Temporal alterations in bovine placental capacity during compromised pregnancies

Zully E. Contreras
zcc802@gmail.com

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Temporal alterations in bovine placental capacity during compromised pregnancies

By

Zully E. Contreras-Correa

Approved by:

Caleb O. Lemley (Major Professor)
Brian J. Rude
Derris Devost-Burnett
Héctor Sánchez-Rodríguez
Jamie Larson (Graduate Coordinator)
Scott Willard (Dean, College of Agriculture and Life Sciences)

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in Agricultural Science
in the Department of Animal and Dairy Sciences

Mississippi State, Mississippi

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The circadian rhythms are not solely regulated by photoperiod but are also influenced by feed regimen. Therefore, maternal nutrient restriction during gestation could potentially impact the fetal circadian rhythm. Melatonin, a circadian rhythm modulator hormone, has shown to act as an antioxidant reducing reactive oxygen species during stress exposure; and two potential mechanisms have been proposed for melatonin causing vasoconstriction and vasodilation regulating blood flow. In livestock species, nutrient restriction during gestation reduces uterine blood flow, limiting nutrients availability to the fetus for growth and development. Therefore, this project aimed to use beef heifers to evaluate the maternal nutrient restriction and/or melatonin supplementation in (1) temporal transcript abundance of clock genes, angiogenic factors and nutrient sensing genes in bovine placenta, (2) temporal alterations of uteroplacental blood flow, vaginal temperatures, and placentome vascularization, and (3) fetal morphometrics. Early maternal nutrient restriction did not alter placental explants gene expression. Furthermore, the maternal portion of the placentome exhibited limit temporal variation, while the fetal tissue exhibited a clear temporal rhythm in the mRNA relative abundance of the genes measured. Additionally, melatonin supplementation during late gestation, showed to increased uterine blood
flow, reduced vaginal temperatures, and rescued fetal weights during compromised pregnancies in a season dependent manner. In conclusion, the bovine placenta exhibited an autonomous circadian rhythm, while the fetal and maternal circadian rhythms appeared to be independent systems. Future research should examine the effects of melatonin supplementation in fetal organ development.
DEDICATION

This work is dedicated to my parents Rafael Contreras and Carmen Correa who taught me the value of hard work. I grew up seeing them making sacrifices so I could obtain the education they did not have the opportunity to receive. Thank you for your unconditional support, love, guidance, and prayers. All of that contributed to the woman I am today.

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# TABLE OF CONTENTS

DEDICATION

ACKNOWLEDGEMENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER

I. REVIEW OF LITERATURE

- Developmental Programming
- Embryonic and Placental Development
- Ruminant Placental Vascularity
- Fetal Growth and Development
- Fetal Undernutrition
- Blood Flow Techniques
- Umbilical and Uterine Blood Flow
- Melatonin
- Circadian Clocks
- Statement of the Problem

REFERENCES

II. TEMPORAL TRANSCRIPT ABUNDANCE OF CLOCK GENES, ANGIOGENIC FACTORS AND NUTRIENT SENSING GENES IN BOVINE PLACENTAL EXPLANTS

- Abstract
- Introduction
- Materials and Methods
  - Animals and Tissue Collection
  - Placental Explants RT-PCR
  - Statistical Analysis
- Results
  - Caruncular mRNA Transcript Abundance
  - Cotyledonary mRNA Transcript Abundance
- Discussion
REFERENCES .................................................................................................................44

III. MELATONIN-INDUCED CHANGES IN BOVINE FETO-PLACENTAL DEVELOPMENT DURING LATE GESTATION MATERNAL NUTRIENT RESTRICTION ........................................................................................................48

Abstract .........................................................................................................................48
Introduction ......................................................................................................................50
Materials and Methods ....................................................................................................52
  Animal Care and Treatments .......................................................................................52
  Color Doppler Ultrasonography and Vaginal Temperatures ...................................54
  Cesarean Sections and Tissue Collection ...................................................................55
  Placentome Perfusion and Macroscopic Blood Vessel Density ...............................56
  Placentome Immunohistochemistry and Microscopic Blood Vessel Density .........56
  Blood Sampling Analysis .........................................................................................57
  Statistical Analysis ......................................................................................................58
Results .............................................................................................................................59
  Maternal Body Weight ...............................................................................................59
  Uterine Artery Hemodynamics and Vaginal Temperatures ....................................59
  Fetal Measurements at Day 240 of Gestation ............................................................61
  Maternal and Fetal Blood Analysis .........................................................................65
  Placentome Vascularity .............................................................................................66
  Discussion .....................................................................................................................68

REFERENCES ...................................................................................................................91

IV. GENERAL DISCUSSION ...........................................................................................94
LIST OF TABLES

Table 2.1  Assays, GenBank accession number, amplicon length, and efficiency (10\(^{-1/slope}\)) of primers used for TaqMan probe-based real-time qPCR of placental explants. ........................................43

Table 3.1  Number of animals allocated into maternal diets and treatments..........................84

Table 3.2  Animal number distribution for Cesarean section timepoints...............................84

Table 3.3  Ipsilateral (IPSI) and contralateral (CON) to the conceptus uterine artery pulsatility index (PI), resistance index (RI), and diameter at day 220 of gestation for animals enrolled in Fall 2019. .................................................................85

Table 3.4  Ipsilateral (IPSI) and contralateral (CON) to the conceptus uterine artery pulsatility index (PI), resistance index (RI), and diameter at day 220 of gestation for animals enrolled in Summer 2020.................................................................86

Table 3.5  Fetal organs and growth parameters at day 240 of gestation for animals in the Fall 2019 trial .................................................................87

Table 3.6  Fetal organs and growth parameters at day 240 of gestation for animals in the Summer 2020 trial .................................................................89
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Scheme representation of the dissertation research</td>
<td>17</td>
</tr>
<tr>
<td>2.1</td>
<td>Caruncular explant transcript abundance of clock genes across 24-h.</td>
<td>38</td>
</tr>
<tr>
<td>2.2</td>
<td>Caruncular explant transcript abundance of angiogenic factors and nutrient sensing genes across 24-h.</td>
<td>39</td>
</tr>
<tr>
<td>2.3</td>
<td>Main effect of breed for (A) <strong>HIF1A</strong> and (B) <strong>VEGFA</strong> transcript abundance in caruncular explant tissues.</td>
<td>40</td>
</tr>
<tr>
<td>2.4</td>
<td>Cotyledonary explant transcript abundance of clock genes across 24-h.</td>
<td>41</td>
</tr>
<tr>
<td>2.5</td>
<td>Cotyledonary explant transcript abundance of angiogenic factors and nutrient sensing genes across 24-h.</td>
<td>42</td>
</tr>
<tr>
<td>3.1</td>
<td>Maternal body weights through weeks of gestation.</td>
<td>75</td>
</tr>
<tr>
<td>3.2</td>
<td>Uterine blood flow at day 220 of gestation.</td>
<td>76</td>
</tr>
<tr>
<td>3.3</td>
<td>Averaged vaginal temperatures recorded at day 220 of gestation.</td>
<td>77</td>
</tr>
<tr>
<td>3.4</td>
<td>Maternal and fetal melatonin concentrations in blood.</td>
<td>78</td>
</tr>
<tr>
<td>3.5</td>
<td>Fluorescent signaling for cotyledonary macroscopic blood vessel density.</td>
<td>79</td>
</tr>
<tr>
<td>3.6</td>
<td>Representation of immunofluorescent images used to determine microscopic blood vessel density in bovine placentomes collected at day 240 of gestation.</td>
<td>80</td>
</tr>
<tr>
<td>3.7</td>
<td>Microscopic blood vessel density for heifers enrolled in Fall 2019.</td>
<td>81</td>
</tr>
<tr>
<td>3.8</td>
<td>Fluorescent signaling for cotyledonary macroscopic blood vessel density in placentomes collected at day 240 of gestation from Summer 2020 heifers.</td>
<td>82</td>
</tr>
<tr>
<td>3.9</td>
<td>Microscopic blood vessel density determination for Heifers enrolled in Summer 2020.</td>
<td>83</td>
</tr>
</tbody>
</table>
CHAPTER I

REVIEW OF LITERATURE

Developmental Programming

After years of epidemiological studies in humans, the “Barker hypothesis” originated, leading to the developmental programming concept. This concept explains that any stimulus or insult experienced during the intrauterine environment has a permanent response on the offspring into adult life (Barker, 2006). Furthermore, exposure during a critical period such as embryonic or fetal development could disrupt the rapid cell division occurring at this timepoint and affect organ growth reducing birth weight (Barker, 1997). There is strong evidence suggesting that low birth weight due to fetal undernutrition is the origin of many metabolic diseases in humans including cardiovascular diseases, hypertension, and diabetes (Barker, 1998). These findings are highly relevant to livestock species because it helps to understand why some offspring with outstanding genetic potential for meat and milk production exhibit poor performance and impaired phenotype.

