each sample were pooled for analysis to represent the average expression of mRNA for all uterine biopsies.

Quantitative RT-PCR

Real time PCR (RT-PCR) was performed using the QuantiNova SYBR Green PCR Kit (Qiagen, Hilden, Germany) instructions in the Rotor Gene Q (Qiagen, Hilden, Germany). Primers for Nitric Oxide Synthase 3 (NOS3), Vascular Endothelial Growth Factor A (VEGFA), and B-Actin were ordered from Invitrogen Custom Primers and the sequences and information are in Table 1. Reactions for each gene were run in duplicate.

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A temperature gradient PCR reaction was run for all of the primer sets to determine the optimal annealing temperatures. According to gradient PCR, the optimal annealing temperature of all primer sets overlapped at 60°C. Correspondingly, the following protocol was designed and applied to all RT-PCR reactions: I) 1 cycle at 95°C for 2 min; II) 40 repeat cycles at 95°C for 5 sec., followed by annealing at 60°C for 10 sec.; III) 60.0°C-95.0°C with melting temperature increasing 0.5°C for each 30 sec. Fluorescence was detected at both step II and III. Real time analysis was enabled at step II, and melt curve data collection and analysis enabled at step III. An amplification efficiency of 95 – 105% was reached for all the RT-PCR reactions in this study. Final data was analyzed through the $2^{-\Delta\Delta Ct}$ method where B-actin was used as a reference gene to normalize all the selected gene expression data.

Tissue nitrite

Total tissue nitrites were determined in uterine biopsy homogenates (prepared as stated above) from days 12, 15, 18, and 21. Total tissue nitrites were determined using the QuantiChrom Nitric Oxide Assay Kit (BioAssay Systems, Hayward, CA, USA). Briefly, homogenized tissues were deproteinized following the manufacturer's recommendations, and samples were quantified following the reduction of total nitrates to nitrites using the Griess method and analyzed against a linear nitrite standard curve (0–100 μM), with a sensitivity of 0.6 μM and interassay coefficient of variation of 7.1%. Tissue nitrites are expressed as micromole per gram of uterine biopsy (μM/g of Ut. Biop.).

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Statistical analysis

Enzyme activity for UGT was expressed relative to milligram of protein and tissue nitrites are expressed as micromole per gram of uterine biopsy. Data were analyzed using repeated measures ANOVA of the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC, USA). Means were separated using the PDIFF option of the LSMEANS statement. The model statement included day of estrous cycle, CL and DF location (respective to each uterine horn), and the respective interactions with day. Pearson correlation coefficients were determined using the CORR procedure of SAS to analyze the relationships between CL and DF location (respective to each uterine horn), endometrial blood perfusion, CL and DF size, and P_4. Statistical significance was declared at $P \leq 0.05$, and tendencies discussed when $P \geq 0.06$ but $P \leq 0.10$.

Results

Serum P_4 concentrations and UGT (Experiment 1)

A significant day effect ($P < 0.001$) was observed from days 0 to 6 and for days 12 to 21 for serum P_4 concentrations (Figure 4A and 4B). Serum P_4 concentrations on days 0 and 3 were not different, but day 6 was greater ($P \leq 0.05$) than both day 0 and 3 for the first estrous cycle (Figure 4A). Days 12 and 15 had greater ($P \leq 0.05$) serum P_4 concentrations as compared to days 18 and 21 of the second estrous cycle (Figure 4B). A significant day effect ($P = 0.0005$) was observed from days 0 to 6 for UGT activity of the first estrous cycle (Figure 4C). Activity of UGT, expressed as mg of protein, on days 0 and 3 were not different, but day 6 was greater ($P \leq 0.05$) than both day 0 and 3 for the first estrous cycle. However, the second estrous cycle exhibited a CL side by day interaction ($P = 0.03$) with the UGT activity tending to be greater in the endometrial

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biopsy samples ipsilateral to the CL on days 15 and 21, ($P = 0.06$ and $P = 0.08$, respectfully; Figure 4D).

Endometrial Perfusion (Experiment 2)

Schematic drawings of the collection of endometrial perfusion are in Figure 5 in relation to the CL (A) and dominant follicle (B). A significant CL side by day interaction ($P = 0.01$) was observed for endometrial perfusion. Endometrial perfusion was greater ($P \leq 0.05$) on the ipsilateral horn to the CL as compared to the contralateral side on day 12. On day 15 endometrial perfusion was not different between the two uterine horns. Endometrial perfusion contralateral to the CL was greater ($P \leq 0.05$) on day 18 as compared to endometrial perfusion ipsilateral to the CL. Endometrial perfusion tended to be greater ($P = 0.08$) on the ipsilateral horn to the CL as compared to the contralateral side on day 21 (Figure 5A). Conversely, no significant dominant follicle side by day interaction was observed for endometrial perfusion across days 12 through 21 of the second estrous cycle in Experiment 2 (Figure 5B).

Angiogenic factors (Experiment 2)

Endometrial expression of VEGFA and NOS3 are illustrated in Figure 6. A significant day effect ($P = 0.009$) was observed from days 12 to 21 for expression of VEGFA (Figure 6A). On day 12 VEGFA expression was greater compared with days 15, 18, and 21. Expression of VEGFA was not different between day 15 and 21. Expression of VEGFA was least on day 18 as compared to days, 12, 15, and 21. A tendency for a day effect ($P = 0.08$) was observed from days 12 to 21 for expression of NOS3 (Figure 6B). Endometrial expression of NOS3 was increased on day 18 compared with day 15 and 21,

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while day 12 was intermediate. Total tissue nitrites in uterine biopsy samples are illustrated in Figure 7. The ipsilateral side to the CL yielded greater concentrations of tissue nitrites ($P = 0.0005$) at day 12 as compared to the contralateral side to the CL.

Discussion

Previous studies have focused on activity of UGT in hepatic tissues; however, our study examined the activity of UGT in endometrial tissues. Activity of UGT displayed similar trends during the first synchronized estrous cycle with circulating serum P_4 concentrations increasing from day 0 to 6. This is supported by a positive correlation ($r = 0.51$, $P = 0.004$) between serum P_4 concentrations and the total activity of UGT. Therefore, as the CL is developing and beginning P_4 synthesis it may be activating steroid metabolizing pathways in the uterus. Activity of UGT enzymes express twice as much activity when exposed to P_4 metabolites as compared to estrogen metabolites in monkey tissues (Albert et al., 2000). However, this relationship between P_4 secretion and endometrial activity was not observed in the second estrous cycle. This differential activity of endometrial activity could be due to the presence of prostaglandins within the uterus, especially on day 15, which is immediately prior to luteolysis, and day 21, which could be due to the increased amount of uterine manipulation. Day 18 of the second estrous cycle occurs during the early proestrus phase (Hurnik et al., 1975) and indicates a low concentration of P_4, which coincides with the current studies circulating serum P_4 concentrations. Greater UGT activity tended to be ipsilateral to the CL on day 21 of the second estrous cycle. Activity of UGT is greatest during the luteal phase, which supports our hypothesis; however, UGT activity tended to be greater ipsilateral to the CL on days

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15 and 21 as compared to the contralateral horn to the CL, which contradicts the hypothesis that UGT activity would be greater ipsilaterally to the CL.

Uterine blood flow is increased when concentrations of estradiol are greatest in cattle within one day of estrus as compared to other days of the estrous cycle (Ford and Chenault, 1981). Ovariectomized ewes, supplemented with estradiol and injected with radiolabeled microspheres (Rosenfeld et al., 1973), also exhibited greater uterine blood flow. Endometrial perfusion, as measured by laser Doppler fluxmetry, has been increasing in popularity in human medicine (Gannon et al., 1997; Sarnik et al., 2007; Barad et al., 2014), and is used as a method of assessing endometrial receptiveness (Yang et al., 1999), and ability for embryonic implantation (Gannon et al., 1997). Conscious human subjects exhibited a varied amount of endometrial perfusion, whereas anaesthetized patients did not exhibit variable perfusion across the regions of the uterus and endometrium (Gannon et al., 1997). Also, endometrial perfusion was greatest during the early follicular and secretory phases of the menstrual cycle, which is most analogous to the estrus and diestrus phases of the cow's estrous cycle (Sherman and Korenman, 1975). In the current experiment, utilizing laser Doppler fluxmetry, a CL side by day interaction was observed. Day 12 endometrial perfusion was greatest on the side ipsilateral to the CL and again tended to be greater on day 21. Increased perfusion on day 12 contradicts previous research, whereby Ford and Chenault (1981) determined a significant negative correlation (r = -0.25, $P < 0.05$) between P_4 and uterine blood flow. When analyzing data that contained the dominant follicle on the same side as the CL, the endometrial perfusion was negatively correlated (r = -0.38, $P = 0.04$) with the size of the dominant follicle. Day 15 perfusion was not different between the ipsilateral and

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contralateral sides of the CL, which could be indicative of the transition between diestrus and proestrus (Hurnik et al., 1975). On day 18, endometrial perfusion was greatest on the contralateral side of the CL, which could be indicative of the prostaglandin F2A (PGF2A), that has been secreted from the endometrium, in greater concentration because the PGF2A secreted on the ipsilateral side to the CL has been shunted to the CL by the counter-current exchange of the ovarian artery (Einer-Jensen and McCracken, 1977; Krzymowski et al., 1982; Oliveira et al., 2008).

Vascularization is greatly dependent upon vasculogenesis and angiogenesis (Bruno et al., 2009) with several key factors assisting with one or both, such as VEGF. Expression of VEGF is variable in intercaruncle tissue across the estrous cycle of cattle, with the greatest expression accompanying the early stages of diestrus during CL formation (Tasaki et al., 2010). The VEGF expression in this experiment was also greatest during the diestrus stage but it was during the mid-diestrus stage (day 12). The lowest VEGFA expression in this experiment was during the proestrus period, which is similar to previous results of VEGFA protein expression in intercaruncle tissue of cattle (Tasaki et al., 2010). Assemblage of uterine biopsies allows for collection and assessment of intercaruncular and caruncular tissues. In this experiment, VEGFA expression was intermediate on days 15 and 21 of the estrous cycle, while previous studies have indicated that VEGF mRNA expression was also greatest during this stage of the estrous cycle (Tasaki et al., 2010).

Nitric oxide synthases are cytochrome enzymes that require three co-substrates (L-arginine, NADPH, and oxygen) and several other cofactors and prosthetic groups to synthesize NO (Nathan and Xie, 1994). Nitric Oxide Synthase 3 is also known as eNOS

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or endothelial, constitutive nitric oxide synthase. Bovine endometrial stromal cells exhibited a lack of stimulation by supplementation of TNFA or oxytocin to increase expression of NOS3, which typically increases the expression of NOS3 in luteal cells (Woclawek-Potocka et al., 2004; Skarzynski et al., 2009). In this experiment, NOS3 expression tended to have a day effect and was greatest at day 18 of the estrous cycle. When analyzing data that contained the dominant follicle on the opposite side as the CL, the NOS3 expression tended to be positively correlated (r = 0.35, *P* = 0.08) with the endometrial perfusion collected via laser Doppler fluxmetry.

Nitric oxide assists with angiogenesis (Nathan and Xie, 1994) and mediates vasodilatory responses in muscular arteries (Ignarro et al., 1987). Supplementation of 17B-estradiol significantly increases uterine blood flow within 2 hours of administration, and is interrelated with the concentration of NO, a metabolite of nitric oxide, in the system (Van Buren et al., 1992). However, in the current study the greatest concentration of NO occurred on day 12 in the biopsies ipsilateral to the CL. Concentrations of NO were correlated (r = 0.28, *P* = 0.04) with the endometrial perfusion collected via laser Doppler fluxmetry across all data collected in Experiment 2. When analyzing data from uterine biopsy samples contralateral the CL, NO concentration was correlated (r = 0.53, *P* = 0.005) with endometrial perfusion collected via laser Doppler fluxmetry. This explains the lack of differences between the NO concentration in uterine biopsy samples ipsilateral or contralateral from days 15 through 21.

Activity of UGT was greater during the luteal phase, as we hypothesized, and mirrored the serum P_4 concentrations between days 0 and 6. Activity of UGT within the uterus and CL indicates localized metabolism of steroids, eicosanoids, and xenobiotics

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throughout the estrous cycle. Endometrial perfusion ipsilateral to the CL follows a similar profile to the main effect of VEGFA expression across days 12 to 21 of the estrous cycle. Endometrial perfusion was greater on day 12 ipsilateral to the CL as well as VEGFA expression and concentration of NO, which support the validity of this novel collection of endometrial perfusion in cattle. Collection of endometrial perfusion provides the ability to gather localized blood flow and could have future implications towards more effective estrous synchronization techniques, improved uterine involution during the post-partum period, or be utilized during breeding soundness exams for future female breeding stock.

Declaration of interest

Authors declare that there are no known conflicts of interest accompanying this publication, and there has been no significant financial assistance that could have influenced the products of this research. All authors read and approved the final version of the manuscript.

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Author's contribution statement

M.P.T. Owen and C.O. Lemley conceived and designed the experiment, ran most of the sample and sample analysis, and wrote the paper. All authors significantly assisted with the performance of the experiment, analyzing the data, and editing the final manuscript. C.O. Lemley provided funding for the project.

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Table 1 Details of genes targeted for RT-PCR analysis in Experiment 2[a].

Gene	Direction	Primer Sequence (5' to 3')	Amplicon Length
NOS3	Forward	GTCCTGTGCATGGATGAGTATG	22
	Reverse	GCTGTTGAAGCGGATCTTGTA	21
VEGFA	Forward	GCAGATTATGCGGATCAAACCT	22
	Reverse	TTCTTTGGTCTGCATTCACATTTGT	25
B-Actin	Forward	GTCGACACCGCAACCAGTT	19
	Reverse	AAGCCGGCCTTGCACAT	17

[a]All primer pairs were designed to amplify efficiently, produce a single amplicon, not generate primer–dimers and produced BLAST specificity to expressed gene. Amplicon length for each primer are reported.

