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Melatonin implants during pregnancy on maternal hemodynamics and growth of offspring in beef cattle

By

Keelee J. McCarty

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

Mississippi State, Mississippi

May 2018

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Keelee J. McCarty

2018

Melatonin implants during pregnancy on maternal hemodynamics and growth of offspring in beef cattle

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Melatonin is a strong antioxidant that has previously been observed to increase uteroplacental blood flow and increase postnatal calf growth when supplemented during gestation. The objective of the current study was to examine the effects of melatonin implants on uterine blood flow and subsequent offspring growth. Commercial beef heifers and cows were artificially inseminated and assigned to one of two treatment groups supplemented with (**MEL**) or without (**CON**) melatonin from days 180 to 240 of gestation. Total uterine artery blood flow was increased in MEL- versus CON-treated cattle. Fetal and birth weight were not different between treatments. However, at castration, body weight was increased in calves from MEL-treated dams compared with CON-treated dams. Further research on placental vascularization and the mechanism in which melatonin impacts angiogenic factors is necessary to understand the relationship between melatonin and compensatory growth that occurs in postnatal offspring.

DEDICATION

I would like to dedicate this thesis to my grandfather, Michael McCarty. He was one of my biggest supporters in agriculture and watched me work with livestock every opportunity he could. I know he is looking down on me as I work towards everything he ever hoped I could accomplish with a smile on his face and a smart quip prepared about work ethic. I want to thank my parents, James and Lori McCarty, for their guidance and endless support throughout my college experience. A thank you towards Russell and Bill Sherwood of Sherwood Family Farms for allowing my curiosity of livestock to grow and cultivate from a young age and the mentorship they provided. Thank you to Dr. Brian Rude for challenging me and introducing me to the world of research and animal handling, for without it I do not believe I would be where I am today. Thank you to Dr. Caleb Lemley and Dr. Megan Owen for representing the best of what Mississippi State University has to offer in terms of mentorship, friendship, and serving as role models throughout my academic career. Thank you to the undergraduates who have assisted with every portion of the project and served as close friends during my stay in Mississippi. Finally, to my brother Dustyn McCarty, you have always been my guardian angel, my protector, and inspiration to persevere. Everyone, I hope I have made you proud.

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CHAPTER I

REVIEW OF LITERATURE

1.1 Developmental Programming

Livestock species intended for meat production spend a majority of their lifetime being supported by the dam in utero. Because of this, as growth and development occur, the fetus is susceptible to changes in the internal and external environment in addition to any genetic predispositions. Programming describes the phenomenon in which a stimulus or insult introduced during a critical period of development leads to permanent consequences later in life including structural, physiological, and metabolic changes (Godfrey, 2002). In particular, this encompasses plasticity in response to environmental or nutritional signals during early life that could lead to potential adverse consequences (Gicquel, 2008). An adaptive change occurs when the fetal environment is deprived of nutrients with optimization of the growth of key body organs, however, these adaptions may lead to adverse effects postnatally (Godfrey and Barker, 2000; Gicquel, 2008). For example, disorders associated with suboptimal fetal growth are caused by changes in the development of key endocrine axes, which may impede physiological mechanisms (Gicquel, 2008). Complications in the livestock industry include increased neonatal morbidity and mortality (Uetake, 2013) and decreased birth weights (Kais et al., 2010). Many models revolve around maternal nutrient supply or how nutritional schemes or

supplementation practices impact fetal development and subsequent offspring growth. In models of maternal over nutrition or undernutrition, growth and development in ruminant species are primarily studied as a result of energy, protein, and fat restriction (Satterfield et al., 2013; Yan et al., 2013). The restriction of these factors contributes to the overall nutrient supply available to the fetus.

Programming varies depending on the stage of development in which an insult or stimulus is applied. In early gestation, maternal nutrition affects adipose tissue development by increasing adipocyte precursor cells or nutrient adaption may advance into metabolic syndrome (Symonds et al., 2009). In late gestation, maternal capacity restricts growth of the fetus as well as the number of offspring (Davies et al., 2005). Increased blood pressure in offspring has been induced in late gestation via glucocorticoid administration, diabetes, and placental dysfunction (Amri et al., 1999; Alexander, 2003; Nehiri et al., 2008). Changes in the postnatal environment contribute to developmental programming of the offspring as well. Bartol et al. (2008) examined the maternal effects of milk on female reproductive tract development in pig offspring. In these studies, relaxin, a morphoregulatory factor, is delivered to neonates as a direct result of nursing to act on relaxin receptors in the neonates to affect development of uterine and cervical tissues (Bartol et al., 2008). The lactocrine hypothesis incorporates the extended effect the maternal body has on the neonate through provision of milk during the perinatal and early postnatal period that impacts development (Bagnell et al., 2009). Developmental programming is a broad subject encompassing prenatal and early postnatal effects on subsequent offspring.

1.2 Uteroplacenta

The placenta is the transient organ that primarily serves to support the developing fetus and provide for exchange between fetal and maternal circulations (Reynolds & Redmer, 1995). The uteroplacenta is pertaining to the placenta and uterus whereas uteroplacental circulation is the circulation of blood of both the dam and fetus via placental membranes. Placental membranes are composed of maternal (derived from endometrial tissue) and fetal (derived from chorionic tissue) portions that interchange or exchange materials such as nutrients, gas, waste, and serve as a site of hormone production. Ruminants have an epitheliochorial type placenta, in which the maternal and fetal epithelium remains intact and separate the maternal and fetal capillary beds (Luckett, 1975).

1.2.1 Uterine Blood Flow

Immediately following fertilization, uteroplacental circulation is one of the first systems to come about in terms of placental and embryonic development (Ramsey, 1985). Efficiency of placental transport is directly related to uteroplacental blood flow due to transport of nutrients, gases, and waste (Reynolds & Redmer, 1995). Uteroplacental blood flow plays a role in successful growth and development of the fetus (Reynolds et al., 2010; Vonnahme et al., 2012; Lemley et al., 2014). Placental function may be insulted by oxidative stress during pregnancy which may induce disorders or disrupt fetal development. Disorders such as intrauterine growth restriction and decreased birth weights stem from placental dysfunction (Robinson, 2017; Sultana et al., 2017). In various models of compromised pregnancy in sheep, such as overfed or underfed

adolescents, heat stressed adults, or multiple pregnancies, there was a decrease in uteroplacental blood flow by at approximately 20% which attributed to impairment of placental and fetal growth(Reynolds et al., 2006).

Transplacental exchange, or exchange through the placenta, relies on increased placental growth during early gestation followed by increased uteroplacental vasculature during late gestation (Reynolds et al., 1995; Meschia, 1983). In ruminants, the predominant sites of exchange are placentomes made up of caruncle (maternal) and cotyledon (fetal) tissues. The caruncle is the glandular site found on the uterus for maternal circulation with a coinciding portion on the placenta for fetal circulation, referred to as the cotyledon. In sheep, the cotyledon undergoes exponential growth during the first portion of pregnancy then tapers off (Naaktgeboren et al., 1974). While in cattle, the cotyledon exhibits progressive growth throughout gestation (Reynolds et al., 1995; Vonnahme et al., 2001).

Therapeutic supplements that impact placental blood flow, such as melatonin and arginine, have been studied in compromised pregnancies and intrauterine growth restriction models (Richter et al., 2009; Scatterfield et al., 2010). Previously, Brockus et al. (2016a) demonstrated that dietary melatonin supplementation to Holstein heifers during mid- to late-gestation increased uterine artery blood flow. In an ovine model of intrauterine growth restriction, melatonin supplementation increased umbilical artery blood flow compared to non-supplemented control ewes (Lemley et al., 2012).

Uterine hemodynamics is influenced by various factors such as maternal nutrition and vascularization. Studies involving maternal nutrition such as nutrient restriction,

overfeeding (Wallace et al., 2004) and protein supplementation (Larson et al., 2009) are currently being evaluated. Undernutrition in early gestation has been associated with placental development and vascularity of the uteroplacenta which is important for growth and development of sheep (Vonnahme et al., 2007). Additionally, overfeeding may lead to negative consequences on organ system development or physiological function such as endocrine systems in the developing fetus. In sheep, the overfeeding of pregnant adolescent sheep leads to placental growth restriction and reduced birth weight (Wallace et al., 1999), and decreased uterine blood flow in early gestation (Wallace et al, 2004). In sheep, early insults alter placental weight; placental growth is greater than fetal growth until approximately 80 days of gestation (Gardner et al., 2007). Later in gestation, insults have no effect on placental weight due to limited placental growth and exponential growth of the fetus (Gardner et al., 2007). In cattle, placental growth and fetal growth are similar until the last third of gestation where fetal growth is greater than placental growth (Ferrell et al., 1976).

1.2.1.1 Ultrasonography

Doppler ultrasonography has been used as a tool to measure blood flow in uterine vessels during pregnancy since the 1980s following Campbell and colleagues (1983) experiments with humans. This ultrasonography uses the principles of the Doppler Effect to measure blood flow through a particular vessel. The technique was intended to replace other uteroplacental perfusion methodologies that were far more invasive and incorporated radioactive material, making them impractical. Previous methods incorporated infusion of gamma-labeled microspheres that would perfuse the vascular

beds. This method, as seen by Ford et al. (1982), was valuable to determine exact location of perfusion in the uterine horns, however, the animals had to be sacrificed to collect data. Doppler ultrasonography uses waveform analysis in order to evaluate arteries in the uterine wall non-invasively, and the animal may be utilized for repeated measurements.

The Doppler Effect is defined as the change in frequency of a wave for an observer moving relative to its source. The ultrasound uses the Doppler-shift frequencies to measure blood flow. Doppler-shift frequencies are the difference of the ultrasound wave and the received echo picked up by the transducer (Pierson & Ginther, 1987). Direction of blood flow towards the probe results in a positive integer whereas blood flow away from the probe results in a negative integer. Ultrasonography originates from crystals, with piezoelectric properties, activated by an electric charge that expand or contract to emit sound waves (Dudwiesus et al., 1993). These waves permeate the body and are reflected back based off of the density of the tissue of interest. When these waves strike moving surfaces (e.g. red blood cells) compared to waves emitted from dense tissue, the frequency emitted forms a Doppler shift.

The angle of isonation is a result of the orientation of the probe when Doppler ultrasonography is being performed. This is the angle at which the ultrasound probe interacts with the vessel and influences the accuracy of the blood flow measurements. If the angle of isonation nears 90°, the blood is traveling perpendicular to the probe and cannot be properly analyzed. Because of this, no Doppler-shift would be detected or

blood flow calculated. To prevent this, an optimal angle would be 45° or less since the smaller angles have greater accuracy.

Color Doppler ultrasonography is a preferred method of evaluation for uterine blood flow in livestock research. Initially, applications of this technique evaluated cyclicity and uterine perfusion in cattle (Bollwein et al., 2002). Color Doppler incorporates the use of color velocity imaging to estimate blood velocity from the Doppler angles. To do this, the shift frequencies are transformed into color pixels representing blood flow. Similar to two-dimensional images, the direction of blood flow towards the probe results in a positive integer (red color) whereas blood flow away from the probe results in a negative integer (blue color). Velocity and hemodynamic measurements are calculated from quantitative color Doppler indices such as resistance index (RI), pulsatility index (PI), mean velocity (MnV), and analysis of the systolic (s) and diastolic (d) waves (Fig. 1.1).

Previous methods that incorporated electromagnetic blood flow probes and authors observed increased uterine artery blood flow in the first trimester of pregnancy (Ford et al., 1979; Farrell & Ford, 1980). A 4.5 fold increase in uterine blood flow and 21 fold for umbilical blood flow between days 137 and 250 of gestation was observed in cows using a steady state diffusion model via injection of radioactive substances (Reynolds & Ferrell, 1987). The diffusion model incorporated transplancental clearance of deuterium oxide (D_2O), where changes in blood flow rates were paralleled by D_2O clearance rates (Reynolds & Ferrell, 1987). Color Doppler ultrasonography is a reliable method in that the results from Bollwein et al. (2002) on three pregnant cows reflected

the findings of Reynolds and Ferrell (1987). Findings from Bollwein et al. (2002) have shown that uterine artery blood flow ranged from approximately 4.0 to 5.5 L/min on the contralateral side and 8.6 to 16.6 L/min on the ipsilateral side at ten months of gestation in cows. Recent studies observed an increase in total uterine artery blood flow in melatonin treated cows (7.2 ± 3.5 L/min) compared with control treated heifers (5.7 ± 3.5 L/min) (Brockus et al., 2016a).

Current Doppler techniques described by Brockus et al. (2016a) include identification of the uterine artery by following the abdominal aorta towards the origin of the external iliac artery (Fig. 1.2). The internal iliac artery is then located by moving the probe caudally where uterine arteries are identified as a major branch off of the internal iliac.

Overall, Doppler ultrasonography is an effective and noninvasive method for analyzing uterine hemodynamics in cattle throughout pregnancy (Campbell et al., 1987; Bollwein et al., 2002; Panarace et al., 2006). Previously, Doppler techniques have been used to predict physiological changes such as preeclampsia, abrupt hypertension and edema, or fetal growth retardation (North et al., 1994). This method allows for assessment of uteroplacental circulation and predicts changes in the developing fetus.

1.2.2 Angiogenic Factors

The impact of exogenous therapeutics on uterine hemodynamics and how they interact with endogenous factors has become a more recent area of study (Yunta et al., 2015; Kennedy et al., 2016). Specifically, how therapeutics could improve placental efficiency via increased gas exchange or nutrient transport or expression of genes related

to vasculature. The presence of endogenous factors, such as angiogenic factors, impact the capacity of uteroplacental blood flow (Reynolds et al., 2006; Reynolds et al., 2007). Placental vascular development has been associated with angiogenic factor expression. Angiogenesis, or development of new blood vessels, require factors to activate the receptors or endothelial cells present on pre-existing blood vessels. Among these, vascular endothelial growth factor (*VEGF*) stimulates the formation of blood vessels via its receptors Fms-like tyrosine kinase (*FLT1*), or *VEGF* receptor 1, and kinase insert domain containing receptor (*KDR*), or *VEGF* receptor 2 (Ribatti et al., 2000). Angiopoietin 1 (*ANGPT1*) is a growth factor specific to vascular endothelium that binds to endothelial cells similar to tyrosine kinase receptors (Ribatti et al., 2000). Whereas *VEGF* works from vasculogenesis, initial formation of blood vessels, and throughout angiogenesis, *ANGPT-1* is restricted to later stages of vascular development (Ribatti et al., 2000).

Vonnahme et al. (2007) observed an increase in mRNA expression of *VEGF* and *FLT1* when beef cows were nutrient restricted from days 30 to 125 of gestation, but no difference in vascularity of the placentome. Similar studies by McLean et al. (2017) observed decreased *VEGF* expression of restricted fed Angus heifers compared with control fed heifers during early gestation. In sheep, numerous models of compromised pregnancies have shown a decrease in vascularity due to underfeeding or overfeeding adolescents, underfeeding adults, or multiple pregnancies (Reynolds et al., 2010). Moreover, an increase in vascularity occurred in sheep when high dietary selenium was introduced or hypoxic stress was induced (Reynolds et al., 2010). In sheep capillary area

density of caruncles have a positive relationship with *VEGF*, but negative relationships with *ANGPT1*, and no differences when compared with cotyledonary density (Borowicz et al., 2007). The relationship of mRNA expression of angiogenic factors and different models of compromised pregnancies have been extensively studied in sheep, however, cattle studies are limited.

