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Investigating Melatonin Supplementation on Maternal Hemodynamics and Offspring Programming

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Investigating melatonin supplementation on maternal hemodynamics and offspring programming

By

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Investigating melatonin supplementation on maternal hemodynamics

and offspring programming

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The objective was to examine effects of melatonin supplementation during late gestation on uterine artery hemodynamics, offspring growth, and endocrine profiles. Prior to day 170 of gestation, heifers were trained to the Calan feeding system. On day 190 of gestation, heifers (n = 20) were blocked by BW and then randomly assigned to one of two dietary treatments: 1) 20 mg of dietary melatonin per day (MEL) or 2) no melatonin supplementation (CON). Supplementation ceased on day 262 of gestation. A main effect ($P < 0.01$) of treatment was observed for total uterine artery blood flow with it being increased in MEL vs. CON. An interaction ($P = 0.008$) was observed in calf body weight increasing at weeks 8 and 9 in MEL vs. CON. Dietary melatonin could be used to potentially increase uterine blood flow and calf body weight.

Key Words: melatonin, gestation, uterine hemodynamics, antioxidants

DEDICATION

I would like to dedicate this thesis to my grandpa, Melvin Stockard. He was always someone that I looked up to and admired. Even though he is no longer with us, I hope that I made him proud. I would like to thank my parents, Larry and Lori Brockus, for all the love and support they have shown throughout my time at Mississippi State. They have always been my biggest fans and never failed to show endless amounts of encouragement. A thank you also goes out to my grandma, Lorene Stockard, for all the phone calls and frozen homemade meals that got me through my time away from home. Thank you for supporting me since day one when I decided to move to Mississippi. Thank you to my brothers, sister, nieces, and nephews for always being there for me when times were tough. Thank you to Dr. Brandi Karisch, Matt Karisch, and baby Karisch for being my Mississippi cattle family. A special thank you is addressed to Dr. Andrea Sexten for introducing me to graduate school and being a great mentor to me. I cannot thank her enough for leading me in the right direction and reminding me that with God all things are possible. I would like to thank all of my friends for their love and support. Even though hundreds of miles were between us, you guys were always just a phone call away. Without each and every one of you, this would not have been possible.

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CHAPTER I

REVIEW OF LITERATURE

Melatonin

American physician, Aaron B. Lerner, at Yale University School of Medicine, first discovered melatonin in 1958. Melatonin (N-actyl-5-methoxytryptamine) is a hormone that is secreted by the pineal gland, which is located at the center of the brain. Melatonin has various effects on the physiological system, which is illustrated in Fig. 1. Melatonin plays an important role in circadian rhythm and is influenced by light and dark detections by the retina (Richter et al. 2009). Melatonin increases blood flow to the fetus (Lemley et al. 2013). This hormone is unique as it has the ability to protect the body against free radicals via direct or indirect pathways (Reiter et al. 1995). Melatonin can bind directly to cellular membranes and aid in the stabilization of the membrane against possible oxidative damage, and it has the ability to protect cells indirectly by helping the body up-regulate its own antioxidant defense system (Reiter et al. 1995). Imbalances between reactive oxygen specie production and antioxidant systems could have a negative impact by creating oxidative stress which, in turn, can negatively impact the reproductive process. With reactive oxygen specie levels being increased during pregnancy, oxidative stress is directly related to several pregnancy disorders, such as spontaneous abortions, embryopathies, preeclampsia, fetal growth restriction, preterm labor, and low birth weight (Kaïs et al. 2010). The links between oxidative stress, the

female reproductive system, and the development of negative pregnancy outcomes results in important concerns in both human and animal reproductive sciences (Kaïs et al 2010).

Fetal Programming

Seventy-five percent of the growth of the ruminant fetus occurs during the last two months of gestation (Robinson et al. 1977). With this in mind, researchers hypothesized that variation in maternal intake would have effects on a variety of production characteristics during late gestation (Funston et al. 2009). Findings by Larson et al. (2009) observed that late gestation protein supplementation and increased global nutrient supply increased calf weaning weights from 225 kg in the non-supplemented to 247 kg in the protein supplemented dams. Martin et al. (2007) also provided a protein supplement during the last one-third of gestation but birth weights of calves were not different from those that were not supplemented. However, weights pre-breeding were greater for heifers from protein-supplemented dams (Martin et al. 2007).

Most fetal programming studies use the model of undernutrition or overnutrition during a critical window of gestation. Restriction during early gestation appears to affect placental development and vascularity, where restriction during late gestation is likely affecting final development of organ systems and nutrient uptake by tissues that are important for growth and reproduction (Vonnahme et al. 2007; Lemley et al. 2012).

A study by Nissen et al. (2003) in pregnant sows had three treatment groups, which included 1) control feeding, 2) ad libitum feeding from day 25 to 50 of gestation, and 3) ad libitum feeding from day 24 to 70 of gestation. This study found no effect of treatment on meat quality traits in the offspring (Nissen et al. 2003). With these findings, it is clear that increasing maternal nutrition does not have a programming effect on the

offspring for either increased meat quality traits or growth characteristics. The fetal period is critical for skeletal muscle development as there is no net increase in the number of muscle fibers following birth (Glore & Layman 1993; Greenwood et al. 2000; Nissen et al. 2003).

While undernutrition can alter uteroplacental development, overfeeding is also of great alarm as some of the same physiological and endocrine responses occur. Reynolds et al. (2006) explains that experimental conditions designed to investigate intrauterine growth restriction and placental efficiency, whether it be through overnutrition, nutrient restriction, hyperthermia, or high altitude, commonly cause reduced uterine and umbilical blood flow. In the overfed pregnant adolescent ewe, uterine blood flow was reduced by 56% at day 90 of gestation, which was before any reduction in fetal or placental weights were observed (Wallace et al. 2005). Thus, overfeeding the dam can lead to a reduction in blood flow, which could have a permanent response in the blood flow of the offspring.

Nitric oxide is an endothelium derived vasorelaxing factor that plays a key role in the regulation of uteroplacental blood flow (Bird et al. 2003). When free radicals exceed the body's antioxidant production capacity, the result is oxidative stress (Castillo et al. 2004). Specifically in dairy cattle, the peripartum and early lactation periods are especially crucial and present considerable physiological challenges to homeostasis by imposing significant metabolic stressors that could contribute to the onset of various disorders (Goff & Horst 1997). Castillo et al. (2005) evaluated oxidative status by analyzing total antioxidant capacity in healthy cows during late pregnancy and lactation onset. Castillo et al. (2005) found that total antioxidant capacity peaked one week after calving followed by a decline thereafter. With total antioxidant capacity peaking one

week after calving, this could possibly lead to an increase in milk letdown of the dam, as oxidative stress is decreased one week post calving.

Numerous livestock models have been used for fetal programming in an attempt to further investigate fetal programming implications in humans. A study in humans observed that low birth weight has been consistently associated with coronary heart disease and type 2 diabetes (Barker et al. 2002). The effects of low birth weight can be slow infant growth and rapid postnatal weight gain during childhood, which can increase the risk of coronary heart disease and type 2 diabetes (Barker et al. 2002). Barker et al. (2002) found that men and women who had birth weights above 4 kg had around half the risk of type 2 diabetes and hypertension when compared to people who had birth weights below 3 kg. These findings could be a result of a negative insult during pregnancy, which can then be programmed in the offspring and have a negative effect on childhood or adult health. With coronary heart disease and type 2 diabetes being two of the most abundant diseases in humans, additional research in fetal programming is crucial in the area of human medicine.

Antioxidant Effects of Melatonin

The relationship between melatonin and total antioxidant capacity is a recent concept that has not been extensively researched until the last fifteen years. Melatonin is an antioxidant that has the ability to affect genomic actions, regulation of expression of genes, antioxidant enzyme activity, and cellular mRNA levels of enzymatic activity. Melatonin is endogenously produced from the pineal gland and scavenges reactive oxygen and nitrogen species (Reiter et al. 2001). Melatonin also plays a pivotal role in the up-regulation of antioxidant pathways (Richter et al. 2009). A number of melatonin

receptors have been identified in mammalian species; however, further research is needed to better understand melatonin receptor pathways.

Antioxidant enzymes form the first line of defense against free radicals (Rodriguez et al. 2003). When equilibrium is unbalanced between free radicals (oxidants) and antioxidant defense systems with the balance favoring oxidants, oxidative stress is formed (Rodriguez et al. 2003). The mechanism by which these antioxidants fight these reactants is by metabolizing them to innocuous byproducts (Rodriguez et al. 2003).

The most abundant reactive oxygen species (ROS) formed in cellular metabolism is the superoxide radical (Rodriguez et al. 2003). Hydrogen peroxide is a product of the of the superoxide anion radical (Reiter et al. 2001). Hydrogen peroxide is not considered a highly reactive agent although; it does have the ability to deactivate certain enzymes (Reiter et al. 2001). A major drawback associated with hydrogen peroxide is its ability to readily cross cell membranes (Reiter et al. 2001). The result of this would be the spreading of additional ROS.

Although the superoxide radical is the most abundant ROS, the hydroxyl radical is the most damaging (Reiter et al. 2001). The destructive actions that the hydroxyl radical produce are typically measured by oxidatively mutilated lipids, proteins, and DNA (Reiter et al. 2001). The hydroxyl radical is such a reactive radical that its diffusion distance is limited (Reiter et al. 2001). Based off this observation, the damage done by the hydroxyl radical appears to be site-specific (Reiter et al. 2001). The results of this damaging radical may lead to diseases such as cancer, neurodegeneration, and autoimmune conditions (Rodriguez et al. 2003).

Melatonin was tested in an attempt to rank its ability to scavenge the hydroxyl radical. Hydrogen peroxide was exposed to 254 nm of ultraviolet light to produce the hydroxyl radical (Reiter et al. 2001). These adducts were inhibited in the presence of melatonin, thus the indole had scavenged the hydroxyl radical (Finkelstein et al. 1980). There is a possibility that melatonin can act as a recycled antioxidant, which would greatly increase its ability to reduce oxidative damage (Stasica et al. 1998).

Just as hydrogen peroxide can pass through cellular membranes with ease, the same is true with nitric oxide. Nitric oxide is important in a plethora of biological regulations within the body. Some of those regulations include blood pressure and destruction of pathogens by immune cells (Snyder et al. 1992). Nitric oxide is a hydrophobic, uncharged molecule that is produced from guanidine nitrogen of L-arginine (Reiter et al. 2001). While nitric oxide has its beneficial properties, negative aspects of nitric oxide are also present. Some of those deterrents are when excessive amounts of nitric oxide are present; it has the ability to damage proteins (Moncada & Higgs et al. 1993; Radi et al. 1996). Nitric oxide also has the ability to damage DNA via protein synthesis (Brune et al. 1994; Wink et al. 1991).

Melatonin plays a vital role in direct free radical scavenging (Tan et al. 1993), as well as regulation of gene transcription (Steinhilber et al. 1995) for antioxidant enzymes. A study was performed in which the pineal gland was surgically removed to analyze melatonin concentrations present within the cells (Menendez-Paleaz et al. 1993; Tan et al. 1999). When analyzing this structure, melatonin was largely found in the nuclei and cytosol (Menendez-Paleaz et al. 1993; Tan et al. 1999). These findings are consistent

with the protection of nuclear DNA against oxidative damage (Menendez-Paleaz et al. 1993; Finnochiarro et al. 1998).

Melatonin can act as an antioxidant when used as a cell protector against diseases. The first publication supporting melatonin having an influence on antioxidant activity was in the mid-1990s (Barlow-Waldon et al. 1995). Antioxidant enzyme activities are known to exhibit endogenous rhythms under normal light:dark conditions (Rodriguez et al. 2003). By manipulating photoperiod, these cycles could be circadian rhythm-dependent (Albarran et al. 2001). If production of endogenous melatonin is altered by a continuous amount of light offered, this will in turn abolish the nighttime rise in antioxidant enzyme activity. According to Albarran et al. (2001) and Pablos et al. (1998), a reduction in night-time activity will increase glutathione peroxidase and superoxide dismutase (SOD) activities in chick tissues. If a decrease in endogenous serum melatonin is observed, the results could lead to a decrease in antioxidant capacity in the resistance of oxidative stress (Manev et al. 1996; Kilic et al. 1999).

Melatonin supplementation during pregnancy increases antioxidant enzyme activity in the fetus (Rodriguez et al. 2003). Melatonin can pass all morphophysiological barriers (Reiter et al. 2001). This finding is especially important during pregnancy as melatonin can pass directly through the placenta and enter fetal cells (Okatani et al. 1998; Wakatsuki et al. 1999). This phenomenon has been found in rats, sheep, and humans. High doses of melatonin administered to pregnant rats caused an increase in the indole in both rat brain and maternal serum (Okatani et al. 2001). Glutathione peroxidase and SOD activities were also increased (Okatani et al. 2001). Thus, melatonin administration could be helpful during times of increased oxidative stress such as pregnancy.

There is variation among studies seeking to understand if melatonin is dosage dependent. According to Kotler et al. (1998), after administering either 50 or 500 μg/kg of melatonin to rats, the lower dosage had a greater stimulatory effect on antioxidant gene expression than the higher dosage. Antolin et al. (1996) reported that melatonin provided protection against in vivo neurotoxicity using a dosage of 500 μg/kg in rats. These studies provide support that both antioxidant enzyme activity and expression are elevated after peripheral administration of melatonin (Kotler et al. 1998; Antolin et al. 1996).

Ultrasonography

Doppler Frequency

Doppler ultrasonography has been an evolving piece of technology within the last decade. Doppler ultrasonography can be used to measure blood flow. The ultrasound machine is based off of Doppler-shift frequencies. These frequencies are defined as the differences between the frequency of ultrasound waves and that of received echoes (Ginther 2007). The shift frequencies are echoes from moving red blood cells either increasing or decreasing in relation to them moving closer or further away from the transducer. These red blood cells are moving at a speed of around 1 meter per second. When the frequency results in a positive integer, the red blood cells are moving toward the transducer. The result will be a negative integer when the red blood cells are moving away from the transducer. The Doppler-shift frequencies from the red blood cells are separated from other received echoes via demodulation.

Transducers

Typically there are two types of transducers used: linear-array and convex array transducers. The linear transducer depicts a rectangular view, whereas the convex array depicts a pie-shaped field of view. The linear array is more frequently used in viewing a large structure near to the transducer in one view (Ginther 2007). The advantage of the convex transducer is ease of use because it is shorter and easier to manipulate.

Doppler Angle

The orientation in which the transducer is being used is crucial in Doppler ultrasonography. If the image is on a part of the artery that transverse directly across, the field of view will be an undesirable angle of 90° and will not be able to accurately detect velocity of blood flow. If the artery is angled slightly towards the transducer, it will result in a smaller and more desirable angle (Ginther 2007). The most desirable angles are between 30° and 60° as this range is the greatest in velocity accuracy; however, this range is also variable depending on the tissue being measured.

There is also a feature on the ultrasound that is an angle cursor, which can be used to show the angle of intersection of the sound beams in relation to blood flow. It has been estimated that an angle length of greater than or equal to 2 cm for placement of the angle cursor is advantageous (Ginther 2007). If the tissue is difficult to see, it can result in a cross section and the angle cannot be detected; therefore, the true velocity will not be obtained. Both the shift frequency and Doppler angle are used for finding blood velocity. Most often the desired measurements are computing systolic and diastolic measurements.

Color-Doppler

Incorporating color into the Doppler technology is also possible. This is commonly referred to as color flow or color-velocity imaging. This estimates blood velocities from the Doppler shifts and angles. Brightness mode (gray scale or B-mode) is normally used to identify and measure structures, but it also has the ability to diagnose physiological status. When using B-mode, the shift frequencies are received from the operator and are then transformed into color-coded spots representing blood flow. Each color-scan line can consist of 8 to 10 Doppler cycles. One cycle represents one pulse along with the resulting echoes. The echoes of the moving reflectors are computed in order to find the average velocity and variance for each cycle. High frequency signals can sometimes be detected. This is commonly referred to as noise and can usually be filtered by settings on the ultrasound machine. Color-Doppler ultrasonography has been impacting human medicine for more than two decades, but the implications that it can have in the future are limitless.

Velocity Measurements

Each waveform represents one cardiac cycle. The maximum point is the systolic velocity (s) and represents the maximum Doppler-shift frequency. The low point is the diastolic velocity (d). A depiction of the waveform showing systolic velocity being the maximum peak and diastolic being the lowest point of the waveform is represented in Fig. 2. There are limitations with determining velocity. If the angle is not obtainable then a true velocity cannot be computed. There is also not a plethora of information available on the relationships of blood velocity and blood flow of the tissue supplied by the artery.