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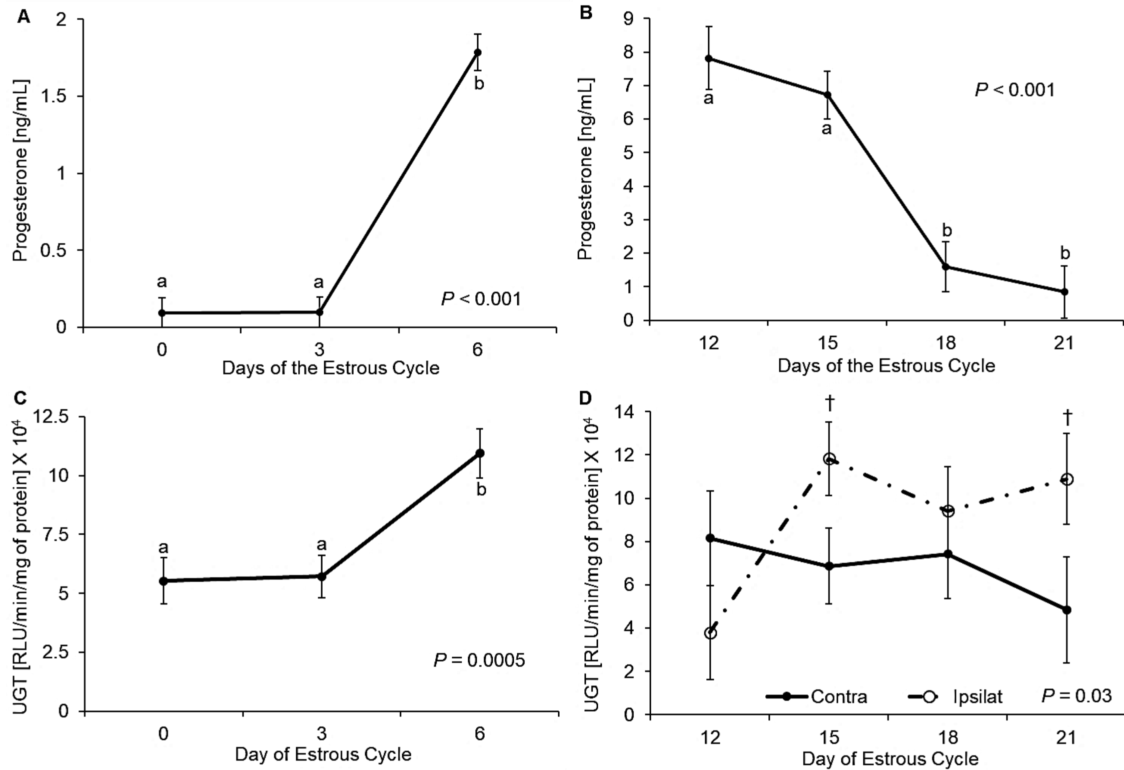

Figure 4 Circulating progesterone profile and UGT activity in endometrial biopsies across the estrous cycle in cattle in Experiment 1.

(A) Progesterone concentrations from day 0 through day 6 of the first estrous cycle and (B) progesterone concentrations from day 12 through day 21 of the second estrous cycle (C) Enzyme activity of UGT in endometrial biopsies from day 0 through day 6 of the first estrous cycle and (D) enzyme activity of UGT in endometrial biopsies from day 12 through day 21 of the second estrous cycle. Means with a different letter represent a significant difference ($P < 0.05$). Means with a dagger (†) represent a tendency ($0.06 \leq P \leq 0.10$) within day.

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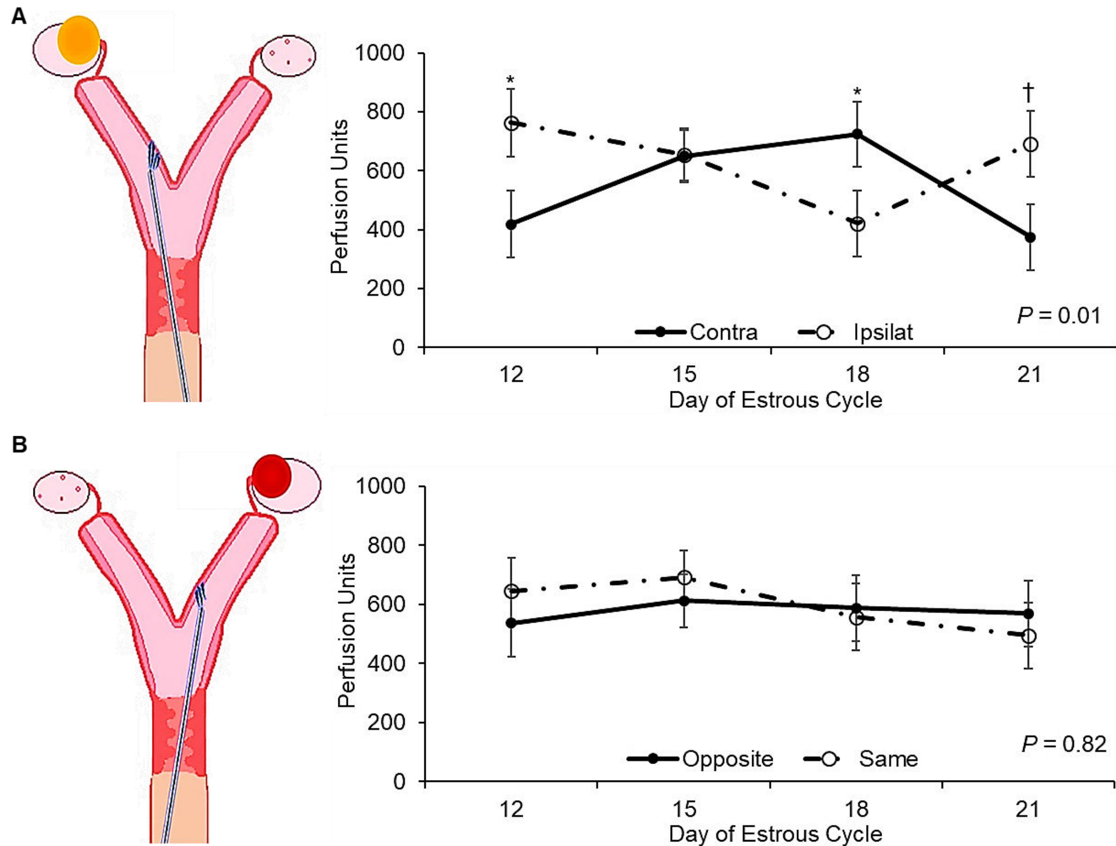

Figure 5 Schematic showing placement of the laser Doppler probe as well as microvascular perfusion data collected in Experiment 2.

(A) Schematic drawing of the uterus with the laser Doppler probe inserted into the uterus on the ipsilateral horn to the CL of cows to collect endometrial blood perfusion that is indicated in blue, and the graph that represents the CL side by day interaction with the asterisk (*) representing a significant difference ($P \leq 0.05$) and a dagger (†) representing a tendency ($0.06 \leq P \leq 0.10$) within day. (B) Schematic drawing of the uterus with the laser Doppler probe inserted into the uterus on the ipsilateral horn to the dominant follicle of cows to collect endometrial blood perfusion that is indicated in blue, and the graph that represents the dominant follicle side by day interaction.

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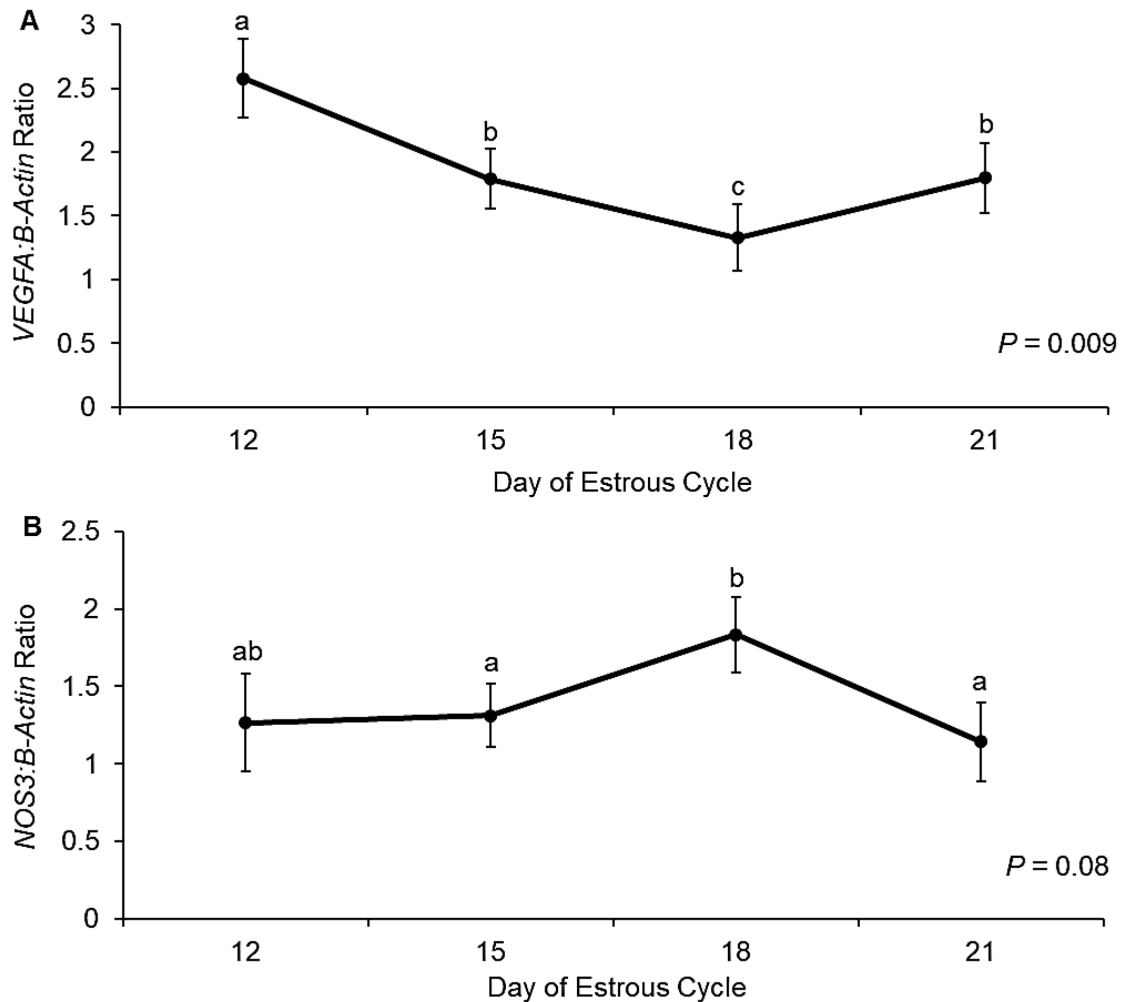

Figure 6 Vascular Endothelial Growth Factor A (VEGFA) and Nitric Oxide Synthase 3 (NOS3) relative expression in endometrial biopsies across the estrous cycle in cattle in Experiment 2.

(A) Vascular Endothelial Growth Factor A (VEGFA) expression in endometrial biopsies with different letters representing a significant difference ($P \leq 0.05$) between days. (B) Nitric Oxide Synthase 3 (NOS3) expression in endometrial biopsies with different letters representing a significant difference ($P \leq 0.05$) between days.

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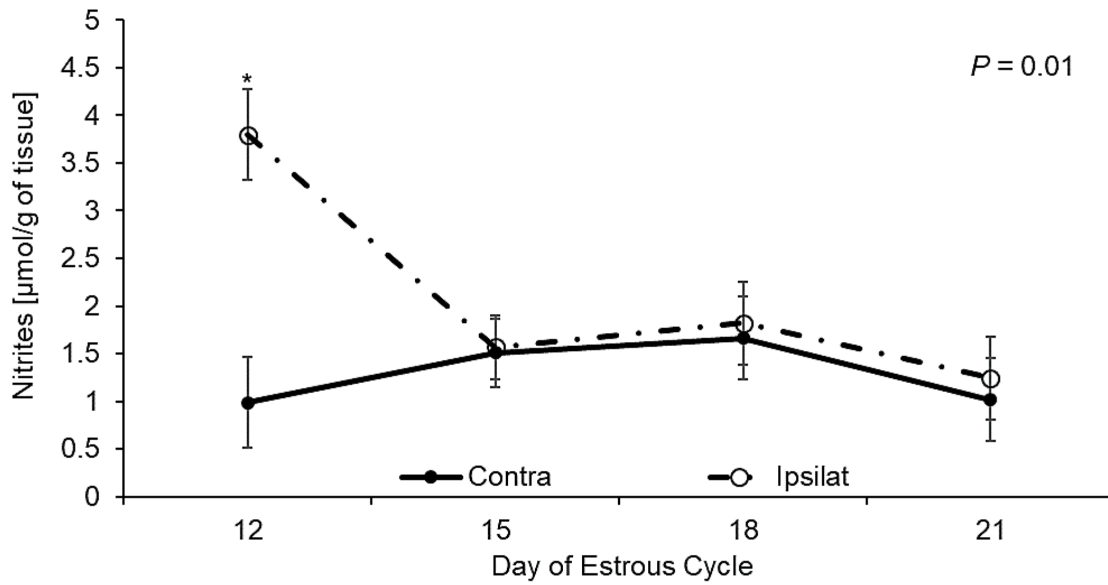

Figure 7 Total nitrites in uterine biopsy samples across the estrous cycle in cattle in Experiment 2.

Nitrites in uterine biopsy samples across the days of the estrous cycle in relation to the side of the CL µmol/gram of tissue. The asterisk (*) represents a significant difference ($P \leq 0.05$) between contralateral and ipsilateral nitrite measurements to the CL.

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CHAPTER III

OXYLIPIN PROFILES AND EICOSANOID METABOLIZING ENZYMES IN BOVINE REPRODUCTIVE TISSUES DURING MATERNAL RECOGNITION OF PREGNANCY

Abstract

The objective was to determine the effects of pregnancy status on hormone metabolizing enzymes and oxylipins profiles in corpora lutea (**CL**) or endometrial (caruncle; **CAR** and inter-caruncle; **IC**) tissues. Cattle were fixed-time artificially inseminated, and were classified as either exhibiting or not exhibiting estrus based on estrus activity. Sixteen days after AI, cattle were euthanized and reproductive tracts collected from 18 non-pregnant and 10 pregnant cows. Activity of cytochrome P450 1A (CYP1A) and UDP-glucuronosyltransferase (UGT) enzymes were determined using specific luminogenic substrates, and oxylipin profiles were obtained. Data were analyzed using MIXED procedure of SAS and model statement included pregnancy status, display of estrus, and the respective interaction. Activity of CYP1A in the CL and UGT in CAR and IC was not different ($P > 0.05$) between pregnant and non-pregnant cattle, as well as cattle that exhibited or failed to exhibit estrus. In the CL, activity of UGT was decreased ($P < 0.05$) in pregnant vs. non-pregnant cattle which exhibited estrus. Corpora lutea of pregnant cattle contained greater ($P < 0.05$) concentrations of 9,10-DiHODE, 15,16-DiHODE, and 9,10-DiHOME. The decrease in CL UGT activity during pregnancy

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indicates alterations in local hormone metabolism. Moreover, the increase in specific oxylipins in the CL may indicate novel pathways of eicosanoid metabolism during maternal recognition of pregnancy.