1.3 Melatonin

Following its discovery in 1958 by Lerner and colleagues, melatonin has been a topic of research in reproductive physiology (Lerner et al., 1958). Melatonin, or 5-methoxy-N-acetyltryptamine, is a hormone released from the pineal gland commonly associated with circadian rhythm (Smits & Nagtegaal, 1999) and seasonal breeding (Wehr, 1997). Melatonin serves several physiological functions in the body as outlined in Fig. 1.3 (redrawn from Dubocovich & Marowska, 1995). In particular, melatonin functions to act on vasculature and contributes to total antioxidant capacity in the body, which are discussed in further detail below.

1.3.1 Antioxidant Effects

Melatonin is a strong antioxidant due to its ability to scavenge free radicals and act on reactive oxygen species (**ROS**; Halliwell & Gutteridge, 1999; Thakor et al., 2010). During pregnancy, ROS can have negative consequences on the dam, placenta, and fetus (Reiter et al., 2009). Reactive oxygen species, such as superoxide radicals, damage the integrity of cellular membranes. Oxidative stress will occur when the free radicals overwhelm the body's antioxidant capacity and cause damage and reduce the membrane

integrity (Castillo et al., 2004). Chronic oxidative stress may induce disorders such as preeclampsia during pregnancy (Reiter et al., 2009; Kais et al., 2010). Melatonin may combat these effects due to its ability to directly bind to the cellular membrane and reduce oxidative damage (Stankov et al., 1991). Melatonin participates in the up-regulation of antioxidant pathways (Richter et al., 2009). Also, melatonin metabolites have antioxidant properties (Hardeland & Pandi-perumal, 2005), and regulate gene expression of antioxidant enzymes (Steinhilber et al., 1995). Additionally, melatonin is one of the few hormones able to cross the placental blood barrier unaltered (Torres-Farfin et al., 2008).

In humans, a relationship has been observed between decreased melatonin concentrations and increased instance of hypertension (Sewerynek, 2002). During pregnancy, maternal plasma melatonin concentrations are elevated, peak at the end of term, then return to basal levels following parturition in women, sheep, and cattle (Yellon et al., 1987; Skrzypczak, 1998; Nakamura et al., 2001). Furthermore, in humans, decreased concentrations of melatonin during scotophase, or times of darkness in a daylight cycle, have been associated with compromised pregnancies (Nakamura et al., 2001). Hence, melatonin may serve as an optimal therapeutic supplement during pregnancy to reduce oxidative stress and increase total antioxidant capacity.

Melatonin increases blood flow by directly modulating local vascular tone and numerous studies have demonstrated both vasoconstrictor and vasorelaxant properties (Reiter et al., 1995). Melatonin has been observed to induce vasodilation of rat and rabbit aorta, iliac, renal, and basilar arteries (Hashimoto et al., 1989; Weekley, 1993) and

vasoconstriction of rat cerebral arteries (Visanathan et al., 1995; Geary et al., 1998). Additionally, increase in umbilical blood flow by melatonin treatment may be due to increased sensitivity of placental vessels to bradykinin induced vasorelaxation (Shukla et al., 2014).

Vasorelaxation takes place directly when melatonin binds to receptors or indirectly via upregulation of nitric oxide (NO) synthesis. The direct action of melatonin occurs via two G protein-coupled receptors, MT_1 and MT_2, (Reiter, 1993; Chemineau et al., 2008). Placental melatonin receptor activation may mediate the increase in fetal blood flow that occurs during melatonin supplementation (Lemley et al., 2013a). Additionally, receptors have been found throughout the reproductive tract, including the male (Frungieri et al., 2005) and female gonads (Soares et al., 2003).

Using melatonergic-specific pathways, melatonin may increase blood flow or induce vasorelaxation via NO synthesis. Thakor et al. (2010) found that melatonin increased umbilical blood flow in sheep, but its effects were negated when a NO clamp was introduced. Nitric oxide functions to regulate uteroplacental blood flow due to vasorelaxation of vascular smooth muscle cells (Simko & Paulis, 2007). Melatonin participates as a vasodilator directly by increasing NO synthesis through MT receptors. It does this by binding to the G-protein coupling receptors on the surface of the endothelial cells and cause an increase in intracellular calcium. This activates nitric oxide synthase (NOS) to convert arginine to NO and be transported to the vascular smooth muscle cells to activate the enzyme soluble guanalyl cyclase (sGC) and activate cyclic GMP (cGMP).

The cGMP acts as a secondary messaging system and induces vasorelaxation in vascular smooth muscle cells.

Independent of melatonergic receptor-specific pathways, melatonin decreases the amount of ROS available to cause damage. Circulating ROS inhibit the production of NO, but the antioxidant action of melatonin reduces the effect of ROS and allows for NO synthesis to occur (Aydogan et al., 2006). Overall, melatonin has the ability to increase uterine or umbilical blood flow via direct or indirect action to induce vasorelaxation.

1.3.2 Hair Growth and Body Temperature

Melatonin supplementation has been associated with core body and distal skin temperature variation as well as hair growth in mammals. In humans, oral administration of melatonin increased distal skin temperature and decreased core body temperature (Cagnacci et al., 1995; Krauchi et al., 1997). In goats, melatonin initiated fiber growth of primary hair follicles (Nixon et al., 1993) while other mammals experience primary follicle growth as a result of photoperiod and melatonin concentration cues (Allain et al., 1981). Melatonin injections in Cashmere wethers resulted in stimulated primary hair follicle growth (Nixon et al., 1993). However, no difference in hair growth or distal skin temperature occurred in calves born to dams supplemented with dietary melatonin (Brockus et al., 2016b). More research on the direct and developmental impacts of melatonin on bovine hair growth and skin temperature change are necessary.

1.3.2.1 Thermal Imaging

As melatonin has been observed to alter core body and distal skin temperature, a non-invasive method of evaluation is preferred to evaluate pregnant cattle. Thermography is an imaging method that registers infrared waves of the electromagnetic spectrum emitted by an object. Digital infrared thermal imaging serves as a noninvasive method that detects surface temperature gradients to evaluate core body temperature of an animal (Purohit et al., 1985). Overall, thermal imaging cameras measure body surface temperature based on the temperature emitted from internal tissues (Fig 1.4). Internal temperature change is influenced by blood flow in addition to the external environment. For animal studies, the image may be taken without the animal being restrained which reduces the animal's stress during the procedure. When an area of a hide is shaved, imaging compares distal skin temperature of the shaved and unshaved areas using the polygon function of an imaging software (Fig. 1.5).

1.3.3 Postnatal Growth

In utero supplementation of melatonin during gestation exhibited effects on postnatal growth in the brain of rats (Watson et al., 2006), gonads in hamsters (Lucas et al., 2000), and oxidative stress of human newborns (Gitto et al., 2009). Melatonin plays a role in regulation of body weight and adiposity (Janesick & Bloomburg, 2011). Brockus et al. (2016b) observed that all calves had similar birth weights, but calves born from melatonin treated Holstein heifers had increased calf growth compared to calves born from control heifers. While melatonin has been studied throughout prenatal and perinatal

life and its programming effects, there is limited research on the postnatal development of subsequent offspring that warrants further investigation.

1.4 Male Development

1.4.1 Melatonin

Melatonin receptors are in the testis of many species such as the mouse, rat, and human (Liu et al., 2009; Vargas et al., 2011; Zhang et al., 2012). In utero development of the male gonads involve the transformation of the sex cords into seminiferous tubules to serve as the primary site of spermatogenesis. The diameter of seminiferous tubules are reduced in mice injected with melatonin (Ng et al., 1990). Within the seminiferous tubules reside the Sertoli cells that specialize in nursing the developing sperm cells through the process of spermatogenesis (Gholami et al., 2017). They permanently reside within the seminiferous tubules and regulate sperm production through inhibin and activin production. Melatonin receptors MT_1 and MT_2 had been found in bovine Sertoli cells. In addition, exogenous melatonin up-regulates gene expression of spermatogenesis-related genes (Yang et al., 2014). In hamsters, injections of melatonin restored Sertoli cell responsiveness to follicle stimulating hormone (Heindel et al., 1984) and acts to inhibit androgen production (Frungieri et al., 2005). Effects of melatonin supplementation varies between photoperiodic and nonphotoperiodic animals. In rodents, subcutaneous melatonin capsules regulate testicular function in periodic rodents but had no effect in nonphotoperiodic rodents (Turek et al., 1976). Essentially, exogenous melatonin supplementation impacts regulation of testicular function when treated ex utero.

1.4.2 Breeding Soundness Exam

Breeding soundness examinations (BSE) are used to assess the breeding potential of a sexually mature male. Breeding soundness refers to a male's ability to impregnate a female. The examination aides to predict bull fertility and exclude those with significant deficits from the breeding pool. The Society for Theriogenology standards (http://www.therio.org) assess the likelihood of a bull establishing pregnancy in ≥ 25 healthy, cycling females within a 65–70 day breeding season (Kastelic et al., 2008).

Breeding soundness exams consist of a physical examination evaluating internal and external reproductive structures, measurement of scrotal circumference, and collection and evaluation of semen. The physical evaluation includes assessment of body condition, structure, overall health, and conformation. Internal and external reproductive structures are palpated to evaluate functionality of the bulls, such as the scrotum, testis dissention and morphometrics, and accessory sex glands. The testis should be symmetrical, similar in size, freely moveable, and firm within the scrotum. Scrotal circumference is determined with a flexible tape measure of the testis when pushed to the bottom of the scrotum. Younger bulls have an acceptable scrotal circumference at 30 cm (minimum), however, the size requirement increases with age. Scrotal circumference is positively correlated with earlier onset of puberty and increased fertility in female offspring (Palasz et al., 1994). Collection of semen for evaluation of motility and morphology takes place following internal palpation of accessory sex glands. Semen morphology evaluates the number of abnormalities found in the ejaculate sample while motility evaluates if the sperm cells exhibit progressive movement.

In order for a bull to pass a comprehensive BSE, they must be sound, in good health, with adequate scrotal circumference, and ≥ 70% normal morphology and ≥ 30% progressive motility. Following evaluation, a bull is classified as a 'satisfactory' or 'unsatisfactory' breeder. However, under recommendation of the technician performing the BSE, the bull may be 'classification deferred' in order for them to have more time to mature or develop and be revaluated at a later date (Bagley & Chapman, 2005).

Implications with BSE are that while they exclude males from the breeding pool that are sterile, they do not exclude all sub-fertile males. The problems of sub-fertile bulls are that they prolong the calving season, decrease conception rates, and increase cull rate of females (Kastelic et al., 2008). However, BSE are a great tool for producers and buyers to use in order to evaluate the breeding ability of bulls.

1.4.3 Computer-Assisted Sperm Analyzer (CASA)

Semen analysis has been used to indicate male fertility using methods such as a hemocytometer. Traditional methods determined semen analysis via ejaculate volume, pH, sperm concentration, motility, and morphology (Johnson et al., 1990). Development of objective assessment practices began with time-lapse photography (Overstreet et a., 1979) and multiple-exposure photomicrography (Makler, 1978), however both seemed too time consuming. Objective sperm motility evaluation by a Computer-Assisted Sperm Analyzer or CASA allows for sperm movement to be analyzed for individual spermatozoa in each ejaculate (Farrell et al., 1998). This system measures specific motion characteristics relative to the functionality of the sperm cell (Kastelic et al., 2008). Sperm analysis with CASA exhibits comparable and reliable results that do not differ

clinically or biologically in comparison with manual analysis procedures (Katz, 1992). This technique allows for assessment of multiple characteristics on a larger sample size that results in high repeatability (Farrell et al., 1995). Analysis of spermatozoa takes into account the motility and path of each sperm cell as well as the kinematic measurements. Combinations of sperm motion characteristics such as beat cross frequency, linearity, average path velocity, straightness and curvilinear velocity are correlated with bull fertility (Farrell et al., 1998). This system is also used to evaluate frozen-thawed samples in bull (Contri et al., 2010) and pig spermatozoa (Broekhuijse et al., 2011).

1.5 Summary

Melatonin is a strong antioxidant that serves several physiological functions in the body, particularly a stimulant of vasodilation or vasoconstriction on vasculature throughout the reproductive tract. Additionally, melatonin's ability to scavenge free radicals and act on ROS (Halliwell & Gutteridge, 1999; Thakor et al., 2010) has the potential to protect the dam, placenta, and fetus from damage to the cell membrane during pregnancy (Reiter et al., 2009). However, melatonin is a hormone that requires further investigation in order to understand how it affects reproductive tissues, specifically the uteroplacental environment. Current research is evaluating melatonin as a therapeutic supplement during compromised pregnancies (Lemley et al., 2012), such as intrauterine growth restriction or multiple pregnancies, in addition to developmental programming via uterine artery blood flow (Brockus et al., 2016a). Melatonin may be used as an economical supplement to improve subsequent growth of offspring due to in utero exposure. Brockus et al. (2016b) observed that calves born from melatonin-treated

Holstein heifers had an increase in calf growth compared to calves born from control heifers but similar birth weights. However, limited studies have been performed to evaluate melatonin treatment on male development in cattle. Exogenous melatonin supplementation impacts regulation of testicular function in mice, rats, and humans (Semercioz et al., 2003; Liu et al., 2009; Mehraein & Negahdar, 2010; Vargas et al., 2011; Zhang et al., 2011). Moreover, further research is needed to attribute the effect of chronic maternal melatonin supplementation in utero on male offspring's testicular development and semen quality in cattle.