Embryonic and Placental Development

After the oocyte is fertilized in the oviduct, the zygote is formed leading to a diploid cell from the contribution of the two parental haploid cells. One of the first critical events in early embryonic developments is the embryonic genome activation (Latham and Schultz, 2001). In early embryonic development, the embryo relies on the oocyte reserves of RNA and proteins, therefore, the maternal genome regulates the first mitotic division and initiates cell fate of the
early embryo (Tadros and Lipshitz, 2009). As development advances, the maternal to embryonic transition is initiated, where the maternal RNA and proteins degrade and the embryonic genome activation is established (Graf et al., 2014). In cattle, the embryonic genome activation occurs around the 4 to 8 cell stage, a crucial period for chromatin remodeling and the occurrence of epigenetic modifications that can affect further gene expression regulating developmental programming (Gad et al., 2012; Handy et al., 2011). Once a morula, the outer cells adhere developing into the trophectoderm and the remaining cells congregate in one pole developing into the inner cell mass (Spencer, 2013). After the blastocoel is formed, which is the fluid filled central cavity in the blastocyst, there is a clear distinction between the inner cell mass which will lead to the embryo and the surrounding trophoblast cells that will result in the placenta (Schlafer et al., 2000). Once the blastocyst is hatched from the zona pellucida the conceptus elongation is initiated where the length and the weight of the trophectoderm increases exponentially (Spencer, 2013). The trophectoderm is further fused with the mesoderm developing the chorion, which is further fused with the allantois resulting in the chorioallantois which is the fetal contribution to the placenta (Schlafer et al., 2000).

**Ruminant Placental Vascularity**

Cows are known to have a cotyledonary placenta where the chorioallantois membrane (cotyledon) attaches to the maternal caruncles which are evenly distributed throughout the endometrium. The fetal villi are interdigitated with the maternal caruncular crypts increasing the surface area and establishing the placentomes which are the functional units of the ruminant placenta. These placentomes are highly vascularized and the oxygen, nutrient, and waste exchange between the maternal and fetal systems are a mixture of crosscurrent and countercurrent patterns (Leiser et al., 1997a; Leiser et al., 1997b). To better understand the
vascular architecture and interconnection of the feto-maternal systems, Leiser et al. (1997b) and Pfarrer et al. (2001) developed a corrosion cast and mapped out the three-dimensional placentome vascularization using scanning electron microscopy. They found that vascularization was simple in early pregnancy and more complex in mid- to late gestation. Maternal vasculature is supplied by a large caruncular stalk that branch into stem arteries and during the third month of gestation, these stem arteries branch into multiple arterioles, converging into terminal capillary loops (Pfarrer et al., 2001); whereas the fetal portion of the placentome is supplied by the cotyledonary arteries (Pfarrer et al., 2001).

The placental functional capacity is dependent on its size, blood supply and transporter abundance assuring an adequate amount of nutrients available to the fetus (Belkacemi et al., 2010). The bovine placenta grows exponentially throughout gestation (Lemley and Vonnahme, 2017); nevertheless, the exponential fetal growth rate during the last-third of pregnancy is more drastic when compared to that of the placenta (Reynolds and Redmer, 1995). This excessive growth rate is supported by uterine blood flow but is not solely explained by Pfarrer et al. (2001) microvasculature system (Lemley and Vonnahme, 2017). Using placental perfusion fixation methods, Borowicz et al. (2007) demonstrated that the caruncular capillary beds grow primarily by increasing capillary size, while the cotyledonary capillary beds grow by branching thereby increasing their capillary number. Recently, Lemley et al. (2018) developed a novel technique to measure the placentome vascular density by perfusing a fluorescent substrate through the cotyledonary artery and photographing the placentome with an in vivo imaging system. This research revealed a compensatory response at the placentome level in nutrient restricted dams indicated by increased fluorescent signal. Furthermore, Lemley et al. (2018) suggested the
existence of capillary sphincters to explain the dramatic blood shunting in the placentome observed at the macroscopic level.

**Fetal Growth and Development**

In sheep, only a 10% of fetal weight is acquired during the first two-thirds of pregnancy, while the remaining 90% of fetal growth occurs during the last third of gestation (Redmer et al., 2004). Similarly, the bovine fetus growth is minimum during early and mid- pregnancy, while 60% of fetal body weight is obtained in the last 2 months of gestation (Bauman and Currie, 1980). The fetal period in cattle begins at day 45 after conception (Inskeep and Daily, 2005) where the initial instantaneous growth rate has been described to be between 5.1 and 7.4% per day and can be explained by exponential equations (Ferrel et al., 1976; Prior and Laster, 1979). Fetal organs develop at different rates (Kahn, 1989) where initially they grow by hyperplasia and advancing in gestation start growing by hypertrophy (Winick and Noble, 1965). Hyperplasia refers to an increase in cell number, while hypertrophy is attributed to increase in cell size (Sainz and Bentley, 1997). Hyperplasia ceases in many vital organs such as heart, brain, kidney, and lung once it has reached maturity lacking the ability to increase the number of cells or regenerate (Goss, 1966).

Understanding the organ hyperplasia and hypertrophy in livestock is of great importance since the intrauterine environment during fetal development can affect economically important tissues. For example, in fetal muscle, there is a vast number of mesenchymal stem cells which are the origin of the 3 cell types (myocytes, adipocytes, and fibroblast) that result in the basic structure of the skeletal muscle (Du et al., 2010a; Du et al., 2010b). In livestock species, primary and secondary myofibers develop during the prenatal stage (Yan et al., 2013) and there is no net increase in postnatal myofiber number (Zhu et al., 2004). Therefore, the fetal period is important
for skeletal muscle development and if myofiber number is decreased or compromised during fetal programming, muscle mass can be permanently affected (Du et al., 2010a). Besides skeletal muscle fibers, adipocytes are also important for profitable meat producing animals, since it contributes to intramuscular fat deposition increasing meat marbling and palatability. Overall, myogenesis occurs during early to mid-gestation, while adipogenesis begins in mid- to late gestation overlapping with the secondary myogenesis (Feve, 2005; Du et al., 2010a). Therefore, it is important to ensure that the fetus is receiving the appropriate amount of nutrients during this period to promote the skeletal muscle formation and to guarantee optimal offspring performance and profitability.

**Fetal Undernutrition**

Fetal nutrient deficiency can result from maternal undernutrition or reduced placental efficiency (Yan et al., 2013), negatively affecting fetal growth and development. Placental efficiency can be defined as the ratio of fetal weight to placental weight, and is indicative of proper placental function. This ratio represents the grams of calf that can be grown per gram of placenta (Lemley et al., 2021). Placental insufficiency accounts for 60% of intrauterine growth restriction cases in humans (Ghidini, 1996), affecting birth weight and further offspring performance. Inadequate placental growth results in negative repercussion such as reduced uteroplacental blood flow and nutrient transport capacity (Redmer et al., 2004). The placenta is the main communication between the maternal and fetal systems and its size will determine the nutrient transport capacity including the metabolic substrates available for prenatal growth (Reynolds et al., 1995).

Compromised pregnancies such as nutrient restriction or maternal heat stress exposure can reduce glucose and amino acid availability in fetal plasma (Harding, 2001; Kown et al.,
During late pregnancy most carbon and nitrogen required for bovine fetal growth come from glucose and amino acids (Bell, 1995). Glucose is utilized by the fetus as a primary source of energy, and it can be stored as glycogen in some tissues, especially in muscle and liver; but it could also be used for synthesis of nonessential amino acids (Battaglia and Meschia, 1978). In ruminants, the placenta metabolizes glucose into fructose to meet the fetal requirements of other carbon sources (Hay, 1995) and sequestered in the amniotic fluid (Rasby et al., 1990). The fructose stored in the amniotic fluid is a readily available source of energy for the fetus (Battaglia and Meschia, 1978). Additionally, the fetus requires adequate amino acids concentration for protein synthesis, hormone regulation and secretion, gene expression and cell signaling (Wu, 2009). The fetal nutrient flux can be calculated using the basics of Fick’s principle where the umbilical blood flow is multiplied by the difference in concentrations of umbilical vein and umbilical artery related to any nutrient (Reynolds and Redmer, 1995). These calculations in compromised or melatonin treated pregnancies will be further discussed below.

**Blood Flow Techniques**

Since the 1800’s there has been curiosity to understand and describe blood flow in humans and animals. The first techniques developed to study blood flow were traumatic and moreover, the procedures included cutting the blood vessels which made it impossible to perform in humans (Folts, 1974). Later in the mid- 1900’s, the electromagnetic flowmeter was developed which was less traumatic, but still very invasive since it required dissection of the blood vessel of interest or fetal exteriorization for umbilical blood flow measurements (Rudolph and Heymann, 1967). The electromagnetic flowmeter uses the Faraday’s physics law and consist of placing an electromagnet around the conductive blood vessel wall and the voltage generated by the liquid flowing through that vessel is picked up by electrodes and is proportional to blood flow.
(Gessner, 1961; Folts, 1974). This technique has the advantage of being precise and can record umbilical blood flow continuously, but the surgical trauma is an inconvenience. Therefore, scientists at that time opted for developing an indirect method such as the steady-state diffusion of a known substrate to determine the transplacental diffusion rate using Fick’s principle without the need of exteriorizing the fetus (Meschia et al., 1967; Rudolph and Heymann, 1967). For umbilical blood flow, this technique consisted of infusing antipyrine at a constant rate until the transplacental diffusion rate remained constant and the aforementioned rate was divided by the arterial to venous difference in the umbilical circulation (Meschia et al., 1967). Even though this technique is performed in utero, it is still invasive and less precise. A more precise technique for measuring the umbilical and uterine blood flow are radioactive microspheres, but a limitation of this technique is that only one time point can be collected and the animals need to be euthanized (Makowski et al., 1968; Ford et al., 1979).