Doppler Indices

Doppler indices are used to measure ratios that are computed from different points on the spectrum. These are relative to the hemodynamics of the tissue that is being supplied by the artery. An increase in resistance index (RI) or pulsatility index (PI) values, indicates increased resistance and decreased perfusion to the distal tissues. When using Doppler ultrasonography, the stationary object is the transducer, and the moving reflectors are red blood cells. The RI is used because it is convenient to relate the negative relationship between the degree of resistance in the tissue and the degree of vascular perfusion. The greater the RI and PI, the lesser the perfusion.

Comparative Hemodynamic Techniques

Earlier studies used various techniques for measuring blood flow. Ferrell & Ford (1980) quantitated blood flow during various stages of gestation in Hereford cows. The technique performed was via electromagnetic blood flow probes that were placed in the middle of the uterine artery. Indwelling catheters were also placed in an artery and vein on the surface of the gravid horn. Blood flow measurements were recorded daily for 15 minutes. Blood samples were collected immediately following the blood flow measurements. From days 178-199, 202-224, and 230-258, blood flow averaged 3.2 L/min, 3.9 L/min, and 3.1 L/min, respectively (Ferrell & Ford 1980). With this technique, there are certain drawbacks that were recorded. Complete data were not recorded as some of the blood flow transducers did not function properly (Ferrell & Ford 1980). Blood flow to the pregnant horn became greater than could be accommodated by the blood flow transducers and a blockage occurred to the uterine horn (Ferrell & Ford 1980). Catheters also became inoperable in some cows (Ferrell & Ford 1980). It is important to record

these complications within the study as this affected retrieving a complete data set from the study. The same laboratory later used a different technique, injection of deuterium oxide, during late gestation. They observed that ipsilateral uterine artery blood flow at day 226 was 2.9 L/min and at day 250 was 13.1 L/min (Reynolds 1986; Reynolds & Ferrell 1987). Moreover, they observed a 4.5-fold increase in ipsilateral blood flow from day 137-250 of gestation (Reynolds 1986; Reynolds & Ferrell 1987). Another disadvantage to these techniques is that they were invasive and required surgical procedures.

One of the first known studies to use Doppler ultrasonography was Bollwein et al. (2001). Two Simmental cows and one Brown Swiss cow were used in the study to analyze uterine artery hemodynamics. Both ipsilateral and contralateral uterine arteries were scanned monthly. Blood flow was determined using the following variables: RI, time-averaged maximum velocity, vessel diameter, and blood flow volume. Resistance index was negatively correlated to all other variables measured; however, positive correlations were found among time-average maximum velocity, vessel diameter, and blood flow volume (Bollwein et al. 2001). As gestation progressed, the RI decreased while vessel diameter and blood flow volume was increased (Bollwein et al. 2001). This study was a foundational for future Doppler ultrasound studies involving uterine blood flow. This is important to note as this technique is non-invasive.

Doppler ultrasonography has not only been performed in cattle but also in various other species. Bollwein et al. (2004) used transrectal Doppler ultrasonography on four mares measuring uterine and umbilical blood flow during pregnancy. Resistance index and blood flow volume of the uterine arteries were evaluated every four weeks. Fetal

blood flow was measured every two weeks on the umbilical arteries using RI. There were no significant differences found in uterine or umbilical blood flow (Bollwein et al. 2004). The mean RI decreased during pregnancy and the mean volume increased almost 400-fold from 69 ± 37 to 27,467 ± 8852 ml/min (Bollwein et al. 2004).

Both studies performed by Bollwein et al. (2001; 2004) are important to the future of Doppler ultrasonography as both studies resulted in decreased RI as gestation progressed and blood flow volume was increased with gestational day. Doppler ultrasonography is a reliable technique as similar findings were found between the two studies even though the studies were performed in different species.

A more recent study conducted by Herzog et al. (2011) performed Doppler ultrasonography and measured uterine artery blood flow in cattle at days 203, 231, 259, and 273 of gestation. This experiment observed a linear increase in total uterine artery blood flow for all days (Herzog et al. 2011). It was also observed that heavier cows had a greater increase in blood flow versus lighter cows (Herzog et al. 2011). Cows of similar weight carrying heavy fetuses had increased blood flow compared to the light fetuses (Herzog et al. 2011).

Since Doppler ultrasonography is such a new technology in research, a study by Menzies-Gow and Marr (2007) was performed in Thoroughbred horses to show the repeatability of using Doppler ultrasonography. According to Menzies-Gow and Marr (2007) the technique is repeatable and sensitive enough to detect changes in blood flow during both physiological and pathological states as well as following pharmacological intervention. With the studies described, it appears the Doppler ultrasonography is repeatable and accurate in blood flow measurements.

Conclusion

Melatonin is a hormone that needs further investigation. The mechanisms and pathways in which melatonin functions as a physiological and pharmacological antioxidant are still incomplete. The function and use of melatonin are still of great interest.

Currently, melatonin has been studied as a feed supplement during both compromised pregnancies as well as non-compromised pregnancies (Lemley et al. 2012). With melatonin's antioxidant properties, research is being conducted in the area of fetal programming along with its effects on uterine artery blood flow. With studies showing an increase in total uterine artery blood flow following melatonin supplementation to the dam, there could be future implications of increased growth in the offspring by further investigating blood distribution and physiological functions of various organs. Improved organ function, as a result of fetal programming with melatonin exposure, could increase longevity of livestock.

As previous researchers suggested, melatonin is a good antioxidant and can aid during stressful time points. More research needs to be conducted in the area of melatonin acting as an antioxidant to better realize its exact function. Melatonin along with its oxidative properties could have a large impact on livestock production, as it is a cost effective supplement costing 2 cents per head per day. With further research, melatonin could have the possibility of being applicable in a production setting.

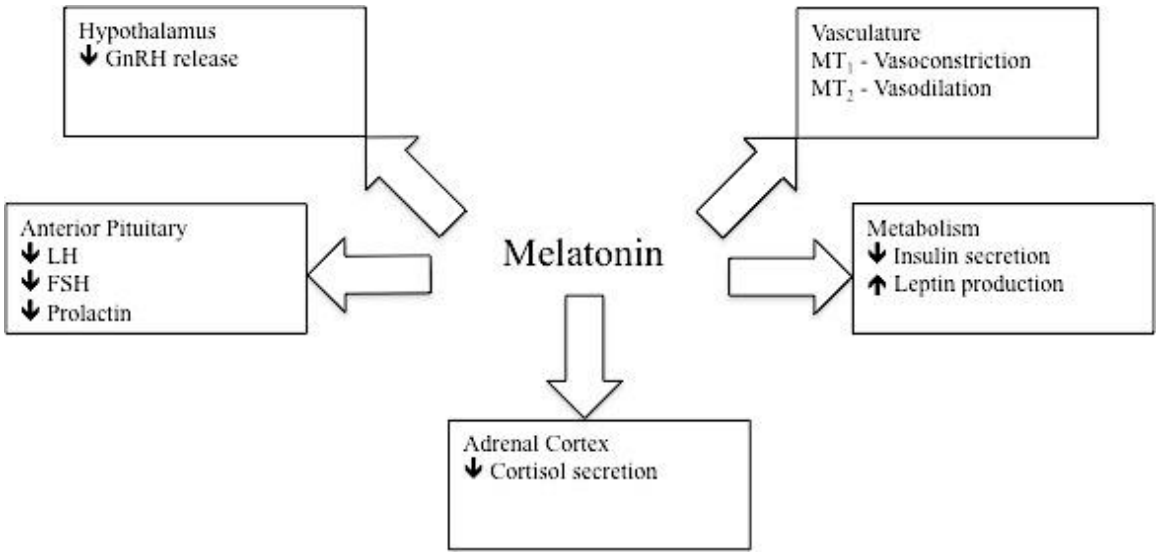

Figure 1 Melatonin's effect on physiological systems

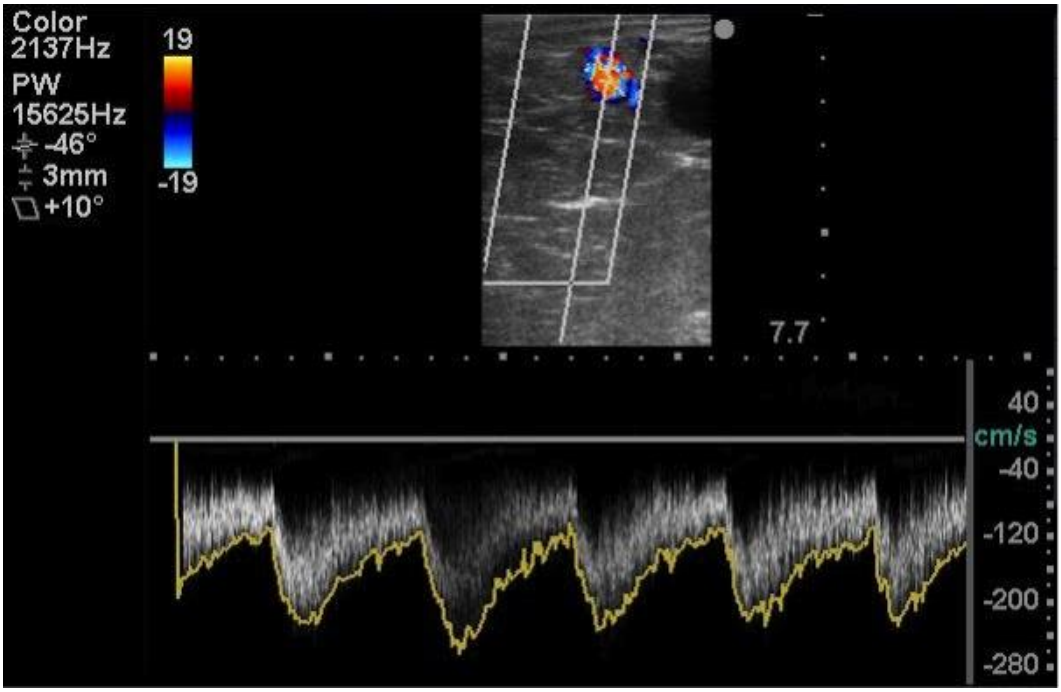

Figure 2 Uterine artery blood flow in Holstein heifers

Uterine artery blood flow with angle cursor representing a 46° angle. The waveform below the image represents a cardiac waveform with systolic being the peak of the waveform and diastolic being the trough of the waveform. The waveform velocity is measured in cm/s, which is located at the bottom right of the image. Blood flow is calculated by taking the mean velocity multiplied by the vessel area multiplied by 60 seconds.

References

Albarran MT, Lopez-Burillo S, and Pablos MI 2001 Endogenous rhythms of melatonin, total antioxidant status and superoxide dismutase activity in several tissues of chicks and their inhibition by light. Journal of Pineal Research **30** 227–233.

Antolin I, Rodriguez C, and Sainz RM 1996 Neurohormone melatonin prevents cell damage: Effect on gene expression for antioxidant enzymes. Federation of American Societies for Experimental Biology **10** 882-890.

Barker DJP, Eriksson JG, Forsen T, and Osmond C 2002 Fetal origins of adult disease: strength of effects and biological basis. International Journal of Epidemiology **31** 1235-1239.

Barlow-Walden LR, Reiter RJ, and Abe M 1995 Melatonin stimulates brain glutathione peroxidase activity. Neurochemistry International **26** 497–502.

Bird IM, Zhang L, and Magness RR 2003 Possible mechanisms underlying pregnancy induced changes in uterine artery endothelial function. American Journal of Physiology- Regulatory, Integrative, and Comparative Physiology **284** 245-258.

Bollwein H, Baumgartner U, and Stolla R 2001 Transrectal Doppler sonography of uterine blood flow in cows during pregnancy. Theriogenology **57** 2053-2061.

Bollwein H, Weber F, Wischée I, and Stolla R 2004 Transrectal Doppler sonography of uterine and umbilical blood flow during pregnancy in mares. Theriogenology **61** 499-509.

Brune D, Dimmeler S, Moline V, and Lapetina EG 1994 Nitric oxide: A signal for ADP-ribosylation of proteins. Life Sciences **54** 61–70.

Castillo C, Hernandez J, Bravo A, Lopez-Alonso M, Pereira V, and Benedito JL 2005 Oxidative status during pregnancy and early lactation in dairy cows. The Veterinary Journal **169** 286-292.

Dubocovich ML & Markowska M 2005 Functional MT_1 and MT_2 Melatonin Receptors in Mammals. Endocrine Journal **27** 101-110.

Ferrell CL & Ford SP 1980 Blood flow steroid secretion and nutrient uptake of the gravid bovine uterus. Journal of Animal Science **50** 1113-1121.

Finkelstein E, Rosen GM, and Rauckman EJ 1980 Spin trapping. Kinetics of the reaction of superoxide and hydroxyl radicals by nitrones. Journal of the American Chemical Society **102** 4994–4999.

Finnochiarro LE & Glikin GC 1998 Intracellular distribution of melatonin in cultured cell lines. Journal of Pineal Research **24** 22–34.

Funston RN, Larson DM, and Vonnahme KA 2009 Effects of maternal nutrition on conceptus growth and offspring performance: Implications for beef cattle production. Journal of Animal Science **88** 205-215.

Ginther OJ 2007 Ultrasononic imaging and animal reproduction: color-doppler ultrasonography. Book 4 Equiservices Publishing Cross Plains, WI 25-84.

Glore SR & Layman 1983 Cellular growth of skeletal muscle in weanling rats during dietary restrictions. Growth **47** 403-410.

Goff JP & Horst RL 1997 Physiological changes at parturition and their relationship to metabolic disorders. Journal of Dairy Science **80** 1260-1268.

Greenwood PL, Hunt AS, Hermanson, and Bell AW 2000 Effects of birth weight and postnatal nutrition on neonatal sheep: II. Skeletal muscle growth and development. Journal of Animal Science **78** 50-61.

Herzog K, Koerte J, Flachowsky G, and Bollwein H 2011 Variability of uterine blood flow in lactating cows during the second half of gestation. Theriogenology **75** 1688-1694.

Kaïs H, Al-Gubory, Fowler PA, and Garrel C 2010 The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. The International Journal of Biochemistry & Cell Biology **42** 1634-1650.

Kilic E, Özdemir YG, Bolay H, Kelestimur H, and Dalkara T 1999 Pinealectomy aggravates and melatonin administration attenuates brain damage in focal ischemia. Journal of Cerebral Blood Flow & Metabolism **19** 511–516.

Kotler M, Rodriguez C, and Sainz RM 1998 Melatonin increases gene expression for antioxidant enzymes in rat brain cortex. Journal of Pineal Research **24** 83-89.

Larson DM, Martin JL, Adams DC, and Funston RN 2009 Winter grazing system and supplementation during late gestation influence performance of beef cows and steer progeny. Journal of Animal Science **87** 1147-1155.

Lemley CO, Meyer AM, Camacho LE, Neville TL, Newman DJ, Caton JS, and Vonnahme KA 2012 Melatonin supplementation alters uteroplacental hemodynamics and fetal development in an ovine model of intrauterine growth restriction. American Journal of Physiology - Regulatory, Integrative, and Comparative Physiology **302** R454-R467.

Manev H, Uz T, Kharlamov A, and Joo J Y 1996 Increased brain damage after stroke and excitotoxic seizures in melatonin-deficient rats. FASEB Journal **10** 1546–1551.

Martin JL, Vonnahme KA, Adams DC, Lardy GP, and Funston RN 2007 Effects of dam nutrition on growth and reproductive performance of heifer calves. Journal of Animal Science **85** 841-847.

Menendez-Pelaez A, Poeggeler B, and Reiter RJ 1993 Nuclear localization of melatonin in different mammalian tissues: immunocytochemical and radioimmunoassay evidence. Journal of Cellular Biochemistry **53** 373–382.

Menzies-Gow NJ & Marr CM 2007 Repeatability of doppler ultrasonographic measurement of equine digital blood flow. Veterinary Radiology & Ultrasound **48** 281-285.

Moncada S & Higgs A 1993 The L-arginine-nitric oxide pathway. New England Journal of Medicine **329** 2002–2011.

Nissen PM, Danielsen VO, Jorgenen PF, and Oksbjerg N 2003 Increased maternal nutrition of sows has no beneficial effects on muscle fiber number or postnatal growth and has no impact on the meat quality of the offspring. Journal of Animal Science **81** 3018-3027.