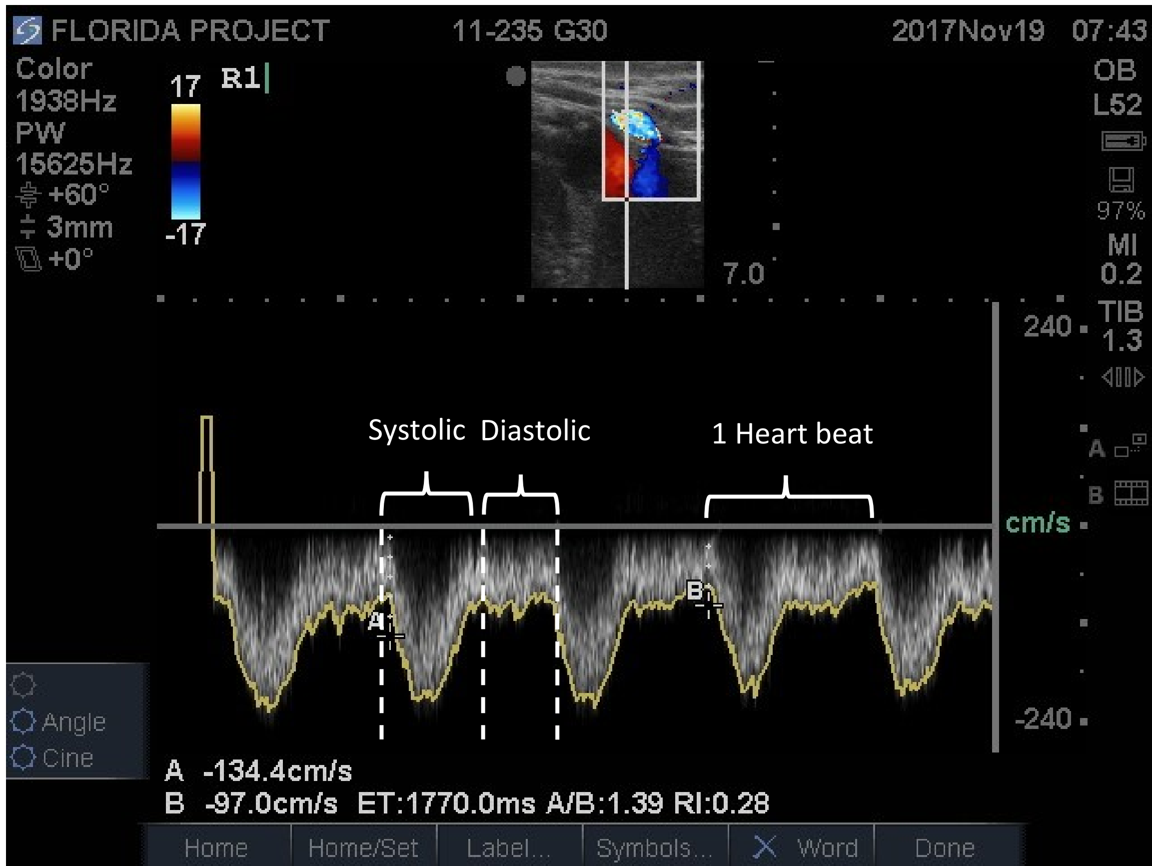

Figure 1.1 Representative waveforms of blood flow in uterine artery vessel in pregnant cattle during mid-gestation using Doppler ultrasonography

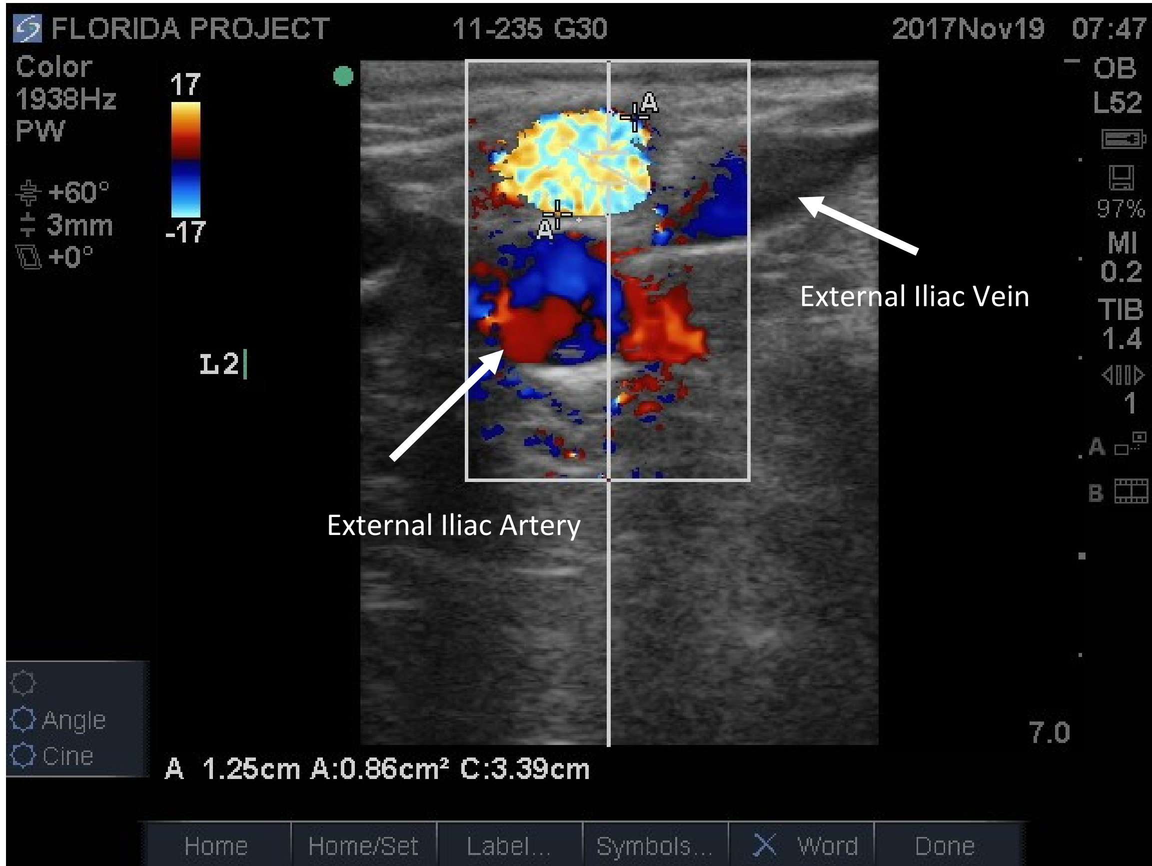

Figure 1.2 Representative image of the uterine artery vessel adjacent to the External Iliac artery and External Iliac vein in pregnant Angus cattle during late gestation using Doppler ultrasonography

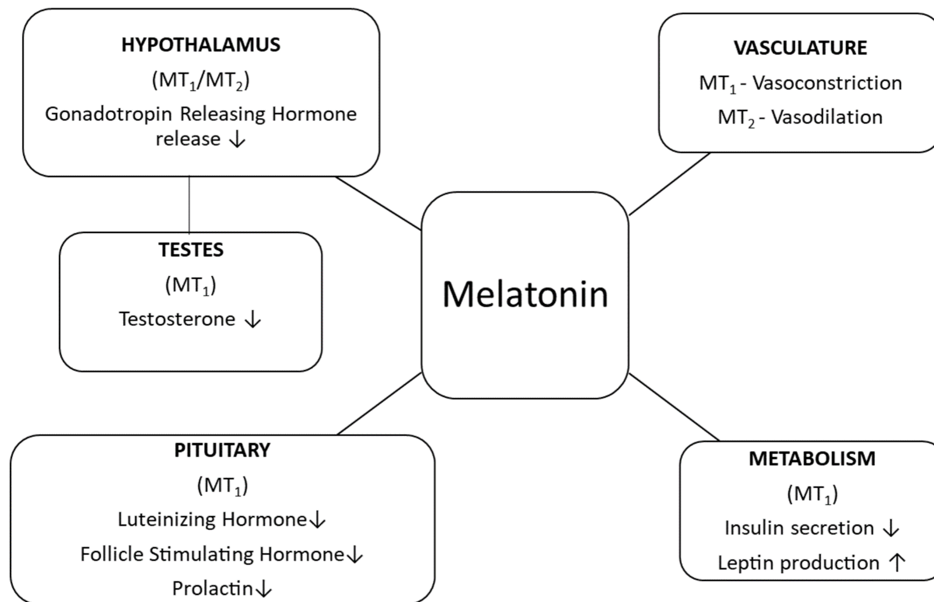

Figure 1.3 Review of melatonin's functions and physiological effects (redrawn from Dubocovich & Marowska, 1995).

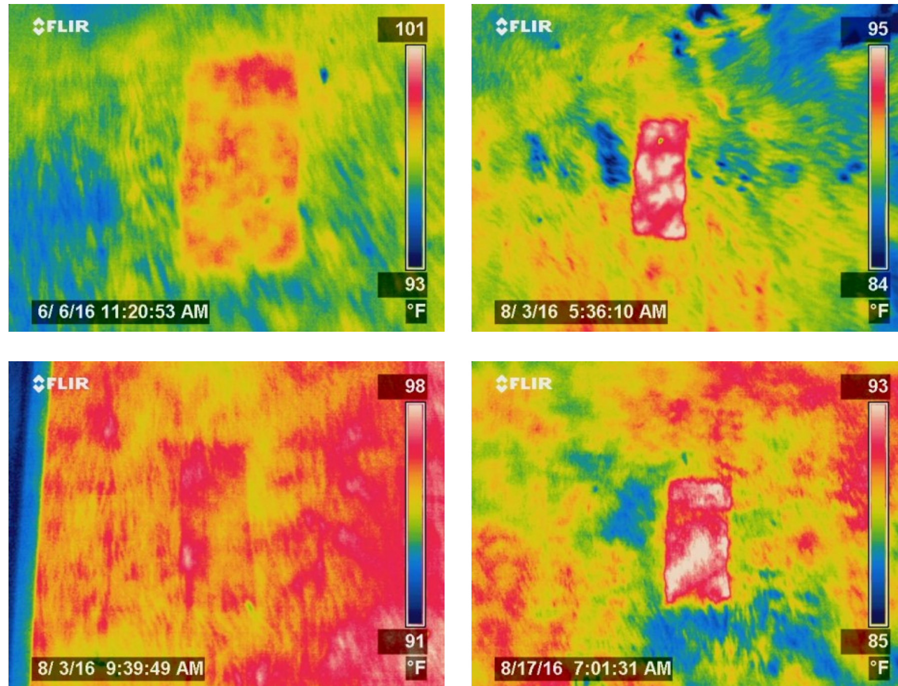

Figure 1.4 Representative thermal image of a shaved and unshaved area on the right thoracic side of pregnant beef cattle from mid to late gestation using Flir ThermaCAM S60 (Flir Systems, Boston, MA, USA). Color gradient scale is shown.

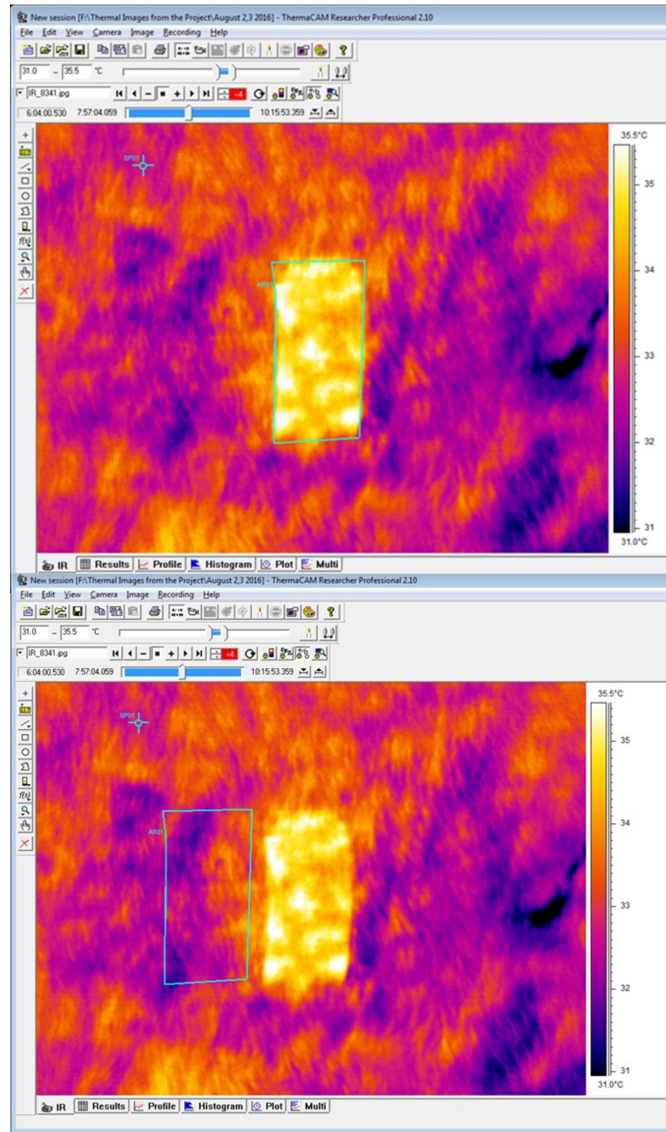

Figure 1.5 Representative thermal image of a shaved and unshaved area on the right thoracic side of an Angus cow using Flir ThermaCAM S60 (Flir Systems, Boston, MA, USA). Comparative analyses of shaved and unshaved areas via polygon function of ThermaCAM Researcher Pro 2.7 software (FLIR Systems). Color gradient scale is shown.

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CHAPTER II

EFFECT OF CHRONIC MELATONIN SUPPLEMENTATION DURING MID- TO LATE-GESTATION ON UTERINE ARTERY BLOOD FLOW, PLACENTAL ANGIOGENIC FACTORS, AND FETAL WEIGHTS IN BEEF CATTLE

2.1 Abstract

Melatonin is a strong antioxidant that has previously been observed to increase uteroplacental blood flow when supplemented in the diet during gestation of Holstein heifers. The objective of the current study was to examine the effects of supplemental melatonin on uterine blood flow from mid- to late gestation in cattle. Commercial beef heifers (*n* = 32) and cows (*n* = 25) were artificially inseminated and assigned to one of two treatment groups supplemented with (**MEL**) or without (**CON**) melatonin delivered as two- 24 mg implants or placebo at d 180, 210, and 240 of gestation. Uterine artery blood flow was determined using color Doppler ultrasonography. A subset of twelve crossbred heifers (*n* = 6 MEL; *n* = 6 CON) underwent Cesarean section on day 243 ± 2 of gestation to allow for placental and fetal tissue collection. In addition, fetal body weight, head circumference, head length, heart girth, abdominal girth, curved crown rump (CCR) length, and ponderal index (BW [kg]/CCR [m]3) were determined. Maternal and fetal serum were collected to analyze melatonin concentrations. Total uterine artery blood flow had a treatment x day interaction (*P* = 0.025) where MEL-treated heifers had increased (*P* = 0.009) blood flow at day 240 compared with CON-treated heifers. Total uterine artery blood flow was increased (*P* = 0.003) in MEL-treated cows compared with CON-treated

cows. Fetal weight, head circumference, head length, heart girth, abdominal girth, CCR, or ponderal index were not different ($P > 0.05$) in fetuses from MEL-treated heifers compared with fetus' from CON-treated heifers. Fetal and maternal concentrations of melatonin increased ($P < 0.03$) in MEL-treated heifers compared with CON-treated heifers. Cotyledonary *ANGPT1* mRNA tended to increase ($P = 0.095$) in MEL-treated heifers compared with CON-treated heifers. There were no differences ($P > 0.05$) in caruncular *ANGPT1, VEGF, KDR*, and *FLT1* or cotyledonary *VEGF, KDR,* and *FLT1* mRNA between treatments. In summary, melatonin supplementation increased uterine blood flow in mid- to late gestating cattle but this was not accompanied by an increase in fetal weight. Additional studies regarding the physiological impact of these hemodynamic changes on nutrient transport and offspring development are warranted.