The Doppler ultrasound is a non-invasive diagnostic tool that helps in the Animal Sciences to study animal reproduction such as uterine and umbilical artery hemodynamics, testicular artery blood flow. The B-mode of the ultrasound machine also help to determine female cyclicity, pregnancy, marbling scores (intramuscular fat and back fat thickness), and pathologies evaluations such as cardiovascular diseases and liver abscesses. The basics of Doppler ultrasound go back to the 19th century when Christian Doppler observed changes in the transmitted wave’s frequency when relative motion exists between the source of the wave and the observer (Maulik, 2005); this phenomenon is referred to as the “Doppler effect”. It was not until the need to detect enemies’ submarines in the World War I and II that the first sonar equipment was developed by Paul Langevin using the piezoelectric crystals that transmit and receive ultrasound waves (Maulik, 2005). Ultrasonography started to be used in the medical field
in the 1960’s, but it was not until 1998 that it was introduced into the Animal Sciences for the study of mares’ cyclicity (Bollwein et al., 1998). The Doppler ultrasound can be used to calculate blood flow velocity during peak systolic and end diastolic, phases of cardiac cycle, allowing to estimate the blood movement through a vessel (Lemley, 2017).

**Umbilical and Uterine Blood Flow**

Proper umbilical and uterine blood flow are vital for adequate delivery of oxygen and nutrients from the dam to the fetus (Thakor et al., 2010; Lemley et al., 2012). The umbilical and uterine blood flow increase exponentially throughout gestation (Reynolds and Redmer, 1995) and represent the primary blood supply for the fetal and maternal portions of the placenta, respectively (Ramsey, 1982; Mossman, 1987). These findings seem accurate since fetal growth and nutrient demands are greater during late pregnancy (Bell et al., 1995), thereby fetal metabolic requirements are met with an increase in uterine and umbilical blood flow (Herzog and Bollwein, 2007). The umbilical cord in calves consist of two umbilical veins that deliver oxygen and nutrient rich blood from the placenta to the fetus, while two umbilical arteries return nutrient depleted blood from the fetus to the placenta. In cattle, the primary blood supply of the uterus is provided by the uterine artery (Budras et al., 2003). The uterine artery originates from the internal iliac artery which subsequently branch into the umbilical artery and can be found within the mesometrium (Bollwein et al., 2002; Budras et al., 2003). Using electromagnetic flow probes, Ferrel and Ford (1990) found an increase of uterine blood flow in the first two trimesters of gestation, while the last period remained constant. Nevertheless, when the Doppler ultrasound was later introduced, Bollwein et al. (2002) found that uterine blood flow increased drastically in the last trimester of gestation, being the uterine horn ipsilateral to the conceptus the one that received a greater blood supply when compared to the contralateral region.
Bollwein et al. (2002) characterized bovine uterine blood flow using Doppler ultrasonography in early, mid- and late gestation in Simmental and Brown Swiss cows. These researchers found that in early pregnancy (days 30 to 90 of gestation) the bovine uterine blood flow is minimum (around 0.10 to 0.30 L/min) and noticed no changes in uterine artery diameter. Nevertheless, while advancing into the second trimester of pregnancy (day 180 of gestation) it was found that the uterine blood flow in the gravid horn was around 4.0 L/min, compared to 1.3 L/min in the non-gravid side. Lastly, in the third trimester, the uterine blood flow ipsilateral to the fetus drastically increased from 5 to 6.0 L/min (days 210 to 240 of gestation) to 10.5 to 13.1 L/min (day 270 to 280 of gestation). The authors attributed this increase in blood flow throughout gestation to an increase in the uterine artery diameter and a decrease in resistance index. The resistance index in inversely proportional to blood flow and can be translated as when the resistance index decreases, the arterial blood flow increases. Clinically, an increase uterine or umbilical vascular resistance along with reduced uterine and umbilical blood flow are common signs of high-risk pregnancies, negatively affecting fetal growth (Reynolds and Redmer, 2001). Compromised pregnancies such a maternal nutrient restriction, have shown aberrations in umbilical cord blood flow, uterine blood flow, and placental vascularity (Lemley et al., 2012) ; while the first studies on beef cattle maternal nutrient restriction showed a 10% increase in neonate mortality rate, while an additional 20% of those animals die prior to weaning (Corah et al., 1975). Therefore, understanding the placental function and fetal programing would allow scientists to determine the appropriate period of gestation when therapeutics should be offered to the dams to improve offspring performance. As an attempt to improve diets that could benefit fetal growth and development, researchers have worked towards improving pastures (Underwood et al., 2008) and increase the amount of protein in diets (Long et al., 2012).
Furthermore, Lassala et al. (2010) supplemented nutrient restricted pregnant ewes with L-arginine and found that this amino acid prevents intrauterine growth restriction. On the other hand, Lemley et al. (2012) found a 17% increase in umbilical blood flow when supplementing pregnant ewes with melatonin, while Brockus et al. (2016) found a 25% increase in uterine blood flow when supplementing dairy heifers with the same hormone. The aforementioned studies aimed at improved placental function to negate the effects of intrauterine growth restriction, whereas it is also important to understand the mechanisms by which these therapeutics could negate the adverse effects of compromised pregnancies.

**Melatonin**

Melatonin is a neuroendocrine hormone secreted by the pineal gland and acts as a circadian rhythm modulator (Tamura et al., 2008). Melatonin has the capacity to cross the placenta and enter the fetal circulation unaltered (Tamura et al., 2008). Previous studies have demonstrated an increase in umbilical blood flow and placental efficiency in ewes supplemented with 5 mg of dietary melatonin during the daytime (Lemley et al., 2012; Lemley et al., 2013). Additionally, Brockus et al. (2016) showed an increase in uterine artery blood flow in dairy heifers supplemented with 20 mg of dietary melatonin during the last trimester of pregnancy. Recently, McCarty et al. (2018) showed that subdermal melatonin implants increased uterine blood flow in commercial beef heifers and cows during late gestation. These results allowed researchers to hypothesize that melatonin controls circadian rhythms in the uteroplacental blood flow. Therefore, further studies are needed to determine the exact time at which livestock should receive melatonin supplementation as a therapeutic mediator in compromised pregnancies.

Melatonin supplementation could increase blood flow by two possible pathways: a) indirectly by acting as an antioxidant to decrease vascular oxidative stress or b) by directly
binding to endothelial melatonin receptors (Paulis and Simko, 2007). Oxidative stress refers to an imbalance between reactive oxygen species (ROS) and antioxidant capacity (Betteridge, 2000). Oxidative damage causes alterations in nucleic acids, lipids, carbohydrates, proteins, and free amino acids (Sies, 1985). Examples of free radicals are superoxide (O$_2$-) and nitric oxide (NO-); (Halliwell et al., 1992). Melatonin directly reduces ROS from mitochondrial respiratory chain electron leakage (Reiter et al., 2016) and helps to preserve antioxidative enzymes functional integrity (Reiter, 2003). Additionally, Pogan et al. (2002) proposed that melatonin binds to melatonin receptor 2 (MT2) in the endothelial cells, causing an increased in cytosolic Ca$^{2+}$, which activates the nitric oxide synthase and stimulates the nitric oxide production. An increased in nitric oxide, also known as the endothelium-derived relaxing factor, leads to vasodilation (Garg and Hassid, 1989). Previous research has shown that NO bioavailability is important for umbilical blood flow by promoting vasodilation (Thakor et al., 2010). Moreover, Thakor et al. (2010) showed that fetal NO blockade prevented the melatonin-induced umbilical blood flow increase. Therefore, melatonin supplementation could increase bovine uteroplacental blood flow by the aforementioned mechanisms. An increased in uteroplacental blood flow could enhance the uteroplacental nutrient transport capacity (Lemley et al., 2013). Lemley et al. (2013) evaluated the effects of dietary melatonin supplementation on ewe uteroplacental nutrient flux, finding an increase of total amino acids and branched chain amino acids in fetal vein and arterial differences. The increase of nutrients delivered to the fetus result in better development and adult performance, therefore the effect of melatonin supplementation should be evaluated in nutrient restricted pregnant animals.

Melatonin supplementation has also shown to reduce core body temperature during daytime in humans (Cagnacci et al., 1992); a drop of 0.30° C was observed with a 5 mg
melatonin supplementation (Dawson et al., 1996). Heat stress is another factor that can compromise pregnancies and animals with higher body temperature present a reduction in uterine blood flow and an increase in peripheral blood flow to compensate through heat dissipation (West, 2003). This blood redistribution to facilitate heat dissipation, negatively affects reproductive performance in cattle limiting fetal growth and placenta vascularity (Hansen, 2009). Maternal hyperthermia in pregnant mice has shown to increase embryonic death via increase in oxidative stress, while melatonin injections alleviated this effect by maintaining a neutral redox status (Matsuzuka et al., 2005). Melatonin hypothermic properties could be a potential benefit to heifers with compromised pregnancies due to heat stress.