Okatani Y, Okamoto K, Hayashi K, Wakatsuki A, Tamura S, and Sagara Y 1998 Maternal-fetal transfer of melatonin in pregnant women near term. Journal of Pineal Research **25** 129–134.

Okatani Y, Wakatsuki A, and Shinohara K 2001 Melatonin stimulates glutathione peroxidase activity in human chorion. Journal of Pineal Research **30** 199–205.

Pablos MI, Reiter RJ, and Ortiz GG 1998 Rhythms of glutathione peroxidase and glutathione reductase in brain of chick and their inhibition by light. Neurochemistry International **32** 69-75.

Radi R 1996 Reactions of nitric oxide with metalloproteins. Chemical Research in Toxicology **9** 828–835.

Reiter RJ, Tan DX, Manchester LC, and Qi W 2001 The University of Texas Health Science Center. Biochemical Reactivity of Melatonin with Reactive Oxygen and Nitrogen Species. Cell Biochemistry and Biophysics **34**.

Reiter RJ, Melchiorri D, Sewerynek E, Poeggeler B, Barlow-Walden L, Chuang J, Ortiz GG, and AcuñaCastroviejo D 1995 A review of the evidence supporting melatonin's role as an antioxidant. Journal of Pineal Research **18.1** 1-11.

Reynolds LP & Ferrell CL 1987 Transplacental clearance and blood flows of bovine gravid uterus at several stages of gestation. American Journal of Physiology **253** R735.

Reynolds LP, Caton JS, Redmer DA, Grazul-Bilska AT, Vonnahme KA, Borowics PP, Luther JS, Wallace JM, Wu G, and Spencer TE 2006 Evidence for altered placental blood flow and vascularity in compromised pregnancies. Journal of Physiology **572** 51-58.

Reynolds LP 1986 Utero-ovarian interactions during early pregnancy: role of conceptus-induced vasodilation. Journal of Animal Science **62** 47-61.

Richter HG, Hansell JA, Raut S, and Giussani DA 2009 Melatonin improves placental efficiency and birth weight and increases the placental expression of antioxidant enzymes in undernourished pregnancy. Journal of Pineal Resarch **46** 357-364.

Robinson JJ, McDonald I, Fraser C, and McHattie I 1977 Studies on reproduction in prolific ewes. I. Growth of the products of conception. Journal of Agricultural Science **88** 539-552.

Rodriguez C, Mayo JC, Sainz RM, Antolin I, Herrera F, Martin V, and Relter RJ 2003 Regulation of antioxidant enzymes: A significant role for melatonin. Journal of Pineal Research **36** 1-9.

Snyder SH & Bredt DS 1992 Biological roles of nitric oxide. Scientific American 68–77.

Stasica P, Ulanski P, and Rosiak JM 1998 Melatonin as a hydroxyl radical scavenger. Journal of Pineal Research **25** 65–66.

Steinhilber D, Brungs M, and Werz O 1995 The nuclear receptor for melatonin repress 5-lipoxygenase gene expression in human B lymphocytes. Journal of Biological Chemistry **270** 7037–7040.

Tan DX, Manchester LC, Reiter RJ, Qi W, Zhang M, Weintraub S, Cabrera J, Sainz RM, and Mayo JC 1999 Identification of highly elevated levels of melatonin in bone marrow: Its origin and significance. Biochimica Biophysica Acta **1472** 206–214.

Tan DX, Chen LD, Poeggeler B, Manchester LC, and Reiter RJ 1993 Melatonin: A potent, endogenous hydroxyl radical scavenger. Endocrine Journal **1** 57–60.

Vonnahme KA, Zhu MJ, Borowics PP, Geary TW, Hess BW, Reynolds LP, Caton JS, Means WJ, and Ford SP 2007 Effect of early gestational undernutrition on angiogenic factor expression and vascularity in the bovine placentome. Journal of Animal Science **85** 2464-2472.

Wakatsuki A, Okatani Y, Izumiya C, and Ikenoue N 1999 Melatonin protects against ischemia and reperfusion-induced oxidative lipid and DNA damage in fetal rat brain. Journal of Pineal Research **26** 147–152.

Wallace JM, Regnault TR, Limesand SW, Hay WW Jr, and Anthony RV 2005 Investigating the causes of low birth weight in contrasting ovine paradigms. Journal of Physiology **565** 19-26.

Wink DA, Kasprzak KS, Maragos CM, Elespura RK, Misra MK, Runams TM, Cebula TA, Koch WH, Andrews AW, and Allen TS 1991 DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science **254** 1001–1003.

CHAPTER II

DIETARY MELATONIN ALTERS UTERINE ARTERY HEMODYNAMICS IN PREGNANT HOLSTEIN HEIFERS

Introduction

The uterine environment during late gestation is vital for assuring a continual delivery of sufficient oxygen and nutrients to the exponentially growing fetus. Based on average lactation numbers in the United States, a Holstein female spends approximately 14% of their life within the uterus. Moreover, livestock used for meat production spend an even larger amount of developmental time within the uterus, whereby the placenta is their sole source of nourishment (Vonnahme & Lemley 2012; Redmer et al. 2004). Melatonin supplementation during a compromised pregnancy may alter uteroplacental blood flow through melatonergic receptor-mediated pathways or indirectly by decreasing oxidative stress in the vascular system (Lemley et al. 2012; Lemley et al. 2013).

Melatonin is unique in that it has the ability to protect the body against free radicals via direct or indirect pathways (Reiter et al. 1995). Firstly, melatonin can bind directly to cellular membranes and help stabilize that membrane against possible oxidative damage. Secondly, melatonin has the ability to protect cells indirectly by helping the body up-regulate its own antioxidant defense system. Imbalances between reactive oxygen specie production and antioxidant systems could have a negative impact in creating oxidative stress which, in turn, can negatively impact the reproductive

process. Oxidative stress is increased during pregnancy, and is directly related to several pregnancy disorders, such as spontaneous abortions, embryopathies, preeclampsia, fetal growth restriction, preterm labor, and low birth weight (Kaïs et al. 2010). The links between oxidative stress, the female reproductive system and the development of negative pregnancy outcomes, results in important concerns in both human and animal reproductive sciences (Kaïs et al. 2010).

Compromised pregnancies experiencing fetal growth restriction have been accompanied by increased oxidative damage. These pathways could be related to nitric oxide, a powerful vasodilator that helps modulate blood flow during pregnancy (Beckman & Koppenol 1996). Free radicals, such as superoxide anion react with nitric oxide and decrease its bioavailability, which can lead to a decrease in blood flow. Melatonin has a part in the regulation of blood pressure, myocardial contractility, and arterial vasoconstriction and vasodilation (Paulis & Simko 2007; Lemley et al. 2012). Melatonin has been known to decrease peripheral resistance but when analyzing isolated arteries, this finding becomes increasingly controversial, as this cascade cannot completely be explained (Paulis & Simko 2007). It is a possibility that melatonin may play a pivotal role in peripheral vascular resistance through melatonin receptors (Lemley et al. 2013). Receptor-mediated vasoconstriction on the vascular system could be counter-balanced by receptor-mediated nitric oxide release, which could further enhance melatonin's antioxidant properties (Paulis & Simko 2007).

Many studies have been conducted involving nutrient restriction during pregnancy; however, few studies addressed uteroplacental hemodynamics of improved fetal growth (Lemley et al. 2012). In addition, supplementing dietary melatonin may have

the ability of altering fetal organ development and functional capacity (Lemley et al. 2012). Dietary melatonin increases total umbilical artery blood flow, as well as increases the cross sectional area of the umbilical cord in the ewe (Lemley et al. 2012). With melatonin's strong antioxidant capacity, melatonin can stimulate peroxidase activity and scavenge peroxyl radicals generated during lipid peroxidation (Reiter et al. 2007). Serving as an antioxidant, melatonin can reduce oxidative stress and partially negate the decreased birth weight in nutrient restricted rats (Richter et al. 2009). Moreover, melatonin supplementation increased placental antioxidant enzyme activity in mid-gestating ewes (Lemley et al. 2013). Our primary objective was to measure uterine artery hemodynamics during late gestation following melatonin supplementation in Holstein heifers. Therefore, we hypothesized that dietary melatonin supplementation would increase uterine artery hemodynamics and total antioxidant capacity in late gestating Holstein heifers.

Materials and Methods

Animal care and use were according to protocols approved by the Mississippi State University Institutional Animal Care and Use Committee.

Animal Model

Dairy heifers were artificially inseminated with sex-sorted semen at the Joe Bearden Dairy Research Center. Artificial insemination dates ranged from December 6, 2012 to January 30, 2013. Twenty singleton pregnant Holstein heifers were selected at day 70 to 90 of pregnancy. The project consisted of a randomized complete block design. The heifers were blocked by both artificial insemination date and body weight. On day

170 of gestation, the heifers were trained to the Calan feeding system (American Calan, Northwood, NH, USA) to monitor dry matter intake. Dietary melatonin supplementation (MEL; n = 10) or no melatonin supplementation (CON; n = 10) started on day 190 of gestation and ended on day 262. Melatonin (Batch 130307, Health Supplement Wholesalers, Red Lion, PA, USA) supplementation was individually provided by top-dressing grain with 2 mL of 10 mg/mL melatonin dissolved in ethanol (20 mg of melatonin per head per day). The melatonin dosage was based on previous dietary melatonin supplementation studies with cattle (Zinn et al. 1988; Sanchez-Barcelo et al. 1991; Dahl et al. 2000). A total mixed ration (Table 1) of 8.9 kg/d was provided based on NRC recommendations for a pregnant Holstein heifer during late gestation along with ad libitum access to water. The total mixed ration was analyzed for dry matter, ash, crude protein, neutral detergent fiber, and acid detergent fiber. Maternal body weights were recorded every 14 days. Maternal body condition was scored on a 1 to 5 scale, with 1 = emaciated and 5 = obese, by two separate evaluators on days 178 and 256 (± 2.5 days; SE) of gestation. Milk yield was recorded for the first 30 days of lactation. Blood samples were collected via venipuncture of the coccygeal vessels on days 180 (baseline), 210, 240, and 262 of gestation. Maternal heart rate (HR) and blood pressure (BP) were collected prior to blood sampling using a digital sphygmomanometer (Omron, Schaumburg, IL, USA). Mean arterial pressure (MAP) was calculated by taking one third of the distance between the systolic pressure and the diastolic pressure. In addition, pulse pressure was calculated by subtracting the diastolic pressure from the systolic pressure.

Color Doppler Ultrasonography

Uterine artery hemodynamics, contralateral and ipsilateral to the conceptus, were determined on days 180 (baseline), 210, 240, and 262 of gestation via color Doppler ultrasonography (MicroMaxx, SonoSite, Inc., Bothell, WA, USA) using a transrectal probe (Sonosite MicroMaxx with a Linear Endorectal L52x probe). Following techniques described by Camacho et al. (2014), 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 where identified as a major branch of the iliac arteries. Moreover, the uterine arteries were palpated to assure pliability and pulsatility, which is easily observed during late gestation. The ultrasound transducer was aligned to the uterine artery at an average angle of insonation of 56 ± 2 degrees (mean ± SE). 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*60s).

Blood Sampling Analysis

A subsample of heifers (n = 5 per treatment) were selected to measure serum melatonin concentrations in peripheral circulation pre-feeding and 1 hour post feeding at day 216 ± 2 (mean ± SE) of gestation. Melatonin concentrations were determined with an ELISA kit (MyBiosource, San Diego, CA, USA). The melatonin ELISA assay was performed following manufacturer's instructions. Briefly, samples were analyzed against

a melatonin standard curve (0 – 1,000 pg/mL), with a sensitivity of 1.0 pg/mL and a spike recovery of 95%. The intra-assay coefficient of variation for the melatonin assay was 8.3%.

Total antioxidant capacity was determined with a colorimetric assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). The antioxidant capacity assay was performed following manufacture's instructions except the serum was diluted with assay buffer to place unknowns within 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 ABTS oxidation product was monitored at 405 nm via a Spectra Max Plus plate reader (Sunnyvale, CA, USA). The kit does not separate aqueous- and lipid-soluble antioxidants; therefore, the combined antioxidant activities of all constituents in maternal serum are reported. The capacity of serum antioxidants in unknown samples to prevent substrate oxidation is compared with that of a tocopherol analogue (Trolox) and is reported as mM Trolox equivalents. Samples were analyzed against a linear Trolox standard curve (0 – 0.33 mM), with a sensitivity of 0.01 mM and intra- and interassay coefficients of variation of 14.4% and 9.6%, respectively.

Total serum nitrites were determined using the 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 the reduction of total nitrates to nitrites using the Griess method and analyzed against a linear nitrites standard curve (0 – 100 μM), with a sensitivity of 0.6 μM and intra- and interassay coefficients of variation of 3.3% and 1.3%, respectively.

Serum progesterone and estradiol were determined using RIA kits (Siemens Healthcare Diagnostics Inc, Los Angeles, CA, USA) following the methods of Hart et al. (2014). For both assays the log concentration of the standard (progesterone or estradiol) was plotted against the logit of percent bound. The intra-assay coefficient of variation for the progesterone RIA was 2.6%. The intra-assay coefficient of variation for the estradiol RIA was 4.15%.

Statistical Analysis

Dependent variables measured over time were analyzed using repeated-measures ANOVA of the MIXED procedure of SAS (SAS software version 9.3, SAS Institute, Cary, NC, USA), and means were separated using the PDIFF option of the LSMEANS statement. The model statement contained dietary treatment, gestational day, and their respective interaction. Main effects of dietary treatment or gestational day are discussed in the absence of significant ($P < 0.05$) treatment by day interactions. Least square means and SEM are reported. Statistical significance was declared at $P < 0.05$.

Results

Maternal Body Condition

Heifer body weight increased ($P < 0.01$) as gestational day increased (Fig. 3); however, melatonin treatment did not influence ($P = 0.34$) heifer body weight. Average daily gain from day 180 to 262 of gestation was similar ($P = 0.64$) between MEL (0.92 ± 0.05 kg/d) versus CON (0.87 ± 0.07 kg/d). Body condition score was not different ($P = 0.61$) between heifers receiving MEL and CON and averaged 3.45 ± 0.08. Dry matter

intake throughout the supplementation period was not different (P = 0.14) between dietary treatment groups and averaged 8.95 ± 0.03 kg/day.

Uterine Artery Hemodynamics

Main effects of gestational day (P < 0.01) and MEL treatment (P = 0.01) were observed for ipsilateral uterine artery blood flow (Fig. 4A). On average ipsilateral uterine artery blood flow was increased by 32% in MEL treated heifers compared to CON. A main effect of gestational day (P = 0.02) was observed for ipsilateral RI, which decreased at days 210, 240, and 262 versus 180 (Fig. 4B). A main effect of gestational day (P < 0.01) was also observed for ipsilateral PI, which was decreased at days 210, 240, and 262 of gestation versus 180 (Fig. 4C). Ipsilateral RI (P = 0.19) and PI (P = 0.29) were not influenced by melatonin supplementation. A main effect of gestational day (P < 0.01) was observed for ipsilateral diameter of the uterine artery, which increased from day 180 to 240 of gestation. Ipsilateral diameter of the uterine artery was not influenced (P = 0.46) by melatonin supplementation. Both MnV and s:d ratio on the ipsilateral side were not different (P ≥ 0.11) across gestational day or melatonin treatment (data not shown).

A main effect of gestational day (P < 0.01) was observed for contralateral uterine artery blood flow, which increased from d 180 to d 240 (Fig. 5A). Contralateral uterine artery RI (Fig. 5B) and PI (Fig. 5C) were not different across gestational day (P > 0.09) or dietary treatment (P > 0.28). Contralateral uterine artery MnV and s:d ratio were not different (P ≥ 0.16) across gestational day or dietary treatment (data not shown). A main effect of gestational day (P < 0.01) was observed for contralateral diameter of the uterine artery, which increased as gestation proceeded. Dietary treatment did not influence (P = 0.87) contralateral diameter of the uterine artery.

A main effect of gestational day and melatonin treatment was observed for total uterine artery blood flow (Fig. 6). Total uterine blood flow was increased ($P < 0.01$) by 25% in the MEL treated heifers compared to the CON. There were no differences across gestational day ($P = 0.81$) or dietary treatment ($P = 0.52$) for maternal mean arterial pressure (Fig. 7A). A dietary treatment by gestational day interaction ($P = 0.02$) was observed for pulse pressure (Fig. 7B), which was increased in MEL-treated heifers vs. CON at day 240 of gestation. There was an increase ($P < 0.01$) in heart rate as gestation proceeded. In addition, MEL-treated heifers had a 9% increase in heart rate when compared to the CON-treated heifers ($P = 0.03$) (Fig. 7C).