Introduction

There are a multitude of factors that affect the display of estrus in cattle (Landaeta-Hernández et al., 2002), but cattle that exhibit estrus yield decreased pregnancy loss and greater concentrations of serum progesterone (P_4) (Pereira et al., 2016). Production of P_4 is the primary function of the CL in livestock species (Kittok et al., 1983), and a lack of P_4 within the uterus creates a less hospitable environment for successful pregnancies and formation of successful extra-embryonic membranes (Nephew et al., 1994). Maternal recognition of pregnancy occurs between days 15 and 17 in cattle, which prolongs the lifespan of the CL by the conceptus secreting interferon-tau (IFNτ) that stimulates the endometrium to secrete pro-embryonic proteins (Bazer, 1992; Cheng et al., 2007). Moreover, using an in vitro model our laboratory has previously shown decreased CYP activity following IFNτ exposure (Gilfeather and Lemley, 2016). However, minimal research has been performed to examine the local steroid metabolism within the CL. Steroid metabolism occurs in two phases: phase I is performed by cytochrome P450 (CYP) enzymes, which are further metabolized in phase II by UDP-glucuronosyltransferase (UGT). However, some studies have identified CYP enzymes within the CL (Irusta et al., 2007; Bogan et al., 2008) and uterus of monkeys (Uno et al., 2006), as well as various reproductive tissues in humans (Yokose et al., 1999; Roman, 2002). Due to the localized and variable control of steroid production based on pregnancy status and expression of estrus, we hypothesized that pregnant cattle that expressed estrus

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would yield decreased activity of CYP and UGT in CL and endometrial tissues. Therefore, the first objective was to determine the activity of CYP and UGT within CL, intercaruncle, and caruncle tissues in pregnant and non-pregnant cattle that have and have not exhibited signs of estrus.

Oxylipins are a family of lipids that result from biosynthesizing polyunsaturated fatty acids which result in products such as eicosanoids, epoxides, diols, thromboxanes, and leukotrienes (Calder, 2010; Gouveia-Figueira et al., 2015; Mavangira and Sordillo, 2017). Production of these oxylipins occurs via CYP enzymes and has been linked to both pro- and anti-inflammatory responses (Calder, 2010; Zivkovic et al., 2012; Mavangira and Sordillo, 2017), such as DHA metabolites that include HDPEs, HETEs, and some EETs. Further metabolism by UGT allows for the inactivation and clearance of these oxylipins (Heemskerk et al., 2014). Some eicosanoids are important for the regression of the CL such as $PGF_{2\alpha}$ (Rekawiecki et al., 2008), which allows for the non-pregnant female to begin another follicular phase of the estrous cycle, but other eicosanoids are important for the maintenance of the CL such as PGE2 to prolong during gestation and produce P_4, especially during maternal recognition of pregnancy period (Weems et al., 2011). The lack of profiling of oxylipins contained within CL in cattle, as well as the significant importance of eicosanoid metabolism during maternal recognition of pregnancy has created a significant gap in knowledge. Moreover, the expression and activity of CYP and UGT enzymes in the CL could lead to oxylipin metabolite production and inactivation during maternal recognition of pregnancy. Therefore, we hypothesized that alterations of localized oxylipins within the CL would be influenced by pregnancy status during the maternal recognition of pregnancy period.

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Materials and Methods

Animal care and sample collection

Animal care and use were according to protocols approved by the South Dakota State University Institutional Animal Care and Use Committee. Cattle were allowed to graze on native pasture (Smooth Brome, Reed Canary, Quack grass, Kentucky Blue, and Switch Grass) and supplemented with approximately 1.36 kg/head/day of a mixed concentrate (containing 50% DDGS and 50% corn) and *ad libitum* access to water. Twenty-Eight Angus cross cattle (between 3 and 13 yr) had their estrous cycles synchronized with the CO-Synch fixed-time AI protocol, and estrus activity was determined from day -2 until AI (day 0). Cattle were classified as either exhibiting or not exhibiting estrus based on visually determined estrus activity (confirmed with peripheral concentrations of estradiol). Sixteen days after AI, all cattle were euthanized and reproductive tracts were collected from 18 non-pregnant and 10 pregnant cows, with pregnancy determined by presence of an embryo. Corpora lutea (CL) were collected and weighed at harvest, then snap frozen. Endometrial (caruncle; CAR and inter-caruncle; IC) tissues contralateral to the CL were collected at harvest and also snap frozen for further analysis.

Tissue processing

Approximately 200 mg of tissue was placed into a polypropylene tube with 1 mL potassium phosphate (KPO_4) buffer (400 mM, pH = 7.4). Samples were then mechanically homogenized using the Tissue Tearor (Biospec Products, Bartlesville, OK, USA) following the manufacturer's protocol. Tissue homogenates were then centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was stored at −80°C until enzyme

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assays were conducted. The protein concentration of the supernatant was determined by a Coomassie Plus (Bradford) protein assay following the manufacturer's protocol (Thermo Scientific, Rockford, IL, USA). Following protein analysis, samples were diluted to 4 mg/mL.

CYP enzyme assays

Assay kits for CYP1A, CYP2C, CYP3A, and NADPH regeneration solution were purchased from Promega Corporation (Madison, WI, USA) and assays conducted according to Hart and others, 2014, (Hart et al., 2014) with minor adaptations. Briefly, reconstitution buffer was added to luciferin detection reagent. Luciferin CEE (CYP1A), luciferin IPA (CYP3A), and luciferin H (CYP2C) were diluted in KPO_4 buffer. Tissue homogenates (30 µg of protein per well) and enzyme-specific luciferin substrate were added to 96-well opaque white plates in duplicate Plates were then pre-incubated for 10 min (CYP1A), 30 min (CYP3A), or 90 min (CYP2C) at 37°C. Following the incubation, NADPH regeneration solution was added to each well and plates were incubated for 30 min (CYP1A and CYP2C) at 37°C or 10 min (CYP3A) at room temperature. After the second incubation, luciferin detection reagent was added to each well and plates were protected from light and incubated for an additional 20 min at room temperature. Plates were then placed into a Promega Multi-Plus plate reader and luminescence was measured. However, no detectable activity of CYP2C or CYP3A in any tissue, as well as CYP1A activity of uterine tissues were obtained.

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UGT enzyme assay

The UGT assay kit was purchased from Promega Corporation and the assay performed according to Hart et al. (2014) with minor adaptations. Briefly, uridine diphosphoglucoronic acid (UDPGA) was added to half the plates to act as reaction wells, and distilled water was added to the other half as control wells. The UGT reaction mixture containing UGT multienzyme substrate was then combined with tissue homogenates (28 μg of tissue protein per well), and the plates were preincubated for 90 min at 37°C. After incubation, detection reagent was added to each of the wells followed by an incubation period of 20 min at room temperature while protected from light. The plates were then analyzed using a Promega Multi- Plus plate reader with luminescence detection mode.

Luteal progesterone assay

Luteal P_4 concentrations were determined using radioimmunoassay procedures based on manufacturer recommendations (MP Biomedicals, Costa Mesa, CA, USA) and Cline et al., 2016 [30]. Approximately 50 mg of luteal tissue was placed into a 15-mL conical tube with 5 mL of phosphate buffered saline (PBS). Samples were then mechanically homogenized using the Tissue Tearor (Biospec Products, Bartlesville, OK, USA) as previously described. Tissue homogenate samples were centrifuged at 1500 × g for 15 min at 4°C, then the supernatant was placed into 5-mL polypropylene tube. The supernatant was vortexed and diluted (1:20) in PBS, which was used to analyze for luteal P_4 concentrations. The inter- and intra-assay coefficients of variation were 3.6 and 4.0%, respectively. Progesterone concentration is reported as μg of P_4 per gram of CL as well as

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total P_4 content of CL, which was calculated by multiplying the P_4 concentration (μg of P_4 per gram of CL) by the CL weight at harvest.

Luteal oxylipin metabolome assay

Luteal oxylipin metabolome profiles were determined using previously published procedures based on Yang et al., 2009 (Yang et al., 2009). Only CL from cattle that exhibited estrus were used for this method of analysis. The formal name and classification of all oxylipins can be found in supplementary Table 2.

Statistical analysis

Enzyme activities were expressed relative to mg of protein or g of tissue. In addition, total activity of the CL was calculated by multiplying activity per g of tissue by CL weight. Data, respective to each tissue, enzyme activity, and CL parameters (CL wt, μg P_4/ gram of CL, and total CL P_4) were analyzed using MIXED procedure of SAS and the model statement included pregnancy status, display of estrus, and the respective interaction. However, data, respective to CL individual and categorized oxylipin profile was analyzed using MIXED procedure of SAS and the model statement included pregnancy status. Pearson correlation coefficients were determined using the CORR procedure of SAS to analyze the relationships between CL weight, μg of P_4/ gram of CL, total CL P_4, and enzyme activity with respect to each tissue. Treatment means were separated using the PDIFF options of the LSMEANS statement. Least square means and standard error are reported. Statistical significance was declared at $P \leq 0.05$, and tendencies discussed when $P \geq 0.06$ but $P \leq 0.10$.

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Results

Effect of estrus and pregnancy on CYP1A and UGT

Activity of CYP1A (RLU/mg of protein, RLU/g of tissue, and total CL) were not different ($P = 0.20$) between pregnant and non-pregnant cattle, as well as between cattle that exhibited or failed to exhibit estrus ($P = 0.53$) and are displayed in **Fig. 8A and 8B,** respectively. Activity of UGT (RLU/g of tissue) among tissues are displayed in **Fig. 9**. In CAR and IC, activity of UGT relative to mg of protein and g of tissue were not different ($P \geq 0.65$) between pregnant and non-pregnant cattle, as well as between cattle that exhibited or failed to exhibit estrus ($P \geq 0.15$; **Fig. 9A and 9B,** respectively). However, activity of UGT in the CL relative to g of tissue [not shown] and CL total (**Fig. 9C**) were decreased ($P \leq 0.05$) in pregnant vs. non-pregnant cattle which exhibited estrus, while it was not different in pregnant vs. non-pregnant cattle that did not exhibit estrus.

Luteal weight, progesterone, and oxylipins

Corpora lutea weights at the time of harvest were not different across all groups (4.37 ± 0.17 g). Luteal concentrations of P_4, µg /g CL, were not different across all groups (50.84 ± 5.23 µg /g CL). Also, total CL P_4, was not different across all groups (222.98 ± 22.48 µg). Concentrations of circulating serum P_4 were not different across all groups (4.05 ± 0.31 ng/mL). A detailed list of the individual oxylipins found in the CL tissues are found in Table 3, and categories of oxylipins are found in Table 4. Corpora lutea from pregnant cattle contained greater ($P \leq 0.05$) concentrations of 9,10-DiHODE, 15,16-DiHODE, and 9,10-DiHOME as compared to non-pregnant cattle. Corpora lutea from pregnant cattle tended ($P = 0.08$) to contain lesser concentration of 13-oxo-ODE, while CL from pregnant cattle tended ($P = 0.06$) to contain greater concentration of

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19,20-DiHDPE as compared to non-pregnant cattle. No differences ($P = 0.21$) were observed between pregnant and non-pregnant concentrations of total oxylipins in CL.

Discussion

One of the more interesting findings from this research was a decrease in Phase II UGT enzyme activity in the CL of pregnant vs non-pregnant cattle. These UGT enzymes are involved in conjugating lipophilic molecules, such as steroids and eicosanoids, for more easy excretion via the urinary system. This experiment exposed the differential activity of UGT between intercaruncle and caruncle tissues; however, no differences were observed between cattle that expressed estrus or not and cattle that were pregnant or not in intercaruncle and caruncular activity of UGT. Data from all cattle in the current study tended to exhibit a negative correlation ($r = -0.36$, $P = 0.06$) between UGT activity in caruncular tissue (RLU/g of tissue) and ng of P_4/g of CL. In addition to activity of UGT in uterine tissues, this study examined the activity of UGT in CL tissues. This activity of UGT in CL was greatest in cattle that expressed estrus and were non-pregnant as compared to non-pregnant cattle that failed to express estrus and pregnant cattle that expressed estrus. A significant negative correlation ($r = -0.50$, $P = 0.02$) existed between CL weight and UGT activity in the CL (RLU/mg of protein), which indicates that CL with lesser weight have greater activity of UGT as compared to their heavier counterparts. However, local UGT activity within the CL did not correlate with the P_4 content of the CL in this study and therefore is not influencing the P_4 content of the CL.

The presence of the phase I steroid metabolizing enzyme, CYP1A, indicates localized metabolism of steroids within the CL of cattle. Contrary to the current study, CYP2C and 3A have been observed in the CL of rhesus monkeys (Irusta et al., 2007), but

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these enzymes were not detectable in the CL collected from cattle. Activity of CYP1A in CL tissue was not different between cattle that expressed estrus or not and cattle that were pregnant. Previous research in our laboratory (Gilfeather and Lemley, 2016) have indicated decreased CYP1A activity in bovine endometrial epithelial cells treated with IFNτ as well as oxytocin and IFNτ as compared to those cells treated with oxytocin and vehicle control. This was the first report of decreased steroid metabolizing enzyme activity following in vitro IFNτ treatment which indicates that during the maternal recognition of pregnancy, the IFNτ secreted from the conceptus induces a localized decrease of steroid and eicosanoid metabolism. This *in vitro* work does not recapitulate the current *in vivo* analysis, due to the inability to detect CYP1A in endometrial tissues. This may be due to external factors affecting the biological functions that affect the activity of the enzymes. Neither pregnancy status nor expression of estrus was different with regards to CL weight, μg of P_4/g of CL, and total CL P_4.

Due to the decreased activity of UGT within the CL of pregnant cattle that expressed estrus and our hypothesis that pregnant cattle that expressed estrus would yield lesser activity of CYP and UGT within the CL, we examined the profile in the CL. A majority of the oxylipins characterized were not different between pregnant and non-pregnant cattle in the present study. Octadecadienoic acids (ODE), 15,16-DiHODE and 9,10-DiHODE, were greater in CL from pregnant cattle as compared to CL from non-pregnant cattle. However, 13-oxo-ODE tended to be lesser in pregnant CL as compared to the non-pregnant CL. These ODE are primarily derived from omega fatty acids (omega 3, 6, and 9), specifically α-linoleic acid, linoleic, and oleic acid (Calder, 2010; Chang et al., 2012; Gouveia-Figueira et al., 2015) and have been identified in human serum and

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plasma, hepatic, and intestinal microsomes (Little et al., 2004; Zivkovic et al., 2012; Gouveia-Figueira et al., 2015; Berkecz et al., 2017), which are concurrent with the locations of CYP and UGT (Chun et al., 1999; Yokose et al., 1999; Khan and Khan, 2013). Supplementation of omega fatty acids have decreased the inflammatory oxylipins (Zivkovic et al., 2012; Bruins et al., 2013), increased density of omega 3 fatty acids in the blood (White et al., 2012), and increase in ODE that have been noted to be involved with cellular proliferation (Reynolds et al., 2006).