**Circadian Clocks**

Circadian rhythms are influenced by environmental factors such as light, feed availability, stress, and activity level. The circadian rhythms allow the organism to anticipate metabolic and physiological requirements facilitating its adaptation to daily needs (Woelfle et al., 2004). For years, circadian rhythms were thought to be controlled only by the suprachiasmatic nuclei of the hypothalamus; but more recently researchers found the existence of semiautonomous clocks located in peripheral tissues throughout the organism and these oscillatory clock genes have shown to function in a tissue-specific manner (Lowrey and Takahashi, 2004). Components of the clock gene network (CLOCK, ARNTL, CRY1-2, and PER1-3) have shown to regulate the expression of transcription factors and proteins that control rate-limiting steps in metabolic pathways (Panda et al., 2002). The clock gene network operates by rhythmic transcription and translation creating an autoregulatory feedback loop (Dolatshad et al., 2010). Briefly, the CLOCK and ARNTL proteins heterodimerize (CLOCK:ARNTL) and bind to the E-box enhancer within the nucleus activating the transcription of the CRY and PER
proteins (Reppert and Weaver, 2002). The activated CRY and PER proteins, dimerize in the cytoplasm and create a complex that causes a negative feedback in the nuclear CLOCK:ARNTL repressing its own transcription (Lowrey and Takahashi, 2004).

The circadian clock controls physiological and behavioral processes; its expression regulates homeostasis, and a disruption could cause metabolic disorders (Wharfe et al., 2011). Most of the circadian rhythm studies have been performed in rodents which are nocturnal animals; due to this reason it seems unreliable to extrapolate those results into our livestock species who are mostly diurnal. Nevertheless, one undeniable advantage of working with mice is the ability to genetically engineer mutant mice such as knock-out or knock-down models for specific genes of interest to evaluate their functions. As reviewed by Harfmann et al. (2015), multiple mice models have been created to study the role of circadian clocks in physiological processes. As for example, mice with a CLOCK mutation are predisposed to obesity, hepatic steatosis and hyperglycemia (Rudic et al., 2004); a CRY and PER mutation can cause a shorter or longer period length or even an arrhythmic behavior (van der Horst et al., 1999; Zheng et al., 2001); lastly, the ARNTL mutation exhibit the most severe pathologies reducing its lifespan significantly (Kondratov et al., 2006).

Even though the circadian pacemaker is located in the suprachiasmatic nuclei of the hypothalamus (Rusak and Zucker, 1979), clock genes have been found in other peripheral tissues such as: mouse skeletal muscle (McCarthy et al., 2007), rat ovaries (Karman and Tischkau, 2006), rat oviduct (Kennaway et al., 2003), mouse uterus (Dolatshad et al., 2008), and more recently the placenta (Ratajczak et al., 2010; Wharfe et al., 2011). The placenta as a multifunctional organ has the capacity of transmitting maternal rhythmicity influencing fetal circadian rhythm (Mark et al., 2017). Nevertheless, circadian rhythm in bovine placental clock
genes has not been evaluated. Therefore, future studies should aim to evaluate the effect of nutrient restriction in clock genes and angiogenic factors in the maternal and fetal placental portions.

Examples of circadian rhythm disruptors are stress, temperature and interruption of light-dark cycle. Although the exact mechanisms remain unknown, Gozeri et al. (2008) found negative effects in fetal-placental development when the light-dark cycle was interrupted. In this same study, fetal weight was reduced in both altered circadian rhythm groups when compared to the control; nevertheless, the constant light exposure group had significantly lower fetal weights than the constant dark counterpart. These results may be due to a disturbance in melatonin synthesis, which maximal secretion occurs at dark hours (Wharfe et al., 2011). Fetus pineal gland is unable to produce melatonin; therefore, it depends on maternal supplies (Yellon and Longo, 1998).

The increase of uterine blood flow leads to placental vascularity growth due to an increment in angiogenic factors in the maternal placental tissue (Reynolds and Redmer, 1995). Angiogenic factors promotes angiogenesis, which is the migration, growth and differentiation of endothelial cells for the formation of new blood vessels. One of the most important growths factors in the placenta is the vascular endothelial growth factor (VEGF), which is essential for embryonic vasculogenesis (Powe et al., 2011) and its expression increases as gestation advances (Cheung, 1997). Using a zebra fish model, Jensen et al. (2012), established that the circadian clock controls VEGF expression, therefore, it regulates angiogenesis and embryonic development.
Statement of the Problem

Beef cattle production is the most important agricultural industry in the United States of America and in 2019 accounted for $66.2 billion in cash receipts (USDA, 2021). Cow-calf operations are responsible for supplying cattle for meat production and the next generation of replacement heifers. Independent of the fate of these offspring, adequate maternal nutrition is needed to assure maximum performance and profitability. Maternal nutrient restriction during mid- to late gestation has shown to cause intrauterine growth restriction, leading to offspring with reduced birth weights and weaning weights affecting their survival rates. It is estimated that calf wastage and mortality cost producers over $1 billion annually (USDA, 2011). Furthermore, maternal undernutrition could potentially impact the fetal circadian rhythm. Circadian rhythms control physiological and behavioral responses in a 24-hour pattern. The circadian rhythms are not solely regulated by photoperiod but are also influenced by feed regimen. Melatonin, a neuroendocrine hormone, along with the clock gene network are known to regulate the circadian rhythms. Furthermore, melatonin has shown to act as an antioxidant reducing ROS caused during stress exposure; and two potential mechanism have been proposed for melatonin causing vasoconstriction and vasodilation regulating blood flow. Adequate uteroplacental blood flow is essential for oxygen and nutrient transport from the dam to the fetus. In livestock species, nutrient restriction during gestation has shown to reduce uterine blood flow, limiting nutrients availability to the fetus for growth and development, while melatonin supplementation has shown to increase uterine blood flow. Thus, there is a critical need to determine how compromised pregnancies affect uteroplacental rhythms and if melatonin properties could alleviate these negative effects. With this continuous gap of knowledge, the producers’
economical losses will be persistent because of the offspring impaired performance. A schematic representation of this dissertation is provided in Figure 1. This project aimed to use beef heifers to evaluate the maternal nutrient restriction and/or melatonin supplementation in (1) temporal transcript abundance of clock genes, angiogenic factors and nutrient sensing genes in bovine placenta, (2) temporal changes in uteroplacental blood flow, vaginal temperatures, and placentome vascularization, and (3) fetal morphometrics.
Circadian rhythms are entrained by factors such as photoperiod and feed availability. Maternal nutrient restriction is used as a compromised pregnancy model reducing uteroplacental blood flow and causing fetal intrauterine growth restriction. This research project evaluates the effect of melatonin supplementation in nutrient restricted heifers. Furthermore, we aim to determine the bovine placental circadian rhythms (uteroplacental blood flow and clock network gene expression) and elucidating the effects of circadian dysregulation during pregnancy on fetal growth and development. Understanding the placental circadian rhythms will allow us to determine at what time of the day supplementation may need to be provided to pregnant animals.
REFERENCES


CHAPTER II
TEMPORAL TRANSCRIPT ABUNDANCE OF CLOCK GENES, ANGIOGENIC FACTORS AND NUTRIENT SENSING GENES IN BOVINE PLACENTAL EXPLANTS

Abstract
Recent research has shown expression of clock genes in peripheral tissue explants, targeting multiple pathways leading to the entrainment of circadian rhythms. Temporal variations are not solely regulated by photoperiod but factors such as maternal feed availability can entrain fetal circadian clock. Currently, a paucity of information exists for clock gene expression and short-term temporal transcript abundance in the bovine placenta which is essential for proper offspring development. Therefore, the objective of this study was to determine the effect of early to mid-gestational nutrient restriction on clock genes, angiogenic factors, and nutrient sensing genes mRNA transcript abundance in placental explants during a 24 h period. Placentomes from adequately fed and nutrient restricted heifers were collected via Cesarean section at day 180 of gestation; separated into caruncular and cotyledonary tissue and placed in culture media for a 24 h period. The mRNA transcript abundance of clock genes (ARNTL, CRY1, and PER2), angiogenic factors (HIF1A and VEGFA), and nutrient sensing genes (NAMPT and NR3C1) was determined every 4 h. Clock genes were expressed in caruncular and cotyledonary explant tissue. The caruncular explant transcript abundance of the clock genes was not influenced by time ($P > 0.05$); while ARNTL abundance decreased over time in the cotyledon explant ($P < 0.05$). A main effect of time was observed for HIF1A, VEGFA, and NR3C1 in the caruncular tissue ($P < 0.05$).
Although, angiogenic factors and nutrient sensing genes in cotyledonary tissue displayed evident temporal variation in transcript abundance ($P < 0.05$). Nutrient restriction did not alter ($P > 0.15$) mRNA transcript abundance of clock genes, angiogenic factors, or nutrient sensing genes in either caruncular or cotyledonary tissue. Interestingly, these data may indicate limited transmission and synchronization of maternal and fetal temporal variations in transcript abundance. These findings demonstrate that multiple timepoint collections are needed in future studies due to the innate existence of temporal oscillations observed in the bovine placenta.