2.2 Introduction

Uteroplacental blood flow is imperative for normal fetal development in livestock species. Oxidative stress during pregnancy may induce disorders or disrupt fetal development such as intrauterine growth restriction and decreased birth weights (Robinson, 2017; Sultana et al., 2017). To combat these disorders, scientists are evaluating the mediators of uterine hemodynamics during pregnancy. Uteroplacental blood flow plays a role in successful growth and development of the fetus (Reynolds & Redmer, 1995; Vonnahme et al., 2012; Lemley et al., 2014). Efficiency of placental transport is directly related to uteroplacental blood flow (Reynolds & Redmer, 1995). Transplancental exchange relies on increased placental growth during early gestation followed by increased uteroplacental vasculature during late gestation (Reynolds et al., 1995). Endogenous factors, such as angiogenic factors, impact the capacity of

uteroplacental blood flow (Reynolds et al., 2005; Reynolds et al., 2006). The impact of exogenous therapeutics on uterine hemodynamics and how they interact with endogenous factors has become a more recent area of study (Yunta et al., 2015; Brockus et al., 2016a; Kennedy et al., 2017). Specifically, those that could improve placental efficiency to increase gas exchange and nutrient transport. Our laboratory has previously investigated how melatonin supplementation impacts uteroplacental hemodynamics and fetal growth in sheep and cattle (Lemley et al., 2012; Brockus et al., 2016a). Previously, Brockus et al. (2016a), demonstrated that dietary melatonin supplementation to Holstein heifers during mid- to late gestation increased uterine artery blood flow. Additionally, calves born from melatonin-treated heifers had an increase in calf growth compared to calves born from control-treated heifers (Brockus et al., 2016b). In an ovine model of intrauterine growth restriction, melatonin supplementation increased umbilical artery blood flow compared to non-supplemented control ewes (Lemley et al., 2012). We hypothesized that chronic melatonin supplementation, via melatonin ear implants, would increase uterine artery blood flow and placental angiogenic factor expression compared with non-supplemented control beef heifers. The objective of the study was to determine the effect of maternal melatonin supplementation during late gestation on uterine blood flow, placental angiogenic factor expression, fetal morphometric measurements, hair growth, and temperature change.

2.3 Materials & Methods

2.3.1 Animal Care and Treatments

Animal care and use were according to protocols approved by the Mississippi State University Institutional Animal Care and Use Committee (#16-036).

All animals were bred using a timed artificial insemination (TAI) protocol. Heifers and cows were inseminated at the H.H. Leveck Animal Research Center at Mississippi State University. Heifers (*n* = 87) were bred on December 10, 2015 and cows (*n* = 65) on December 21, 2015. At approximately 120 days post-AI, both groups underwent ultrasonography to determine pregnancy to artificial insemination, and those who conceived were enrolled in the study; 32 heifers and 25 cows. Breeds consisted of 8 Hereford, 28 Angus, and 21 crossbred.

A randomized complete block design was utilized to block animals based on breed and body weight. On day 180 of gestation, heifers and cows were assigned to one of two treatments: a melatonin implant (**MEL**; *n* = 29) or a no melatonin implant control (**CON**; *n* = 28) starting on day 180 of gestation and ending on day 270. Melatonin treatment consisted of two sub-dermal ear implants containing 24 mg of melatonin each (Melatonin Implants, Conroe, Texas, USA) to slow-release melatonin into peripheral circulation over a 30-day period. Implants were administered every 30 days, occurring on day 180, day 210, and day 240 of gestation. The last implant, at day 240 of gestation, was expected to provide an increased concentration of melatonin until day 270 of gestation. Dams did not receive any further treatment after final administration at day 240. Non-treatment control animals involved sticking the ear with a sterilized needle.

2.3.2 Color Doppler Ultrasonography

Hemodynamic measurements of the uterine artery both ipsilateral and contralateral to the conceptus were determined on days 180, 210, and 240 of gestation. Color Doppler Ultrasonography (MicroMaxx; SonoSite, Inc., Bothell, WA, USA) was performed using a transabdominal probe (MicroMaxx; SonoSite with a Phased array P17

1-5 MHz 17 x 17 mm probe). Examinations were approximately 30 min per animal between 05:00 and 12:00 hr. Following the techniques described by Brockus et al. (2016a), briefly, the uterine artery was identified by following the abdominal aorta towards the origin of the external iliac artery. The internal iliac artery was located by moving the probe caudally. The left and right uterine arteries were identified as a major branch of the iliac arteries. Additionally, the uterine arteries were palpated to assure pliability and pulsatility. The ultrasound transducer was aligned to the uterine artery at an average angle of isonation of 69 ± 0.4 degrees (mean ± SE) of all animals. Three cardiac cycle waveforms from two independent ultrasound scans were used to calculate systolic velocity (s; cm/s), diastolic velocity (d; cm/s), s:d ratio, pulsatility index (PI), and resistance index (RI) using preset functions on the Doppler ultrasound. Mean velocity (MnV) was calculated using the equation: (s – d)/PI. Blood flow was calculated using the equation: (MnV*vessel area*60 s). Total uterine artery blood flow was calculated as the summation of both the right and left uterine arteries. Ipsilateral and contralateral uterine artery blood flow is presented as the vessels on the same and opposite side of the fetus, respectively.

2.3.3 Cesarean Sections

A subset of twelve crossbred heifers (n = 6 MEL; n = 6 CON) underwent Cesarean section on day 243 ± 2 of gestation to allow for placental and fetal tissue collection for further analysis. A blood sample was collected from all heifers via venipuncture of the coccygeal vein immediately preceding surgery. Briefly, samples were centrifuged at 10,000x g for 15 min at 4° C. The supernatant was aliquoted and stored at -80° C long term.

Surgeries were performed at the H. H. Leveck Animal Research Center (Mississippi State, MS); therefore, animals were not transported prior to surgery. Cesarean sections were performed with the dam standing following a paravertebral or inverted-L block with 2% lidocaine. After the skin surrounding the incision site was prepared for aseptic surgery, an incision was made 10 to 15 cm ventral to the transverse processes of the lumbar vertebrae midway between the last rib and the tuber coxae and extended sufficiently to allow extraction of the fetus. A left oblique celiotomy approach was utilized for standing cows. The abdominal wall incision extended cranioventrally at a 45 degree angle. This surgical approach permitted easier access to the gravid uterus than more traditional vertical incisions in the paralumbar fossa. One of the fetal limbs was identified and used as a handle to deliver the uterus to the abdominal incision. After the uterus was incised, the umbilical cord was located and clamped off in two locations approximately 10 cm from the fetus and 10 cm from the major branch points feeding the cotyledons and cut between the two clamps. Further processing of the fetus began immediately following successful removal of the fetal calf. In addition, a placentome was immediately excised from the uterine wall following successful removal of the fetal calf. All placentomes were separated into caruncle (maternal) and cotyledon (fetal) portions and snap frozen in liquid nitrogen and stored at -80ºC for later processing of angiogenic factor mRNA expression.

2.3.4 Fetal Tissue Collection

Fetal body weight (pre-exsanguination) and morphometric measurements such as head circumference, head length, curved-crown rump (CCR) length, heart girth, and abdominal girth were collected and ponderal index (BW [kg]/CCR $[m]^3$) determined

immediately following extraction from the dam. Fetal blood samples were collected from the umbilical artery and vein via venipuncture in addition to blood collected at exsanguination to be evaluated for peripheral concentrations of melatonin.

2.3.5 Blood Analysis

Blood samples were analyzed from fetuses of crossbred heifers (*n* = 6 per treatment), as well as the associated dam, to measure serum concentrations of melatonin in peripheral circulation. Melatonin concentrations were determined with an ELISA kit (TECAN, Morrisville, NC, USA) following manufacturer's instructions. Samples were compared to a melatonin standard curve (0 – 1000 pg/mL), with a sensitivity of 1.0 pg/mL. The intra-assay coefficient of variation for the melatonin assay was 11.8%.

Total antioxidant capacity was determined with a colorimetric assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). The total antioxidant assay was performed following manufacturer's instructions except for the serum being diluted in assay buffer to place unknowns in the range of the standard curve. Antioxidant capacity of serum was determined based on the inhibition of ABTS (2,2'- Azino-di-[3-ethylbenzthiazoline sulphonate]) oxidation by metmyoglobin. The amount of ABTS produced was monitored at 405 nm via a Spectra Max Plus Plate reader (Sunnyvale, CA, USA). The combined antioxidant activity of all constituents in maternal serum were reported due to the assays lack of ability to separate aqueous- and lipid- soluble antioxidants. The capacity of serum antioxidants in the unknown serum samples were compared to a trocopherol analogue known as Trolox. Antioxidant capacity, reported as mM Trolox equivalents, were analyzed against a Trolox standard curve (0 – 0.33 mM), with a sensitivity of 0.01 mM. The intra-assay coefficient of variation for the total antioxidant assay was 14.6%.

2.3.6 Tissue Analysis

Total nitrates of the caruncle (*n* = 6 per treatment) and cotyledon (*n* = 6 per treatment) were determined using QuantiChrom Nitric Oxide Assay Kit (BioAssay Systems, Inc. Haward, CA, USA) following the methods of Lemley et al. (2013). Briefly, samples were deproteinized and quantified following reduction of total nitrates to nitrites using the Griess method and analyzed against a linear nitrites standard curve (0-100 μM) and the intra-assay coefficient of variation was 5.4%.

2.3.7 RT-PCR

Gene expression (mRNA abundance) analysis was performed on caruncle (*n* = 12) and cotyledon (*n* = 12) tissues collected on day 243 ± 2 of gestation. The RNA transcript abundance for angiogenic factors and their receptors was determined using RT-PCR. Approximately 1 g of caruncle and cotyledon tissue was homogenized with 1 mL of PBS followed by RNA extraction of homogenate using miRNeasy Mini Kit (QIAGEN, Germantown, MD, USA). Reverse transcription of 20 μL of RNA homogenate was used to form cDNA via a High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific, Auburn, AL, USA). RT-PCR was performed using Custom TaqMan Gene Expression Assays (Thermo Fischer Scientific, Auburn, AL, USA) via protocols provided by the manufacturer. Polymerization and amplification reactions were carried out in triplicates in a 96-well PCR plate at a final volume of 10 μL using a QuantiStudio 3 system (Applied Biosystems, Foster City, CA, USA). Duplexing of genes were executed per manufacturer's instruction; briefly, 1 μL of Taqman Gene was substituted for 1 μL of nuclease free water per reaction mixture. Final data were

analyzed through the $2^{-\Delta\Delta Ct}$ method where the geometric mean of GAPDH and B Actin was used as a reference to normalize all the selected gene expression data

2.3.8 Thermal Imaging and Hair Collection

Thermal imaging using a Flir ThermaCAM S60 (Flir Systems, Boston, MA, USA) infrared thermography camera and hair weight were collected on days 180 (baseline) and 240 of gestation. A hair sample was collected from a 5.08 x 10.16 cm area on the left thoracic region right behind the shoulder of the animal. Hair samples were placed in a pre-weighed bag, then the total weight subtract the bag weight resulted in the actual hair weight from the shaved area. Images were taken of the shaved 5.08 x 10.16 cm area and adjacent unshaved areas. Temperatures of both the shaved and unshaved areas were analyzed using ThermaCAM Researcher Pro 2.7 software (FLIR Systems, Boston, MA, USA). Average temperature of the shaved area was determined by constructing a rectangular polygon that corresponded to the shaved area and then analyzing temperature gradient change of the polygon. The same polygon was relocated to an adjacent unshaved area to calculate an average surface temperature. To ensure a standard surface area was being measured between both the shaved and unshaved areas, the same polygon was used. The ambient temperature was not different ($P = 0.27$) and averaged 31 ± 1°C; however, average humidity was increased on d 240 (82 ± 4%) vs 180 (67 ± 5%).

2.3.9 Statistical Analysis

Uterine blood flow was analyzed using repeated measures of ANOVA (SAS software version 9.4, SAS Institute, Cary, NC, USA). The model statement included: day

of gestation, treatment, day by treatment interaction, breed, and calf sex. Autoregressive was the type of covariance structure selected based on the fit of statistical parameters in the model. The cows and heifers were analyzed separately due to a confounding effect of breed between the two parities. Least square means and standard error of the means are reported. Fetal blood profiles were analyzed using ANOVA (SAS software version 9.4, SAS Institute, Cary, NC, USA). Where the model statement included: treatment, breed, fetal sex, and their respective interactions. Fetal morphometric measurements were analyzed using ANOVA (SAS software version 9.4, SAS Institute, Cary, NC, USA) where the model statement included: treatment, breed, and calf sex. Least square means and standard error of the means are reported. Gene expressions were analyzed using ANOVA (SAS software version 9.4, SAS Institute, Cary, NC, USA). The model statement included: treatment and fetal sex. Gestation length, fetal weight, and placentome weight served as covariates. Least square means and standard error of the means were reported. Statistical significance was declared at $P \leq 0.05$ while a tendency was declared at $0.05 < P \leq 0.10$.

2.4 Results

2.4.1 Maternal Body Weight

Heifer and cow body weight increased ($P < 0.0001$) as gestational day increased (data not shown). Heifer body weight was not different ($P = 0.951$) between treatments and averaged 512.0 ± 3.9 kg. Cow body weight tended to be increased ($P = 0.086$) in MEL-treated cows (580.0 ± 16.3 kg) compared with CON-treated cows (572.0 ± 16.5 kg) (data not shown).

2.4.2 Uterine Artery Hemodynamics

2.4.2.1 Ipsilateral Uterine Artery Blood Flow

Ipsilateral uterine artery blood flow tended to have a treatment x day interaction ($P = 0.060$) where MEL-treated heifers had increased ($P = 0.023$) blood flow at day 240 compared with CON-treated heifers (Fig. 2.1A). No effect of treatment was observed for ipsilateral MnV ($P = 0.3293$) in heifers. A main effect of gestational day ($P = 0.0005$) was observed for ipsilateral MnV in heifers where day 240 was increased compared with day 210 (Fig. 2.1B). Diameter of the ipsilateral uterine artery tended to have a treatment x day interaction ($P > 0.060$) where treatments did not differ at day 210 ($P = 0.309$), however, MEL-treated heifers had larger ($P = 0.003$) diameter compared with CON-treated heifers at day 240 (Fig. 2.1C). No effect of treatment was observed for s/d ratio ($P = 0.516$), RI ($P = 0.587$), or PI ($P = 0.621$) of the ipsilateral uterine artery of heifers (data not shown).

Ipsilateral uterine artery blood flow was increased ($P = 0.01$) in MEL-treated versus CON-treated cows (Fig. 2.1D). A main effect of gestational day ($P < 0.0001$) was observed for ipsilateral uterine artery blood flow which increased ($P < 0.0001$) as gestational day increased in cows (Fig. 2.1D). Ipsilateral MnV was increased ($P = 0.020$) in MEL-treated versus CON-treated cows (Fig. 2.1E). Diameter the ipsilateral uterine artery tended to have a treatment x day interaction ($P > 0.07$) where MEL-treated cows tended to have larger ($P = 0.078$) diameter compared with CON-treated cows at day 210, however, did not differ ($P = 0.991$) at day 240 (Fig. 2.1F). No effect of treatment was observed for s/d ratio ($P = 0.678$), RI ($P = 0.557$), or PI ($P = 0.669$) of the ipsilateral uterine artery of cows (data not shown).