Epoxide hydrolase metabolites 9,10-DiHOME and 19,20-DiHDPE were greater in CL from pregnant cows as compared to CL from non-pregnant cattle in this study. Concentrations of 9,10-DiHOME have been differentially expressed in the cow brain, heart, and liver, with the liver containing the greatest quantity and the brain with the least (Gouveia-Figueira and Nording, 2015b). Contrary to the ODE oxylipins mentioned above, 9,10-DiHOME and 19,20-DiHDPE decreased in serum when supplemented with omega fatty acids (Zivkovic et al., 2012). Supplementation of cancer cells with docosahexaenoic acid (DHA) were converted to 19,20-DiHDPE which decreased angiogenic factors, tumor growth, and endothelial cell migration; however, 19,20-DiHDPE in healthy adipose tissue from humans have been noted to suppress inflammation (Bruins et al., 2013; Heemskerk et al., 2014). The increase of 19,20-DiHDPE in CL from pregnant cattle as compared to non-pregnant animals could indicate the suppression of inflammation that luteolysis induces; however, further investigations are needed to prove this pathway.

Activity of UGT in this experiment was observed within intercaruncle and caruncle tissues with no differences observed between cattle that expressed estrus or not

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nor cattle that were pregnant or not. This implies that deactivation and clearance of the eicosanoids and steroid metabolites that are produced could be acted upon via UGT. Also, the identification of local metabolism of steroids could allow researchers to develop a more efficient estrous synchronization protocol or even a therapy for those cattle that are reproductively incompetent. The detection of activity of UGT in CL indicates that there is localized metabolism of steroids and eicosanoids within the CL, and were differentially expressed between pregnant and non-pregnant cattle as well as cattle that expressed or failed to express estrus. Furthermore, characterization of oxylipins in CL of cattle that expressed estrus in this study allows for further research to be conducted to obtain a greater understanding of the pro- and anti-inflammatory and embryonic factors that affect the CL and could affect the health, production, and longevity of this transient organ to maintain pregnancy.

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Table 2 Full oxylipin profile name within CL tissues of cattle at day 16 post insemination and the classification of each in pmol per gram of CL tissue ± standard error.

Oxylipin abbreviation	Full oxylipin name	Classification
10(11)-EpDPE	10,11-epoxy Docosapentaenoic Acid	EH
11(12)-EpETE	11,12-epoxy-5Z,8Z,14Z,17Z-eicosatetraenoic acid	EET
11(12)-EpETrE	11,(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid	EET
11,12-DiHETE	11,12-dihydroxy-5Z,8Z,14Z,17Z-eicosatetraenoic acid	HETE
11,12-DiHETrE	11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid	EET
12(13)-EpODE	12(13)-epoxy-9Z,15Z-octadecadienoic acid	ODE
12(13)-EpOME	12(13)epoxy-9Z-octadecenoic acid	EH
12,13-DiHODE	12,13-dihydroxy-9Z,15Z-octadecadienoic acid	ODE
12,13-DiHOME	12,13-dihydroxy-9Z-octadecenoic acid	EH
13,14-DiHDPE	13,14-dihydroxy-4Z,7Z,10Z,16Z,19Z-docosapentaenoic acid	HDPE
13-HODE	13-hydroxy-9Z,11E-octadecadienoic acid	ODE
13-oxo-ODE	13-oxo-9Z,11E-octadecadienoic acid	ODE
14(15)-EpETE	14,15-epoxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid	EET
14(15)-EpETrE	14(15)-epoxy-5Z,8Z,11Z-eicosatrienoic acid	EET
14,15-DiHETE	14,15-dihydroxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid	HETE
14,15-DiHETrE	14,15-dihydroxy-5Z,8Z,11Z-eicosatrienoic acid	EET
15,16-DiHODE	15,16-dihydroxy-9Z,12Z-octadecadienoic acid	ODE
15-HEPE	15-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid	mFA
15-oxo-ETE	15-oxo-5Z,8Z,11Z,13E-eicosatetraenoic acid	Leukotoxin
16,17-DiHDPE	16,17-dihydroxy-4Z,7Z,10Z,13Z,19Z-docosapentaenoic acid	HDPE
17(18)-EpETE	17,18-epoxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid	EET
17,18-DiHETE	17,18-dihydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid	HETE
19(20)-EpDPE	19,20-epoxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid	EH
19,20-DiHDPE	19,20-dihydroxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid	EH
5(6)-EpETrE	5,6-epoxy-8Z,11Z,14Z-eicosatrienoic acid	EET
6-keto-PGF1a	6-oxo-9α,11α,15S-trihydroxy-prost-13E-en-1-oic-3,3,4,4-acid	Prostaglandin
7(8)-EpDPE	(4Z)-6-[3-(2Z,5Z,8Z,11Z)-2,5,8,11-tetradecatetraen-1-yl-2-oxiranyl]-4-hexenoic acid	EH
8(9)-EpETE	8,9-epoxy-5Z,11Z,14Z,17Z-eicosatetraenoic acid	EET
8-HEPE	8-hydroxy-5Z,9E,11Z,14Z,17Z-eicosapentaenoic acid	mFA

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Table 2 (continued)

9(10)-EpODE	9(10)-epoxy-12Z,15Z-octadecadienoic acid	Leukotoxin
9(10)-EpOME	9(10)epoxy-12Z-octadecenoic 9,10,12,13-acid	Leukotoxin
9,10,13-TriHOME	9,10,13-Trihydroxy-11-octadecenoic acid	ODE
9,10-DiHODE	9,10-dihydroxy-12Z,15Z-octadecadienoic acid	ODE
9,10-DiHOME	9,10-dihydroxy-12Z-octadecenoic-9,10,12,13-acid	EH
9,12,13-TriHOME	9,12,13-Trihydroxy-10-octadecenoic acid	ODE
9-HODE	9-hydroxy-10E,12Z-octadecadienoic-9,10,12,13-acid	mFA
9-HOTrE	9-hydroxy-10E,12Z,15Z-octadecatrienoic acid	mFA
LTB5	Leukotriene B5; 5S,12S-dihydroxy-6Z,8E,14Z,17Z-eicosapentanenoic acid	Leukotoxin
PGD2	Prostaglandin D_2; 9α,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid	Prostaglandin

Table 3 Individual oxylipin profile within CL tissues of cattle at day 16 post insemination comparing pregnancy status in pmol per gram of CL tissue ± standard error.

	Pregnancy Status		
Oxylipin (pmol/g tissue)	**Non-Pregnant**	**Pregnant**	***P*-Value**
10(11)-EpDPE	6.98 ± 1.97	6.12 ± 3.97	0.16
11(12)-EpETE	41.79 ± 7.92	47.84 ± 30.43	0.21
11(12)-EpETrE	300.95 ± 63.02	350.32 ± 217.82	0.21
11,12-DiHETE	1.11 ± 0.10	1.14 ± 0.15	0.88
11,12-DiHETrE	12.99 ± 2.31	13.72 ± 1.60	0.80
12(13)-EpODE	17.25 ± 2.42	18.39 ± 8.93	0.28
12(13)-EpOME	147.16 ± 28.93	148.88 ± 79.14	0.16
12,13-DiHODE	2.13 ± 0.29	3.19 ± 0.63	0.16
12,13-DiHOME	35.86 ± 1.98	47.55 ± 6.50	0.12
13,14-DiHDPE	1.07 ± 0.26	1.30 ± 0.13	0.21
13-HODE	309.05 ± 253.02	60.03 ± 20.32	0.77
13-oxo-ODE	35.74 ± 14.61	15.59 ± 7.32	0.08
14(15)-EpETE	33.60 ± 7.30	43.04 ± 25.86	0.21
14(15)-EpETrE	250.56 ± 60.12	296.31 ± 174.64	0.21
14,15-DiHETE	18.54 4.51	21.99 ± 4.74	0.61
14,15-DiHETrE	90.90 ± 7.65	109.39 ± 23.76	0.48
15,16-DiHODE	5.82 ± 0.73	8.53 ± 0.91	0.04
15-HEPE	4.33 ± 0.46	4.77 ± 0.67	0.60

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Table 3 (continued)

15-oxo-ETE	262.95 ± 232.97	14.82 ± 2.17	0.36
16,17-DiHDPE	32.29 ± 6.44	38.30 ± 12.06	0.88
17(18)-EpETE	61.68 ± 10.05	65.00 ± 38.95	0.21
17,18-DiHETE	11.35 ± 0.99	10.61 ± 1.03	0.62
19(20)-EpDPE	10.13 ± 2.54	9.11 ± 5.61	0.21
19,20-DiHDPE	4.75 ± 0.36	6.38 ± 0.22	0.06
5(6)-EpETrE	1067.25 ± 266.23	1239.26 ± 815.08	0.21
6-keto-PGF1a	12.03 ± 4.02	35.56 ± 13.87	0.11
7(8)-EpDPE	90.80 ± 27.79	78.73 ± 51.00	0.28
8(9)-EpETE	37.05 ± 5.96	37.68 ± 23.72	0.11
8-HEPE	122.77 ± 35.56	214.77 ± 154.67	0.55
9(10)-EpODE	41.16 ± 7.02	40.85 ± 19.65	0.28
9(10)-EpOME	148.59 ± 32.90	135.94 ± 67.05	0.28
9,10,13-TriHOME	10.60 ± 3.18	11.85 ± 1.37	0.73
9,10-DiHODE	0.48 ± 0.09	0.72 ± 0.08	0.05
9,10-DiHOME	4.75 ± 0.36	6.38 ± 0.22	0.003
9,12,13-TriHOME	18.50 ± 5.81	21.04 ± 2.23	0.69
9-HODE	72.77 ± 43.65	31.01 ± 7.78	0.77
9-HOTrE	20.96 ± 17.34	2.25 ± 0.44	0.28
LTB5	2.10 ± 0.40	1.62 ± 0.32	0.37
PGD2	2.15 ± 0.80	5.66 ± 3.12	0.88
Total	3358.30 ± 777.69	3208.74 ± 1691.26	0.21

Table 4 Categorical oxylipin profile within CL tissues of cattle at day 16 post insemination comparing pregnancy status. Concentration in pmol per gram of CL tissue ± standard error.

Oxylipin Category (pmol/g tissue)	Pregnancy Status		*P*-Value
	Non-Pregnant	**Pregnant**	
Prostaglandin	14.18 ± 4.81	41.22 ± 16.47	0.16
HETE	30.99 ± 5.25	33.74 ± 5.53	0.73
EH	300.85 ± 58.23	300.15 ± 133.67	0.21
HDPE	33.36 ± 6.67	39.60 ± 12.15	0.88
EET	1896.77 ± 416.83	2202.56 ± 1314.83	0.21
mFA	227.80 ± 61.01	258.91 ± 156.13	0.36
Leukotoxin	454.80 ± 232.45	193.23 ± 88.04	0.11
ODE	399.57 ± 264.09	139.33 ± 27.52	0.65

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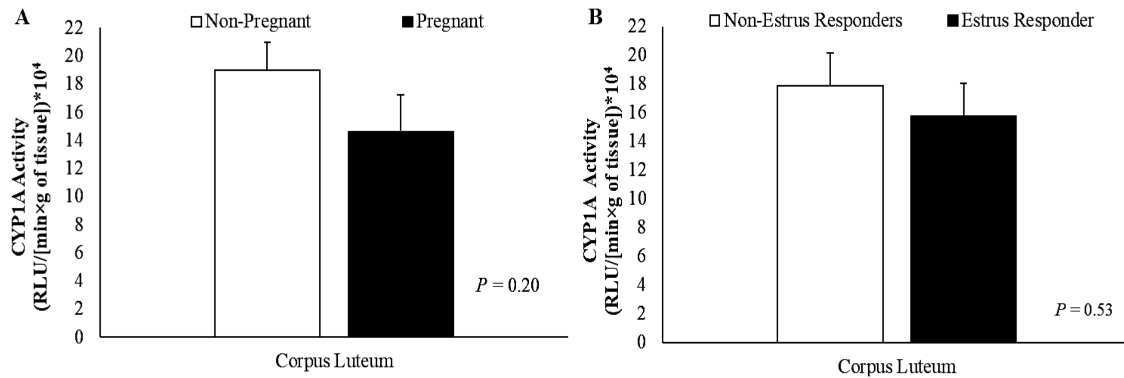

Figure 8 Activity of CYP1A in CL tissue in cattle based on pregnancy status and estrus expression.

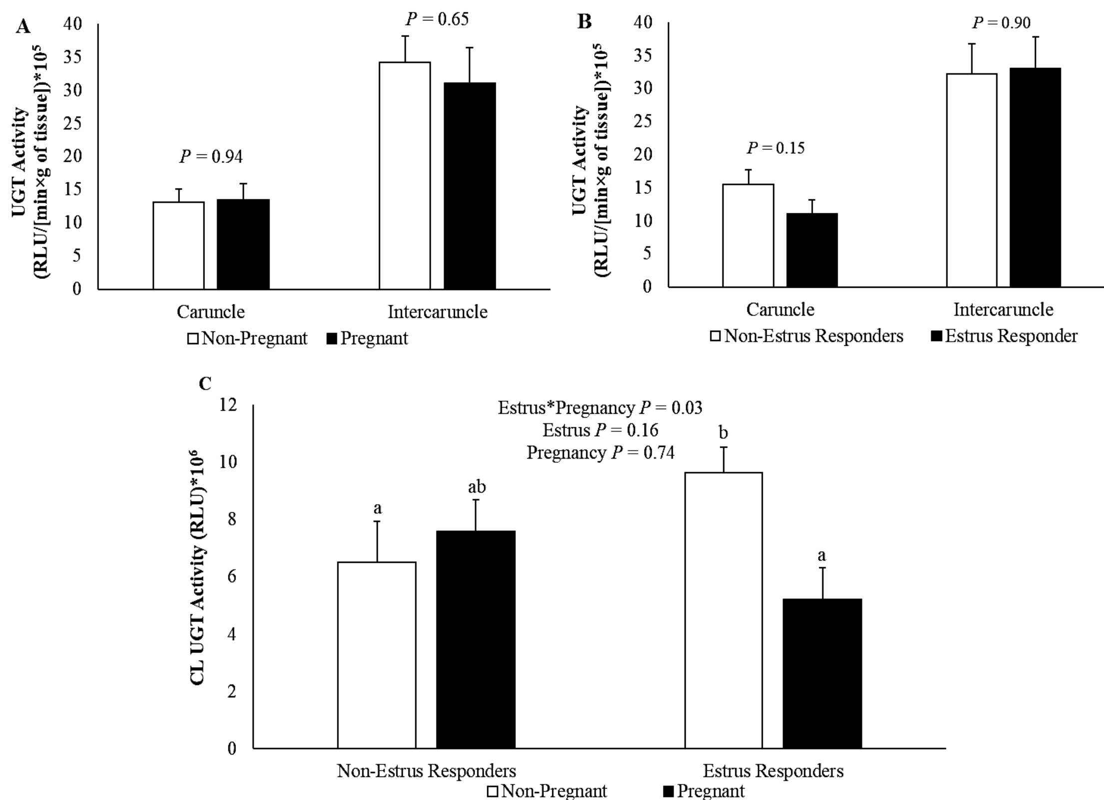

Figure 9 Activity of UGT in caruncle, intercaruncle, and CL tissues in cattle based on pregnancy status and estrus expression. Least square means with different letters within row represent significant differences ($P \leq 0.05$).