Maternal Serum Analysis

Melatonin concentrations showed a dietary treatment by hour interaction ($P < 0.01$). At hour 0 there was no difference between the MEL treated heifers vs. CON treated heifers; however, at hour 1 post-supplementation there was an 8-fold increase in serum concentrations of melatonin in the MEL-treated heifers when compared to the CON-treated heifers (Fig. 8).

A main effect of gestational day ($P < 0.01$) was observed for maternal concentrations of estradiol-17β (Fig. 9A). There was no main effect of dietary treatment ($P = 0.06$) observed for maternal concentrations of estradiol-17β. Estradiol-17β increased with increasing gestational age with the exception of no difference from day 180 to 210. In addition, estradiol-17β was not different in MEL-treated heifers vs. CON treated heifers. A main effect of gestational day ($P < 0.01$) and dietary treatment ($P = 0.02$) were also observed for concentrations of progesterone (Fig. 9B). Progesterone was increased from day 180 to 210 followed by no difference from day 210 to 240 and ending with a

decrease in concentrations of progesterone from day 240 to day 262. There was also a decrease in progesterone concentrations in MEL-treated heifers vs. CON-treated heifers.

Main effects of gestational day ($P < 0.01$) and dietary treatment ($P < 0.01$) were observed for total serum antioxidant capacity (Fig. 10A). Total serum antioxidant capacity was increased on days 210, 240, and 262 of gestation compared to day 180. There was also a main effect of dietary treatment ($P < 0.01$) when examining total serum antioxidant capacity, which was increased by 39% in MEL-treated heifers when compared to CON. Maternal concentrations of total serum nitrites increased ($P < 0.01$) as gestation proceeded (Fig. 10B). There was no main effect of dietary treatment ($P = 0.83$) when examining total serum nitrites.

Parturition and Lactation

Duration of gestation was not different ($P > 0.50$) between treatments and averaged 275 ± 2 days. Birth weight ($P = 0.63$) was not different between treatments and averaged 35 ± 2 kg. A treatment by day interaction ($P < 0.01$) was observed for milk yield (Fig. 11), whereby milk yield was increased by 41% and 33% on days 2 and 3 of lactation in MEL vs. CON heifers, respectively.

Discussion

In the present study, ipsilateral uterine artery blood flow, total uterine artery blood flow, and total antioxidant capacity were increased in pregnant heifers supplemented with dietary melatonin. In addition, no main effects or interactions of melatonin supplementation were observed for heifer body weight, daily feed intake, contralateral blood flow, or gestational length. Melatonin has vasodilation capabilities and could have

the potential of increasing blood flow and antioxidant capacity in pregnant heifers. Additional studies addressing uteroplacental hemodynamics in models of improved fetal growth are critical as this research is novel.

A limited number of studies have utilized Doppler ultrasonography to examine reproductive tract blood flow measurements in cattle. One of the first studies examining uterine artery hemodynamics with Doppler ultrasonography included two Simmental cows and one Brown Swiss cow that were both uniparous and nonlactating (Bollwein et al. 2001). Uterine artery resistance index (RI) values located ipsilateral to the pregnant uterine horn were decreased with the exception of months 1 and 8 (Bollwein et al. 2001). Resistance index has been negatively correlated with time averaged maximum velocity (TAMV) of blood flow and the diameter (d) of the uterine artery (Bollwein et al. 2001). At 7 months of gestation, ipsilateral uterine artery blood flow was approximately 5 L/min, while contralateral uterine artery blood flow was approximately 1.5 L/min (Bollwein et al. 2001). Using surgically implanted electromagnetic flow probes, Ferrell and Ford (1980) observed approximately 3.7 L/min at 7 months of gestation. Reynolds and Ferrell (1987) using the steady-state diffusion method (injection of radioactive substances) observed approximately 7 L/min at 7 months of gestation. The current study observed approximately 5 L/min in the ipsilateral horn with the heifers that were treated with melatonin compared to the controls having approximately 3 L/min at 7 months of gestation. In conclusion, various techniques can be performed in order to calculate blood flow and the observations from the current study are closely related to these previous reports.

When analyzing blood flow in sheep, on day 90 of gestation melatonin-infused dams had an increase in umbilical artery blood flow as well as fetal descending aorta blood flow (Lemley et al. 2013). At day 90 of gestation, umbilical artery blood flow and umbilical artery blood flow relative to placental weight was increased in the melatonin treated dams (Lemley et al. 2013). Therefore, dams supplemented with melatonin typically have increased feto-placental blood flow, and as a result can lead to increased placental efficiency. When analyzing blood flow, heart rate is also a common variable of interest as these measurements can be directly related to cardiac output. According to previous research, fetal heart rate was not different in ewes treated with melatonin (Lemley et al. 2013); however it is important to note that in the present study, maternal heart rate was increased by approximately 9% in heifers supplemented with dietary melatonin.

The increase in blood flow in melatonin-supplemented sheep may be partially mediated via melatonergic receptor-specific pathways (Lemley et al. 2013). Melatonin supplementation has also been associated with improved oxidative status (Paulis & Simko 2007). Melatonin has the ability to increase vascular relaxation through antioxidant properties, which could, in turn, increase the scavenging of superoxide free radicals resulting in increased bioavailability of nitric oxide (Reiter et al. 1995; Jauniaux et al. 2006). Lemley et al. (2012) showed that dietary melatonin supplementation caused a 20% increase in umbilical artery blood flow; however there was no change in uterine artery blood flow. Thakor et al. (2010) imposed a nitric oxide clamp during melatonin infusion, which then verified that these responses in umbilical blood flow were being mediated by nitric oxide production and/or bioavailability.

Melatonin secretion is known to follow a diurnal pattern, with melatonin concentrations being increased during night-time hours compared to day-time hours. During pregnancy, these same responses are maintained but peak plasma concentrations may vary at different stages of pregnancy (Kennaway et al. 1987; Pang et al. 1987). Rat offspring prior to post-natal day 10 do not have the pineal enzymes to synthesize melatonin (Reppert & Klein 1978). While these enzymes are absent until day 10, melatonin still crosses the placenta and can be transferred in maternal milk (McMillen & Nowak 1988; Reppert et al. 1979; Reppert & Klein, 1978). With these findings, it is evident to see that the dam serves as a natural source of melatonin for the offspring during gestation and into early lactation (Jahnke et al. 1999). Lastly, melatonin is known to mediate maternal-fetal transfer of information related to circadian rhythm and photoperiod (Williams et al. 1991; Velàzquez et al. 1992).

Feeding exogenous melatonin to female adult rats can cause disruption of normal estrous cycles and reduced fertility, due to the suppression of luteinizing hormone and resulting in the suppression of ovulation (Rivest 1987; Vaughan et al. 1976; Ying and Greep 1973; Walker et al. 1982). Using a rodent model, Jahnke et al. (1999), examined mammary tissue and serum hormone concentrations of term rats supplemented with melatonin on days 6 through 19 of gestation. Mammary glandular tissue and serum hormone concentrations of term pregnant rats analyzing mammary glandular tissue, serum concentrations of estradiol-17β, progesterone, and luteinizing hormone on day 20 of gestation are not found to be different (Jahnke et al. 1999). In contrast, the present study found a decrease in progesterone, whereas estradiol-17β concentration tended to decrease in those that were supplemented with melatonin verses the CON. The variation

between the two studies could be due to specie differences or the dosage of melatonin provided. Sprague-Dawley-derived rats that were exposed to 0, 50, 100, or 200 mg/kg of BW/d of melatonin on gestation days 6 through 19 showed no difference in gravid uterine weight (Jahnke et al. 1999). Although the current study did not determine gravid uterine weight, it did measure blood flow, which is highly correlated with placental efficiency (Lemley et al. 2013).

Additional studies were performed in mice (Sanchez-Barcelo et al. 1990; San Martín et al. 1995) and red deer (Asher et al. 1994) showing that melatonin inhibits mammary gland development in vivo and in vitro; therefore, suggesting a direct inhibition of growth during lobuloaveolar development (Sanchez-Barcelo et al. 1990; San Martín et al. 1995). In order for lobuloalveolization to develop, an increase in serum concentrations of estrogen, progesterone, prolactin, and glucocorticoids must occur (Dembinski & Shiu 1987; Ichinose & Nandi 1966; Vonderhaar 1987). Prolactin is known to be a mediator in mammary gland growth (Asher et al. 1994). It is important to note that these observations were seen in mice and deer rather than dairy animals that were used in the current study. The mouse strain used in the experiments previously mentioned was lacking the enzymes necessary for melatonin synthesis (Ebihara et al. 1986; Ebihara et al. 1987). It is possible that since these mice do not carry the ability to make endogenous melatonin, perhaps the mammary growth tissue did not respond in the same fashion as cattle.

A study performed by Dahl et al. (2000) tested the hypothesis that dietary melatonin supplementation in cattle mimicking a short-day pattern of release would decrease milk yield during an established lactation. The cows were exposed to 18 h of

light and 6 h of darkness. Melatonin-supplemented cows were treated with melatonin in the middle of the photophase for 8 weeks and found no difference in milk yield between treatment groups (Dahl et al. 2000). The experiment was then repeated during early lactation, but the same results were found with no effect on milk yield (Dahl et al. 2000). In contrast, the current study showed an increase in milk yield on days 2 and 3. The differences in responses could have been due to Dahl et al. (2000) using mature cows and the current study using heifers. The Dahl et al. (2000) study also supplemented melatonin by a daily oral bolus, whereas the current study top-dressed grain with melatonin. This difference in supplementation could result in a different rate of melatonin diffusion into the blood stream. There are a number of factors that regulate mammary gland development and milk yield that need further research.

There are several implications that melatonin supplementation could have on the possibility of altering uterine artery hemodynamics through negating the consequences of fetal growth restriction. Compromised pregnancies have a decrease in uterine blood flow, which is commonly associated with fetal growth restriction. This study utilized a technique that can have the potential to negate these compromised pregnancies by supplementing melatonin and improving uterine artery hemodynamics. Increasing uterine blood flow during late gestation could be a potential benefit to producers by having the potential for healthier calves at birth while not affecting gestation length or birth weight. Increased uterine blood flow has the potential to increase placental transport capacity supporting fetal growth and metabolism. With the observed increase in total uterine artery blood blow, there could be future implications of increased growth of offspring by further investigating blood distribution and physiological functions of various organs.

The improved antioxidant capacity during melatonin supplementation could be directly related to the increase in uterine artery blood flow. These antioxidant capabilities could result in less stress during pregnancy resulting in the possibility of having fewer compromised pregnancies. With the observed increase in milk yield, this could have a large impact on the dairy industry. This increase in milk yield can result in more pounds of milk to sell benefiting both the consumer and the producer. Early lactation is a vital time point in a calf's life and if it can be manipulated by melatonin supplementation this could have an impact on calf survival and overall health. In the future, replication of this experiment in beef cattle could have the potential of increasing milk availability, which could result in health benefits following parturition. Future studies using melatonin along with other therapeutics is of utmost importance in better understanding future implications.

Table 1 Ingredient and average nutrient composition of diets fed to pregnant heifers

Item	Total mixed ration
Ingredient (% of dry matter)	
Corn silage	49
Cotton seed	5
Dry cow grain[1]	10
Baleage	26
Hay	10
Diet Composition (% of dry matter)	
Crude protein	12.6
Neutral detergent fiber	53.6
Acid detergent fiber	30.4
Fat	3.0
Ash	7.8

[1]Dry cow grain consisted of 21.9% wheat middling, 21% soy hulls, 17% ground corn, 13.5% cottonseed meal, 11.3% soybean meal, 5.21% fishmeal, 4.8% calcium carbonate, 1.35% magnesium oxide, 0.75% salt, 0.71% mono-dical, 0.68% mag-pot, 0.58% 20,000 IU vitamin E, 0.34% selenium, 0.29% zinc-pro, 0.25% fat (grease). Dry cow grain was calculated not analyzed.

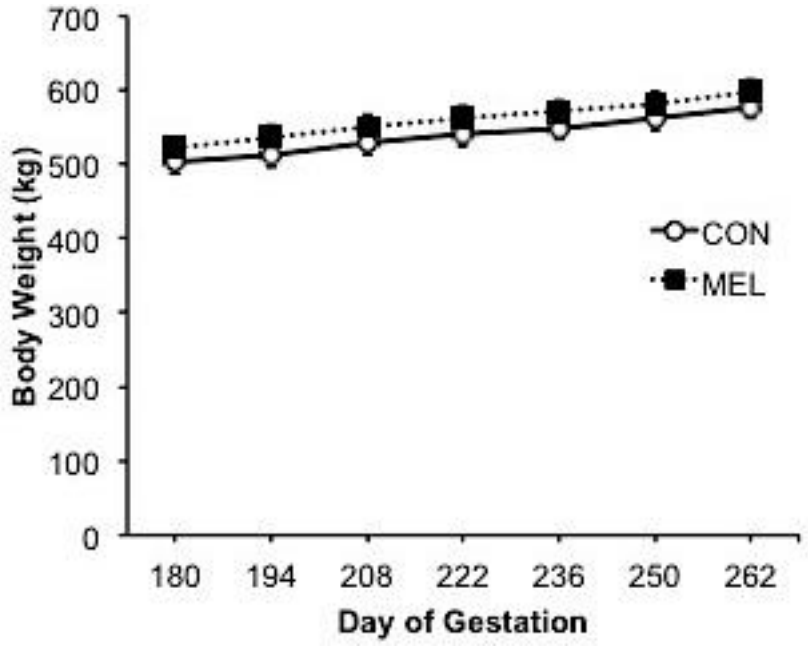

Figure 3 Maternal body weights in heifers treated with (MEL) or without dietary melatonin (CON) from day 190 to 262 of gestation

A main effect of gestational day was observed ($P < 0.01$), while melatonin treatment did not influence body weight ($P = 0.34$).

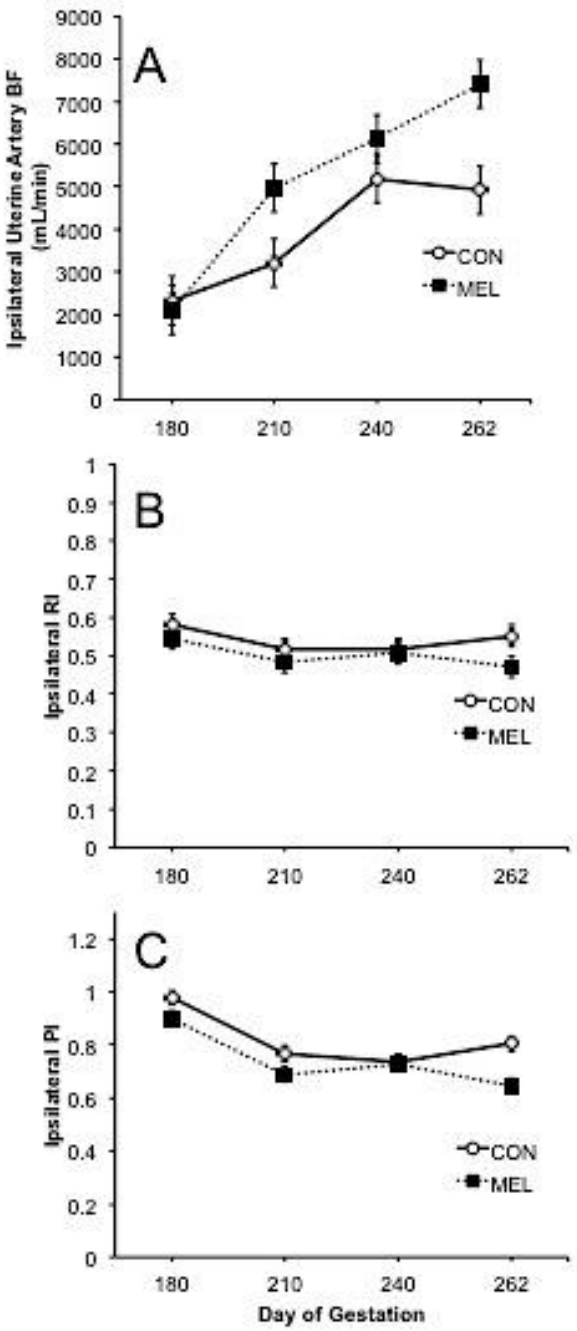

Figure 4 Ipsilateral uterine artery blood flow RI and PI in heifers treated with (MEL) or without dietary melatonin (CON) from day 190 to 262 of gestation

Main effects of gestational day ($P < 0.01$) and melatonin treatment ($P = 0.01$) were observed for ipsilateral uterine artery blood flow (A). A main effect of gestational day ($P = 0.02$) was observed for ipsilateral RI (B). A main effect of gestational day ($P < 0.02$) was also observed for ipsilateral PI (C). Ipsilateral RI ($P = 0.19$) and PI ($P = 0.29$) were not influenced by melatonin supplementation.