2.4.2.2 Contralateral Uterine Artery Blood Flow

Contralateral uterine artery blood flow had a treatment x day interaction (P = 0.032) where MEL-treated heifers had increased (P = 0.012) blood flow at day 240 compared with CON-treated heifers (Fig. 2.2A). No effect of treatment (P = 0.288) was observed for contralateral uterine artery MnV. A main effect of gestational day (P = 0.047) was observed for contralateral MnV in heifers where day 240 was increased (P < 0.0001) compared with day 210 (Fig. 2.2B). Diameter of the contralateral uterine artery tended (P = 0.09) to be increased in MEL-treated versus CON-treated heifers (Fig. 2.2C). Diameter of the contralateral uterine artery increased (P = 0.022) as gestational day increased (Fig. 2.2C). No effect of treatment was observed on s/d ratio (P = 0.222), RI (P = 0.218), or PI (P = 0.229) of the contralateral uterine artery of heifers (data not shown).

Contralateral uterine artery blood flow (Fig. 2.2D), MnV (Fig. 2.2E), and uterine artery diameter (Fig. 2.2F) were not different (P > 0.10) between treatments or gestational day in cows. No effect of treatment was observed for s/d ratio (P = 0.966), RI (P = 0.624), or PI (P = 0.663) of the contralateral uterine artery of cows (data not shown).

2.4.2.3 Total Uterine Artery Blood Flow

Total uterine artery blood flow had a treatment x day interaction (P = 0.025) where MEL-treated heifers had increased (P =0.009) blood flow at day 240 compared with CON-treated heifers (Fig. 2.3A). Heart rate tended to have a treatment x day interaction (P = 0.096) where heart rate did not differ (P = 0.603) at day 210, however, MEL-treated heifers had increased (P = 0.028) heart rate compared with CON-treated heifers at day 240 (Fig. 2.3B)

Total uterine artery blood flow was increased (*P* = 0.003) in MEL-treated cows compared with CON-treated cows (Fig. 2.3C). Total uterine artery blood flow increased (*P* < 0.0001) as gestational day increased in cows (Fig. 2.3C). Additionally, heart rate tended to increase (*P* = 0.059) as gestational day increased in cows (Fig 2.3D).

2.4.3 Fetal Morphometrics

Fetal weight was not different (*P* = 0.561) between treatments and averaged 23.5 ± 6.8 kg (Table 2.1). Head circumference, head length, heart girth, abdominal girth, CCR, or ponderal index were not different (*P* > 0.05) in fetus' from MEL-treated heifers compared with fetus' from CON-treated heifers (Table 2.1).

2.4.4 Blood Analysis

Fetal and maternal concentrations of melatonin increased (*P* < 0.03) in MEL-treated heifers compared with CON-treated heifers (Table 2.2). Total antioxidant capacity (TAC) was not different (*P* > 0.10) in fetal or maternal serum between treatments (Table 2.2).

2.4.5 Tissue Analysis

Nitrate concentration of cotyledon and caruncle tissues were not different (*P* > 0.05) in MEL-treated heifers compared with CON-treated heifers (Table 2.3).

Caruncular *ANGPT1, VEGF, KDR*, and *FLT1* mRNA were not different *(P* > 0.05) between treatments (Table 2.4). Cotyledonary *ANGPT1* mRNA tended to increase (*P* = 0.100) in MEL-treated heifers compared with CON-treated heifers (Table 2.4). Cotyledonary *VEGF, KDR,* and *FLT1* mRNA were not different *(P* > 0.05) between treatments (Table 2.4). Cotyledonary *KDR* mRNA increased (P = 0.036) in female fetus'

compared with male fetus's (data not shown). The weight of sampled placentomes were not different (*P* > 0.05) between treatments and averaged 66.6 ± 20.1 g (data not shown).

2.4.6 Thermal Imaging and Hair Collection

Rectal temperature was not different between MEL-treated vs. CON-treated on d 180 (*P* = 0.336) or d 240 (P = 0.547; Table 2.5). Temperature of the shaved areas on d 180 (*P* = 0.923) and 240 (*P* = 0.277) were not different between treatments (Table 2.5). Temperature of the unshaved area was not different on d 180 (*P* = 0.754); however, on d 240 temperature of the unshaved area was decreased (*P* = 0.055) in MEL-treated (32.7 ± 0.4°C) vs. CON-treated (33.5 ± 0.3°C). Hair weight of the shaved areas on d 180 (*P* = 0.911) and d 240 (*P* = 0.634) were not different between treatments (Table 2.5).

2.5 Discussion

In the present study, total uterine artery blood flow was increased in melatonin treated compared with control treated heifers and cows. Additionally, ipsilateral and contralateral uterine artery blood flow was increased in melatonin treated compared with control treated heifers. While ipsilateral uterine artery blood flow was increased in melatonin treated compared with control treated cows, there was no difference between treatments for contralateral uterine artery blood flow.

Similar results were observed by Brockus et al. (2016a), after dietary melatonin supplementation to Holstein heifers during mid- to late-gestation where total uterine artery blood flow was increased in melatonin treated heifers compared with non-treated control. An increase in uterine blood flow is vital in late gestation to maintain oxygen supply and nutrient delivery to the growing fetus in sheep (Ford, 1995; Redmer et al.,

2004; Reynolds et al., 2006). An increase in placental blood flow could combat the negative consequences of pregnancy disorders such as preeclampsia or intrauterine growth restriction. For example, in an ovine model of intrauterine growth restriction, melatonin supplementation increased umbilical artery blood flow compared to non-supplemented control ewes (Lemley et al., 2012). Therapeutic supplementation of melatonin to increase uterine artery hemodynamics is a field in which requires further study.

Melatonin concentrations in some livestock species participates as a biological clock to mediate onset of puberty, seasonal breeding and reproductive cycles (Yellon & Longo, 1987). In humans, decreased scotophase concentrations of melatonin have been associated with compromised pregnancies (Nakamura et al., 2001). In the current study, following supplementation, melatonin concentration increased 98% in fetal serum and 73% in maternal serum of melatonin treated heifers compared with control treated heifers.

Maternal body weight increased as gestational day progressed and no differences were observed due to treatment. Similarly, fetal weight was not different, regardless of sex or treatment. We hypothesized that an increase in uterine blood flow would have a concomitant increase in fetal body weight. However, similar results were observed when dietary melatonin was supplemented to Holstein heifers and calf birth weight was not different between treatments (Brockus et al., 2016b). Melatonin supplementation was found to negate the decrease in body weight in nutrient restricted rats (Richter et al., 2009), but was thought to be due to antioxidant capacity. An improved antioxidant capacity provided by melatonin could result in reduced oxidative stress during gestation,

thus, overall reduced occurrences of compromised pregnancies. Total antioxidant capacity was not different in fetal or maternal serum following melatonin supplementation. Melatonin is a strong antioxidant due to its ability to scavenge free radicals and act on reactive oxygen species (Halliwell & Gutteridge, 1999; Thakor et al., 2010). During pregnancy, ROS can have negative consequences on the dam, placenta, and fetus (Reiter et al., 2009). When dietary melatonin was supplemented to Holstein heifers, a 43% in total serum antioxidant capacity was reported (Brockus et al., 2016a). Although calves born from melatonin treated or control treated Holstein heifers total antioxidant capacity did not differ (Brockus et al., 2016b). The current study did not find any differences in maternal or fetal serum total antioxidant capacity. Additionally, melatonin contributes to the bioavailabilty of NO (Thakor et al., 2010). In this study, nitrate concentration of cotyledon and caruncle tissues were not different in melatonin treated heifers compared with control treated heifers. Similar results were found in dams (Brockus et al., 2016a) and calves (Brockus et al., 2016b) where nitrate concentration was not different irrespective of melatonin treatment.

Melatonin increases blood flow by directly modulating local vascular tone and numerous studies have demonstrated both vasoconstrictor and vasorelaxant properties (Reiter et al., 1995). Melatonin has been observed to induce vasodilation of rat and rabbit aorta, iliac, renal, and basilar arteries (Hashimoto et al., 1989; Weekley, 1993) and vasoconstriction of rat cerebral arteries (Visanathan et al., 1995; Geary et al., 1998). Additionally, increase in umbilical blood flow by melatonin treatment may be due to increased sensitivity of placental vessels to bradykinin induced vasorelaxation (Shukla et al., 2014).

Placental vascular development has been associated with angiogenic factor expression. In the current study, cotyledonary *ANGPT1* mRNA tended to increase in melatonin treated heifers compared with CON treated heifers. In mice, increased uterine blood flow due to estrogen treatment upregulated VEGF mRNA concentrations (Cullinan-Bove & Koos, 1993). Vonnahme et al. (2007) observed an increase in transcript abundance of *VEGF* and *FLT1* in both caruncle and cotyledon tissues when beef cows were nutrient restricted from days 30 to 125 of gestation, but no difference in vascularity of the placentome. Similar studies by McLean et al. (2017) observed decreased *VEGF* expression in the intercaruncle of restricted fed Angus heifers compared with control fed heifers during early gestation. In sheep, numerous models of compromised pregnancies have decreased vascularity due to underfeeding or overfeeding adolescents, underfeeding adults, or multiple pregnancies (Reynolds et al., 2010). However, nutrient restriction in ewes caused an increase in cotyledon and caruncle morphological change to occur earlier in gestation, thus, altering placental formation (Vonnahme et al., 2006), as well as increased angiogenic factors in caruncle tissue (Vonnahme et al., 2007).

Melatonin supplementation did not alter rectal temperature whereas in humans, oral administration of melatonin increased distal skin temperature and decreased core body temperature (Cagnacci et al., 1995; Krauchi et al., 1997). Additionally, for the current study, melatonin supplementation did not alter temperature of the shaved area, but decreased temperature of the unshaved area. This study observed no difference in hair growth of heifers or cows treated with or without melatonin. This is supported by Brockus et al., (2016a) where no difference in hair growth or distal skin temperature

occurred in calves of Holstein heifer's supplemented dietary melatonin during gestation. Conversely, in goats, melatonin initiated fiber growth of primary hair follicles (Nixon et al., 1993) while other mammal's experience primary follicle growth as a result of photoperiod and melatonin concentration cues (Allain et al., 1981). Melatonin injections in Cashmere wethers resulted in stimulated primary hair follicle growth (Nixon et al., 1993). Seasonality may participate in the effectiveness of melatonin activity on hair growth. This experiment took place over the summer months in Mississippi with expected fall calving. Overall, the weather and environment may influence hair growth.

Melatonin supplementation increased uterine blood flow in mid- to late- gestating cattle but this was not accompanied by an increase in fetal weight. Although angiogenic factor expression in caruncle and cotyledon tissues did not significantly differ between treatments, determining vascularity of the tissues warrants investigation. Studies of melatonin in cattle for altering hemodynamics is lacking compared to other areas of livestock research. Additional studies regarding melatonin and the physiological impact of uterine hemodynamics on placental angiogenic factor expression, fetal growth, and development are needed.

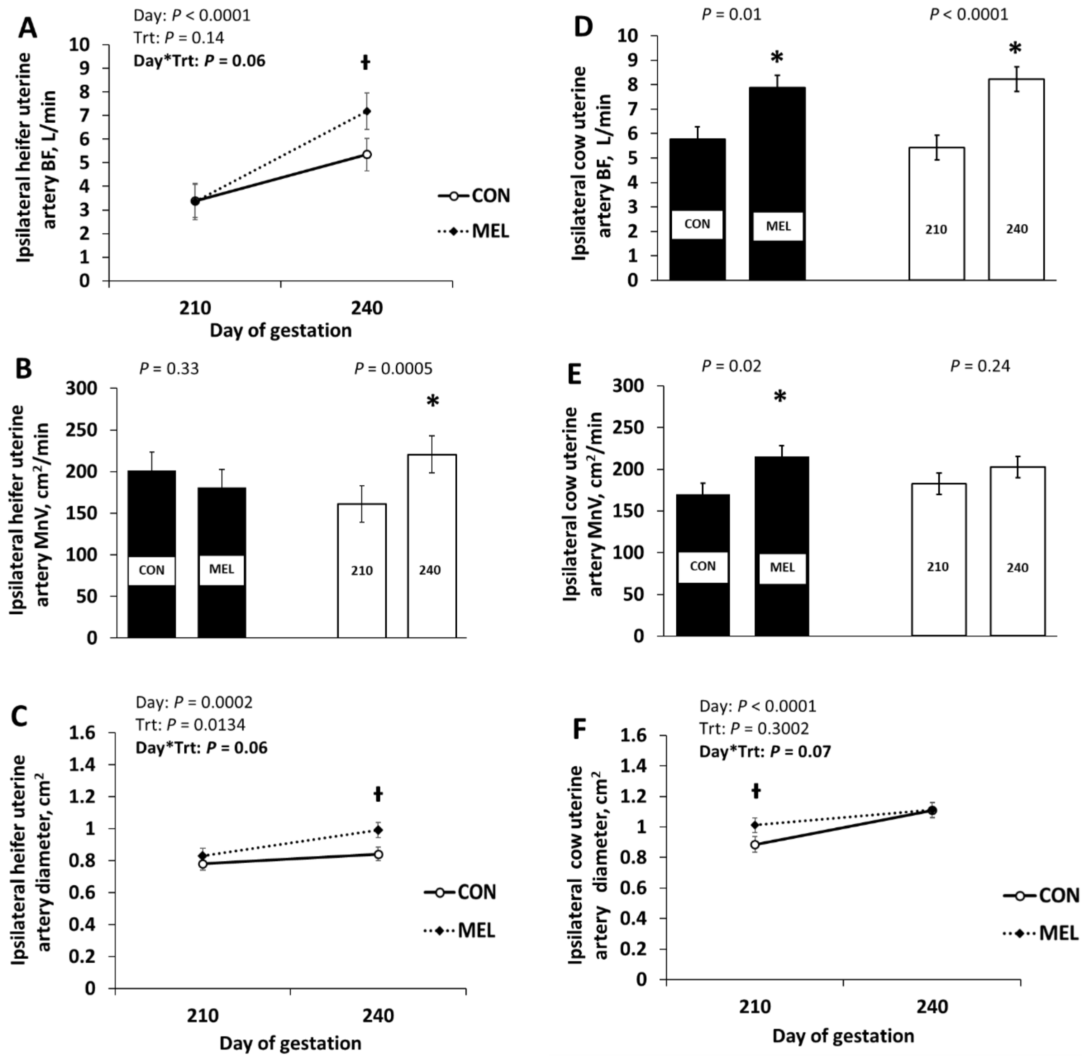

Figure 2.1 Ipsilateral uterine artery blood flow (**A**), MnV (**B**), and vessel diameter (**C**) in heifers and blood flow (**D**), MnV (**E**), and vessel diameter (**F**) in cows treated with (**MEL**) or without dietary melatonin (**CON**) from day 180 to 270 of gestation. An asterisk (*****) signifies a significant difference at $P \leq 0.05$ while a dagger (ǂ) signifies a tendency at $0.05 < P \leq 0.10$.