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CHAPTER IV

CHARACTERIZATION OF FETAL LIVER AND PLACENTOME STEROID AND EICOSANOID METABOLIZING ENZYMES IN HEIFERS SUPPLEMENTED WITH MELATONIN

Abstract

Melatonin supplementation influences the metabolism of steroids and eicosanoids. The objective was to examine fetal liver and placental steroid and eicosanoid enzyme activity during maternal melatonin supplementation. Commercial beef heifers (*n* = 12) were bred via AI and delegated to receive two, 24 mg sub-dermal melatonin implants (**MEL;** *n* = 6) or act as part of a non-implant control group (**CON;** *n* = 6). Treatments were administered at d 180, 210, and 240 of gestation. Heifers underwent Caesarian removal of the fetus at 243 ± 2 d of gestation to harvest fetal liver and placental tissues. The placental samples were collected and separated into maternal (caruncle; **CAR**) and fetal (cotyledon; **COT**) portions. Cytochrome P450 1A (**CYP1A**), 2C (**CYP2C**), 3A (**CYP3A**), and uridine 5'-diphospho-glucuronosyltransferase (**UGT**) activities were determined via luminogenic substrates. Activities were expressed per mg of protein, and data were analyzed, using the Wilcoxon rank sum test, with treatment, fetal sex, and the interaction thereof in the model. Statistical significance was declared at $P \leq 0.05$. Maternal and fetal morphometric measurements were not different between treatments. Activity of CYP1A and UGT in fetal liver was greater from dams treated with

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MEL as compared to CON. Activity of CYP3A was not different between treatments while activity of CYP2C was not detected in fetal liver tissues. Activity of CYP1A, CYP2C, and UGT were not different between treatments in CAR tissues while activity of CYP3A was not detected in CAR tissues. Activity of CYP1A and UGT were not different between treatments in COT tissues while activity of CYP2C and CYP3A was not detected in COT tissues. In conclusion, melatonin supplementation increased CYP1A and UGT in fetal liver; however, hormone metabolizing enzymes of the placenta were not different between treatments. Increased activity of CYP1A and UGT in fetal liver during maternal melatonin supplementation indicates an increase in steroid and eicosanoid metabolism during the fetal period.

Introduction

Increasing the viability and ultimately profitability of calves is the goal of all producers, and research has led to applying various therapies to do so. Therapeutic supplements applied to dams have been utilized to impact future reproductive capabilities of the offspring. Reduced maternal nutrition has decreased cellular proliferation in fetal gonads and longer attainment of puberty in both cattle and sheep (Funston et al., 2010). Adequate maternal nutrition has yielded greater lamb and calf weights, which has been associated with more offspring being more vigorous and having a better chance of survival (Godfrey, 2002; Wu et al., 2004; Meyer et al., 2010; Meyer et al., 2013). Supplementation of melatonin to dams has been observed to influence not only placental characteristics, but also affects the fetal organs in both sheep and cattle (Forcada et al., 1995; Lemley et al., 2012; Lemley et al., 2013a; Eifert et al., 2015). Melatonin is a neurohormone secreted primarily by the pineal gland that serves several physiological

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functions in the body including reproductive cyclicity in seasonal breeding animals and influences pubertal development (Reiter et al., 2006; Pandi-Perumal et al., 2008; Russel J. Reiter et al., 2009).

Supplementation of melatonin to ewes negated the effects of nutrient restriction as compared to those ewes that were not nutrient restricted as measured by the number of viable embryos per CL and overall pregnancy rates (Vázquez et al., 2013). Dietary enhancement of melatonin throughout the last third of gestation increased uterine blood flow, heart rate, and total antioxidant capacity, but it decreased maternal serum estradiol-17β and P_4 concentrations (Brockus et al., 2016b). Melatonin supplementation to bovine endometria cells has also increased the activity of phase I and II enzymes that metabolize steroids and eicosanoids as compared to control treatments (Brockus et al., 2016b). These phase I enzymes include cytochrome P450 (CYP) including CYP1A and CYP3A that metabolizes estrogens and several eicosanoids, as well as CYP2C and CYP3A which metabolizes progestins and several eicosanoids (Canby-Hagino and Thompson, 2005). The primary phase II enzyme responsible for further metabolism of phase I metabolites is UDP-glucoronosyltransferase (UGT) in order to allow for clearance of these molecules (Kim et al., 1997). This leads to the objective to examine fetal liver and placental steroid and eicosanoid enzyme activity during maternal melatonin supplementation in the last third of gestation. We hypothesized that fetal liver and placental tissues from melatonin treated dams would contain greater activity of steroid and eicosanoid metabolizing enzymes.

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Materials and Methods

Animal care and treatments

Animal care and use was conducted according to protocols approved by the Mississippi State University Institutional Animal Care and Use Committee. Twelve - Angus cross heifers were synchronized using a modified 7-day CO-Synch + CIDR protocol. Briefly, a 2 ml, i.m. injection of GnRH (Factrel, Fort Dodge Animal Health, Fort Dodge, Iowa, USA) was administered, and a cattle CIDR (Eazi-Breed™ CIDR® Cattle Insert, Zoetis, Parsippany, NJ, USA) was inserted for 7 days, upon CIDR removal cattle were administered a 5 ml, i.m. injection of Lutalyse (dinoprost injection, Zoetis, Parsippany, NJ, USA), and estrus was detected in 60 ± 6 hrs. Heifers were AI to the same bull and enrolled in the study upon pregnancy verification via rectal ultrasonography on d 127. Heifers were designated to one of two treatments, a control group (CON; n = 6) that acted as a placebo non-implant group, or a melatonin treatment group that would receive two, 24 mg sub-dermal implants (Melatonin Implants LLC, Conroe, TX, USA; MEL; n = 6). Heifers underwent Cesarean removal of the fetus at 243 ± 2 d of gestation where a single placentome was harvested.

Sample collection and treatment application

Heifers designated to the MEL group received two, 24 mg sub-dermal melatonin implants in the backside of the ear using a syringe implant applicator (Melatonin Applicator, Melatonin Implants LLC, Conroe, TX, USA), alternating implant sides each application period. Heifers designated to the CON group were stuck with syringe implant applicator to simulate the mode of receiving an implant. Sampling and treatment application began on d 180 of gestation and were applied again on d 210 and 240 of

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gestation. Each implant was designed to supply sufficient amounts of melatonin for 30 days. The entire treatment period was expected to last for the final 90 days of gestation. Immediately prior to Cesarean section blood samples were collected via coccygeal venipuncture and heifers were weighed. At 243 ± 2 d of gestation heifers underwent Cesarean section removal of the fetus via standing left paralumbar celiotomy. Cattle were initially sedated (5 mg butorphanol, 10 mg xylazine, and 20 mg ketamine) and were injected with lidocaine (100 mL total) in an inverted L-shape to ensure local anesthesia. An incision was made vertically in the middle of the paralumbar fossa, starting approximately 10 cm ventral to the transverse processes of the lumbar vertebrae and continuing ventrally, far enough to allow removal of the calf.

Just prior to complete removal of the calf the umbilical cord was occluded with Kelly forceps to ensure no blood loss. Once the calf was removed, a single placentome was collected approximately 5 cm from the incision site. Incisions were then closed and heifers were administered 50 mL Liquamycin® LA-200® (oxytetracycline injectable solution; Zoetis Services LLC., Parsippany, NJ, USA) and 10 mL Banamine (flunixin meglumine, Merck & Co., Inc., Kenilworth, NJ, USA) i.m. Calves were weighed and euthanized via jugular exsanguination and fetal blood was collected at this time. Carcass weight was collected which includes head, hide, and carcass weights that does not include internal organs. The fetal liver was collected and weighed. Concurrently, the placentome was separated into maternal (caruncle; CAR) and fetal (cotyledon; COT) portions and snap frozen in liquid nitrogen.

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Blood and tissue processing

Serum tubes were incubated at room temperature for at least 30 min prior to being centrifuged and plasma tubes were inverted several times and held on ice for transportation. Centrifugation was performed at 4°C for 15 min at 10,000 x g. Serum was harvested and subsequently stored at -80°C until assayed. Approximately 100 mg of tissue was placed into a polypropylene tube with 1 mL of potassium phosphate (KPO_4) buffer (400 mM, pH = 7.4). Samples were then mechanically homogenized using the Tissue Tearor (Biospec Products, Bartlesville, OK, USA) following the manufacturer's protocol. Tissue homogenate samples were placed into microcentrifuge tubes and then centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was stored at −80°C until enzyme assays were conducted. The protein concentration of the supernatant was determined by a Coomassie Plus (Bradford) protein assay following the manufacturer's protocol (Thermo Scientific, Rockford, IL, USA).

Serum P_4 assay

Serum P_4 concentrations were determined using radioimmunoassay procedures based on manufacturer recommendations (MP Biomedicals, Costa Mesa, CA, USA) and Cline et al., 2016. Briefly, serum samples and standards were vortexed and 100 µL was pipetted into each respective coated tube, in duplicate. Following standard and serum sample allocation 1.0 mL of P_4-I^{125} was pipetted into each tube and briefly vortexed before incubating at 37°C for 2 hours. All standard and sample tubes were decanted and tubes were counted in a Cobra II gamma counter (Packard, Meriden, CT, USA). The intra-assay coefficient of variation is 5.6%, and concentration of P_4 is expressed as

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ng/mL. Serum P_4 concentrations were determined only within the maternal serum due to the fetal serum not containing enough P_4 to yield detectable readings.

CYP enzyme assays

Assay kits for CYP1A, CYP2C, and CYP3A, and NADPH regeneration solution were purchased from Promega Corporation (Madison, WI, USA) and assays conducted according to Hart et al. (2014) with minor adaptations. Briefly, reconstitution buffer was added to luciferin detection reagent. Luciferin CEE (CYP1A), luciferin IPA (CYP3A), and luciferin H (CYP2C) were diluted in KPO_4 buffer. Tissue homogenates (30 μg of protein per well) and enzyme- specific luciferin substrate was added to 96-well plates in duplicate. Plates were then preincubated for 10 min (CYP1A), 30 min (CYP3A), or 90 min (CYP2C) at 37°C. Following the incubation, NADPH regeneration solution was added to each well and plates were incubated for 30 min (CYP1A and CYP2C) at 37°C or 10 min (CYP3A) at room temperature. After the incubation, luciferin detection reagent was added to each well and plates were protected from light and incubated for an additional 20 min at room temperature. Plates were then placed into a Promega Multi-Plus plate reader and luminescence was measured.

UGT assay

The UGT assay kit was purchased from Promega Corporation and the assay performed according to Hart et al. (2014) with minor adaptations. Briefly, uridine diphosphoglucoronic acid (UDPGA) was added to half the plates to act as reaction wells, and distilled water was added to the other half as control wells. The UGT reaction mixture containing UGT multienzyme substrate was then combined with tissue

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homogenates (28 μg of tissue protein per well), and the plates were preincubated for 90 min at 37°C. After incubation, detection reagent was added to each of the wells followed by an incubation period of 20 min at room temperature while protected from light. The plates were then analyzed using a Promega Multi- Plus plate reader with luminescence detection mode.

Statistical analysis

The MIXED procedure of SAS (SAS Inst. Inc, Cary, NC, USA) was used to test the effect of treatment within each tissue type. Fetal body weight was used as a covariant and removed from the model if P-value of > 0.20. Treatment means were separated using the PDIFF options of the LSMEANS statement. Least square means and standard error are reported. Pearson correlation coefficients were determined using the CORR procedure of SAS to analyze the relationships between organ weights, concentration of serum P_4, and enzyme activity with respect to each tissue. Statistical significance was declared at $P \leq 0.05$, and tendencies discussed when $P \geq 0.06$ but $P \leq 0.10$.

Results

Effect of treatment on P_4 and weight parameters

Concentrations of circulating serum P_4 were not different ($P = 0.47$) between MEL treated dams and CON treated dams (4.65 and 3.83 ± 0.77 ng/mL, respectively). Effects of the supplementation of subdermal melatonin during the last third of gestation on body and organ weights are outlined in Table 5. Melatonin supplementation for 90 d, during the last third of gestation, had no effect on maternal BW ($P = 0.27$). Fetuses

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collected from dams that were treated with melatonin or control dams had no effect on body wt (P = 0.51), carcass wt (P = 0.53), or liver wt (P = 0.70).

Activity of CYP and UGT enzymes in fetal liver and placentome components

Enzymatic activity of CYPs and UGT in fetal liver tissues are outlined in Table 6. Activity of CYP1A (RLU/ [min*mg protein]) was greater in fetal livers collected from dams that were supplemented with melatonin. However, activity of CYP1A (RLU/ (min/gram) was not different in fetal liver. Activity of CYP2C was not detected in the fetal liver. Activity of CYP3A (RLU/ [min*mg protein] and RLU/ [min/gram]) in fetal liver was not different (P = 0.20) between fetuses that were collected from control or melatonin treated dams. Activity of UGT (RLU/ [min*mg protein] and RLU/ [min/gram]) was greater in fetal livers from dams that were supplemented with melatonin versus control. Enzymatic activity of CYPs and UGT in placentome tissues are outlined in Table 7. Activity of CYP1A, CYP2C, and UGT (RLU/ [min*mg protein] and RLU/ [min/gram]) in caruncular tissue was not different (P > 0.05) between control or melatonin treated dams. Activity of CYP1A and UGT (RLU/ [min*mg protein] and RLU/ [min/gram]) in cotyledonary tissue was not different (P > 0.05) between control or melatonin treated dams. Caruncular tissue did not express activity of CYP3A, while cotyledonary tissue did not express activity of CYP1A and UGT.

Discussion

Application of melatonin in the current study did not increase dam or fetal weights, and previous research in our lab demonstrated similar birth weights in cattle orally supplemented with melatonin. However, the calves born to melatonin

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supplemented dams increased in BW and heart girth by 9 wk of age compared to calves born to control dams (Brockus et al., 2016a). Supplementation of melatonin to ewes during the middle third of gestation failed to change fetal carcass weights (Lemley et al., 2013b), similar to the current study. This lack of increase in fetal weight and morphometric measurements indicates that supplementation of melatonin to dams, especially heifers, will not increase the likelihood of dystocia due to increased fetal size.