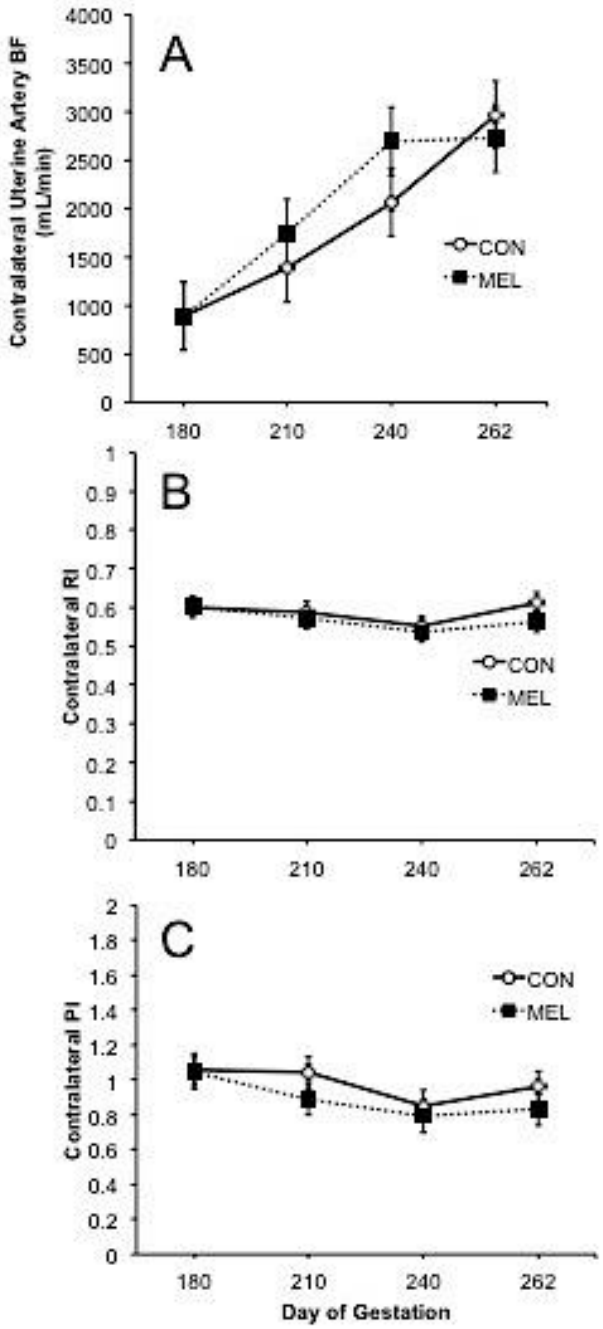

Figure 5 Contralateral uterine artery blood flow, RI, and PI in heifers treated with (MEL) or without dietary melatonin (CON) from day 190 to 262 of gestation

A main effect of gestational day ($P < 0.01$) was observed for contralateral uterine artery blood flow, with blood flow increasing from d 180 to d 240 (A). Contralateral uterine artery RI (B) and PI (C) were not different across gestational day ($P = 0.10$; $P > 0.09$) or dietary treatment ($P = 0.43$; $P = 0.28$), respectively.

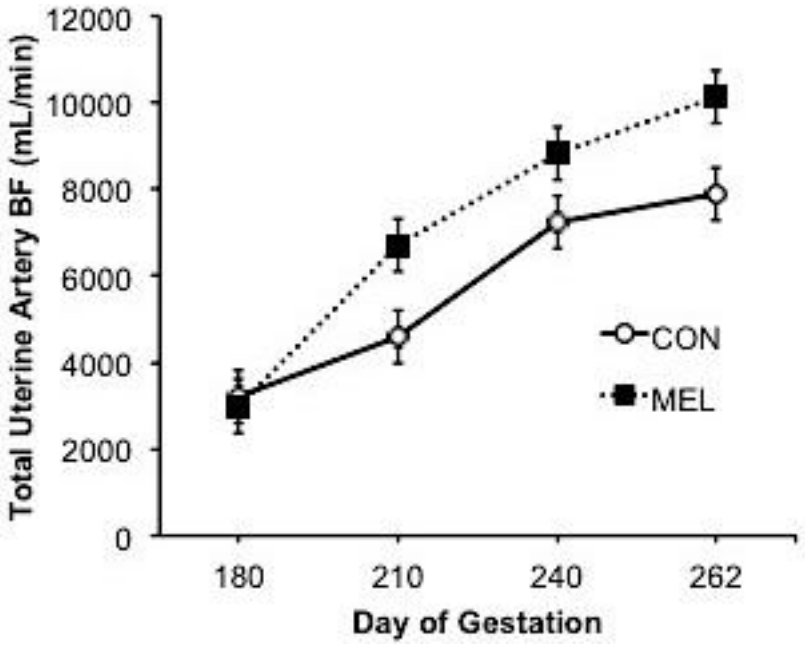

Figure 6 Total uterine artery blood flow in heifers treated with (MEL) or without dietary melatonin (CON) from day 190 to 262 of gestation

Main effects of gestational day ($P < 0.01$) and melatonin treatment ($P < 0.09$) were observed for total uterine blood flow.

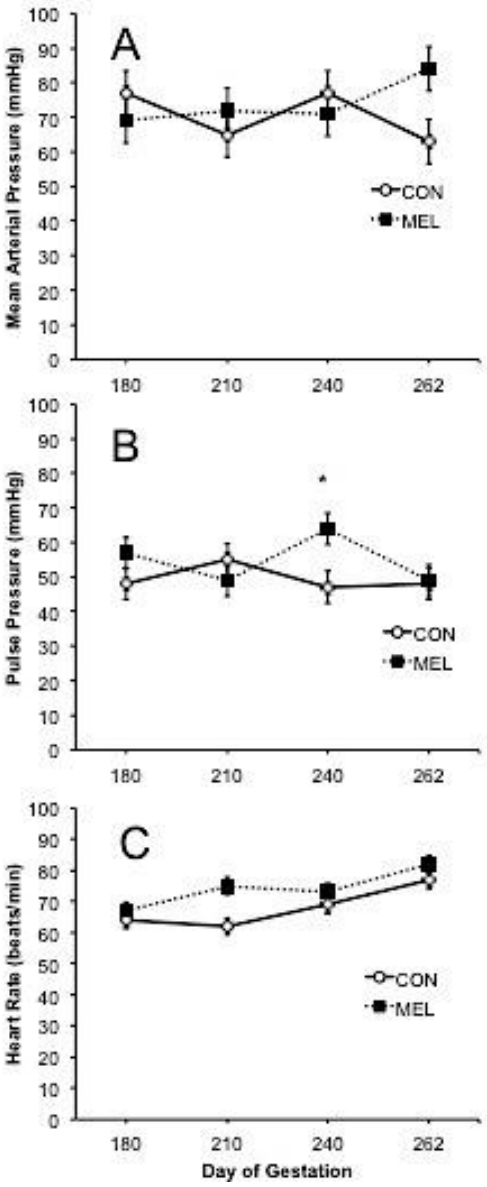

Figure 7 Maternal mean arterial pressure, pulse pressure, and heart rate in heifers treated with (MEL) or without dietary melatonin (CON) from day 190 to 262 of gestation

There were no differences across gestational day (P = 0.81) or dietary treatment (P = 0.52) for maternal mean arterial pressure. A treatment by gestational day interaction (P = 0.02) was observed for pulse pressure (B). Asterisk (*) at day 240 representing the increase in MEL versus CON at day 240 of gestation. A main effect of gestational day (P < 0.01) and dietary treatment (P = 0.03) was observed for maternal heart rate (C).

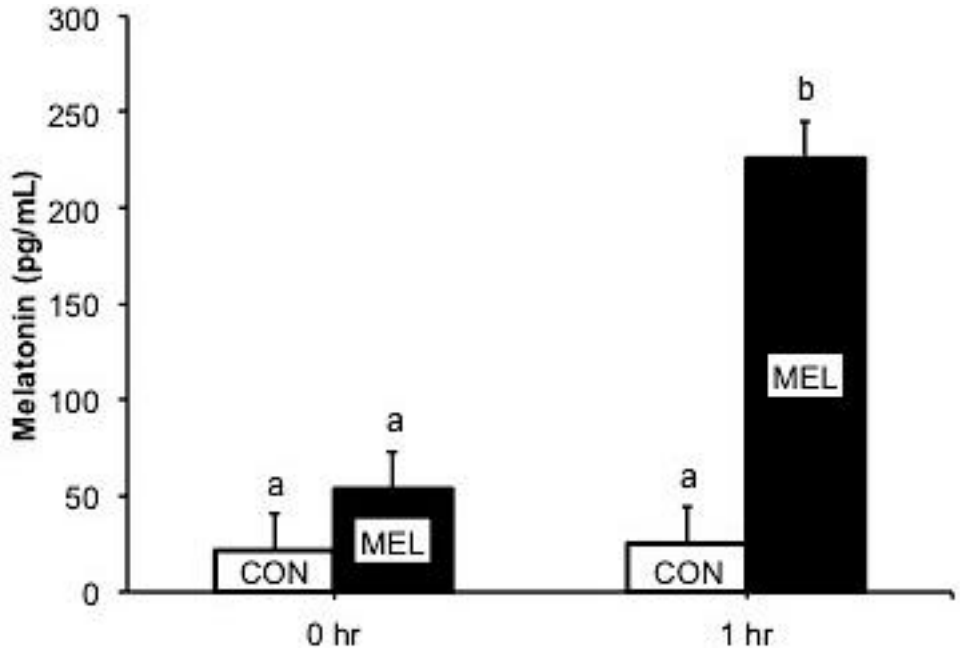

Figure 8 Maternal serum melatonin concentrations treated with (MEL) or without dietary melatonin (CON) in heifers at hour 0 (pre-melatonin supplementation) and hour 1 (post-melatonin supplementation)

A dietary treatment by hour interaction ($P < 0.04$) was observed. Different letters represent a significant difference ($P < 0.05$).

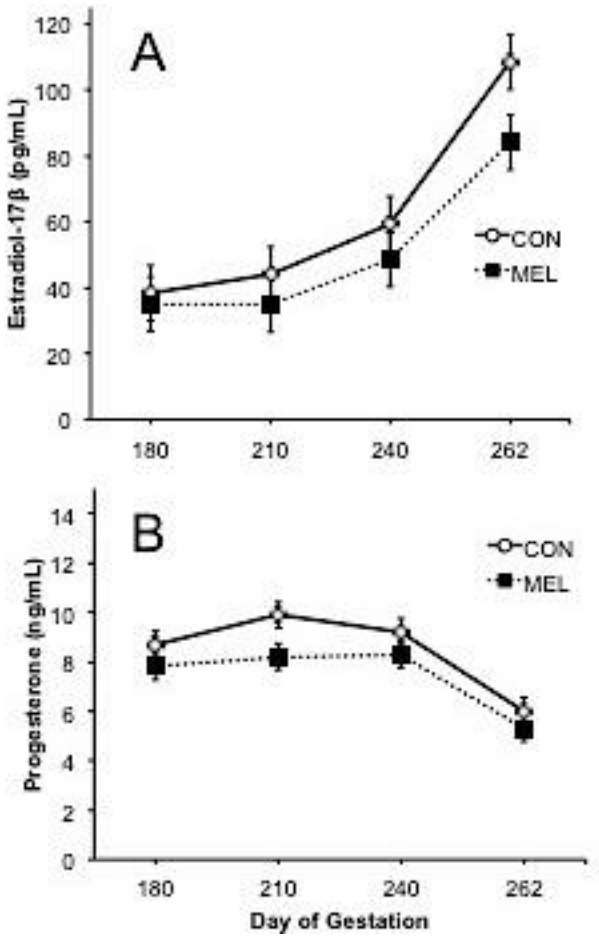

Figure 9 Maternal serum concentrations of estradiol-17β and progesterone in heifers treated with (MEL) or without dietary melatonin (CON) from day 190 to 262 of gestation

A main effect of gestational day ($P < 0.01$) was observed for maternal concentrations of estradiol-17β (A). A main effect of gestational day ($P < 0.01$) and melatonin supplementation ($P < 0.05$) was observed for concentrations of progesterone (B).

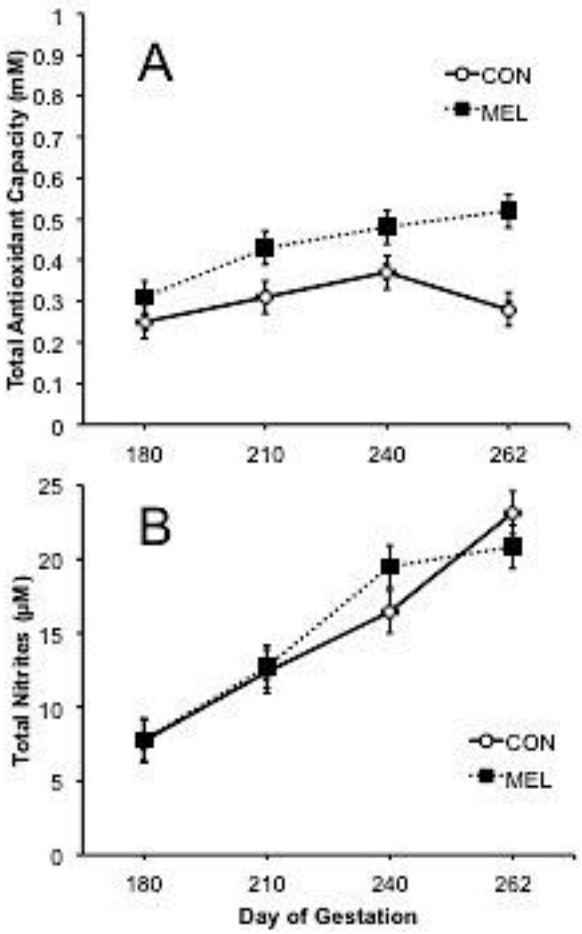

Figure 10 Maternal total antioxidant capacity and total nitrites in heifers treated with (MEL) or without dietary melatonin (CON) from day 190 to 262 of gestation

Main effects of gestational day ($P < 0.02$) and dietary treatment ($P < 0.01$) were observed for total serum antioxidant capacity (A). A day effect ($P < 0.01$) was observed for total serum nitrites increasing with gestational age (B).

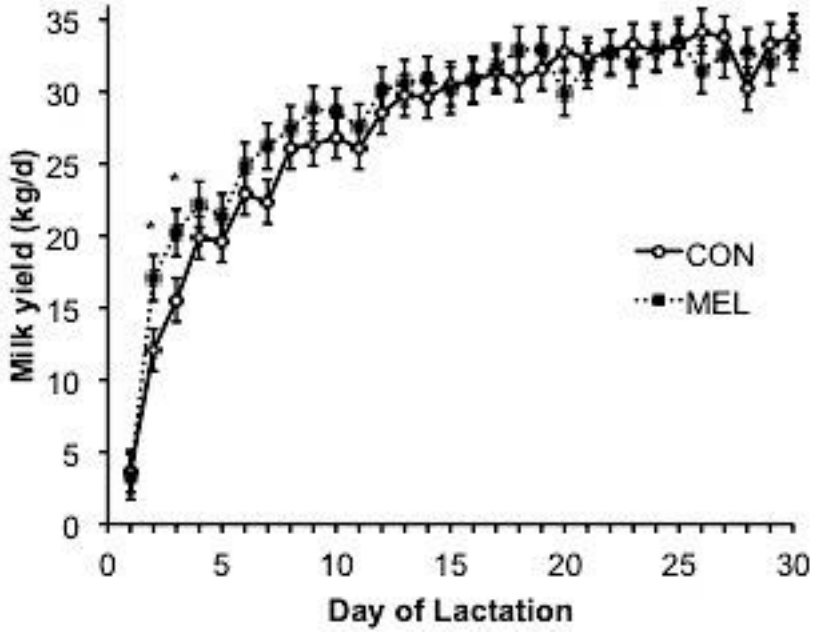

Figure 11 Maternal milk yield through day 30 of lactation in heifers treated with (MEL) or without melatonin (CON) from day 190 to 262 of gestation

A treatment by day interaction ($P < 0.01$) was observed for milk yield. Asterisk (*) represents a significant difference ($P < 0.05$) between treatments within a day.