**Introduction**

The placenta, as a multifunctional organ, is responsible for gas, nutrient, and waste exchange between the maternal and fetal systems throughout gestation (Reynolds and Redmer, 1995). In domestic livestock species, maternal nutrient restriction has been shown to compromise pregnancies due to reduced uteroplacental blood flow (Reynolds et al., 2006; Lemley et al., 2018) and limited nutrient availability to the fetus. Placental insufficiency results in offspring with reduced birth weight and can negatively impact their survival rates (Lemley and Vonnahme, 2017). Fetal growth approaches exponential rates during late gestation which is mirrored by increases in uteroplacental blood flow and vascularity (Reynolds and Redmer, 1995). One of the most important angiogenic factors in the placenta is the vascular endothelial growth factor (VEGF), which is essential for embryonic vasculogenesis (Powe et al., 2011) and its expression increases as gestation proceeds (Cheung, 1997). Clock genes and the hypoxia-inducible factor 1 (HIF1A) control the expression and transcription of VEGF, therefore, the clock network regulates angiogenesis and embryonic development (Forsythe et al., 1996; Jensen and Cao, 2013).
The circadian clock controls physiological and behavioral processes such as body temperature, sleep-wake cycle, hunger, and corticosteroids production (Reiter et al., 2014) its expression regulates homeostasis and is responsible for targeting multiple transcription factors in peripheral tissue. The clock operates by rhythmic transcription and translation of multiple genes establishing an oscillatory feedback loop (Dolatshad et al., 2010) based on a 24-hour period. Even though the circadian pacemaker is located in the suprachiasmatic nuclei of the hypothalamus (Rusak and Zucker, 1979), clock genes have been found in other peripheral tissues such as: rat oviduct and ovaries (Kennaway et al., 2003; Karman and Tischkau, 2006), mice skeletal muscle and uterus (McCarthy et al., 2007; Dolatshad et al., 2008), and more recently, mice placenta (Rtajczak et al., 2010; Wharfe et al., 2011).

The fetal suprachiasmatic nucleus development and maturation may be influenced by maternal feeding regimens (Reiter et al., 2013). Previous research has shown the maintenance of in vitro circadian rhythms in liver, brain, embryonic, and placental explant tissues (Stokkan et al., 2001; Abraham et al., 2005; Dolatshad et al., 2010; Brew and Sullivan, 2017). Moreover, recent research culturing human adipose explants for 24 h have been able to identify temporal variations in clock genes (Gomez-Abellan et al., 2012). Nevertheless, short-term temporal variations in bovine placental transcript abundance have not been evaluated. Furthermore, nutrient restriction in dairy cattle has shown up- and down regulation of liver gene expression (Loor et al., 2006), but the effect of nutrient restriction in the placenta transcript abundance have not been studied. Therefore, the objective of this study was to determine the effect of early to mid-gestational nutrient restriction on clock genes, angiogenic factors, and nutrient sensing gene mRNA transcript abundance in bovine placental explants during a 24-hour period.
Materials and Methods

Animals and Tissue Collection

Heifers were housed and underwent c-section surgeries at the H. H. Leveck Animal Research Center (Mississippi State, MS) in accordance with the guidelines of the Mississippi State University Institutional Animal Care and Use Committee (#16-461). All animal management and tissue collection procedures have been described previously (Lemley et al., 2018). Briefly, at day 50 of gestation, 6 Angus (Bos taurus taurus) and 6 Brahman (Bos taurus indicus) pregnant heifers were equally assigned to 1 of 2 dietary treatments consisting of 100% (control; n = 6) or globally restricted to the 60% (restricted; n = 6) of net energy requirements (according to the National Research Council for Beef Cattle). Placentomes adjacent to the umbilical cord entry were collected via Cesarean section at day 180 of gestation. Immediately after removal, placentomes were submerged in 1 x PBS and transported to the Wise Center (Mississippi State, MS) for further processing. A single placentome was manually separated into the caruncular crypts and fetal villi. Placental tissues were cut using a biopsy punch (Acuderm inc., Ft. Lauderdale, FL) with a 5 mm internal diameter. Tissue fragments were plated to a density of 25% of the well in small pieces. Explants proceeded at 37°C in 5% CO2/air in D-MEM/F-12 culture media with the addition of 10% fetal bovine serum, 500 IU/mL of penicillin, and 500 ug/mL of streptomycin for 24 h. The explants were placed in the culture media at 1600h (0h) and were harvested at 2000h (4h), 2400h (8h), 0400h (12h), 0800h (16h), 1200h (20h), and 1600h (24h). The scotophase or in vivo dark phase was determined to be from 1800 to 0600 h (2 to 14h) and the photo phase or light phase was determined to be from 0600- 1800 h (14 to 2h). Placental tissue was snap frozen in liquid nitrogen and stored at -80 °C for RNA extraction.
**Placental Explants RT-PCR**

RNA was extracted from frozen placental explants (caruncular and cotyledonary tissue) for each cultured timepoint. Approximately 25 mg of tissue were homogenized in 0.35 mL of RLT Lysis buffer (QIAGEN, Hilden, Germany) and RNA were purified using RNeasy Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer’s recommendations. Total RNA extraction quality was assessed and quantified using a NanoDrop One spectrophotometer (Thermo Scientific, Waltham, MA) and stored at -80 °C. The High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Vilnius, Lithuania) was used to reverse transcribe 2 µg of total RNA into cDNA and stored at -20 °C. The gene expression levels were obtained using TaqMan probe-based assays (Applied Biosystems, Pleasanton, CA) and a QuantStudio 3 (Applied Biosystems, Foster City, CA) real-time qPCR system. The thermal cycling parameters were previously published (Lemley et al., 2018). The genes of interest for caruncular and cotyledonary tissue were clock genes (ARNTL, CRY1, PER2), angiogenic factors (HIF1A, VEGFA), and nutrient sensing genes (NR3C1 and NAMPT). Beta actin (ACTB), Glyceraldehyde-3-phosphate dehydrogenase (GADPH), ribosomal protein L19 (RPL19), and splicing factor 3a subunit 1 (SF3A1) were measured as housekeeping genes. The geNorm algorithm was used to evaluate the reference genes stability (Vandesompele et al., 2002). The most suitable reference genes for the caruncular tissue were RPL19 and SF3A1, and the most stable genes for the cotyledonary tissue were GADPH, RPL19 and SF3A1. Assays were validated prior to qPCR and efficiencies were considered acceptable if between 1.85 and 2.15 (92.5% to 107.5%) (Table 2.1). The efficiencies were calculated using a formula previously described (Lemley et al., 2018). Samples were run in duplicate and CT values were averaged and used for relative quantification using the 2-ΔΔCT method. The geometric mean of the most
stable housekeeping genes was calculated and used to normalize the transcript abundance of target genes in the corresponding tissue.

**Statistical Analysis**

Data normality was tested using the Shapiro-Wilks statistics of the UNIVARIATE procedure in the SAS software version 9.4 (SAS Institute, Cary, NC). The mRNA relative transcript abundance measured over time was analyzed using repeated measurements of ANOVA of the MIXED procedure of SAS. The model included the fixed effect of treatment, time, breed, and corresponding interactions. Fetal sex was included as a covariate and removed from the model if determined to have no influence (P > 0.25). Means were not separated across fetal sex and therefore, not reported. The fetal sex distribution for each breed and treatment was Angus-CON (n = 2 male and n = 1 female), Brahman-CON (n = 1 male and n = 2 female), Angus-RES (n = 2 male and n = 1 female), and Brahman-RES (n = 1 male and n = 2 female). The best fit of the variance-covariance structure was determined to be compound symmetry. Means were separated using the PDIFF option of the LSMEANS statement. Statistical differences were considered significant if P ≤ 0.05. Data is presented as means ± pooled SE.

**Results**

Early gestational nutrient restriction did not alter (P > 0.15) mRNA relative transcript abundance of clock genes (ARNTL, CRY1, and PER2), angiogenic factors (HIF1A and VEGFA), or nutrient sensing genes (NAMPT and NR3C1) in either caruncular or cotyledonary explant tissue. Additionally, all interactions of dietary treatment by time and dietary treatment by breed for caruncular and cotyledonary mRNA expression were not significant (P > 0.05).
Therefore, the main effect of time, breed, and time by breed interaction are reported when significant.

**Caruncular mRNA Transcript Abundance**

The caruncular explant transcript abundance of ARNTL, CRY1, and PER2 was not influenced \((P > 0.05)\) by time (Fig. 2.1). Nevertheless, a main effect of time was observed for HIF1A \((P = 0.003)\), VEGFA \((P = 0.047)\), and NR3C1 \((P < 0.0001)\) in the caruncular tissue. The mRNA transcript abundance of HIF1A displayed temporal variations, increasing \((P = 0.001)\) from 0 to 8 h and decreasing \((P = 0.012)\) from 8 to 12 h (Fig. 2.2A). Maternal expression of VEGFA and NR3C1 decreased \((P < 0.025)\) over time (Fig. 2.2B and C). A time by breed interaction was observed \((P < 0.001)\) for the caruncular transcript abundance of NAMPT, which was increased \((P < 0.05)\) in Angus \((2.934 \pm 1.075)\) vs. Brahman heifers \((1.099 \pm 1.075)\) at 16 h and increased \((P < 0.05)\) in Brahman \((6.2092 \pm 1.1865)\) vs. Angus heifers \((1.626 \pm 1.075)\) at 20 h; the abundance of NAMPT remained increased in Brahman \((8.664 \pm 1.075)\) vs. Angus tissue \((1.179 \pm 1.187)\) at 24 h \((P < 0.05);\) Fig. 2.2D). A main effect of breed was observed in maternal transcript abundance of HIF1A, which was increased \((P = 0.019)\) in Angus \((2.097 \pm 0.238)\) vs. Brahman \((1.035 \pm 0.240)\) (Fig. 2.3A). Similarly, breed influenced the expression of VEGFA which was increased \((P = 0.023)\) in Angus \((1.002 \pm 0.115)\) when compared to Brahman \((0.543 \pm 0.116)\) caruncular tissue (Fig. 2.3B). Lastly, the most significant correlation was found between HIF1A and VEGFA mRNA transcript in the caruncular tissue \((r = 0.81452; P < 0.0001)\).