Activity of CYP1A (RLU/ mg protein) was increased in fetal liver tissues collected from MEL treated dams in the current study. Supplementation of melatonin to pregnant mice and rats assisted in mitigating the effects of inflammatory challenges within the fetal liver based on the concentrations of TNFα, IL-6, and IL-1β, which are inflammatory mediators (Perez et al., 2007; Xu et al., 2007). This indicates that melatonin supplementation to the dams positively affects the abilities of the fetus to overcome adverse biological processes. Activity of CYP2C was not detected within fetal liver in the current experiment, which is in agreement with previous research (Lekatz et al., 2015). This indicates that there must be a signal to the liver to activate this enzyme to begin metabolizing hormones and xenobiotics. Whereas activity of CYP3A was not different between treatments. Activity of UGT in fetal liver tissues was 2.25-fold greater ($P < 0.05$) in fetuses collected from melatonin treated dams as compared to control. This increase could indicate that the fetus's liver is already beginning to inactivate not only steroids, but also eicosanoids. In addition to melatonin supplementation increasing fetal liver UGT activity, increased metabolizable protein administered to ewes during gestation increases UGT activity within fetal liver tissues (Lekatz et al., 2015).

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Activity of CYP1A, CYP2C, and UGT in caruncular tissues was not different between treatments, but the CYP2C activity within the caruncle was negatively correlated ($r = -0.60$, $P = 0.05$) with fetal weight. Activity of CYP3A was not detected within caruncle or cotyledon tissues in the current experiment while activity of CYP2C was not detected within cotyledon tissues in the current experiment. Activity of CYP1A and UGT in cotyledon tissues was not different between treatments. These findings do not support our hypothesis and are not correlated to the maternal serum P_4.

Supplementation of melatonin to pregnant animals could provide for a viable method to increase post-natal weight gain and help prevent oxidative stress. Melatonin supplementation did not alter organ morphometric measurements at d 240 of gestation which indicates that this supplement may not increase the incidence of dystocia due to increased calf birth weights. Increased CYP1A and UGT enzyme activity within fetal liver implies that the fetus is preparing itself for greater metabolism of steroids, eicosanoids, and xenobiotics. However, melatonin's ability to increase blood flow and alterations in-utero have been linked to future health complications and future research is needed to extrapolate these consequences. Melatonin supplementation did not affect the activity of CYP or UGT as hypothesized and this could indicate that melatonin does not alter the typical metabolism performed by the caruncle and cotyledon during gestation.

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Table 5 Measurements of heifer body weight, fetal body weight, and fetal organ weight following maternal melatonin (MEL) supplementation on d 180, 210, and 240 of gestation.

Dependent variable	CON	MEL	SE	*P*-Value
Heifer				
BW, kg[1]	511.8	525.3	8.0	0.27
Fetal				
BW, kg[2]	24.0	22.9	1.2	0.51
Carcass wt, kg[3]	18.9	18.0	1.0	0.53
Liver wt, kg	0.50	0.48	0.03	0.70
g/kg of ECW	0.026	0.027	0.001	0.79

Least square means and pooled standard error is reported. Probability values (*P*-values) are indicated.

Table 6 Activity of cytochrome P450 1A (CYP1A), cytochrome P450 3A (CYP3A), and uridine 5'diphosphate-glucuronosyltransferase (UGT) in fetal liver tissue of heifers treated with melatonin (MEL) or control (CON) at 180, 210, and 240 d of gestation.

Dependent variable	CON	MEL	*P*-Value
Fetal liver			
CYP1A			
RLU/(min*mg protein)	109.6 ± 54.9^a	207.4 ± 50.3^b	0.03
RLU/(min/gram) x 10^2	39.1 ± 21.5	57.3 ± 13.5	0.22
CYP3A			
RLU/(min*mg protein) x10^2	99.7 ± 54.4	288.4 ± 107.0	0.20
RLU/(min/gram) x 10^4	34.7 ± 21.4	87.7 ± 35.9	0.20
UGT			
RLU/(min*mg protein) x 10^4	68.2 ± 8.2^a	153.3 ± 21.8^b	0.0002
RLU/(min/gram) x 10^6	22.4 ± 4.0^a	42.4 ± 5.9^b	0.03

Least square means with different letters within row represent significant differences ($P \leq 0.05$). Activity is reported in relative light units (RLU) per mg of protein and per gram of tissue. Dam treatment is separated with standard errors for each, and probability values (*P*-values) are indicated.

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Table 7 Activity of cytochrome P450 1A (CYP1A), cytochrome P450 2C (CYP2C), and uridine 5'diphosphate-glucuronosyltransferase (UGT) in caruncle and cotyledon tissues collected from cattle that were treated with either sub-cutaneous melatonin implants (MEL) or control group (CON) at 180, 210, and 240 d of gestation.

Dependent variable	CON	MEL	*P*-Value
Caruncle			
CYP1A			
RLU/(min*mg protein) x 10^2	30.3 ± 9.6	42.1 ± 20.4	0.88
RLU/(min/gram) x 10^4	10.1 ± 3.0	16.1 ± 8.1	0.88
CYP2C			
RLU/(min*mg protein)	89.2 ± 15.3	74.7 ± 12.4	0.73
RLU/(min/gram) x 10^2	29.3 ± 3.9	27.8 ± 4.5	0.87
UGT			
RLU/(min*mg protein) x 10^4	20.1 ± 6.2	12.7 ± 1.9	1.00
RLU/(min/gram) x 10^5	47.8 ± 8.4	42.1 ± 9.1	1.00
Cotyledon			
CYP1A			
RLU/(min*mg protein)	208.3 ± 30.8	562.5 ± 311.5	0.39
RLU/(min/gram) x 10^3	7.2 ± 1.2	22.0 ± 13.2	0.49
UGT			
RLU/(min*mg protein) x 10^3	72.1 ± 22.8	60.8 ± 28.7	0.49
RLU/(min/gram) x 10^5	24.4 ± 8.7	21.3 ± 9.9	0.61

Least square means are reported. Activity is reported in relative light units (RLU) per mg of protein and per gram of tissue. Dam treatment is separated with standard errors for each, and probability values (*P*-values) are indicated.

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CHAPTER V

CONCLUSION

Pregnancy status will differentially result in activity of CYP and UGT enzymes. Endometrial biopsies collected from cattle throughout the estrous cycle only expressed activity of UGT (Owen et al., Unpublished), as depicted in Figure 10. Activity of UGT was localized in both intercaruncle and caruncle tissues of non-pregnant and pregnant cattle that either failed to express or expressed estrus at d 16, with no differences observed ($P \geq 0.05$; Owen et al., Unpublished). In addition, activity of CYP1A and UGT was localized in CL tissues of non-pregnant and pregnant cattle that either failed to express or expressed estrus at d 16, with no differences observed ($P \geq 0.05$; Owen et al., Unpublished) as depicted in Figure 10. In mid-gestation activity of CYP1A was localized in cotyledonary tissue, caruncle tissue yielded activity of both CYP1A and CYP2C, activity of CYP1A, CYP2C and UGT was found within CL tissue, activity of CYP1A and UGT was found within fetal liver, and CYP1A, CYP2C, CYP3A, and UGT activity was observed within maternal liver (Owen et al., Unpublished c). Finally, during late gestation, activity of CYP1A, CYP3A, and UGT was observed within fetal liver, activity of CYP1A and UGT was localized in cotyledonary tissue, and the caruncle tissue yielded activity of CYP1A, CYP2C, and UGT. Based on these previous data, we can observe a progression of enzyme activity in the reproductive tissues through gestation. Also, from d 85 to 240 of gestation activity of CYP1A and UGT increased in fetal liver and also gained the activity of CYP3A from mid- to late gestation (Owen et al., Unpublished c).

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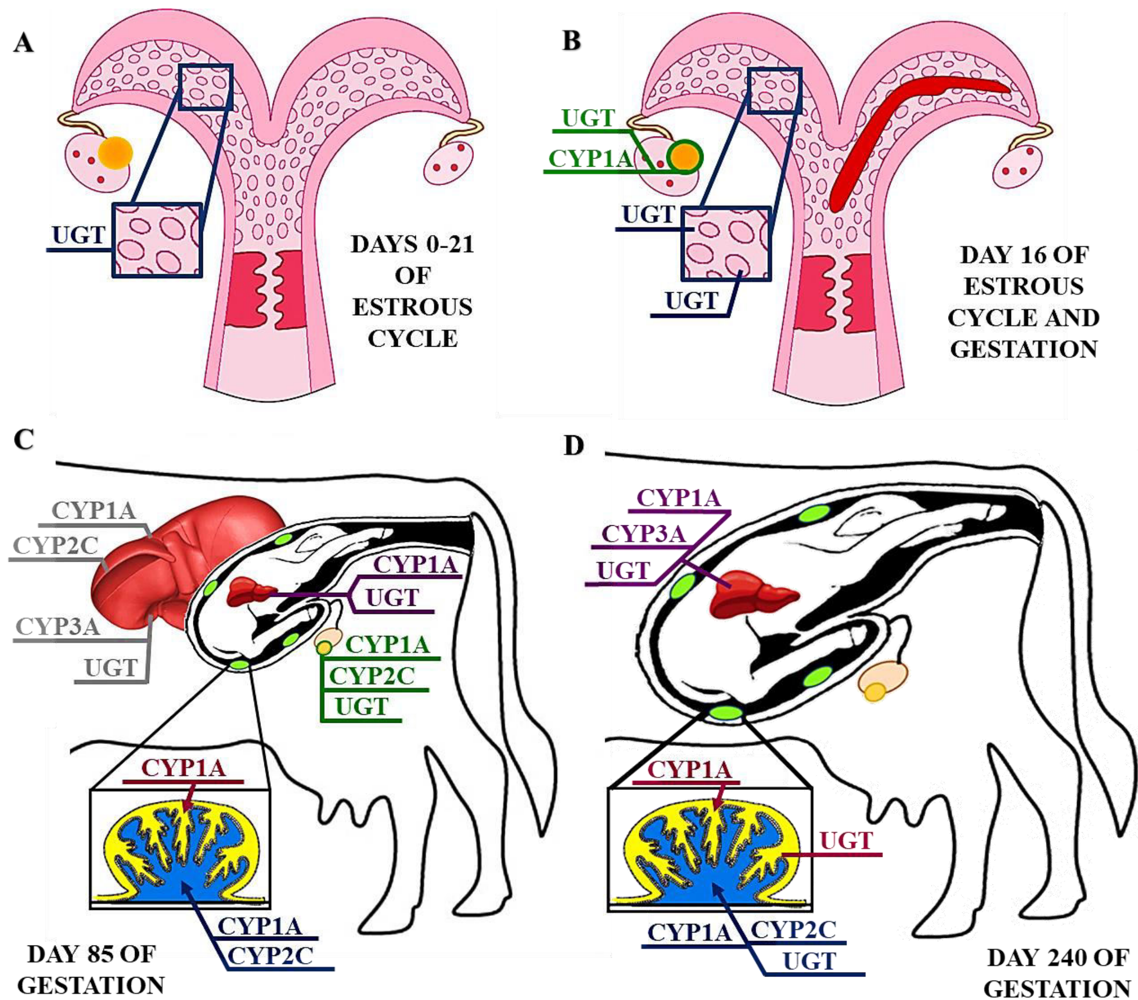

Figure 10 Activity of cytochrome P450 1A (CYP1A), cytochrome P450 2C (CYP2C), cytochrome P450 3A (CYP3A), and uridine 5'diphosphate-glucuronosyltransferase (UGT) in tissues collected from beef cattle during the estrous cycle or gestation.

(A) Localization of UGT within endometrial tissues throughout the estrous cycle of cattle. (B) Localization of enzymes within intercaruncle, caruncle, and corpora lutea tissues at day 16 of non-pregnant and pregnant cattle. (C) Localization of enzymes within maternal liver, fetal liver, corpora lutea, cotyledon, and caruncle tissues of day 85 of gestation of cattle. (D) Localization of enzymes within fetal liver, cotyledon, and caruncle tissues of day 240 of gestation of cattle. Activity of enzymes within the endometrial tissues, including intercaruncle and caruncle tissues, are indicated in dark blue. Activity of enzymes within the corpora lutea is indicated in green. Activity of enzymes within the fetal liver is indicated in purple. Activity of enzymes within the cotyledon tissue is indicated in maroon. Activity of enzymes within the maternal liver is indicated in gray.

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APPENDIX A

EFFECTS OF BODY CONDITION SCORE (BCS) ON STEROID AND EICOSANOID METABOLIZING ENZYME ACTIVITY IN VARIOUS MARE TISSUES DURING WINTER ANESTROUS

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Abstract

The objective of the current study was to determine the activity of steroid and eicosanoid metabolizing enzymes in horses with varying BCS. The BCS of twenty non-pregnant, anestrous mares were determined prior to euthanasia, and tissue samples were collected from the liver, kidney, adrenal gland, ovary, and endometrium. Cytochrome P450 1A (CYP1A), 2C (CYP2C), 3A (CYP3A), and uridine 5'-diphospho-glucuronosyltransferase (UGT) activities were determined using luminogenic substrates. The MIXED procedure of SAS was used to test the effect of BCS on enzyme activity and differences between tissues. Activity of CYP1A in adrenals was increased ($p \leq .05$) in BCS 5 versus BCS 4 and 6. Activity of CYP1A in liver was increased ($p = .05$) in BCS 4 versus BCS 5 and 6. Activity of CYP1A was 100-fold greater ($p < .0001$) in the liver than in the adrenal, ovary, and kidney. Activity of CYP2C was 100-fold greater ($p < .0001$) in the liver than in the adrenal, ovary, and endometrium. Activity of CYP3A was only detectable in the liver. Activity of UGT in the kidney was decreased ($p = .02$) in BCS 4 versus BCS 5 and 6. Activity of UGT was 3-fold greater ($p < .0001$) in the liver than in the kidney; whereas activity of UGT was 9-fold greater ($p < .0001$) in the kidney than in the ovary and endometrium. In general, BCS did not alter the activity of steroid and eicosanoid metabolizing enzymes in horses. However, tissue differences of these enzymes indicated abundant hepatic metabolism in horses, which is similar to other livestock species.