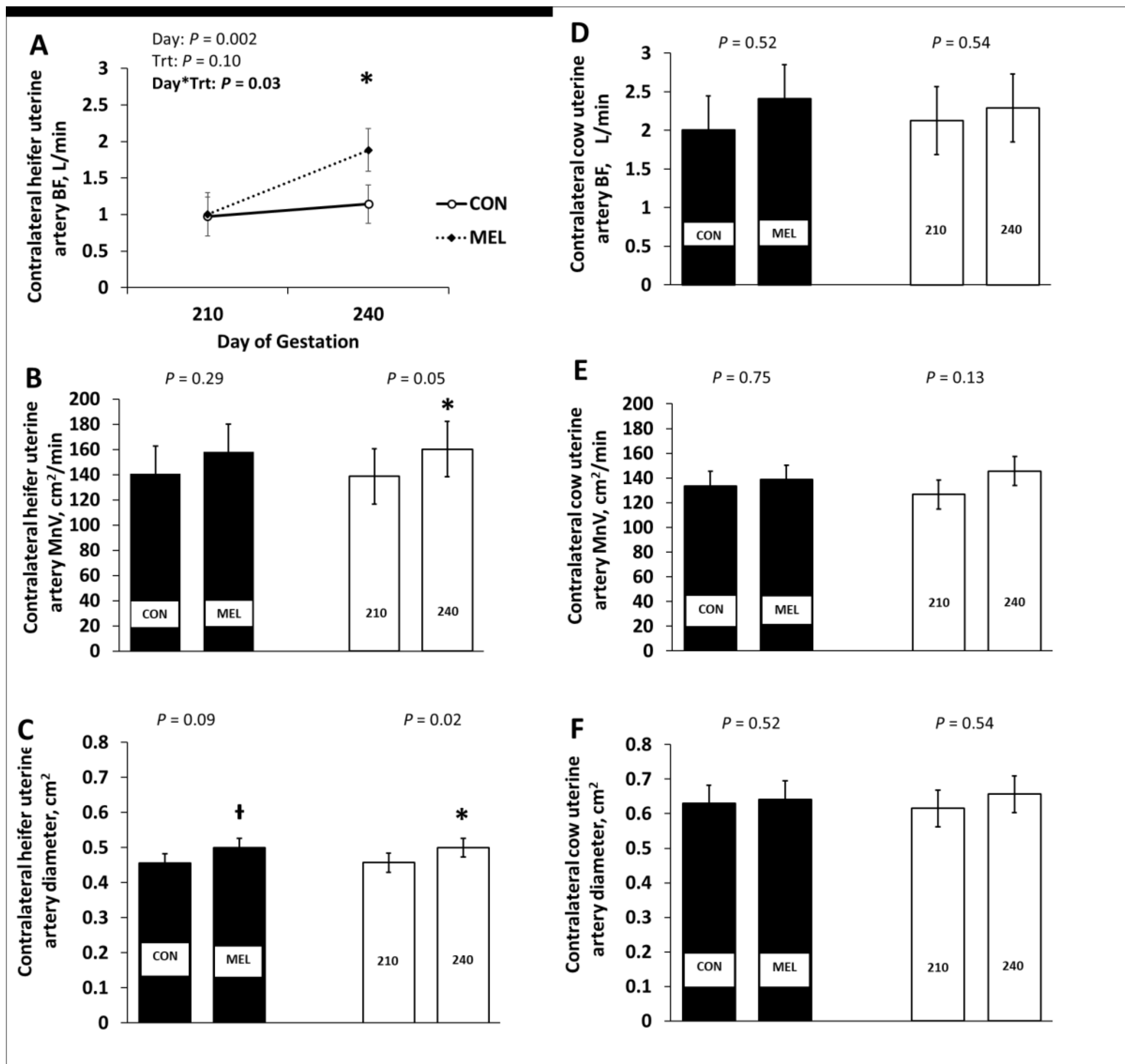

Figure 2.2 Contralateral uterine artery blood flow (**A**), MnV (**B**), and vessel diameter (**C**) in heifers and blood flow (**D**), MnV (**E**), and vessel diameter (**F**) in cows treated with (**MEL**) or without dietary melatonin (**CON**) from day 180 to 270 of gestation. An asterisk (*) signifies a significant difference at $P \leq 0.05$ while a dagger (ɫ) signifies a tendency at $0.05 < P \leq 0.10$.

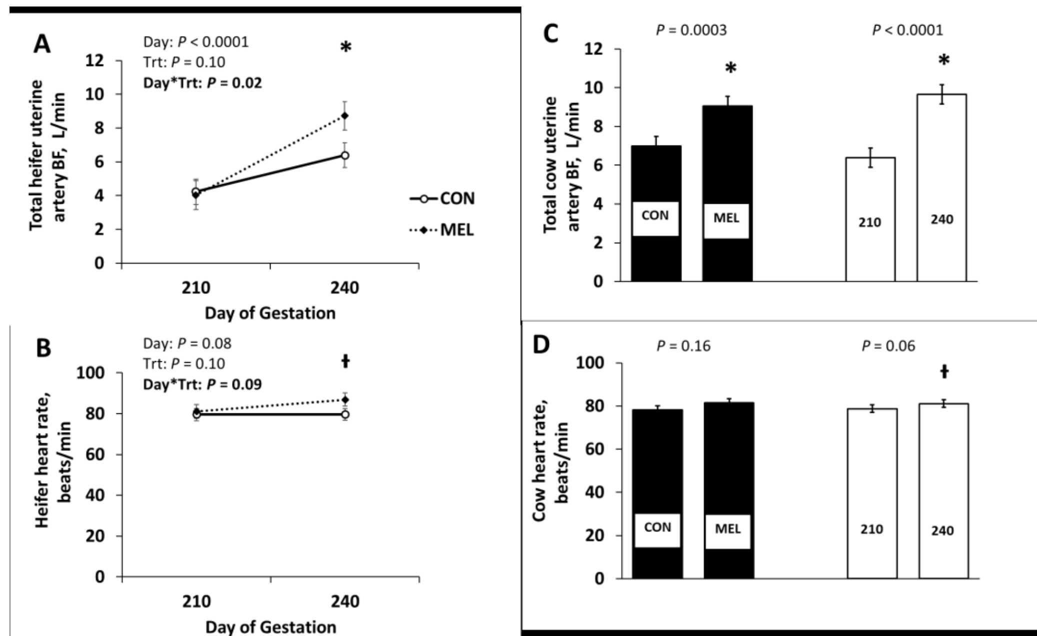

Figure 2.3 Total uterine artery blood flow (**A**) and heart rate (**B**) in heifers and blood flow (**C**) and heart rate (**D**) in cows treated with (**MEL**) or without dietary melatonin (**CON**) from day 180 to 270 of gestation. An asterisk (*) signifies a significant difference at $P \leq 0.05$ while a dagger (ƚ) signifies a tendency at $0.05 < P \leq 0.10$.

Table 2.1 Morphometric measurement of fetus' from heifers treated with (MEL; n = 6) or without melatonin (CON; n = 6) at 243 ± 2 days of gestation

Dependent Variable	CON	MEL	SE	***P* - value** Trt
BW, kg	24.0	22.9	1.9	0.561
Head circumference, cm	44.0	44.6	1.1	0.777
Head length, cm	18.4	18.0	1.1	0.719
Heart girth, cm	62.0	60.9	1.4	0.479
Abdominal girth, cm	64.6	62.3	2.7	0.424
CCR[1], cm	78.8	77.8	2.3	0.683
Ponderal index, BW [kg]/CCR [m]^3	124,150.0	124,393.0	5,364.8	0.965

[1]Curved-crown rump length (CCR)

Table 2.2 Maternal and fetal serum analysis from C-section heifers treated with (MEL; $n = 6$) or without melatonin (CON; $n = 6$) from day 180 to 243 ± 2 of gestation

Dependent Variable	CON	MEL	SE	*P* – value
				Trt
Fetal				
Melatonin, pg/mL	25.16	49.83	9.64	0.028
TAC[1], mM	0.80	0.81	0.12	0.948
Maternal				
Melatonin, pg/mL	15.76	27.34	4.22	0.021
TAC, mM	1.06	1.00	0.03	0.224

[1]Total antioxidant capacity (TAC) in serum

Table 2.3 Cotyledon and caruncle homogenate analysis from C-section heifers treated with (MEL; $n = 6$) or without melatonin (CON; $n = 6$) from day 180 to 243 ± 2 of gestation

Dependent Variable	CON	MEL	SE	*P* – value
				Trt
Cotyledon				
Nitrates, pg/mg	0.057	0.063	0.014	0.782
Caruncle				
Nitrates, pg/mg	0.026	0.023	0.004	0.570

Table 2.4 Relative transcript abundance of angiogenic factors of cotyledon and caruncles from C-section heifers treated with (MEL; $n = 6$) or without melatonin (CON; $n = 6$) from day 180 to 243 ± 2 of gestation

Dependent Variable	CON	MEL	SE	*P* – value
				Trt
Caruncle				
ANGPT1[1]	1.08	1.26	0.39	0.649
VEGF[2]	4.58	0.90	2.25	0.141
KDR[3]	4.15	1.27	2.58	0.297
FLT1[4]	0.88	1.12	0.30	0.459
Cotyledon				
ANGPT1[1]	0.87	1.41	0.29	0.100
VEGF[2]	1.10	1.14	0.17	0.810
KDR[3]	1.07	1.16	0.10	0.451
FLT1[4]	1.09	0.90	0.19	0.346

[1]Angiopoeitin1 (*ANGPT1*)
[2]Vascular Endothelial Growth Factor (*VEGF*)
[3]Kinase insert domain containing receptor (*KDR*)
[4]Fms-like tyrosine kinase (*FLT1*)

Table 2.5 Thermal imaging analysis from heifers and cows treated with (MEL; $n = 29$) or without melatonin (CON; $n = 28$) on day 180 ± 0 and 240 ± 0 of gestation

Dependent Variable	CON	MEL	SE	*P* – value
				Trt
Day 180				
BW, kg	553.81	552.16	17.26	0.924
Hair weight, g	0.27	0.26	0.03	0.911
Rectal temperature, ºC	38.38	38.21	0.17	0.336
Shaved area temperature, ºC	34.25	34.28	0.33	0.923
Unshaved area temperature, ºC	33.15	33.28	0.42	0.754
Day 240				
BW, kg	580.22	579.15	15.81	0.946
Hair weight, g	0.20	0.21	0.02	0.634
Rectal temperature, ºC	38.70	38.81	0.18	0.537
Shaved area temperature, ºC	34.42	34.06	0.34	0.277
Unshaved area temperature, ºC	33.49	32.72	0.39	0.055

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CHAPTER III

EFFECT OF MATERNAL MELATONIN SUPPLEMENTATION DURING MID- TO LATE-GESTATION ON SUBSEQUENT OFFSPRING GROWTH AND TESTICULAR DEVELOPMENT

3.1 Abstract

Melatonin is a strong antioxidant that has been found to increase postnatal calf growth when supplemented to dairy heifers during gestation. The objective of the current study was to examine the effects of chronic melatonin supplementation in mid- to late-gestating beef cattle on their offspring's subsequent postnatal growth and development, as well as offspring semen quality after sexual maturity. Commercial beef heifers *(n* = 32) and cows (*n* = 25) were artificially inseminated and assigned to one of two treatment groups supplemented with (**MEL**) or without (**CON**) melatonin delivered as two- 24 mg implants or placebo at d 180, 210, and 240 of gestation. Morphometric measurements and blood profiles were collected at birth. At weaning (195 ±2 days of age), bull calves (*n* = 15) were castrated and testicular tissue harvested for seminiferous tubule analysis. A subset of six bulls remained intact until sexual maturity for semen collection. Curved-crown rump length was increased (*P* = 0.018) and ponderal index decreased (*P* = 0.027) in calves from melatonin treated dams compared to calves from control treated dams. Birth weight, heart girth, and abdominal girth were not different (*P* > 0.05) between treatments. Blood concentrations of alkaline phosphatase were increased (*P* = 0.039) and

cholesterol decreased (*P* = 0.021) in calves from melatonin treated dams compared with calves from control treated dams. At weaning, rib eye area, rib eye area per cwt, intramuscular fat, fat thickness, and rump fat were not different (*P* > 0.05) between treatments. Seminiferous tubule diameter and area were not different (*P* > 0.05) between treatments. Semen analysis revealed the percent of distal droplets was decreased (*P* = 0.033), and total percent of sperm for motility (*P* = 0.066), progressive (*P* = 0.096), and slow sperm (*P* = 0.079) tended to be increased in bulls from melatonin treated dams compared to bulls from control treated dams. Kinematics of sperm were not different (*P* > 0.05) between treatments. In summary, melatonin supplementation from mid- to late gestation did not alter offspring birth weight, however, at weaning, bull calves born to melatonin treated dams were heavier compared to bull calves born to control dams. Additional studies investigating the impacts of gestational melatonin supplementation on the subsequent milk yield of dams are warranted.

3.2 Introduction

Postnatal growth and development hinges on the development and integration of organ systems during gestation which are influenced by genetic and environmental parameters. Programming describes the phenomenon in which a stimulus or insult introduced during a critical period of development leads to permanent consequences later in life such as structural, physiological, and metabolic changes (Godfrey, 2002). Common models of programming revolve around maternal nutrition during gestation, specifically, overnutrition or undernutrition. An adaptive change occurs when the fetal environment is deprived of nutrients with optimization of the growth of key body organs, however, these adaptions may lead to adverse effects in postnatal development (Gicquel,

2008). Any growth restriction of the fetus can lead to negative consequences in meat quality and body composition in the postnatal animal (Wu et al., 2004). Metabolic programming studies in sheep examined the impact of undernutrition during late gestation on subsequent offspring that had reached adulthood which resulted in stunted intermediary metabolism and disrupted glucose-insulin homeostasis (Gardner et al., 2005). Mossa et al. (2013) elicited the effects of maternal undernutrition of cattle during early gestation and observed diminished reproductive and cardiovascular systems in the female offspring. While programming has been extensively researched throughout prenatal and perinatal life, there is limited research on the postnatal development of male offspring in cattle.

Our laboratory has previously investigated how dietary melatonin supplementation impacts fetal growth in sheep and cattle (Lemley et al., 2012; Brockus et al., 2016b). Previously, Brockus et al. (2016b), demonstrated that dietary melatonin supplementation resulted in an increase in body weight of calves born from melatonin treated heifers compared with calves born from control heifers. This experiment treated Holstein heifers with 20 mg per day of dietary melatonin from days 190 to 262 of gestation (Brockus et al., 2016b). In male testis, exogenous melatonin up-regulates gene expression of spermatogenesis-related genes (Yang et al., 2014). The diameter of seminiferous tubules are reduced in mice injected with melatonin (Ng et al., 1990). Melatonin injections restored Sertoli cell responsiveness to follicle stimulating hormone in hamsters (Heindel et al., 1984) and acts to inhibit androgen production (Frungieri et al., 2005). We hypothesized that chronic maternal melatonin implantation during late gestation would increase progeny growth, improve carcass quality, and increase

seminiferous tubule development in the testis during postnatal development. The objective of the study was to determine the effect of maternal melatonin supplementation during mid- to late-gestation on morphometric measurements at birth and carcass characteristics at weaning while male offspring were evaluated for semen quality following sexual maturity.