**Cotyledonary mRNA Transcript Abundance**

A main effect of time was observed for the clock gene ARNTL \((P < 0.0001)\) and its transcript abundance decreased \((P < 0.0001);\) Fig. 2.4A) over time in the cotyledonary explant
tissue. The transcript abundance of CRY1 and PER2 was not different over time ($P > 0.05$; Fig. 2.4B and C). Temporal variations were observed ($P < 0.05$) in angiogenic factors and nutrient sensing genes (Fig. 2.5). The mRNA transcript abundance of HIF1A displayed a double oscillation through the 24 h in the culture media; one oscillation corresponded to the in vivo scotophase and the second oscillation corresponded to the in vivo photo phase (Fig. 2.5A). The first oscillation of HIF1A increased ($P < 0.0001$) from 0 to 8 h and decreased ($P = 0.004$) from 8 to 12 h, while the second oscillation increased ($P < 0.001$) from 12 to 16 h and decreased ($P = 0.044$) from 16 to 20 h (Fig. 2.5A). The transcript abundance of VEGFA in the fetal portion of the placenta, decreased ($P < 0.001$) from 0 to 12 h, corresponding to the in vivo scotophase, and increased ($P < 0.0001$) from 12 to 24 h, corresponding to the in vivo photo phase (Fig. 2.5B). Similar to the VEGFA expression, NR3C1 transcript abundance exhibited a decrease ($P = 0.0001$) during 0 h to 12 h and an increase ($P < 0.0001$) from 12 to 24 h corresponding to the in vivo scotophase and photophase, respectively (Fig. 2.5C). Lastly, the cotyledonary transcript abundance of NAMPT, increased ($P < 0.001$) from 0 h to 8 h, decreased ($P = 0.006$) from 8 h to 12 h, and remained constant ($P > 0.05$) from 12 h to 24 h (Fig. 2.5D).

**Discussion**

To our knowledge, this is the first work demonstrating the expression of clock genes in the maternal and fetal portions of the bovine placenta. Furthermore, the mRNA transcript abundance of the angiogenic factors and nutrient sensing genes measured in this study displayed temporal variations in the cotyledonary tissue. The current study was performed using cultured placental explants, which have been extensively utilized as an effective model to study placental functions corresponding to the in vivo physiology (Brew and Sullivan, 2017).
Wharfe et al. (2011) showed the expression of the canonical clock genes in the rat placenta (junctional and labyrinth) with a limited circadian variation. The clock network is composed of the CLOCK, ARNTL (also known as BMAL1), three Period (PER1-3), and two Cryptochrome (CRY1 and CRY 2) genes (Reppert and Weaver, 2002). Heterodimerization of CLOCK and ARNTL (CLOCK:BMAL1) potentiates the activation and transcription of PER and CRY genes; subsequently, dimerization of PER and CRY proteins inhibit the CLOCK:BMAL1 transcriptional activity in the nucleus (Reppert and Weaver et al., 2002; Mark et al., 2017). CRY1, a potent circadian repressor, has been found to inhibit HIF1A binding site and reduce its half-life in hypoxic mouse embryonic fibroblasts (Dimova et al., 2019). Deficiency of CRY1 in mouse embryonic fibroblast cells, increases HIF1A accumulation and induces cellular proliferation and migration, while knocking out hypoxia inducible factors by CRISPR/Cas9 has shown to reverse this effect (Dimova et al., 2019). These results confirm that the circadian clock negative feedback loop manages the hypoxic inducible factor protein levels (Peek et al., 2017). In addition, these previous results could help to understand why the evaluated clock genes in the current study did not exhibit temporal variations in the bovine placenta.

The HIF1A gene actively participates through all the stages of placental differentiation and growth, ensuring placental function (Patel et al., 2010). In the sheep placenta, HIF1A has a well-known role in angiogenesis by promoting the extent of vascular bed formation (Borowicz et al., 2007). Interestingly, HIF1A was the only mRNA transcript that showed temporal oscillations for both caruncular and cotyledonary tissue, indicating its potential importance in short-term temporal feto-maternal placental function. Under low oxygen conditions, HIF1A activates multiple transcription factors including VEGF which promotes angiogenesis and vascularization in the placenta (Agaoglu et al., 2015). In the cotyledon, even though the HIF1A displayed a
double oscillation, only the oscillation corresponding to the photo phase appears to have a positive effect on vascular endothelial growth factor A (VEGFA) transcript abundance. Recent observations from our laboratory using the same animals, found an increase in cotyledonary blood vessel density in Brahman heifers when compared to the Angus counterparts (Lemley et al., 2018) and it was attributed to an increase in the angiogenic factors transcript abundance. In the same study, there was no difference in the VEGFA transcript abundance between breeds in the caruncular tissue when sampling at a single timepoint. Nevertheless, it is important to notice that the mRNA expression in this study were performed on multiple timepoints during a 24 h period. In contrast with the previous study, Angus heifers had an increase HIF1A and VEGFA mRNA abundance in the caruncular tissue when compared to their Brahman counterparts. These results confirm the importance of multiple sample collections due to the existence of temporal oscillations.

The nicotinamide phosphorybosyltransferase (NAMPT), also known as vistafin, has been implicated in the modulation of lipid, glucose, and insulin metabolism (Reverchon et al., 2016). NAMPT is the rate limiting enzyme in the NAD+ biosynthetic pathway, where the NAD+/NADH ratio is an indicator of the cell energy status (Nkahat et al., 2009). NAMPT has been related with the circadian clock feedback cycle (Ramsey et al., 2009) showing that mice deficient in ARNTL have reduced NAMPT expression. Furthermore, inhibition of NAMPT has been shown to stimulate PER2 oscillations (Ramsey et al., 2009). The lack of oscillation of PER2 in the caruncular and cotyledonary tissue observed in our study may be related to the NAMPT activity in the placenta. Using MCF7 cells, a breast cancer line, previous research showed that hypoxia and overexpression of HIF1A significantly increase NAMPT mRNA and protein levels (Bae et al., 2006). In the current study, HIF1A and NAMPT indicate a similar
oscillation during the scotophase in the cotyledonal tissue. Similar to our study, Ramsey et al. (2009) identified a sharp circadian oscillation in NAMPT RNA from wild-type mouse liver and white adipose tissue, peaking at the scotophase. In addition, NAMPT comparable to HIF1A has shown to induce angiogenesis in human umbilical endothelial cells by upregulation of VEGF (Adya et al., 2007) and this may help to elucidate the role of NAMPT in the placenta.

The nuclear receptor subfamily 3, group C, member 1 (NR3C1) is a glucocorticoid receptor found in the central and peripheral nervous systems, which responds to adrenal glucocorticoids (Oster et al., 2016). The adrenal cortex secretes corticosterone in rodents and cortisol in primates and ruminants to modulate stress, gluconeogenesis, lipid metabolism, and immune responses (Lefcourt et al., 1993; Son et al., 2008). It has been shown that the circadian rhythm exhibited in adrenal glucocorticoid synthesis is controlled by the CLOCK:ARNTL heterodimer (Son et al., 2008). Plasma concentrations of corticosterone have a robust circadian rhythm in mice, which decreased overnight and increased over daylight hours, with a peak in the night onset (Son et al., 2008). Differing from rodents, peripheral cortisol concentration in cows do not exhibit a circadian variation (Lefcourt et al., 1993; Eley et al., 1992). Nevertheless, the NR3C1 transcript abundance in the cotyledonal tissue observed in this study decreased during scotophase and increased during photo phase displaying temporal variation. In ruminants, glucocorticoids increase towards term and cortisol is essential for fetal organ maturation such as lungs, liver, kidney, and gut (Fowden et al., 1998). However, excessive exposure to cortisol during late gestation increases uteroplacental glucose uptake and decreases umbilical glucose uptake (Ward et al., 2004), causing intrauterine growth restriction (Greenwood and Bell, 2003).

In conclusion, the clock genes measured in this study were present in the placenta but limited temporal variation was observed in their transcript abundance. Based on the mRNA
transcript abundance it appears that the maternal and fetal temporal oscillations are not synchronized, rather they are two independent systems. This could be related to the differences between the caruncular and cotyledonary cells and tissue. Moreover, the lack of temporal variations in the caruncular tissue may be attributed to an increase in connective tissue which could limit viability in culture media. Nevertheless, HIF1A displayed temporal oscillations in both tissues during the scotophase demonstrating its importance in placental function and development. In the cotyledonary tissue, HIF1A appears to influence angiogenic factors and nutrient sensing genes, while regulating clock genes. Lastly, with the confirmation of temporal oscillations in the bovine placenta, it is suggested that multiple collection timepoints are needed to avoid variability in results due to the natural changes in transcript abundance within hours.