References

Asher GW, Veldhuizen FA, Morrow CJ, and Duganzich DM 1994 Effects of exogenous melatonin on prolactin secretion, lactogenesis, and reproductive seasonality of adult female red deer. Journal of Reproduction and Fertility **100** 11-19.

Beckman JS & Koppenol WH 1996 Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly. American Journal of Physiology-Cell Physiology **40.5** C1424.

Bollwein H, Baumgartner U, and Stolla R 2001 Transrectal Doppler sonography of uterine blood flow in cows during pregnancy. Theriogenology **57** 2053-2061.

Camacho LE, Lemley CO, Prezotto LD, Bauer ML, Freetly HC, Swanson KC, and Vonnahme KA 2014 Effects of maternal nutrient restriction followed by re-alimentation during mid-gestation on uterine blood flow in beef cows. Theriogenology **81** 1248-1256.

Dahl GE, Buchanan BA, and Tucker HA 2000 Photoperiodic effects on dairy cattle: A review. Journal of Dairy Science **83** 885-893.

Dembinski TC & Shiu RP 1987 Growth factors in mammary gland development and function. The Mammary Gland: Development, Regulation, and Function 355-373.

Ebihara S, Marks T, Hudson DJ, and Menaker M 1986 Genetic control of melatonin synthesis in the pineal gland of mice. Science **231** 491-493.

Ebihara S, Hudson DJ, Marks T, and Menaker M 1987 Pineal indole metabolism in the mouse. Brain Research **416** 136-140.

Ferrell CL & Ford SP 1980 Blood flow steroid secretion and nutrient uptake of the gravid bovine uterus. Journal of Animal Science **50** 1113-1121.

Hart CG, Camacho LE, Swanson KC, Vonnahme KA, and Lemley CO 2014 Maternal nutrient restriction followed by realimentation during early to mid-gestation on hepatic steroid inactivation in beef cows. Domestic Animal Endocrinology **49** 31-38.

Ichinose RR & Nandi S 1966 Influence of hormones on lobulo-alveolar differentiation of mouse mammary glands in vitro. Journal of Endocrinology **35** 331-340.

Jahnke G, Marr M, Myers C, Wilson R, Travlos G, and Price C 1999 Maternal and developmental toxicity evaluation of melatonin administered orally to pregnant sprague-dawley rats. Toxicological Sciences **50** 271-279.

Jauniaux E, Poston L, and Burton GJ 2006 Placental-related diseases of pregnancy: Involvement of oxidative stress and implications in human evolution. Human Reproduction **12** 747-755.

Kaïs H, Al-Gubory, Fowler PA, and Garrel C 2010 The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. The International Journal of Biochemistry & Cell Biology **42** 1634-1650.

Kennaway DJ, Matthews CD, and Seamark RF 1981 Pineal function in pregnancy: Studies in sheep and man. Pineal Function 123-126.

Lemley CO, Meyer AM, Camacho LE, Neville TL, Newman DJ, Caton JS, and Vonnahme KA 2012 Melatonin supplementation alters uteroplacental hemodynamics and fetal development in an ovine model of intrauterine growth restriction. American Journal of Physiology - Regulatory, Integrative, and Comparative Physiology **302** R454-R467.

Lemley CO, Camacho LE, and Vonnahme KA 2013 Uterine infusion of melatonin or melatonin receptor antagonist alters ovine feto-placental hemodynamics during midgestation. Biology of Reproduction **89** 1-9.

McMillen IC & Nowak R 1988 Maternal pinealectomy abolishes the diurnal rhythm in plasma melatonin concentrations in the fetal sheep and pregnant ewe during late gestation. Journal of Endocrinology **120** 459-464.

National Research Council 2000 Nutritional requirements of dairy cattle. 7th rev. National Academy Press.

Pang SF, Tang PL, Tang GK, Yam AC, and Ng KW 1987 Plasma levels of immunoreactive melatonin, estradiol, progesterone, follicle stimulating hormone, and beta-human chorionic gonadotropin during pregnancy and shortly after parturition in humans. Journal of Pineal Research **4** 21-31.

Paulis L & Simko F 2007 Blood pressure modulation and cardiovascular protection by melatonin: potential mechanisms behind. Physiological Research **56** 671-684.

Reynolds LP & Ferrell CL 1987 Transplacental clearance and blood flows of bovine gravid uterus at several stages of gestation. American Journal of Physiology **253** R735.

Redmer DA, Wallace JM, and Reynolds LP 2004 Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. Domestic Animal Endocrinology **27** 199-217.

Reiter RJ, Melchiorri D, Sewerynek E, Poeggeler B, Barlow-Walden L, Chuang J, Ortiz GG, and AcuñaCastroviejo D 1995 A review of the evidence supporting melatonin's role as an antioxidant. Journal of Pineal Research **18.1** 1-11.

Reiter RJ, Melchiorri D, Sewerynek E, Poeggeler B, Barlow-Walden L, Chuang J, Ortiz GG, and AcunaCastroviejo D 2007 A review of the evidence supporting melatonin's role as an antioxidant. Journal of Pineal Research **18** 1-11.

Reppert SM & Klein DC 1978 Transport of maternals [^{3}H]-melatonin in the neonatal rat. Endocrinology **102** 582-588.

Reppert SM, Chez RA, Anderson A, and Klein DC 1979 Maternal-fetal transfer of melatonin in the nonhuman primate. Pediatric Research **13** 788-791.

Richter HG, Hansell JA, Raut S, and Giussani DA 2009 Melatonin improves placental efficiency and birth weight and increases the placental expression of antioxidant enzymes in undernourished pregnancy. Journal of Pineal Research **46** 357-364.

Rivest RW 1987 The female rat as a model describing patterns of pulsatile LH secretion during puberty and their control by melatonin. Gynecology Endocrinology **1** 279-293.

Sanchez-Barcelo EJ, Mediavilla MD, and Tucker HA 1990 Influence of melatonin on the mammary gland growth: In vivo and in vitro studies. Proceedings of the Society for Experimental Biology and Medicine **194** 103-107.

Sanchez-Barcelo, Mediavilla MD, Zinn SA, Buchanan BA, Chapin LT, and Tucker HA 1991 Melatonin suppression of mammary growth in heifers. Biology of Reproduction **44** 875-879.

San Martín M, Recio J, Sánchez-Barceló EJ, and Mediavilla MD 1995 Melatonin inhibits mammary gland development during pregnancy. Neuroendocrinology Letters **17** 245-250.

Thakor AS, Herrera EA, Seron-Ferre M, and Giussani DA 2010 Melatonin and vitamin C increase umbilical blood flow via nitric oxide-dependent mechanisms. Journal of Pineal Research **49** 399-406.

Vaughan MK, Reiter RJ, and Vaughan GM 1976 Fertility patterns in female mice following treatment with arginine vasotocin or melatonin. International Journal of Fertility and Sterility **21** 65-68.

Velàzquez E, Esquifino AI, Zueco JA, Ruiz Alusac JM, and Blazquez E 1992 Evidence that circadium variations of circulating melatonin levels in fetal and suckling rats are dependent on maternal melatonin transfer. Neuroendocrinology **55** 321-326.

Vonderhaar BK 1987 Prolactin: Transport, function, and receptors in mammary gland development and differentiation. The Mammary Gland: Development, Regulation, and Function 383-417.

Vonnahme KA & Lemley CO 2012 Programming the offspring through altered uteroplacental hemodynamics: How maternal environment impacts uterine and umbilical blood flow in cattle, sheep, and pigs. Reproduction, Fertility, and Development **24** 97-104.

Walker RF, McCamant S, and Timiras PS 1982 Melatonin and the influence of the pineal gland on timing of the LH surge in rats. Neuroendocrinology **35** 37-42.

Williams LM, Martinoli MG, Titchener LT, and Pelletier G 1991 The ontogeny of central melatonin binding sites in the rat. Endocrinology **128** 2083-2090.

Ying SY & Greep RO 1973 Inhibition of ovulation by melatonin in the cyclic rat. Endocrinology **92** 333-335.

Zinn SA, Chaplin LT, Enright WJ, Schroeder AL, Stanisiewski EP, and Tucker HA 1988 Growth, carcass composition and plasma melatonin in postpubertal beef heifers fed melatonin. Journal of Animal Science **66** 21-27.

CHAPTER III

EFFECTS OF SUPPLEMENTING DIETARY MELATONIN DURING LATE GESTATION ON GROWTH AND CARDIOVASCULAR MEASUREMENTS OF OFFSPRING

Introduction

Fetal growth and development are complex biological events that can be influenced by genetic, epigenetic, maternal maturity, environment, and other factors (Redmer et al. 2004; Gootwine 2005). Fetal growth restriction can occur as the result of an insult to one of the previous variables mentioned. When fetal growth restriction occurs, the result can be permanent stunting of post-natal growth, decrease in feed efficiency, negative effects on whole body composition and meat quality, as well as long-term health effects and athletic performance (Wu et al. 2006). When endocrine or nutritional status is altered, developmental adaptions may occur that permanently change the structure, physiology, metabolism, and post-natal growth of the offspring (Wu et al. 2006).

Undernutrition or overnutrition can also play critical roles in intrauterine growth restriction. From this research, a field of study called fetal programming has been derived in an effort to further investigate intrauterine growth restriction via programming the fetus during gestation. Fetal programming is when the intrauterine environment of the conceptus may alter expression of the fetal genome and have lifelong consequences (Wu

et al. 2006). Recently, our laboratory began investigating potential therapeutics to stimulate offspring growth and development during late gestation (Lemley et al. 2012). For example, melatonin supplementation during a compromised or normal pregnancy may alter uteroplacental blood flow via melatonergic receptor-mediated pathways or indirectly by decreasing oxidative stress in the vascular system (Lemley et al. 2012; Brockus et al. 2015).

Melatonin is unique in that it has the ability to protect the body against free radicals via direct or indirect pathways (Reiter et al. 1995). Oxidative stress is increased during pregnancy and is directly related to several pregnancy disorders, such as spontaneous abortions, embryopathies, preeclampsia, fetal growth restriction, preterm labor, and low birth weight (Kaïs et al. 2010). The links between oxidative stress, the female reproductive system, and the development of negative pregnancy outcomes, results in important concerns in both human and animal reproductive sciences (Kaïs et al. 2010).

Close relationships between melatonin and the cardiovascular system exist (Sewerynek 2002). Moreover, melatonin supplementation increased distal skin temperature and decreased core body temperature (Kräuchi et al. 1997) as well as initiated primary hair follicle growth (Nixon et al. 1993). With these findings, the current study measured offspring blood pressure, heart rate, liver blood flow, skin temperature, and hair growth to analyze if supplementing melatonin to the dams would have an effect on these variables during post-natal development. Previously, our lab showed that supplementing melatonin increased uterine artery blood flow (Brockus et al. 2015); therefore, we were interested to see if this finding would be programmed in the offspring.

Based off of previous fetal programming studies and melatonin's antioxidant effects, the current study supplemented dietary melatonin to dams and followed post-natal growth and cardiovascular measurements of their offspring. Our primary objective was to measure hepatic portal blood flow and growth following melatonin supplementation in Holstein heifers during late gestation. Therefore, we hypothesized that supplementing dietary melatonin to the dams would increase post-natal growth and cardiovascular measurements.

Materials and Methods

Animal care and use were according to protocols approved by the Mississippi State University Institutional Animal Care and Use Committee.

Animal Model

The establishment of pregnancy and maternal treatments were previously outlined in Brockus et al. (2015). Briefly, heifers were inseminated to sex-sorted semen between December 6, 2012 and January 30, 2013. On day 170 of gestation, the heifers were trained to the Calan feeding system to monitor individual dry matter intake. Dietary melatonin supplementation (MEL; n =10) or no melatonin supplementation (CON; n =10) started on day 190 of gestation and ended on day 262. Melatonin (Batch 130307, Health Supplement Wholesalers, Red Lion, PA, USA) supplementation was provided by top-dressing grain with 2 mL of 10 mg/mL melatonin dissolved in ethanol (20 mg of melatonin per head per day). A total mixed ration of 8.9 kg/d was provided based on NRC recommendations for a pregnant Holstein heifer during late gestation along with ad libitum access to water. Maternal endocrine and uterine hemodynamic measurements

were collected during melatonin supplementation and were previously reported (Brockus et al. 2015).

At birth, calves were separated from their dams and given 3.8 L of colostrum. Calves were managed identically with no further treatments. Calves were fed 5.7 L of whole milk daily and offered 0.9 kg/d of starter grain. Starter grain was increased by 0.9 kg/d when orts were 0 kg. Two calves were aborted during the fall calving, one from a melatonin treated dam and one from a control treated dam. Therefore, measurements were recorded on (n = 9) calves born to melatonin treated dams (7 heifer calves; 2 bull calves) and (n = 9) calves born to control dams (8 heifer calves; 1 bull calf). Calf (n = 18) body weight and heart girth were collected weekly from weeks 0 through 9. Curved crown rump length, abdominal circumference, hip height, wither height, blood pressure using a digital sphygmomanometer (Omron, Schaumburg, IL, USA), heart rate, and a blood sample were collected on weeks 0, 1, 2, 3, and 4 of age.

Thermal imaging using a Flir ThermaCAM® S60 (Flir Systems, Boston, MA, USA) infrared thermography camera and hair weight were collected on week 1. Firstly, a hair sample was collected in a pre-weighed bag by shaving a 5.08 x 10.16 cm area on the animals' right thoracic region directly behind the shoulder. The hair plus the pre-weighed bag was then weighed again to calculate hair weight. The hair weight was then subtracted from the pre-weighed bag weight to find the actual hair weight. Next, images were taken of the 5.08 x 10.16 cm shaved area and adjacent unshaved area (Fig. 12). Temperatures for both the shaved and unshaved areas were then analyzed using ThermaCAM Researcher Pro 2.7 software (FLIR Systems). Ambient temperature (P = 0.76) and humidity (P = 0.36) were not different at the time of thermal imaging between calves

born to MEL-treated dams versus CON-treated dam. Average temperature of the shaved area was determined by constructing a rectangular polygon corresponding to the spot and analyzing temperatures with this area. The exact polygon was transferred to the unshaved area and an average surface temperature was calculated for the unshaved area. The same polygon was used to ensure a standard surface area was being measured.

Doppler Ultrasonography

Hepatic portal blood flow was determined on weeks 0 and 4 of age via Doppler ultrasonography (MicroMaxx, SonoSite, Inc., Bothell, WA, USA) using a transabdominal probe (Sonosite MicroMaxx with a Phased array P17 1-5MHz 17x17mm probe; Fig. 13). The liver was first identified on the right side of the calf. The hepatic portal vein transfers blood from the gastrointestinal tract to the liver. The superior mesenteric vein and the splenic vein form the portal vein. The portal vein was located by scanning through the liver at the 10^{th} intercostal space. The ultrasound transducer was aligned to the portal vein at an average angle of insonation of 66 ± 9 degrees. Mean velocity (MnV) was calculated using the equation: (systolic velocity – diastolic velocity)/pulsatility index. Blood flow was calculated using the equation: (MnV*vessel area*60 sec).

Blood Sampling Analysis

Blood serum was collected on weeks 0, 1, 2, 3, and 4 of age and analyzed for total antioxidant capacity, nitrites, and cortisol concentrations. Insulin-like growth factor one (IGF-1) was analyzed from blood serum on weeks 0 and 4.

Total antioxidant capacity was determined with a colorimetric assay kit (Cayman Chemical Co. Ann Abor, MI, USA). The antioxidant capacity assay was performed

following manufactures' instructions except that the serum was diluted with assay buffer to place unknowns within 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. All serum samples were diluted in a 1:4 dilution. The ABTS oxidation product was monitored at 750 nm via a Spectra Max Plus plate reader (Sunnyvale, CA, USA). The kit does not separate aqueous- and lipid-soluble antioxidants; therefore, the combined antioxidant activities of all constituents in maternal serum are reported. The capacity of serum antioxidants in unknown samples to prevent substrate oxidation is compared with that of a tocopherol analogue (Trolox) and is reported as mM Trolox equivalents. Samples were analyzed against a linear Trolox standard curve (0 – 0.33 mM), with a sensitivity of 0.01 mM and intra- and interassay coefficients of variation were 13.3% and 5.9%, respectively.