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Introduction

Reproductive capabilities are dictated by the ability to maintain an adequate body condition, as measured by body condition scores (BCS), indicating nutritional maintenance has been met. However, excessive body condition, or amount of adipose cover, can also cause reproductive failures in various livestock species. These reproductive competencies can be heavily influenced by the production and metabolism of hormones within the body that are vital throughout the estrous cycle and gestation. Moderately conditioned, non-pregnant, cycling mares (approximate BCS of 5) yielded greater serum concentrations of luteinizing hormone compared to fat conditioned (approximate BCS of 7) mares (Cavinder, Vogelsang, Gibbs, Forrest, & Schmitz, 2007). Serum progesterone and estradiol-17β concentrations in gestating ewes were greatest in restricted fed ewes, whereas they were median in adequately fed ewes and lowest in excessively fed ewes (Lemley et al., 2014). Both production and metabolism of steroid hormones can be altered by nutritional status, which can alter BCS.

Steroid hormone metabolism occurs in two phases and have primarily been identified in the liver of several species including humans (Kuehl, Lampe, Potter, & Bigler, 2005), mice (Collier et al., 2012), cattle (Lemley, Wilmoth, Tager, Krause, & Wilson, 2010), and sheep (Lemley et al., 2013). Post-menopausal women have expressed uridine 5'-diphospho-glucuronosyltransferase (UGT) mRNA in endometrial tissues, which indicates these metabolizing enzymes are still active through a reproductively quiescent time (Duguay et al., 2004). Horses that were nutrient-restricted, which resulted in low BCS (3 to 3.5) during the winter anestrous period, failed to ovulate or form any type of luteal tissues; which was different as compared to mares that were

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maintained to a high BCS (7.5 to 8.5) that began recruiting follicles to ovulate normally (Gentry et al., 2002). Pregnant cattle undergoing nutrient restriction had decreased steroidogenic enzyme activity, cytochrome P450 (CYP) 1A and CYP2C, as well as an increased concentration of estradiol (Hart, Camacho, Swanson, Vonnahme, & Lemley, 2014). No study had identified tissue distribution and relative activity of these enzymes throughout the horse's body. In addition, minimal information exists on the relationship between steroidogenic enzyme activity and BCS in mares. Based on previous data in other livestock species we hypothesized a concomitant increase in BCS and activity of steroidogenic enzymes in mares. Therefore, the objective of the current study was to determine the activity of steroid and eicosanoid metabolizing enzymes in horses with varying BCS. In addition, we examined tissue distribution of steroid and eicosanoid metabolizing enzymes in mares during winter anestrous.

Materials and Methods

Animal care and treatments

Animal care and use was conducted according to protocols approved by the Mississippi State University Institutional Animal Care and Use Committee (study #15093). Twenty non-pregnant mares were selected during the anestrous period of January and ranged in age from 7 to 23 years. Due to these horses under the influence of the winter anestrus period, peripheral hormone analysis was not conducted. Horses were selected based on 3 primary criteria (geriatric status, lameness issues, and/or unsafe due to behavioral attributes) and were allowed *ad libitum* access to water and native grass pasture. Mares were separated into two groups: 1) 10 mares were maintained solely on native grass pasture, and 2) 10 additional mares were supplemented with a commercial

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concentrate (Nutrena SafeChoice, Cargill, Inc., Minneapolis, MN) to achieve the desired BCS for this study 4 (n = 6), 5 (n = 9), and 6 (n = 5), respectively. Approximately 20 hr prior to administering euthanasia, horses were weighed and evaluated for a numerical BCS (1-9), by two separate appraisers, based on the non-modified Henneke Body Condition Scoring method (Henneke, Potter, & Kreider, 1984), with 1 being extremely emaciated and 9 being extremely fat and obese. Horses were housed in a dry lot with *ad libitum* access to water for approximately 10 hr prior to euthanasia.

Euthanasia, tissue collection, and processing

Horses were initially sedated with xylazine, at a dose of 1.1 mg/kg intravenously. Once horses were under adequate sedation, ketamine was administered at a dose of 2.2 mg/kg intravenously to induce anesthesia. Potassium chloride solution was administered intravenously, with an average volume of 750 mL, to cease cardiac function. Horses were then exsanguinated by severing the carotid and jugular veins. After approximately 20 min of exsanguination, horses were transported to the Mississippi State University School of Veterinary Medicine Necropsy Laboratory where the exsanguinated weight was recorded.

Immediately after transportation, mares were eviscerated and organs were removed from the general viscera, fat was detached, and organs were weighed. If the organ was part of a pair, the pair of organs was weighed together (e.g. both kidneys). Approximately 2 g of tissue was snap-frozen in liquid nitrogen and stored at -80°C. A liver sample from the cranial portion of the right lobe on the diaphragmatic side was collected which included the hepatic capsule. An adrenal gland sample was collected from a single adrenal gland that was towards the central most portion and included the adrenal capsule, adrenal medulla, and adrenal cortex. Kidney samples were collected

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from the lateral border of the right kidney, approximately 2 cm from the apex. Endometrial samples were collected by opening the lumen of the uterus and collecting the endometrial tissue directly adjacent (approximately 0.3 mm deep) to the lumen and only in the left horn of the uterus. Ovaries were weighed and a portion of the left ovary was sectioned, which had some antral follicle development. Approximately 100 mg of tissue was placed into a polypropylene tube with 1 mL of potassium phosphate buffer (400 mM, pH = 7.4). Samples were then mechanically homogenized using the Tissue Tearor (Biospec Products, Bartlesville, OK, USA). Tissue homogenates were centrifuged at 10,000 × g for 10 min at 4°C, and the supernatant was stored at −80°C until enzyme assays were conducted. Protein concentration of the supernatant was determined by a Coomassie Plus (Bradford) protein assay following the manufacturer's protocol (Thermo Scientific, Rockford, IL, USA).

Cytochrome P450 enzymes

Assay kits for CYP1A (P450-Glo CYP1A1 Assay) CYP3A (P450-Glo CYP3A4 Assay (Luciferin-IPA)), and CYP2C (P450-Glo CYP2C9 Assay) and NADPH regeneration solution (NADPH Regeneration System) were purchased from Promega Corporation (Madison, WI, USA) and assays conducted according to Hart et al. (2014) with minor modifications. Briefly, reconstitution buffer was added to luciferin detection reagent. Luciferin CEE (CYP1A), luciferin IPA (CYP3A), and luciferin H (CYP2C) were diluted in KPO_4 buffer. Tissue homogenates (30 µg of protein per well) and enzyme-specific luciferin substrate were added to 96-well opaque white plates in duplicate. Plates were then pre-incubated for 10 min (CYP1A), 30 min (CYP3A), or 90 min (CYP2C) at 37°C. Following the incubation, NADPH regeneration solution was added to each well

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and plates were incubated for 30 min (CYP1A and CYP2C) at 37°C or 10 min (CYP3A) at room temperature. After the incubation, luciferin detection reagent was added to each well and plates were protected from light and incubated for an additional 20 min at room temperature. Plates were then placed into a Promega Multi-Plus plate reader (Promega Corporation, Madison, WI, USA) and luminescence was measured. A linear relationship was determined between the rate of luminescence in relative light units (RLU) versus the concentration of protein from the various tissues.

Uridine 5'-diphospho-glucuronosyltransferase (UGT)

The UGT assay kit was purchased from Promega Corporation (Madison, WI, USA) and the assay performed according to Hart et al. (2014) with minor modifications. Briefly, uridine diphosphoglucoronic acid (UDPGA) was added to half of the wells of a 96-well opaque white plate to act as reaction wells, and nuclease-free water was added to the remaining wells to act as control wells. The UGT reaction mixture containing UGT multienzyme substrate was then combined with tissue homogenates (28 μg of protein per well), and the plates were preincubated for 90 min at 37°C. After incubation, detection reagent was added to each of the wells followed by an incubation period of 20 min at room temperature while being protected from light. The plates were then analyzed using a Promega Multi- Plus plate reader with luminescence detection mode. Activity of UGT was calculated by following the manufacturer's recommended formulas.

Statistical analysis

The MIXED procedure of SAS (SAS Inst. Inc, Cary, NC, USA) was used to test the effect of BCS within a given tissue as well as enzyme activity of these between

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tissues. Live body weight, age, and group were also in the model as covariates and only removed if they did not yield a *p*-value of $\leq .20$. Data found to be nonnormally distributed were tested using the Wilcoxon rank sum test. Treatment means were separated using the PDIFF options of the LSMEANS statement. Least square means and standard error of the mean are reported. Statistical significance was declared at $p \leq .05$.

Results

Effect of body and tissue weights and tissue activity

Final live body weight, exsanguinated, eviscerated, and organ weights are displayed in Table 1. No differences were observed across BCS in live body, exsanguinated, eviscerated, liver, adrenal, kidney, reproductive tract, and ovary weights. Activity of enzymes (RLU/mg of protein) among tissues are displayed in Figure 1. Activity of CYP1A, CYP2C, and UGT were greatest in hepatic tissues ($p < .0001$) compared to extra-hepatic tissues. Activity of CYP1A was 100-fold greater ($p < .0001$) in the liver than in the adrenal, ovary, and kidney; whereas activity of CYP1A was undetectable in the endometrium (Figure 1A). Activity of CYP2C was 100-fold greater ($p < .0001$) in the liver than in the adrenal, ovary, and endometrium; whereas activity of CYP2C was undetectable in the kidney (Figure 1B). Activity of CYP3A was only detectable in the liver (Figure 1C). Activity of UGT was 3-fold greater ($p < .0001$) in the liver than in the kidney; whereas activity of UGT was 9-fold greater ($p < .0001$) in the kidney than in the ovary and endometrium (Figure 1D). Similar results were observed when enzyme activity was expressed relative to gram of tissue or total tissue weight (data not shown).

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Effect of body condition score

Activity of CYP1A, per mg of protein, were not different (p = .06) among BCS within liver tissue (Table 2). However, activity of CYP1A (RLU/g of tissue) was different (p = .05) among BCS within liver tissue, where horses with BCS 4 was increased (p = .02) compared to horses with BCS 5 (Table 2). Total liver activity of CYP1A was not different (p = .20) among BCS (Table 2). Activity of CYP2C, CYP3A, and UGT were not different ($p \geq .41$) among BCS within liver tissue (Table 2).

Activity of CYP1A, relative to mg of protein, per gram of tissue, and total activity of the adrenal gland, were different ($p \leq .02$) among BCS, where horses with BCS 5 were increased compared to horses with BCS 4 and 6, respectively (Table 3). Activity of CYP2C was not different ($p \geq .71$) among BCS within adrenal tissue (Table 3). Activity of CYP1A was not different ($p \geq .32$) among BCS within kidney tissue (Table 3). Activity of UGT, relative to mg of protein and per gram of tissue, was different ($p \leq .02$) among horses with BCS within kidney tissue, where horses with BCS 4 were decreased (p = .005) compared to horses with BCS 5, but horses with BCS 4 were not different ($p \geq$ .10) compared to horses with BCS 6 (Table 3). However, total activity of UGT in the kidney was not different (p = .36) among BCS (Table 3). Mare BCS did not alter activity of CYP1A, CYP2C, or UGT within ovarian tissue (Table 4). Similarly, mare BCS did not alter activity of CYP2C or UGT within endometrial tissue (Table 4).

Discussion

Enzyme activity was greatest in hepatic tissues ($p < .0001$), which reflects the results of several other studies conducted with monkeys (Uno, Fujino, Kito, Kamataki, & Nagata, 2006), mice (DeLozier et al., 2004), and sheep (Dupuy, Larrieu, Braun,

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Alvinerie, & Galtier, 2001). This is due to the liver acting as the primary organ of metabolism within the body. In addition, there is a differential effect of species on the extent of the enzyme activity between organs, which is evident through the exponentially greater CYP1A activity in cattle as compared to pigs, goats, and sheep (Hart et al., 2014). Hepatic CYP1A activity (per gram of tissue) from the mare's in this experiment was increased 20-fold as compared to cattle, and these horses have 150-fold greater hepatic CYP1A activity as compared to ewes (Kennedy et al., 2016; Lekatz et al., 2015). Horse hepatic CYP2C activity (per mg of protein) from this study was increased 6100-fold as compared to cattle and was increased 3.5-fold as compared to sheep (Kennedy et al., 2016; Lekatz et al., 2015). Horse hepatic CYP3A activity (per mg of protein) from this study have 2100-fold greater activity than that of cattle, but have 1.5-fold lesser activity than that of sheep (Kennedy et al., 2016; Lekatz et al., 2015). Horse hepatic UGT activity (per mg of protein) from this study was increased 4.3-fold and 3.6-fold as compared to cattle and sheep (Kennedy et al., 2016; Lekatz et al., 2015), respectively.

The enzyme activity within the liver of cattle is supported by greatest mRNA expression of specific isoforms of the three enzymes, CYP1A2, CYP2C9, and CYP3A4 in hepatic tissues (Darwish, Ikenaka, El-Ghareeb, & Ishizuka, 2010). However, CYP1A1, an isoform of CYP1A, yielded greatest mRNA expression within the kidney of cattle (Darwish et al., 2010), which could explain the greatest extra-hepatic activity of CYP1A in the kidney tissue from the mares in the current study. Expression of mRNA of these steroid metabolizing enzymes is highly species-dependent, which is evident through the differential expression of isoforms of CYP1A and UGT in hepatic tissues between sheep and cattle (Girolami et al., 2016). In cattle, Darwish et al. (2010) showed the greatest

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expression of UGT1A1 mRNA within the kidney followed by hepatic tissues. In contrast, the current study found that UGT enzyme activity in horses were greatest in hepatic tissue followed by the kidney. However, of the 5 horse tissues examined, only hepatic tissue expressed CYP3A activity. Cultured bovine endometrial cells had an 1800-fold increase in CYP2C activity as compared to the endometrial samples from the horses in the current study (Gilfeather & Lemley, 2016). Conversely, endometrial activity of CYP2C from bovine endometrial biopsies had no detectable activity of CYP2C, which is similar to the current results in the horse (Owen and Lemley, unpublished data). The horse endometrium exhibited activity of UGT, whereas the cultured bovine endometrial cells did not express UGT activity (Gilfeather & Lemley, 2016). Ovarian tissue from the current study did not express differences in CYP1A, CYP2C, or UGT between BCS, which is probably due to the reproductive status of the mares, winter anestrous, at the time of harvest.

The primary steroid that CYP1A metabolizes is estrogen, which should not have been in large quantities due to the quiescence of the ovaries under winter anestrous. In addition to steroids, cytochrome P450 enzymes metabolize several eicosanoids. This eicosanoid metabolism could explain the BCS differences seen in the adrenal gland across all indices of CYP1A. However, this does not explain the lack of differences expressed in CYP2C indices. More research is needed to elucidate the mechanisms of CYP1A within the adrenal gland. While the horses utilized in the current study were all stock type horses, there were various breeds including quarter horse, paint, and thoroughbred. Ewes that were given 60%, 80%, and 100% of their metabolizable protein between days 90 and 130 of gestation did not have different liver weights ($p = .66$),

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which was reflected by no differences in final body weight (Lekatz et al., 2015). This differential expression and activity of these steroid and eicosanoid enzymes between species and breeds could explain the results of the enzyme activity of these horse tissues.