Identifying temporal dependent changes of angiogenic factors and nutrient sensing genes may allow us to determine the appropriate time to provide pregnant animals with dietary supplementation to ameliorate the consequences of a decrease in these factors and prevent intrauterine growth restriction in livestock species.
Figure 2.1  Caruncular explant transcript abundance of clock genes across 24-h.

Main effect of time for (A) ARNTL ($P = 0.075$) (B) CRY1 ($P = 0.061$), and (C) PER2 ($P = 0.117$). Values were normalized using the geometric mean of the housekeeping genes ($RPL19$ and $SF3A1$). Data presented as mean ± pooled SE. Arrows (↑) represent in vivo feeding time for heifers ($n = 12$). Hour 2 to 14 correspond to the in vivo scotophase (1800 - 0600 h) and hour 14 to 2 correspond to the in vivo photophase (0600 – 1800 h).
Figure 2.2  Caruncular explant transcript abundance of angiogenic factors and nutrient sensing genes across 24-h.

A main effect of time was observed for (A) HIF1A ($P = 0.003$), (B) VEGFA ($P = 0.047$), and (C) NR3C1 ($P < 0.0001$). A time by breed interaction was found for (D) NAMPT ($P < 0.001$). Relative transcript abundance was normalized with housekeeping genes ($RPL19$ and $SF3A1$) geometric mean. Data presented as means ± pooled SE; means with different letters represent significant differences ($P < 0.05$). Asterisk (*) represent a difference ($P < 0.05$) between Angus ($n = 6$) versus Brahman ($n = 6$) heifers within the same time point. Arrows (↑) represent in vivo feeding time for heifers. Hour 2 to 14 correspond to the in vivo scotophase (1800 – 0600 h) and hour 14 to 2 correspond to the in vivo photophase (0600 – 1800 h).
Figure 2.3  Main effect of breed for (A) HIF1A and (B) VEGFA transcript abundance in caruncular explant tissues.

Time by breed interaction for angiogenic factors (HIF1A and VEGFA) was not significant ($P > 0.05$). Asterisk (*) represent a difference ($P < 0.05$) in transcript abundance between Angus ($n = 6$) versus Brahman ($n = 6$) heifers.
Figure 2.4  Cotyledonary explant transcript abundance of clock genes across 24-h.

A main effect of time was observed for (A) ARNTL ($P < 0.0001$). The transcript abundance of (B) CRY1 and (C) PER2 was not influenced by time ($P = 0.121$ and $P = 0.545$; respectively). Cotyledonary relative mRNA abundance was normalized by GADPH, SF3A1, and RPL19 geometric mean. Data presented as means ± pooled SE; means with different letters represent significant differences ($P < 0.05$). Arrows (↑) represent in vivo feeding time for heifers. Scotophase refers to the in vivo dark phase (1800 - 0600 h).
Figure 2.5  Cotyledonary explant transcript abundance of angiogenic factors and nutrient sensing genes across 24-h.

A main effect of time was observed for (A) \(HIF1A\) \(P < 0.0001\), (B) \(VEGFA\) \(P < 0.0001\), (C) \(NR3C1\) \(P < 0.001\), and (D) \(NAMPT\) \(P = 0.011\). Fetal placenta mRNA transcript abundance was normalized using \(GADPH\), \(SF3A1\), and \(RPL19\) as housekeeping genes. Data are presented as means ± pooled SE; means with different letters represent significant differences \((P < 0.05)\). Arrows (↑) represent in vivo feeding time for heifers. Scotophase refers to the in vivo dark phase (1800 - 0600 h) and photo phase correspond to hours 14 to 2 (0600- 1800 h).
Table 2.1 Assays, GenBank accession number, amplicon length, and efficiency (10^-1/slope) of primers used for TaqMan probe-based real-time qPCR of placental explants.

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ARNTL, aryl hydrocarbon receptor nuclear; CRY1, cryptochrome circadian regulator 1; PER2, period circadian regulator 2; HIF1A, hypoxia inducible factor 1 subunit alpha; VEGFA, vascular endothelial growth factor A; NR3C1, nuclear receptor subfamily 3 group C; NAMPT, nicotinamide phosphoribosyltransferase
REFERENCES


45


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CHAPTER III
MELATONIN-INDUCED CHANGES IN BOVINE FETO-PLACENTAL DEVELOPMENT
DURING LATE GESTATION MATERNAL NUTRIENT RESTRICTION

Abstract

The objectives were to examine melatonin mediated changes in temporal uterine blood flow (UBF), vaginal temperatures (VT), and placentome vascularization along with fetal morphometric changes in 54 commercial Brangus heifers (Fall, n=29; Summer, n=25) during compromised pregnancy. At d160 of gestation, heifers were assigned to 1 of 4 groups consisting of adequately fed (ADQ-CON; 100% NRC; n=13), global nutrient restricted (RES-CON; 60% NRC; n=13), and ADQ or RES supplemented with 20 mg of melatonin (ADQ-MEL, n=13; RES-MEL, n=15). In the morning (0500h; AM) and afternoon (1300h; PM) of d220 of gestation, UBF was determined via Doppler ultrasonography while temperature dataloggers attached to progesterone-free CIDRs were used to record VT. At d240 of gestation, heifers underwent Cesarean sections at either 0500h or 1300h for fetal removal and collection of 2 placentomes. One placentome was perfused through the cotyledonary artery with AlexaFluor 647 Con A conjugate for macroscopic blood vessel assessment, whereas the other placentome was cryosectioned and used for microscopic capillary immunofluorescence labelling. The UBF and VT data were analyzed using repeated measures of ANOVA. The macroscopic and microscopic placentome blood vessel density and the fetal organ weights were analyzed by the MIXED procedure of SAS. A significant difference was found in all measured variables between Fall
and Summer groups \((P<0.05)\); therefore, data were individually analyzed. In Fall, a nutrition by treatment interaction was found \((P=0.039)\), where the RES-CON heifers exhibited reduced total UBF compared to ADQ-CON \((5.67\pm0.68 \text{ vs. } 7.97\pm0.54 \text{ L/min})\). In Summer, MEL heifers exhibited increased total UBF compared to the CON counterparts \((8.16\pm0.73 \text{ vs. } 6.00\pm0.70 \text{ L/min}; \ P=0.048)\) while there was no difference between nutritional plane \((P=0.390)\). Moreover, there was a nutrition by treatment by time interaction in VT for Fall and Summer heifers \((P\leq0.005)\). In Fall, all groups had decreased VT in the morning compared to the afternoon \((P<0.05)\). Whereas, in Summer, VT increased for ADQ-CON, RES-CON, and ADQ-MEL \((P<0.0001)\) from morning to afternoon, while the RES-MEL remained constant throughout the day \((P=0.648)\). Furthermore, the RES-MEL-PM exhibited decreased VT compared to ADQ-CON-PM \((38.91\pm0.09 \text{ vs. } 39.26\pm0.09^\circ \text{C}; \ P=0.018)\). In Fall, there were no interactions or treatment differences \((P>0.05)\) for macroscopic blood vessel density analysis. However, the time of day significantly impacted the placentome fluorescent signal radiance, displaying an increase in macroscopic cotyledonary blood vessel density in the afternoon compared to the morning. In the afternoon of Fall animals, the average capillary size and the percent capillary area were decreased \((P<0.05)\) in the placentomes from RES-CON dams compared to ADQ-CON and RES-MEL. Placentomes collected in the afternoon of the Summer animals from RES-CON dams displayed increased macroscopic cotyledonary blood vessel density when compared to ADQ-CON and RES-MEL \((P<0.05)\). In Summer, the placentomes from RES dams had increased average capillary size compared to ADQ. Lastly, in Fall, a main effect of nutrition was observed on fetal weights, where the RES dams had fetuses with decreased body weight when compared to ADQ \((24.08\pm0.62 \text{ vs. } 26.57\pm0.64 \text{ kg}; \ P=0.0087)\). In Summer, a nutrition by treatment interaction was observed on fetal weights where the RES-CON dams had fetuses with reduced
weight when compared to ADQ-CON and RES-MEL \((P<0.05)\). In summary, nutrient restriction decreased UBF and melatonin supplementation increased UBF depending on the season. Additionally, melatonin appeared to decrease VT and rescue fetal weights in the Summer. Lastly, nutrient restricted dams showed altered placentome vascularity which may indicate a compensatory mechanism. Future studies should evaluate the potential benefits of melatonin supplementation during warm months or long days in farm animals.

**Introduction**

Optimum placental functionality is necessary to maintain a healthy communication between the maternal and fetal systems throughout gestation. Maternal nutrient restriction has shown to reduce utero-placental blood flow in ruminants (Lemley et al., 2018; Reynolds et al., 2006), compromising the pregnancy by reducing the amount of oxygen and nutrients available to the conceptus (Lang et al., 2003). Fetal growth and organ development are impaired under compromised pregnancies where these offspring exhibit reduced birth weight which increases neonatal morbidity and mortality (Lemley and Vonnahme, 2017; Redmer et al., 2005). Sixty percent of the observed intrauterine growth restriction is accounted for by placental insufficiency (Ghidini, 1996), resulting in poor placental vascular development (Reynolds et al., 2013). In ruminants, 60 to 90% of the fetal weight is obtained in the last third of pregnancy where angiogenesis at the placentome is needed to support the dramatic increase in blood flow and fetal growth (Bauman and Currie, 1980; Reynolds and Redmer, 1995).