Total serum nitrites were determined using the 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 the reduction of total nitrates to nitrites using the Griess method and analyzed against a linear nitrites standard curve (0 – 100 μM), with a sensitivity of 0.6 μM and intra- and interassay coefficients of variation of 1.3% and 2.27%, respectively.

Cortisol was determined using the Coat-a-Count cortisol RIA kit (Siemens Healthcare Diagnostics Inc., Los Angeles, CA, USA). The cortisol assay was performed following manufacture's instructions. Briefly, following the addition of calibrators and samples to their respective tubes, 1mL of ^{125}I cortisol was added and samples incubated for 45 minutes at 37°C in a water bath. The tubes were then decanted thoroughly and

analyzed using a Packard gamma counter (Meriden, CT, USA). Intra-assay coefficient of variation was 4.15%.

Insulin-like growth factor 1 was determined using an ELISA Kit (MybioSource, Inc., San Diego, CA, USA). The IGF-1 assay was performed following manufacture's instructions. Briefly, this assay used the competitive inhibition enzyme immunoassay technique. Standards or sample was added to the appropriate plate wells with an antibody specific for IGF-1 and Horseradish Peroxidase (HRP) conjugated IGF-1. The competitive inhibition reaction is launched between HRP labeled IGF-1 and unlabeled IGF-1 with the antibody. A substrate solution was then added to the wells and color develops in opposite to the amount of IGF-1 in the sample. Lastly, optical density was determined using a Spectra Max Plus plate reader set to 450 nm. Intra-assay coefficient of variation was 10.16%.

Statistical Analysis

Dependent variables were analyzed using repeated measures ANOVA of the mixed procedure of SAS (SAS software version 9.3, SAS Institute, Cary, NC, USA). Means were separated using the PDIFF option of the LSMEANS statement. The model statement contained dietary treatment, age, and their interaction. Main effects of dietary treatment or age are discussed in the absence of significant treatment by day interactions. Least square means and SEM are reported. Statistical significance was declared at $P < 0.05$.

Results

Calf Growth Measurements

A treatment by age interaction ($P < 0.01$) was observed for calf body weight, which was increased in MEL-treated dams versus CON-treated dams at weeks 8 and 9 (Fig. 14A). A treatment by age interaction ($P < 0.01$) was also observed for heart girth, which was decreased in MEL-treated dams versus CON-treated dams at week 2; however heart girth was increased in MEL-treated dams versus CON-treated dams at week 9 (Fig. 14B). A main effect of age ($P < 0.01$) was observed for curved crown rump length, which was increased with age except from weeks 3 to 4 (data not shown). A main effect of age ($P < 0.01$) was observed for abdominal circumference, hip height, and wither height, which were all increased with age (data not shown).

Cardiovascular Measurements

Calf hepatic portal blood flow was not different across age ($P = 0.06$) and averaged 2.4 ± 0.4 L/min at week 0 and 3.4 ± 0.5 L/min at week 4 (Fig. 15A). In addition, calf hepatic portal blood flow was not different between treatments ($P = 0.94$) and averaged 2.8 ± 0.5 L/min in the offspring born to MEL-treated dams and 2.9 ± 0.5 L/min in the offspring born to CON-treated dams. Calf hepatic portal blood flow was also not different by age or treatment when expressed relative to calf body weight (Fig. 15B). No differences were observed among treatment ($P = 0.99$) or age ($P = 0.50$) for mean velocity. The mean velocity averages were 55.2 ± 6 cm/sec at week 0 and 60.4 ± 6 cm/sec at week 4. Mean velocity among treatments was 58 ± 7 cm/sec in both offspring born to MEL-treated dams and CON-treated dams. No main effects of treatment ($P = 0.22$) or age ($P = 0.50$) were observed for vessel diameter (data not show). The vessel

diameter averages were 1.2 ± 0.05 cm at week 0 and 1.3 ± 0.05 cm at week 4. Vessel diameters were 1.2 ± 0.05 cm in offspring born to MEL-treated dams and 1.3 ± 0.05 cm in offspring born to CON-treated dams.

Pulse pressure was not different across age ($P = 0.73$) or treatment ($P = 0.79$) (Fig. 16A). There was a main effect of age ($P = 0.04$) when observing mean arterial pressure, which was increased from week 0 to 1 followed by a decrease from week 1 to 2 and from week 1 to 4 (Fig. 16B). Mean arterial pressure was not different across treatments ($P = 0.19$). A main effect of age ($P < 0.01$) was observed for heart rate with a decrease from week 1 to 4 (Fig. 16C). Systolic blood pressure was not different across age ($P = 0.15$) or treatment ($P = 0.28$; data not shown). However, there was a main effect of age for diastolic blood pressure ($P = 0.03$), which increased from week 0 to 1 followed by a decrease from week 1 to 2 and week 1 to 4 (data not shown).

Calf Serum Analysis

Insulin-like growth factor 1 was not different between treatments ($P = 0.43$). A main effect of age ($P = 0.02$) was observed for IGF-1 concentrations, which was decreased from week 0 to 4 (Fig. 15C). An age by treatment interaction ($P = 0.04$) was observed for concentrations of cortisol, which was decreased in the MEL-treated dams compared to the CON-treated dams at week 2 (Fig. 17). Total antioxidant capacity was not different between treatments ($P = 0.14$). A main effect of age ($P < 0.01$) was observed for total antioxidant capacity, which peaked at week 1 and was lower at all other weeks (Fig. 18A). Nitrites were not different between treatments ($P = 0.69$). A main effect of age ($P < 0.01$) was observed for nitrites, which peaked at week 1 and was lower at all other weeks (Fig. 18B).

Thermal Imaging and Hair Sampling

There were no treatment differences (P = 0.71) in hair weight or adjusted weight of the hair (P = 0.53). Average temperature of the unshaved skin was not different between treatments (P = 0.59). Averaged shaved skin temperature was not different between treatments (P = 0.11).

Discussion

In the present study, there was no difference in calf weight at birth. This finding is unexpected as we previously reported a 25% increase in total uterine artery blood flow in MEL-treated dams vs. CON-treated dams (Brockus et al. 2015). Considering that 75% of the growth of the ruminant fetus occurs during the last two months of gestation (Robinson et al. 1977), the increase observed in uterine blood flow would be expected to increase birth weights.

Maternal melatonin supplementation ended at day 262 of gestation. Following this period all dams and calves were treated identically to examine in utero developmental programming. Interestingly, offspring born to MEL treated dams showed an increase in body weight at weeks 8 and 9 of age compared to calves born to CON treated dams. This increase in body weight could be a melatonin-programmed response that could possibly change skeletal muscle development and increase weight gain. If this is indeed a fetal programming response, this is an important find as there is no net increase in the number of muscle fibers following birth (Glore & Layman 1993; Greenwood et al. 2000; Nissen et al. 2003).

The increase in body weight of the offspring born to the MEL treated dams could also be due to the increase in blood flow in utero even though there was not a difference

in birth weights. The increase in blood flow could have impacted organ development and therefore caused an increase in feed intake or feed efficiency of the offspring born to MEL-treated dams.

Melatonin has been well documented as a hormone that has the ability to alter cardiovascular function (Sewerynek 2002; Paulis & Simko 2007). The current study examined post-natal alterations in cardiovascular function following melatonin supplementation in utero. Lemley et al. (2013) showed an increase in fetal aorta blood flow following maternal melatonin supplementation. Interestingly, the current study observed no differences in hepatic portal blood flow between offspring born to MEL-supplemented vs. CON dams. The presence of vascular melatonin receptors/binding sites have been demonstrated and linked with vasoconstriction or vasodilatory effects (Sewerynek 2002). We hypothesized that melatonin receptors could be altered, in utero, causing the offspring to also show an increase in organ blood perfusion, such as the hepatic portal blood flow. However, it does not appear that this melatonin mediated modulation in the cardiovascular system carried over into the offspring for hepatic portal blood flow.

Impaired melatonin production has been intimately linked to hypertension (Paulis & Simko 2007). This could be related to the extraordinary antioxidant activity that melatonin possesses. However, the exact mechanism leading to blood pressure modulation following melatonin exposure is unknown (Paulis & Simko 2007). In humans, those with hypertension normally have decreased melatonin secretion (Sewerynek 2002). Humans administered as little as 1 mg of melatonin had reduced blood pressure after 90 minutes (Sewerynek 2002). In contrast, the current study

observed no treatment differences in offspring blood pressure. In our previous work (Brockus et al. 2015), maternal melatonin supplementation during late gestation did not alter mean arterial pressure. These findings were unforeseen as a number of studies show that melatonin decreases blood pressure. This could be a result of the dosage that was given to the dams not being enough to observe alterations in maternal and offspring blood pressure. Since heart rate is a function of cardiac output, the differences that were not seen in hepatic portal blood flow do match up with no differences observed in heart rate.

Melatonin's effects on body temperature and hair growth are important in this study as we observed the temperature of both the calf's skin as it was shaven or unshaven. Studies in humans observe that following oral administration of melatonin, an increase in distal skin temperature, decrease in core body temperature, heart rate, and proximal temperature were observed (Kräuchi et al. 1997; Cagnacci et al. 1995). However, the temperatures of shaved and unshaved skin were not different between offspring born to MEL-treated dams vs. CON-treated dams. This difference in responses could be due to the specie variation in humans versus dairy cattle. Cashmere wethers received a subcutaneous injection of 70 mg of melatonin in a controlled drug release formation to see if initiation of fiber growth in hair follicles was influenced by melatonin (Nixon et al. 1993). Histological examinations of skin biopsies were taken over 14 days (Nixon et al. 1993). It was found that primary hair follicles of goats supplemented with melatonin had initiated fiber growth, whereas primary follicles of untreated goats largely remained in the quiescent stage (Nixon et al. 1993). It was also observed in many mammals that fiber growth initiation is cued by photoperiod or concentrations of melatonin (Allain et al. 1981). In contrast to these studies, the current study observed no

treatment differences in hair weight. This could once again be due to specie differences; however, it is important to note that the dosage given to the melatonin-treated dams were at the physiological level whereas the goats were treated with supraphysiological dosages.

Melatonin has been investigated as being a great modulator for cortisol and stress. Cortisol was of importance to measure in this study as a decrease in stress could result in an increase in blood flow to vital organs such as the liver. A study by Guesdon et al. (2013) using adult ewes characterized stress responses during the day when melatonin is decreased and at night when melatonin is increased. They found less cortisol during the night as compared to the day (Guesdon et al. 2013). In order to further isolate if this response was due to melatonin or darkness, they submitted ewes to familiar conspecific withdrawal during the night with the lights on (Guesdon et al. 2013). They found that ewes infused with melatonin showed decreased plasma cortisol as compared to ewes that were infused with saline (Guesdon et al. 2013). This would support that melatonin has a calming effect in stressful situations. Similarly, we found a decrease in plasma cortisol at week 2 in the offspring of the dams who were supplemented with MEL vs. CON. Future investigations with more intensive blood sampling for cortisol will need to be accomplished to further verify this response.

Insulin-like growth factor 1 was measured in the current study as it is secreted from the liver and could be correlated with alterations in blood flow through the liver. A study by Auldist et al. (2006) used a slow-release melatonin implant in dairy cows to determine whether administration of melatonin would alter the yield and composition of milk in the summer. This study also looked at concentrations of IGF-1 twice before

treatment and three times during the treatment (Auldist et al. 2006). The melatonin-treated cows had no difference in IGF-1 concentrations (Auldist et al. 2006). Dahl et al. (2000) put forth the theory that increases in milk yield observed when photoperiod was increased might be mediated by IGF-1. Dahl et al. (1997) showed that feeding melatonin resulted in decreased concentrations of IGF-1. Previous studies in our lab showed that feeding melatonin increased milk yield on days 2 and 3 of lactation (Brockus et al. 2015). Similarly to the Auldist et al. (2006) study, the current study observed no differences in concentrations of IGF-1 in offspring born to MEL-treated dams versus CON-treated dams. Concentrations of IGF-1 were more intensively analyzed in the Auldist et al. (2006) study, and this could be a reason as to why the differences in observations were found.

There are several implications that dietary melatonin supplementation to the dams could have on the possibility of altering growth and development of the offspring. With the increase in total uterine artery blood flow of melatonin-treated dams, there could be future implications of increased growth in the offspring by further investigating blood distribution and physiological functions of various organs. If melatonin did have the capability of having these fetal programming consequences, then this could alter organ function, which could then lead to alterations in animal productivity or longevity. In order for future implications of fetal programming in the offspring to be observed, additional research will need to be further investigated.

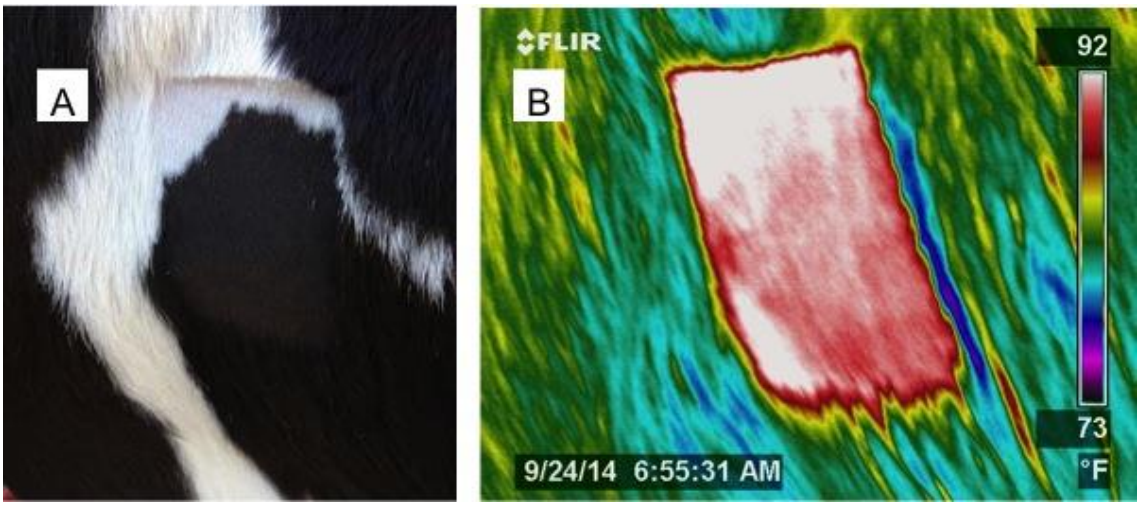

Figure 12 Non-thermal image with shaven area (A) and thermal image with shaven area (B)

Images of a 5.08 x 10.16 cm shaven area on the animals' right thoracic region directly behind the shoulder. Thermal colors across image represent various degrees of temperature on calf skin (B). Colors correspond to bar on right of image which are represented in degrees Fahrenheit.

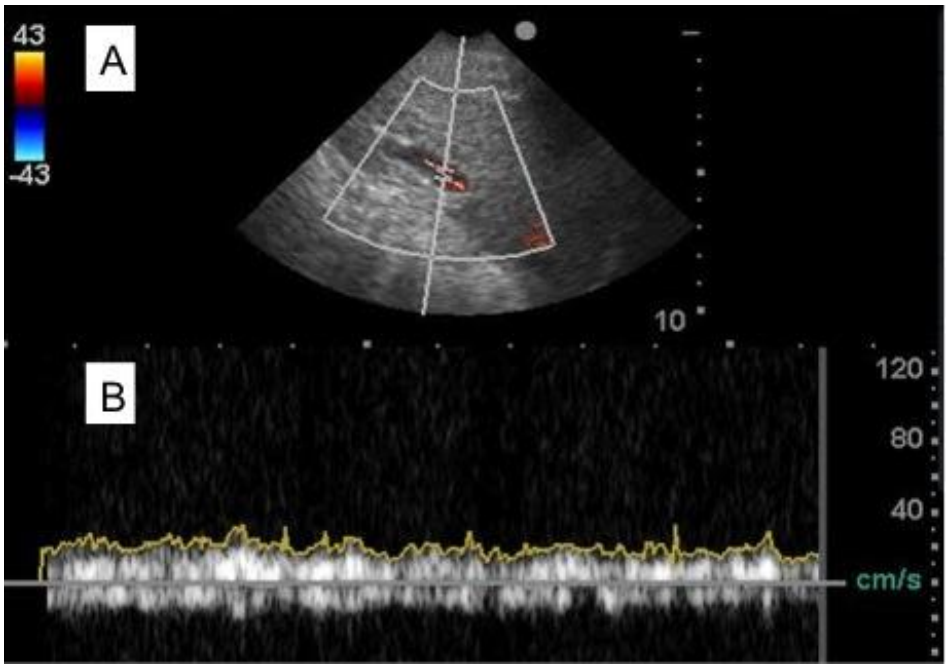

Figure 13 Hepatic portal vein image in Color Doppler ultrasonography (A) and below is a non-pulsatile vein waveform (B)

Sample curser showing the angle in which blood flow is moving through the portal vein (A). A non-pulsatile waveform of the hepatic portal vein with the velocity of blood flow measured in cm/s located on the right side of the image (B).