3.3 Materials & Methods

3.3.1 Animal Care and Treatments

Animal care and use were according to protocols approved by the Mississippi State University Institutional Animal Care and Use Committee (#16-036).

The establishment of pregnancy and maternal treatments were previously outlined in McCarty et al. (2018). Briefly, animals were bred using a timed artificial insemination (TAI) protocol where heifers (*n* = 87) were bred on December 10, 2015 and cows (*n* = 65) on December 21, 2015. Breeds consisted of 8 Hereford, 28 Angus, and 21 crossbred. Treatments included a melatonin implant (**MEL**; *n* = 29) or a no melatonin implant control (**CON**; *n* = 28) starting on day 180 of gestation and ending on day 270. Melatonin treatment consisted of two sub-dermal ear implants containing 24 mg of melatonin each (Melatonin Implants, Conroe, TX, USA) to slow release melatonin into peripheral circulation over a 30-day period. Implants were administered every 30 days, occurring on day 180, day 210, and day 240 of gestation. The last implant, at day 240 of gestation, was expected to provide an increased concentration of melatonin until day 270 of gestation. Dams did not receive any further treatment after final administration at day 240. Non-treatment controls involved sticking the ear with a sterilized needle.

At birth, calves of melatonin treated dams (*n* = 23) and control treated dams (*n* = 22) were all given tag and tattoo identification, naval iodine dip, and evaluated for overall health. Postnatal body weight was collected on 0 (12 – 24 hours after birth) and 195 days of age. Blood samples were collected via venipuncture of the jugular vessels on days 0 (birth). Morphometric measurements recorded included heart girth, abdominal girth, hip height, and curved-crown rump length (CCR). Ponderal index was calculated from CCR and body weight measurements as (BW [kg]/CCR $[m]^3$).

Two calves, one from a melatonin treated dam and one from a control treated dam were lost due to predation. Two sets of twins, one from a melatonin treated dam and one from a control treated dam were removed from the statistical analysis. Additional calves removed from the study due to illness or were lost due to health-related issues (CON *n* = 3; MEL *n* = 4).

3.3.2 Blood Analysis

Blood samples were collected via venipuncture of the jugular vessels at day 0 (birth). A 2 mL aliquot of serum from calves (*n* = 37) were sent to Clinical Pathology at the Mississippi State University for a large animal profile.

3.3.3 Carcass Characteristics

At 180 ± 2 days of age, remaining calves (*n* = 35) underwent ultrasonography to evaluate carcass characteristics. An ALOKA 500V ultrasound machine and Beef Image Analysis (BIA) software (Designer Genes Technologies, Inc.; Harrison, AR, USA) was used in collection of ultrasound images and images were collected by an Ultrasound Guidelines Council certified field and lab technician. Rib eye area (REA), percent

intramuscular fat (IMF), and rump fat were evaluated on the ribs of the right thoracic side of the animal.

3.3.4 Castration

Male offspring (n = 15) were castrated at 195 ± 2.3 days of age. Calves were restrained in a squeeze chute and scrotal circumference was measured prior to castration. An epidural administration of lidocaine (0.17 mg/kg) was performed. The distal one-third of the scrotum was transected, and each testicle removed by slow, continuous traction. Exposed scrotal area was sprayed with commercially available wound spray. Calves received an oral dose of 1 mg/kg of Meloxicam before leaving the chute. Testicular morphometrics such as weight, average length, and total width were taken immediately following castration.

3.3.5 Testicular Analysis

A cross section of testicular parenchyma was placed in a microvette histology cassette (Thermo Fisher Scientific, Auburn, AL, USA). Cassettes were placed in 10% formalin for short-term storage. Tissue sections were prepared for histological processing. Briefly, paraffin sections were sliced into 5 µm sections then stained with hematoxylin eosin to determine seminiferous tubule diameter and area. Approximately 100 circular seminiferous tubules were measured and averaged per animal.

3.3.6 Breeding Soundness Evaluations and Sperm Analyses

Intact bulls from dams treated with (MEL; n = 2) or without melatonin (CON; n = 4) underwent breeding soundness exams and semen collection at approximately 399 ± 4, 416 ± 4, and 456 ± 4 days of age. Bulls underwent a physical examination of internal and

external reproductive structures, measurement of scrotal circumference, and collection and evaluation of semen.

Ejaculate was collected via electroejaculation and the gel-free fraction of the ejaculate was evaluated for sperm concentration and percentage of motile sperm. Additionally, sperm concentration was evaluated using the SpermaCue Photometer (Minitube of America, Mount Horeb, WI).

Using a fresh sample, sperm motility was evaluated by a Computer-Assisted Sperm Analyzer (CASA; HTM-IVOS Hamilton-Thorne Biosciences, Version 12.3, Beverly, MA, USA) as described previously (Palmer and Magistrini, 1992). Briefly, sperm aliquots (80×10^6 spermatozoa/ml) were diluted then loaded (2 µl) into pre-warmed caffeine-free microscope chamber slides (Standard Count 4-chamber Slide Leja®, 20 microns, Nieuw Vennep, The Netherlands). Four fields per chamber-slide were used to analyze the motility characteristics of spermatozoa from each treatment group, using CASA. A total of 16 fields (2 replicates × 2 doses of semen × 4 fields/chamber), averaging 423 ± 10 spermatozoa per field were considered for analyses, using pre-set values of the CASA machine (60 frames/sec; VAP and STR of progressive cells: 45 µm/sec and 45%, respectively; VAP of static cells: 4 µm/sec; VAP and VSL cutoffs of slow cells: 20 and 5 µm/sec, respectively; magnification: 1.3×, and temperature of 37°C).

3.3.7 Statistical Analysis

Calf morphometric measurements, carcass characteristics, and blood profiles were analyzed using ANOVA (SAS software version 9.4, SAS Institute, Cary, NC, USA). The model statement included: treatment, breed, and calf sex. The gestation length of each calf served as a covariate. Least square means and standard error of the means are

reported. Testicular morphometrics and seminiferous tubule measurements were analyzed using ANOVA (SAS software version 9.4, SAS Institute, Cary, NC, USA). The model statement included: treatment and breed. Sperm analysis measurements were analyzed using ANOVA (SAS software version 9.4, SAS Institute, Cary, NC, USA). The model statement included: treatment, breed, and collection number. Statistical significance was declared at $P \leq 0.05$ while a tendency was declared at $0.05 < P \leq 0.10$.

3.4 Results

3.4.1 Morphometric Measurements

Curved-crown rump length was increased ($P = 0.018$) and ponderal index decreased ($P = 0.027$) in calves from MEL-treated dams compared to calves from CON-treated dams (Table 3.1). Hip height tended to increase ($P = 0.061$) in calves from MEL-treated dams compared to calves from CON-treated dams (Table 3.1). Birth weight averaged 29.2 ± 1 kg and was not different ($P = 0.615$) in calves from MEL- treated dams compared to calves from CON-treated dams (Table 3.1). Heart girth and abdominal girth were not different ($P > 0.05$) between treatments (Table 3.1).

3.4.2 Blood Analysis

Alkaline phosphatase concentrations in serum were increased ($P = 0.039$) in calves from MEL-treated dams compared with calves from CON-treated dams (Table 3.3). Cholesterol concentrations in serum were decreased ($P = 0.021$) in calves from MEL-treated dams compared with calves from CON-treated dams (Table 3.3). Creatine kinase concentrations in serum tended to be decreased ($P = 0.095$) in calves from MEL-treated dams compared with calves from CON-treated dams (Table 3.3). Peripheral blood

concentrations were not different ($P > 0.05$) for sodium, potassium, chloride, CO_2, anion gap, glucose, blood urea nitrogen, creatine, asparate amino transferase, gamma-glutamyl transferase, total protein, albumin, globulin, albumin:globulin, calcium, phosphorus, osmolarity, total bilirubin, and magnesium in calves from MEL-treated dams compared with calves from CON-treated dams (Table 3.3).

3.4.3 Carcass Characteristics

At weaning, body weight was increased ($P = 0.046$) in calves from MEL-treated dams compared to calves from CON-treated dams (Table 3.2). Rib eye area, intramuscular fat, fat thickness, and rump fat were not different ($P > 0.05$) between treatments (Table 3.2).

3.4.4 Testicular Morphometrics and Seminiferous Tubule Analysis

At castration, body weight was increased ($P = 0.006$) in bull calves from MEL-treated dams compared to bull calves from CON-treated dams (Table 3.4). Scrotal circumference was increased ($P = 0.032$) in calves from MEL-treated dams compared to calves from CON-treated dams (Table 3.4). Total testis weight, average length, and total width were not different ($P > 0.05$) between treatments (Table 3.4). A representative image of seminiferous tubules from bull calves can be found in Fig. 3.1. Seminiferous tubule diameter and area were not different ($P > 0.05$) between treatments (Table 3.4).

3.4.5 Semen Analysis

Number of spermatozoa per collection was not different ($P = 0.148$) between treatments in bulls from MEL-treated dams compared to bulls from CON-treated dams and averaged 372.1 ± 19 spermatozoa x 10^6/mL (Table 3.5). Total percent of sperm for

motility ($P = 0.066$), progressive ($P = 0.096$), and slow sperm ($P = 0.079$) tended to be increased in bulls from MEL-treated dams compared to bulls from CON-treated dams (Table 3.5). The velocity average path, straight line velocity, curvelinear velocity, straightness, linearity, beat cross frequency, lateral head displacement, and wobble were not different ($P > 0.05$) between treatments (Table 3.5). Percent of distal droplets was decreased ($P = 0.033$) in bulls from MEL-treated dams compared to bulls from CON-treated dams (Table 3.5). Percent of proximal droplet, bent tail, and coiled tail were not different ($P > 0.05$) between treatments (Table 3.5). Scrotal circumference was not different ($P > 0.05$) between treatments and averaged 30.9 ± 10.2 cm (data not shown).

3.5 Discussion

We have previously observed that melatonin increased uterine artery blood flow in melatonin treated compared with control treated cows, but no difference in fetal body weights between treatments were observed (McCarty et al., 2018). In the current study, birth weights of beef calves were similar, irrespective of treatment with or without maternal melatonin supplementation. However, at weaning and castration, bull calves born to melatonin treated dams were heavier compared to bull calves born to control dams. This supports previous findings of Brockus et al. (2016b) where dairy calves born from melatonin treated heifers had similar birth weights and an increase in calf growth compared to calves born from control heifers. In contrast to our previous work with dairy heifers, beef calves from the current study were allowed to remain with the dam until weaning whereas previously the calves of Holstein heifers were immediately removed and fed milk replacer (Brockus et al., 2016b). Milk yield of the dam could contribute to the increase in body weight of the calves, which will be investigated in future studies.

In humans, ponderal index is used for newborns to calculate wasting or disproportionate growth in addition to assessment of intrauterine growth restriction (Vintzileos et al., 1986). Similarly, this index is used to evaluate any growth retardation in sheep (Dwyer et al., 2005) and pigs (Poore et al., 2002). In the current study, curved-crown rump length was increased and ponderal index decreased in calves from melatonin treated dams compared to calves from control treated dams at birth. Overall, calves exposed to chronic supplementation of melatonin in utero were longer and leaner compared to calves from control treated dams, with no difference in body weight between treatments.

At birth, increased alkaline phosphatase and decreased cholesterol concentrations were observed in calves from melatonin treated dams compared with calves from control treated dams. Alkaline phosphatase serves as a dephosphorylating enzyme in the body and plays a role in liver metabolism and bone development. Abnormally high levels of alkaline phosphatase could be indicative of issues such as liver disease or bone disorders. Additionally, primordial germ cells are rich in alkaline phosphatase (Setchell, 1978). Cholesterol is an abundant sterol in the body that serves as the primary 'parent' compound for steroidogenesis, and thus downstream production of sex hormones. Cholesterol's relationship with adiposity in mammals has been studied in humans (Walker et al., 1953), rabbits (Lukefahr et al., 1989), pigs (Rauw et al., 2012), horses (Frank et al., 2006), sheep (Bell et al., 1989), and cattle (Rule et al., 2002). Inhibition of cholesterol synthesis is teratogenic in nature. Decreased cholesterol concentrations reflect the decreased ponderal index of calves from this study. Moreover, melatonin

supplementation in utero may be involved in decreased cholesterol synthesis and the leanness of the offspring.

Offspring carcass characteristics near weaning were not different between maternal treatments. Findings by Zinn et al. (1988) observed an increased percent of rib fat and longissimus muscle in beef heifers fed 4 mg/100 kg body weight from 58 to 133 days. However, during the current study when calves born from dams treated with melatonin, no difference in muscle size or fat thickness was observed.

Melatonin receptors have been found in the testis of many species such as the mouse, rat, and human (Liu et al., 2009; Vargas et al., 2011; Zhang et al., 2012). In utero development of testis begin shortly after sexual differentiation due to the presence of the SRY protein derived from the Y chromosome. As the male gonad is formed, testosterone secretion aids in cell differentiation to form Sertoli and Leydig cells. The sex cords transform into seminiferous tubules which houses the Sertoli cells and serves as the primary site of spermatogenesis. During postnatal development of testis, a 10 fold increase in weight from birth to 3 months of age (Michatach, 1933) and a 4 fold increase in tubule diameter from birth to 18 months of age in Hostein bulls occur (Fossland, 1954). The seminiferous tubules of beef bulls finish developing postnatally by 30 weeks of age (Evans et al., 1996). In Holstein bulls, the most Sertoli cells existed at 27 weeks of age followed by a decline (Al-Haboby, 1986).

At time of castration, scrotal circumference was increased in calves from melatonin treated dams compared to calves from control treated dams. When breeding soundness exams were performed, scrotal circumference was not different irrespective of treatment, breed, or time of collection. Moreover, testicular morphometrics and

seminiferous tubule diameter and area were not different between treatments. Conversely, previous observations of tubule diameter observed an increase with age and a close relationship between increased testis weight and tubule diameter (Abdel-Raouf, 1960). Lodhi (1987) reported the seminiferous tubule diameter 123.2 µm at 190 days of age in Holstein bull calves, whereas the seminiferous tubule diameter at 195 days of age in Angus bull calves from this study averaged 154 ± 40 µm.

Melatonin has been proposed as a recovering agent for testicular damage, but limited studies have evaluated the direct action of melatonin on testicular histology in cattle. For example, in sheep melatonin reduces oxidative injury in seminiferous tubules that occurs during transplantation (Gholami et al., 2017). Prior experiments in mice examined similar histological properties and determined melatonin reduced harmful effects on seminiferous tubule structure due to the freezing and re-thawing process (Gholami et al., 2015). Sertoli cells play a significant role in spermatogenesis (Gholami et al., 2017) within the seminiferous tubules. Melatonin receptors MT1 and MT2 had been found in bovine Sertoli cells in addition to exogenous melatonin up-regulates gene expression of spermatogenesis-related genes (Yang et al., 2014). In hamsters, melatonin injections restored Sertoli cell responsiveness to follicle stimulating hormone (Heindel et al., 1984) and acts to inhibit androgen production (Frungieri et al., 2005). Essentially, exogenous melatonin supplementation impacts regulation of development of bovine Sertoli cells and seminiferous tubules.