Restricted feeding regimes during gestation in cattle significantly decreased the activity of CYP1A and CYP2C in the liver (Hart et al., 2014), which does not match up with the current data set, as horses with BCS 4 yielded the greatest activity of CYP1A when compared to horses with BCS 5 (ideal), while horses with BCS 6 were intermediate. However, these tissues were collected during the winter anestrous period, which was verified by a lack of luteal structures on the ovaries; therefore, these horses were not under the influence of progestins. Moreover, during this winter anestrous period, mares grow small cohorts of follicles which will not produce large amounts of estrogens, which are metabolized by and upregulate CYP1A. The adrenal gland produces steroids in the body, which could also explain the differences seen between mare BCS with regards to the CYP1A activity within the adrenal gland. Metabolism of steroids and eicosanoids is done to inactivate and increase the polarity for the molecules in order to excrete them from the body. One method of excretion is through the urine which is filtered and excreted to the bladder via the kidney. Differences in the activity of UGT could be due to the varying amount of phase II metabolism between BCS of the mares in this study. Greater feed intake not only increases liver blood flow but also increases steroid metabolism in lactating and non-lactating dairy cattle (Sangsritavong, Combs, Sartori, Armentano, & Wiltbank, 2002; Vonnahme et al., 2013). Horses are a unique livestock species due to their additional dependence on 5α-dihydroprogesterone as a vital progestin, different from other livestock species relying solely on progesterone (Scholtz

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et al., 2014). Ovariectomized mares treated with sub-physiological 5α-dihydroprogesterone resulted in glandular development that mirrored mares under a normal luteal cycle (Scholtz et al., 2014), which indicates that 5α-dihydroprogesterone is required for adequate glandular development to prepare for impending gestation. Reduced BCS by chronic feed restriction significantly decreases peripheral concentrations of progesterone (Gentry et al., 2002). Activity of CYP1A expressed as mg of protein, per gram of tissue, and total activity were lowest ($p < .01$) in ewes fed 60% of their metabolizable protein as compared with ewes fed 80 and 100% (Lekatz et al., 2015).

These data support previous research that horses metabolize hormones in the liver, similar to other species (Dupuy, Larrieu, Braun, Alvinerie, & Galtier, 2001; DeLozier et al., 2004; Uno, Fujino, Kito, Kamataki, & Nagata, 2006). Also, these data can be used to further examine the enzyme profile for use in xenobiotic metabolism, which is also performed by the CYP and UGT enzymes. This could assist in the knowledge about the clearance of anti-inflammatory medications, such as Dexamethasone, from extra-hepatic tissues. This extra-hepatic metaboolism is influenced, in some tissues, by BCS of horses and could provide information for dosages based not only on weight, but BCS as well. Finally, these data could help better understand the metabolic impact that BCS can have on the body of horses that can influences the endocrine profiles.

Conclusion

Activity of steroid and eicosanoid metabolizing enzymes does not provide an implication of BCS effects in horses. A wider range of horse BCS might provide a greater amount of diversity to detect BCS influences on hormone metabolizing enzyme activity.

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This is supported by the lack of differences observed in exsanguinated and eviscerated weights. Tissue differences of these enzymes indicated abundant hepatic metabolism followed by renal metabolic activity in horses. Finally, horses that are not in winter anestrous may yield different results because of the nature of steroid metabolizing enzymes.

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Author contributions

Dr. Caleb Lemley and Megan Owen significantly contributed to the experimental design, collection, laboratory analysis, and statistical analysis of the data, and writing of the manuscript. Emily Ferjak and Dr. Clay Cavinder contributed to the experimental design and collection of data for this project. Keelee McCarty, Kalisha Yankey, and Caitlin Hart assisted with the tissue collection, processing, and analysis. Dr. Derris Burnett and Dr. Thu Dinh contributed to the experimental design and collection of data for this project.

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Conflict of interest

Authors declare that there are no known conflicts of interest accompanying this publication, and there has been no significant financial assistance that could have influenced the products of this research.

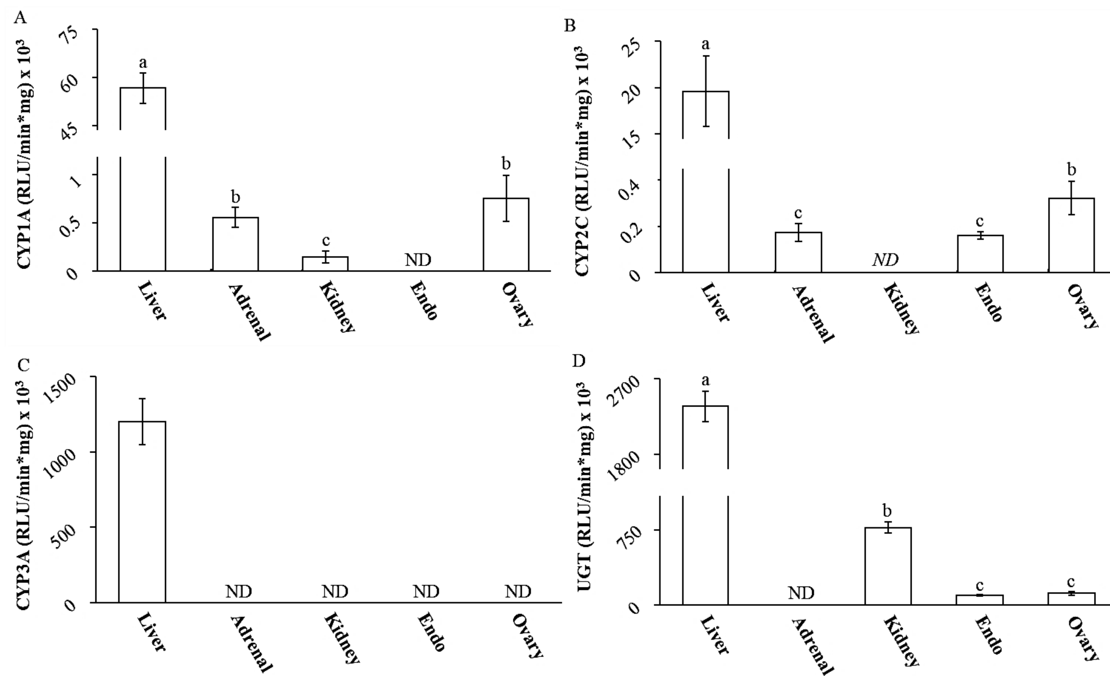

Figure 11 Activity of cytochrome P450 1A (CYP1A; A), 2C (CYP2C; B), 3A (CYP3A; C), and uridine 5'diphospho glucuronosyltransferase (UGT; D) across different horse tissues. ND indicates that no activity was detected.

Least square means with different letters represent significant differences ($p \leq .05$).

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Table 8 Measurements of several body and organ weights in horses across body condition score (BCS) 4, 5, and 6. Data are reported as the mean ± standard error.

Dependent variable	BCS 4	BCS 5	BCS 6	*p*-Value
Live body weight, kg[†]	443 ± 21	455 ± 11	493 ± 12	.10
Exsanguinated body weight, kg[‡]	392 ± 18	406 ± 10	444 ± 12	.53
Eviscerated body weight, kg[§]	287 ± 11	318 ± 21	312 ± 11	.39
Liver weight, kg	4.9 ± 0.9	5.0 ± 0.6	4.3 ± 0.4	.74
Adrenal weight, g	30.5 ± 5.1	35.8 ± 3.3	28.4 ± 2.6	.18
Kidney weight, kg	1.8 ± 0.4	1.6 ± 0.1	1.6 ± 0.2	.88
Reproductive tract weight, kg[¶]	1.5 ± 0.2	1.3 ± 0.1	1.5 ± 0.2	.42
Ovary weight, g	31.9 ± 5.1	39.3 ± 4.9	49.1 ± 12.3	.57

p-values for the main effect of BCS are reported. Least square means with different letters represent significant differences ($p < .05$). [†]Live body weight was obtained 24 hr prior to anesthesia and euthanasia via exsanguination. [‡]Exsanguinated weight = live body weight – blood volume. [§]Eviscerated weight = exsanguinated weight – (internal organ weight + hide weight + front leg weight + mane weight + tail weight). [¶]Reproductive tract weight = mare reproductive tract (ovaries + oviduct + uterus + cervix + vagina) + bladder.

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Table 9 Activity of cytochrome P450 1A (CYP1A), cytochrome P450 2C (CYP2C), cytochrome P450 3A (CYP3A), and uridine 5'diphosphate-glucuronosyltransferase (UGT) in horse liver tissue. Activity is reported in relative light units (RLU) per mg of protein, per gram of tissue, and total tissue activity ± standard error.

	BCS			
Dependent variable	4	5	6	*p*-Value
LIVER				
CYP1A				
RLU/(min*mg protein)x10^3	72.1 ± 8.4	48.4 ± 6.6	53.1 ± 8.0	.06
RLU/(min/gram) x10^5	28.0 ± 2.9[a]	18.4 ± 2.4[b]	20.1 ± 2.8[ab]	.05
RLU/(min*liver) x10^8	131.7 ± 18.7	95.7 ± 20.4	85.7 ± 13.2	.20
CYP2C				
RLU/(min*mg protein)x10^3	15.8 ± 7.7	24.7 ± 6.4	15.0 ± 4.3	.71
RLU/(min/gram) x10^4	75.5 ± 38.3	174.0 ± 58.1	75.8 ± 28.0	.41
RLU/(min*liver) x10^8	7.2 ± 1.9	54.9 ± 27.6	22.0 ± 11.8	.43
CYP3A				
RLU/(min*mg protein)x10^5	13.6 ± 2.2	11.0 ± 1.1	11.5 ± 1.2	.75
RLU/(min/gram) x10^6	53.1 ± 7.9	41.9 ± 4.3	43.9 ± 5.4	.49
RLU/(min*liver) x10^{10}	22.7 ± 3.6	19.0 ± 1.7	18.2 ± 2.5	.61
UGT				
RLU/(min*mg protein)x10^5	21.7 ± 3.2	25.2 ± 2.6	23.5 ± 4.5	.58
RLU/(min/gram) x10^6	85.3 ± 13.8	96.6 ± 10.6	90.9 ± 18.9	.78
RLU/(min*liver) x10^{10}	41.6 ± 8.9	43.5 ± 3.0	39.4 ± 9.3	.85

p-values for the main effect of BCS are reported. Least square means with different letters represent significant differences ($p < .05$).

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Table 10 Activity of cytochrome P450 1A (CYP1A) and cytochrome P450 2C (CYP2C) in adrenal tissue. Activity of CYP1A and uridine 5'diphosphate-glucuronosyltransferase (UGT) in kidney tissue. Activity is reported in relative light units (RLU) per mg of protein, per gram of tissue, and total tissue activity ± standard error.

	BCS			
Dependent variable	4	5	6	*p*-Value
ADRENAL GLAND				
CYP1A				
RLU/(min*mg protein)	279 ± 99.2[a]	897 ± 152.7[b]	300 ± 109.4[a]	.02
RLU/(min/gram) x10^3	10.3 ± 3.6[a]	36.1 ± 7.2[b]	11.1 ± 4.1[a]	.01
RLU/(min*adrenal) x10^4	26.9 ± 10.7[a]	109.5 ± 22.3[b]	28.3 ± 8.6[a]	.01
CYP2C				
RLU/(min*mg protein)	206 ± 72	136 ± 62	164 ± 58	.71
RLU/(min/gram) x10^2	65.1 ± 21.1	45.1 ± 18.5	53.2 ± 18.8	.74
RLU/(min*adrenal) x10^4	18.1 ± 5.9	16.3 ± 7.1	16.2 ± 6.6	.82
KIDNEY				
CYP1A				
RLU/(min*mg protein)	104 ± 35.9	236 ± 141.2	41.9 ± 21.6	.53
RLU/(min/gram) x10^2	38.6 ± 13.9	93.7 ± 58.3	14.6 ± 8.2	.64
RLU/(min*kidney) x10^5	47.1 ± 14.4	107.9 ± 60.1	23.8 ± 15.6	.32
UGT				
RLU/(min*mg protein)x10^4	56.8 ± 6.7[a]	81.9 ± 4.7[b]	91.4 ± 14.7[b]	.02
RLU/(min/gram) x10^6	21.5 ± 2.4[a]	32.2 ± 2.0[b]	34.9 ± 5.8[b]	.01
RLU/(min*kidney) x10^9	36.7 ± 7.0	51.8 ± 5.6	54.8 ± 7.5	.36

p-values for the main effect of BCS are reported. Least square means with different letters represent significant differences ($p < .05$).

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Table 11 Activity of cytochrome P450 1A (CYP1A), cytochrome P450 2C (CYP2C), cytochrome P450 3A (CYP3A), and uridine 5'diphosphate-glucuronosyltransferase (UGT) in mare reproductive tissues. Activity is reported in relative light units (RLU) per mg of protein, per gram of tissue, and total tissue activity ± standard error.

Dependent variable	BCS 4	BCS 5	BCS 6	*p*-Value
OVARY				
CYP1A				
RLU/(min*mg protein)x10^2	14.0 ± 0.8	7.0 ± 3.2	1.9 ± 1.2	.84
RLU/(min/gram) x10^3	41.1 ± 5.6	25.3 ± 10.6	7.0 ± 5.1	.55
RLU/(min*ovary) x10^4	166 ± 79.4	123 ± 67.6	28 ± 19.0	.27
CYP2C				
RLU/(min*mg protein)	385 ± 112	326 ± 137	187 ± 36	.33
RLU/(min/gram) x10^3	10.9 ± 4.2	11.0 ± 4.8	6.0 ± 1.2	.74
RLU/(min*ovary) x10^4	41.6 ± 24.0	36.9 ± 14.3	28.0 ± 7.0	.86
UGT				
RLU/(min*mg protein)x10^4	14.6 ± 4.8	10.1 ± 1.5	9.7 ± 2.5	.94
RLU/(min/gram) x10^5	30.0 ± 4.3	35.2 ± 5.9	33.0 ± 7.9	.97
RLU/(min*ovary) x10^7	10.3 ± 2.8	14.3 ± 3.5	12.2 ± 2.2	.82
ENDOMETRIUM				
CYP2C				
RLU/(min*mg protein)	156 ± 28	169 ± 25	120 ± 34	.73
RLU/(min/gram) x10^2	54.3 ± 8.7	60.3 ± 8.3	36.5 ± 9.0	.36
UGT				
RLU/(min*mg protein)x10^3	82.6 ± 16.8	108.4 ± 16.5	81.4 ± 18.8	.68
RLU/(min/gram) x10^5	30.5 ± 6.0	36.9 ± 3.1	29.3 ± 6.5	.65

p-values for the main effect of BCS are reported.

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