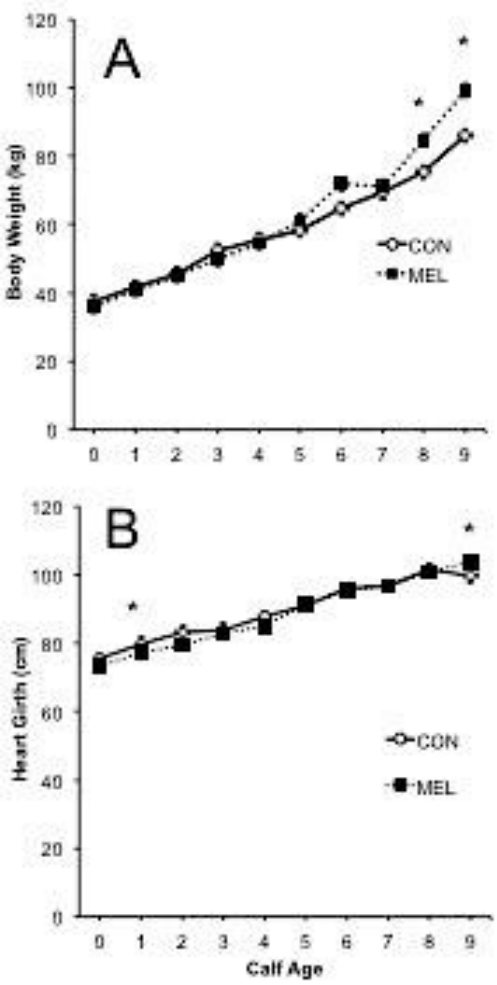

Figure 14 Body weight (A) and heart girth (B) in offspring born to dams treated with (MEL) or without melatonin (CON) during late gestation

An age by treatment interaction was observed for offspring body weight ($P < 0.01$) and heart girth ($P < 0.01$). Asterisk (*) represents a significant difference ($P < 0.05$) between treatment groups within a given age.

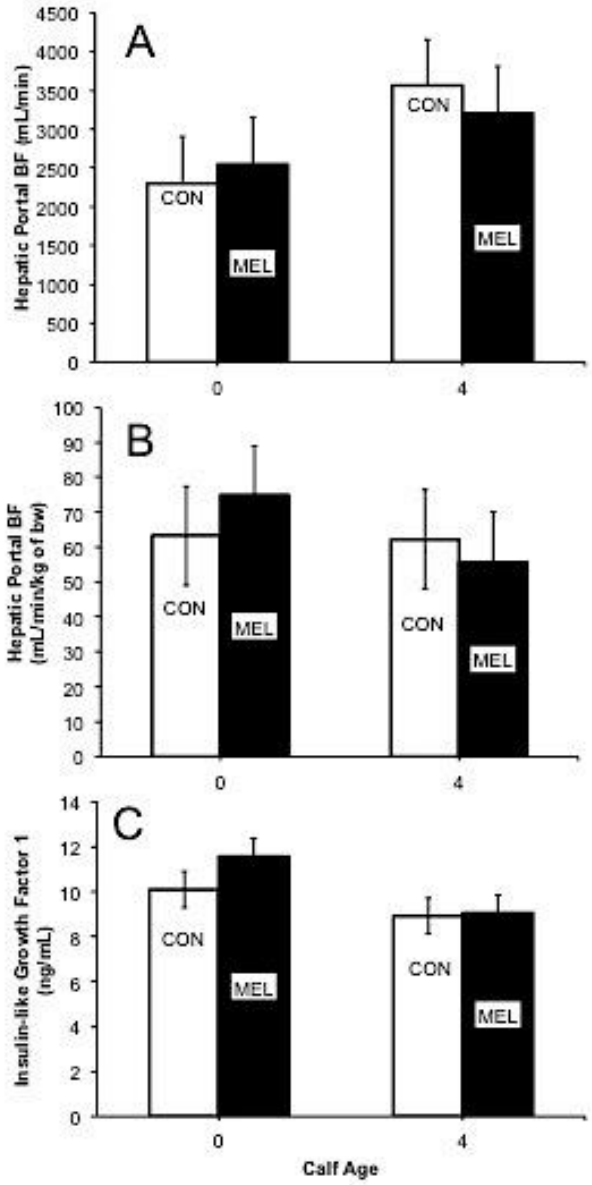

Figure 15 Absolute hepatic portal bf (A), hepatic portal blood flow by bw (B), and Insulin-like growth factor 1 (C) in offspring born to dams treated with (MEL) or without melatonin (CON) during late gestation

No main effects of age (P = 0.06) or treatment (P = 0.94) were observed for absolute hepatic portal blood flow. No main effects of age (P = 0.39) or treatment (P = 0.87) were observed for hepatic portal blood flow by body weight. A main effect of age (P = 0.02) was observed for IGF-1. Insulin-like growth factor 1 decreased from week 0 to 4 of age. Insulin-like growth factor1 was not different among treatments (P = 0.43).

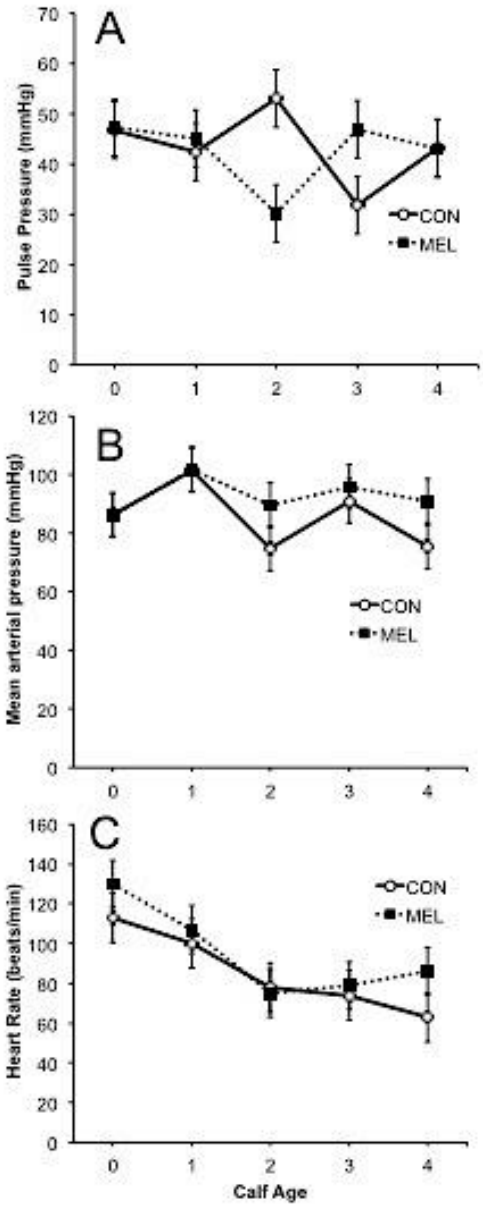

Figure 16 Pulse pressure (A), mean arterial pressure (B), and heart rate (C) in offspring born to dams treated with (MEL) or without melatonin (CON) during late gestation

No main effects of age ($P = 0.73$) or treatment ($P = 0.79$) were observed for pulse pressure. A main effect of age ($P = 0.04$) was observed for mean arterial pressure. Mean arterial pressure was not different among treatments ($P = 0.19$). A main effect of age ($P < 0.01$) was observed for heart rate. Heart rate decreased as calf age increased. Heart rate was not different among treatments ($P = 0.26$).

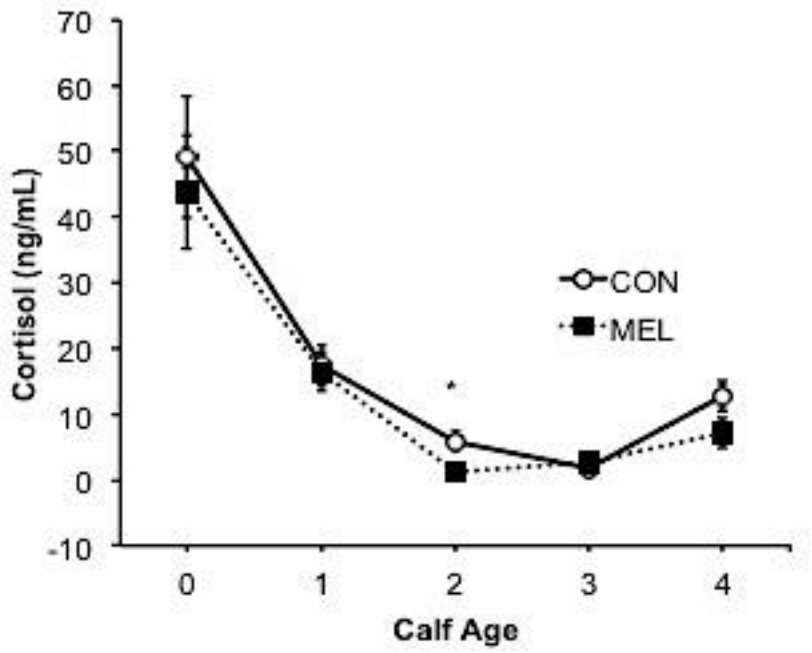

Figure 17 Cortisol in offspring born to dams treated with (MEL) or without melatonin (CON) during late gestation

An age by treatment interaction ($P = 0.03$) was observed for cortisol. Asterisk (*) represents a significant difference ($P < 0.05$) between treatment groups within a given age.

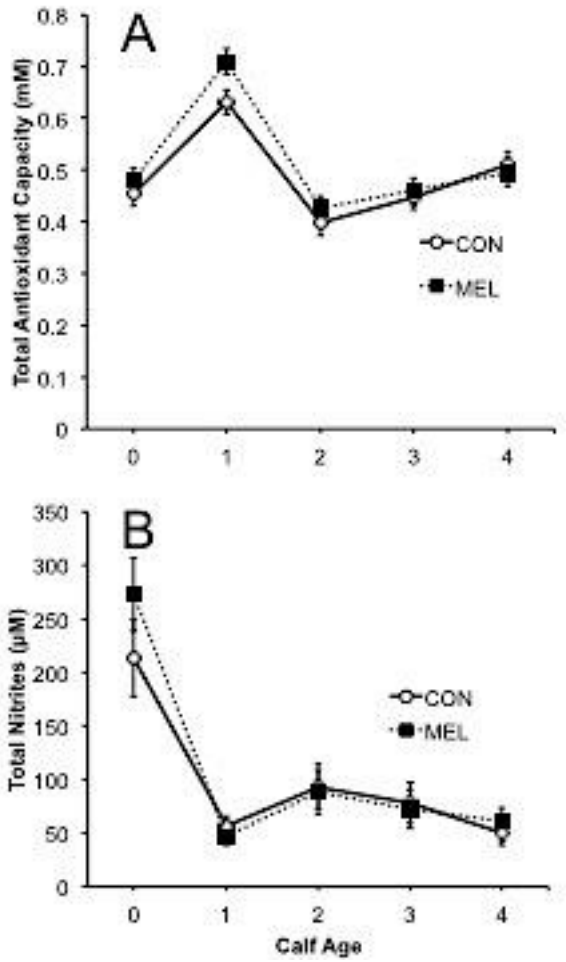

Figure 18 Total antioxidant capacity and total nitrites in offspring born to dams treated with (MEL) or without melatonin (CON) during late gestation

A main effect of age ($P < 0.01$) was observed for total antioxidant capacity. Total antioxidant capacity peaks at week 1 and is lower at all other weeks. Total antioxidant capacity was not different among treatments ($P = 0.14$). A main effect of age ($P < 0.01$) was observed for nitrites, which peaked at week 1 and is lower at all other weeks. Nitrites were not different across treatments ($P = 0.69$).

References

Auldist MJ, Turner S, McMahon CD, and Prosser CG 2006 Effects of melatonin on the yield and composition of milk from grazing dairy cows in New Zealand. Journal of Dairy Research **74** 52-57.

Brockus KB, Hart CG, Fleming BO, and Lemley CO 2015 Dietary melatonin alters uterine artery hemodynamics in pregnant Holstein heifers. (In preparation).

Cagnacci A, Soldani R, and Yen SC 1995 Hypothermic effect of melatonin and nocturnal core body temperature decline are reduced in aged women. Journal of Applied Physiology **78** 314-317.

Dahl GE, Elsasser TH, Capuco AV, Erdman RA, and Peters RR 1997 Effects of a long daily photoperiod on milk yield and circulating concentrations of insulin-like growth factor-1. Journal of Dairy Science **80** 2784-2789.

Dahl GE, Buchanan BA, and Tucker HA 2000 Photoperiodic effects on dairy cattle: A review. Journal of Dairy Science **83** 885-893.

Glore SR & Layman 1983 Cellular growth of skeletal muscle in weanling rats during dietary restrictions. Growth **47** 403-410.

Gootwine E 2005 Variability in the rate of decline in birth weight as litter size increases in sheep. Journal of Animal Science **81** 393-398.

Greenwood PL, Hunt AS, Hermanson, and Bell AW 2000 Effects of birth weight and postnatal nutrition on neo-natal sheep: II. Skeletal muscle growth and development. Journal of Animal Science **78** 50-61.

Guesdon V, Malpaux B, Delagrange P, Spedding M, Cornilleau F, Chesneau D, Haller J, and Chaillou E 2013 Rapid effects of melatonin on hormonal and behavioral stressful responses in ewes. Psychoneuroendocrinology **38** 1426-1434.

Kaïs H, Al-Gubory, Fowler PA, and Garrel C 2010 The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. The International Journal of Biochemistry & Cell Biology **42** 1634-1650.

Kräuchi K, Cajochen C, Möri D, Graw P, and Wirz-Justice A 1997 Early evening melatonin and S-20098 advance circadian phase and nocturnal regulation of core body temperature. American Journal of Physiology – Regulatory, Integrative, and Comparative Physiology **272** R1178-R1188.

Lemley CO, Meyer AM, Camacho LE, Neville TL, Newman DJ, Caton JS, and Vonnahme KA 2012 Melatonin supplementation alters uteroplacental hemodynamics and fetal development in an ovine model of intrauterine growth restriction. American Journal of Physiology - Regulatory, Integrative, and Comparative Physiology **302** R454-R467.

Lemley CO, Camacho LE, and Vonnahme KA 2013 Uterine infusion of melatonin or melatonin receptor antagonist alters ovine feto-placental hemodynamics during midgestation. Biology of Reproduction **89** 40, 1-9.

Nissen PM, Danielsen VO, Jorgenen PF, and Oksbjerg N 2003 Increased maternal nutrition of sows has no beneficial effects on muscle fiber number or postnatal growth and has no impact on the meat quality of the offspring. Journal of Animal Science **81** 3018-3027.

Nixon AJ, Choy VJ, Parry AL, and Person AJ 1993 Fiber growth initiation in hair follicles of goats treated with melatonin. The Journal of Experimental Zoology **267** 47-56.

Paulis L & Šimko F 2007 Blood pressure modulation and cardiovascular protection by melatonin: Potential mechanisms behind. Physiological Research **56** 671-684.

Redmer DA, Wallace JM, and Reynolds LP 2004 Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. Domestic Animal Endocrinology **27** 199-217.

Reiter RJ, Melchiorri D, Sewerynek E, Poeggeler B, Barlow-Walden L, Chuang J, Ortiz GG, and AcuñaCastroviejo D 1995 A review of the evidence supporting melatonin's role as an antioxidant. Journal of Pineal Research **18.1** 1-11.

Robinson JJ, McDonald I, Fraser C, and McHattie I 1977 Studies on reproduction in prolific ewes. I. Growth of the products of conception. Journal of Agricultural Science **88** 539-552.

Sewerynek E 2002 Melatonin and the cardiovascular system. Neuro Endocrinology Letters **23** 79-83.

Wu. G, Bazer FW, Wallace JM, and Spencer TE 2006 Board-invited review: Intrauterine growth retardation: Implications for the animal sciences. Journal of Animal Science **84** 2316-2337.