Morphology of the sperm cells exhibited a decrease in the percent of distal droplets in bulls from melatonin treated dams compared to bulls from control treated. While percent of proximal droplet, bent tail, and coiled tail were not different between

treatments. A reduction in secondary abnormalities, such as proximal and distal droplets, would allow for a higher instance normality of the sperm and increased chance of bulls passing their BSE. Increased occurrence of droplets have been associated with younger animals that are still reaching sexual maturity. Distal droplets are established when the proximal droplet cannot be shaken free as the sperm is maturing and developing in the epididymis (Cooper et al., 2005). Thus, maternal melatonin may partake in the reduction of abnormalities when subsequent offspring begin producing sperm. Similarly, in a study by Koskal and colleagues (2012), rats with testicular ischemia injuries had reduced sperm abnormalities due to melatonin treatment. Sperm analysis for motility indicated a tendency for the percent of sperm motility, progressive, and slow movement to be increased in bulls from melatonin treated dams compared to bulls from control treated dams. Kinematics of sperm and motion of the sperm head did not differ between treatments. Whereas in Ashrafi et al. (2011), ram spermatozoa exposed to melatonin had increased motility following freezing. In terms of preservation of sperm, melatonin has been observed to improve the quality of thawed bovine sperm (Ashrafi et al., 2013). Melatonin administration in humans produced no change in semen quality, but did cause reduced semen concentration (Luboshitzky et al., 2002). Furthermore, abnormal melatonin concentrations in serum and semen have been associated with the sperm parameters and hormonal profile of infertile men (Awad et al., 2006).

In summary, melatonin supplementation from mid- to late gestation did not alter subsequent offspring birth weight, however, bull calves born to melatonin treated dams were heavier compared to bull calves born to control dams at weaning. This response could be related to fetal programming brought about by an increase in uterine blood flow

or it could be related to an increase in milk output from dams treated with melatonin during their pregnancy. At birth, decreased cholesterol concentrations and decreased ponderal index of calves were observed. While calves at weaning differed in body weight between treatments, no differences in carcass quality parameters were observed. Finally, in utero melatonin supplementation did not alter testicular development, specifically, the seminiferous tubules. Moreover, bulls that reached sexual maturity experienced decreased distal droplets when subjected to maternal melatonin supplementation. Compared to previous studies in our laboratory involving the impacts of melatonin supplementation, additional studies are warranted to determine if milk yield of the dams contributed to the change in body weight and composition observed in subsequent offspring growth. Moreover, larger number of offspring will need to be studied during development to fully understand these effects.

Table 3.1 Morphometric measurement of calves from heifers and cows treated with melatonin (MEL; *n* = 18) or without (CON; *n* = 20), analyzed at birth

				***P* - value**
Dependent Variable	**CON**	**MEL**	**SE**	**Trt**
BW, kg	29.2	30.3	1.9	0.615
Heart girth, cm	183.1	185.7	4.6	0.578
Abdominal girth, cm	201.7	203.7	5.8	0.727
CCR[1], cm	199.4	214.4	5.8	0.018
Hip height, cm	182.4	191.3	4.6	0.061
Ponderal index, BW [kg]/CCR [m]^3	61,699.0	53,032.0	3,712.7	0.027

[1]Curved-crown rump length (CCR)

Table 3.2 Carcass characteristics of calves from heifers and cows treated with melatonin (MEL; *n* = 16) or without (CON; *n* = 17), analyzed at 180 ± 2 days of age

				***P* - value**
Dependent Variable	**CON**	**MEL**	**SE**	**Trt**
BW, kg	188.89	200.67	5.60	0.046
Rib eye area, cm^2	41.39	43.78	1.72	0.179
Intramuscular fat, %	2.68	2.96	0.21	0.198
Fat thickness, cm	0.23	0.27	0.02	0.116
Rumpfat, cm	0.28	0.30	0.03	0.517

Table 3.3 Serum analysis of calves at birth from heifers and cows treated with (MEL; *n* = 17) or without melatonin (CON; *n* = 18) from day 274 ± 1 of age

				***P* - value**
Dependent Variable	**CON**	**MEL**	**SE**	**Trt**
Sodium	141.0	139.7	1.2	0.278
Potassium	4.6	4.6	0.2	0.734
Chloride	104.7	103.2	1.1	0.199
CO_2	20.7	21.9	1.0	0.258
ANGAP[1]	20.2	19.3	0.9	0.347
Glucose	98.7	100.8	8.9	0.815
BUN[2]	12.4	10.6	1.1	0.123
Creatine	1.7	1.6	0.1	0.517
AST[3]	59.4	55.6	4.7	0.425
ALP[4]	360.0	495.2	62.6	0.039
GGT[5]	735.4	973.3	232.5	0.314
Total protein	7.0	7.4	0.5	0.402
Albumin	2.1	2.0	0.1	0.146
Globulin	4.9	5.4	0.4	0.341
AG[6]	0.5	0.4	0.1	0.817
Calcium	11.2	11.2	0.2	0.791
Phosphorus	6.9	7.1	0.3	0.536
OSMO[7]	272.1	269.3	2.2	0.215
Tbili[8]	1.2	1.1	0.2	0.655
CK[9]	159.3	131.1	16.3	0.095
Magnesium	2.2	2.2	0.1	0.833
Cholesterol	39.9	34.2	2.4	0.021

[1]Anion Gap (ANGAP)
[2]Blood Urea Nitrogen (BUN)
[3]Asparate Aminotransferase (AST)
[4]Alkaline Phosphatase (ALP)
[5]Gamma-glutamyl Transferase (GGT)
[6]Albumin:Globulin (AG)
[7]Osmolality (OSMO)
[8]Total bilirubin (Tbili)
[9]Creatine Kinase (CK)

Table 3.4 Testicular morphometrics on calves from heifers and cows treated with melatonin (MEL; n = 6) or without (CON; n = 9), castrated at 195 ± 2 days of age

Dependent Variable	**CON**	**MEL**	**SE**	***P* - value** **Trt**
BW, kg	195.5	221.5	7.3	0.006
Scrotal circumference, cm	18.7	19.6	0.4	0.032
Testis				
Total weight, g	129.6	148.0	11.0	0.128
Average length, mm	68.4	71.0	3.2	0.424
Total width, mm	66.3	70.5	2.5	0.113
Seminiferous tubule				
Diameter, µm	157.4	148.2	11.1	0.428
Area, μm^2	20,228.0	19,098.0	2,285.6	0.633

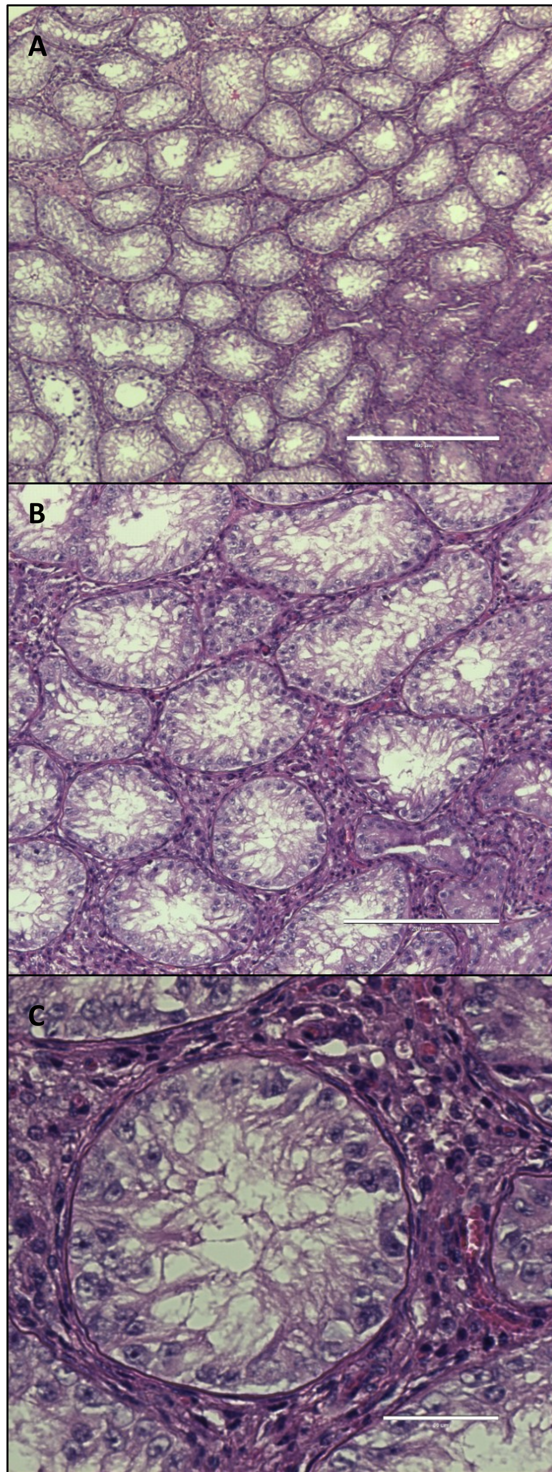

Figure 3.1 A representative image of d 195 seminiferous tubules in bull calves at 195 ± 2 days of age. The white scale bar is (**A**) 400 µm (**B**) 200 µm (**C**) 60 µm

Table 3.5 Average collection sperm analysis of bulls from heifers and cows treated with melatonin (MEL; n = 2) or without (CON; n = 4)

Dependent Variable	CON	MEL	SE	Range		*P* - value
				MIN	MAX	Trt
Count, spermatozoa x 10^6/mL	386.9	357.2	19.3	281.9	458.6	0.148
Motility						
Motility, %	57.0	71.4	7.2	43.6	76.1	0.066
Progressive, %	42.7	53.6	6.1	28.9	70.8	0.096
Slow, %	4.6	8.2	1.9	2.1	16.3	0.079
Kinematics						
VAP[1], µm/s	91.5	91.3	5.8	78.4	105.8	0.980
VSL[2], µm/s	83.4	83.6	5.1	71.4	100.8	0.973
VCL[3], µm/s	135.8	132.2	10.4	124.2	143.2	0.735
STR[4], %	87.8	88.8	1.6	81.2	94.5	0.565
LIN[5], %	60.5	62.8	2.6	50.8	76.0	0.388
BCF[6], Hz	36.6	34.9	1.9	33.1	41.5	0.402
ALH[7], µm	5.2	4.8	0.5	3.7	6.3	0.485
WOB[8], %	67.3	69.4	2.1	60.4	79.9	0.324
Morphology						
Proximal droplet, %	8.7	5.8	2.9	1.2	20.5	0.356
Distal droplet, %	9.0	6.3	1.1	3.2	14.1	0.033
Bent tail, %	3.9	2.7	0.9	1.9	5.3	0.178
Coiled tail,%	1.7	1.3	2.3	0.3	3.7	0.876

[1]Velocity average path (VAP)
[2]Straight line velocity(VSL)
[3]Curvelinear velocity (VCL)
[4]Straightness (STR)
[5]Linearity (LIN)
[6]Beat cross frequency (BCF)
[7]Amplitude lateral head displacement (ALH)
[8]Wobble (WOB)

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CHAPTER IV

GENERAL DISCUSSION

Melatonin is a strong antioxidant that serves several physiological functions in the body, particularly a stimulant of vasodilation or vasoconstriction on vasculature throughout the reproductive tract (Reiter et al., 1995). Additionally, melatonin's ability to scavenge free radicals and act on reactive oxygen species (Halliwell & Gutteridge, 1999; Thakor et al., 2010) has the potential to protect the dam, placenta, and fetus from damage to the cell membrane during pregnancy (Reiter et al., 2009). The placenta is the transient organ that supports the developing fetus and serves to provide nutrients, waste removal, gas exchange, and hormone production. The efficiency of placental transport is directly related to uteroplacental blood flow that plays a role in successful growth and development of the fetus (Reynolds & Redmer, 1995; Reynolds et al., 2010; Vonnahme et al., 2012; Lemley et al., 2014). Placental function may be insulted by oxidative stress during pregnancy and induce disorders or disrupt fetal development. Livestock species are susceptible to an abundance of ROS overwhelming the body throughout gestation due to environmental factors such as heat stress, hypoxia, or metabolic disorders impeding endogenous production of enzymes responsible for ROS defense (Gospodaryov & Lushchak, 2012). Chronic oxidative stress may induce disorders such as preeclampsia or hypertension that could attribute to increased neonatal morbidity and mortality or decreased birth weights (Reiter et al., 2009; Kais et al., 2010; Uetake, 2013).

Previously, dietary melatonin supplementation has been observed to increase umbilical blood flow in sheep and uterine blood flow in dairy heifers when supplemented from mid- to late gestation (Lemley et al., 2012; Brockus et al., 2016a). Furthermore, calves from melatonin treated dairy heifers displayed increased calf growth and body weight compared with calves from control treated dairy heifers (Brockus et al., 2016b). Although, birth weights of the calves from melatonin treated and control treated dairy heifers did not differ, regardless of the increase in uterine blood flow (Brockus et al., 2016b).

In this study, melatonin administration from mid- to late gestation increased total uterine artery blood flow compared with control treated heifers and cows. However, increased uterine blood flow was not accompanied by an increase in fetal weight. While melatonin concentrations were increased in maternal and fetal circulations, there was no concomitant increase in total antioxidant capacity as would be expected. Placental gene expression of angiogenic factors did not differ between treatment groups; however, histology of the placenta may warrant further investigation to observe if capillary density would be affected by melatonin supplementation.

While maternal melatonin supplementation did not alter subsequent offspring fetal weight or birth weight, but it did alter other indicators of offspring size at birth such as CCR and ponderal index. Although, bull calves born to melatonin treated dams were heavier compared to bull calves born to control dams at weaning. This response could be related to fetal programming brought about by the increase in uterine blood flow or it could be related to an increase in milk output from dams treated with melatonin during their pregnancy. At birth, decreased cholesterol concentrations reflected decreased

ponderal index of calves as inhibition of cholesterol synthesis which may be associated with the increased leanness of melatonin treated calves. While calves at weaning differed in body weight between treatments, no differences in carcass quality were observed. Finally, *in utero* melatonin supplementation did not alter testicular development, specifically, the seminiferous tubules. Though, bulls that reached sexual maturity experienced decreased distal droplets when subjected to maternal melatonin supplementation.

Studies of melatonin in cattle for altering hemodynamics is lacking compared to other areas of livestock research. Further research on placental vascularization and the mechanism in which melatonin impacts angiogenic factors is necessary to understand the relationship between melatonin and the compensatory growth that occurs postnatally. Compared to previous studies in our laboratory involving the impacts of melatonin supplementation, additional studies are warranted to determine if milk yield of the dams contributed to the change in body weight and composition observed in subsequent offspring growth. Further investigation is warranted as to how *in utero* melatonin administration may program male development and affect semen quality in subsequent offspring once they have reached sexual maturity.

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