In addition to nutrient restriction, heat stressed cattle have shown a reduction in uterine blood flow (Roman-Ponce et al., 1977) also causing intrauterine growth restriction. Researchers have suggested that this reduction in uterine blood flow is caused by an increase in peripheral blood flow to facilitate heat dissipation in these animals with high body temperatures (West,
Elevated maternal body temperatures make fetal heat exchange difficult which is largely through the fetal-placental circulation and the uterine wall (Tao and Dahl, 2013), creating an unfavorable environment to the fetus. Vaginal temperature data loggers are of great accuracy and represent the core body temperature of the animals without the influence of water and feed intake when compared to ruminal temperature boluses (Ammer et al., 2016). Nevertheless, to the authors’ knowledge, the association between uterine blood flow and vaginal temperatures during gestation has not been described before in the literature. Furthermore, the temporal changes of the aforementioned variables remain elusive creating a gap of knowledge of what time of the day is more beneficial to supplement pregnant animals to increase uterine blood flow or decrease vaginal temperatures.

Recent studies have evaluated dietary melatonin supplementation during day time as a possible mediator to increase umbilical and placental efficiency in ewes (Lemley et al., 2012; Lemley et al., 2013). Additionally, Brockus et al. (2016) showed an increase in uterine artery blood flow in dairy heifers supplemented with dietary melatonin during the last trimester of pregnancy. Melatonin is a neuroendocrine hormone secreted by the pineal gland and has the capacity to cross the placenta and enter the fetal circulation unaltered (Tamura et al., 2008); while it is also known to be a circadian rhythm modulator (Arendt, 2006). Among its properties, it has the capacity to reduce reactive oxygen species, which may overcome fetal growth restriction caused by oxidative damage during compromised pregnancies (Collier et al., 1982; Brockus et al., 2016). Moreover, it has been proposed that melatonin could cause arterial vasoconstriction and vasodilation by binding to melatonin receptors in endothelial and smooth muscle cells (Lemley and Vonnahme, 2017).
Thus, this research project aimed to use beef heifers to evaluate the effect of maternal nutrient restriction and/or melatonin supplementation during late gestation on: (1) uteroplacental blood flow, vaginal temperature, and placentome vascularization temporal changes, and (2) fetal growth and organ development. We hypothesized that maternal nutrient restriction during late gestation would decrease uteroplacental blood flow and melatonin supplementation during daytime will mitigate the adverse effects of compromised pregnancy. Furthermore, we hypothesized temporal changes in vaginal temperatures while melatonin supplemented animals would exhibit reduced vaginal temperatures. Additionally, we hypothesized that nutrient restriction would reduce fetal weights and impair organ morphometrics, whereas melatonin supplementation would negate these effects. Lastly, we hypothesized that the nutrient restricted dams would exhibit increased placentome perfusion and microscopic blood vessel density as a compensatory mechanism compared to adequately fed and melatonin supplemented dams.

Materials and Methods

Animal Care and Treatments

Animal care and use was approved by the Mississippi State University Institutional Animal Care and Use Committee (#17-709). During 2019 (March 27 to April 12) and 2020 (January 15 to January 30), a total of 180 commercial Brangus heifers underwent a 7-day Co-synch + CIDR estrous synchronization protocol and were timed artificially inseminated 55 hours later using commercially available semen from a single Angus sire. In both years, animals were intentionally distributed among 6 different breeding dates to facilitate further data collection. Pregnancy confirmation was performed 35-d post breeding via transrectal ultrasonography. Pregnant heifers were enrolled in the study for years 2019 (n = 29) and 2020 (n = 25). All
animals remained under the same environmental conditions meeting their nutritional requirements for early gestation (NRC, 2000).

At day 140 of gestation, animals underwent a 20-d acclimation period where they were trained to acquire feed from a Calan® electronic feeding system (American Calan, Northwood, NH) prior to treatment initiation. Initial bodyweights were collected and at day 160 of gestation animals were stratified by body weight and assigned to 1 of 4 treatments in a 2 x 2 factorial design consisting of adequately fed (ADQ-CON; 100% NRC), nutrient restricted (RES-CON; 60% NRC), adequately fed supplemented with 20 mg of melatonin (ADQ-MEL), and nutrient restricted supplemented with 20 mg of melatonin (RES-MEL). The animal number distribution per treatment for both years is outlined in Table 3.1. The adequately fed heifers received 100% of the net energy recommendations for maternal and fetal growth (NRC, 2000), while the nutrient restricted counterparts received 60% of their calculated control diet. Control diets were fed at a total of 2.4% of BW/day with a target gain of 1.0 kg/d. Heifers were provided with a TMR (71.65 % DM, and 11.27 % protein, 3.65 % fat, 4.66 % crude fiber, and 38.95 % ADF on DM basis) at a rate of 1.4 % of BW/day and chopped hay (90.5 % DM, and 7.1 % protein, 73 % NDF, 42.4 % ADF on DM basis) at a rate of 0.8 % of BW/day. The TMR consisted in cottonseed hulls (24.26 %), ground corn (14.77 %), soybean hulls (30.33 %), wheat middling (6.06 %), and pre-mix cattle pellets (24.26 %). Additionally, all heifers (independent of their treatment group) received 0.90 kg/day of a grain mix (7.65 % DM, and 22.64 % protein, 8.40 % ADF, 7.42 % fat) used to top dress the treatments. The grain mix contained on DM basis 2.36 % calcium, 0.92 % potassium, 0.78 % chloride, 0.57 % phosphorus, 0.52 % sodium, 0.24 % magnesium, 0.06 % vitamin E, and 9,133 IU/kg of vitamin A. Melatonin (#14427; Cayman Chemical Company, Ann Arbor, MI) was dissolved in absolute ethanol at a concentration of 10
mg/mL and 2 mL were top dressed in the vitamin mix grain, whereas the control groups received 2 mL of absolute ethanol as their treatment. The dietary melatonin concentration was determined by previous research (Brockus et al., 2016). Heifers were fed once daily at 0900 h. The treatments were provided first and after its total consumption (around 15 minutes) the TMR was offered. Diets were adjusted weekly based on maternal body weight changes. Data were collected during different seasons for each year of study, with the last trimester of pregnancy corresponding to November 1st to December 5th in Fall 2019 and August 21st to September 29th in Summer 2020.

**Color Doppler Ultrasonography and Vaginal Temperatures**

On day 220 of gestation, 60 days post-treatment initiation, uteroplacental hemodynamics and vaginal temperatures were recorded at 0500 and 1300 h. The uterine artery hemodynamics were assessed similar to Brockus et al. (2016) and Lemley et al. (2018). Briefly, the uterine arteries ipsilateral and contralateral to the conceptus were located via the color function on the Doppler ultrasound (M-Turbo, Sonosite Inc., Bothell, VA) and using a trans-rectal probe (Linear Endorectal L52x probe; Sonosite Inc., Bothell, VA) three independent cardiac cycle waveforms were used for further calculations. The hemodynamics of interest were systolic velocity (s; cm/s), diastolic velocity (d; cm/s), s:d ratio, pulsatility index (PI), and resistance index (RI). Additionally, the ellipse preset function in the ultrasound was used to determine the blood vessel diameter, area, and circumference. The mean velocity was calculated using the equation: \((s - d)/PI.\) Blood flow was determined using the equation: \(\text{mean velocity} \times \text{vessel area} \times 60\) sec. Uterine artery ultrasonography measurements took approximately 10 min per animal. For vaginal temperatures, TidbiT v2 water temperature data loggers (Onset Computer Corporation; Bourne, MA) were attached to a non-progesterone CIDR and placed in the heifer’s vagina with a CIDR.
applicator 24-h before data collection. Thermometers were programmed to record vaginal temperature every 5 minutes starting at 0600 h of the day before ultrasonography and were removed approximately around 1400 h with the last ultrasound. The vaginal temperature values from the day of ultrasonography were average from 0400 to 0600 h (Morning; 0500h) and 1200 to 1400 h (Afternoon; 1300 h) for further analysis. Lastly, maternal blood samples were collected via coccygeal venipuncture at both times of data collection.

**Cesarean Sections and Tissue Collection**

At day 240 of gestation, 80 days post-treatment initiation, heifers underwent Cesarean sections for placentome and fetal tissue collection according to Lemley et al. (2018). Surgeries were performed at the H.H. Leveck Animal Research Center where animals were housed. Coccygeal maternal blood was collected before surgery initiation. Surgeries took approximately 25 min and were performed at either 0500 h or 1300 h. The animal number distribution corresponding to each timepoint for each year is outlined in Table 2. Briefly, Cesarean sections were performed with the dam standing in the chute following a paravertebral block with 2% lidocaine. The left oblique was prepared for aseptic surgery and a 10 to 15 cm incision was made ventral to the transverse processes of the paralumbar fossa. The fetal frontal limbs were identified and used as a handle to deliver the gravid uterus to the abdominal incision. After the uterus was incised, the umbilical cord was located and clamped off to cut it approximately 10 cm from the fetus. After fetal removal, two placentomes adjacent to the umbilical cord were collected. One placentome was submerged in cold 1 x PBS and immediately transported to the laboratory for placentome blood vessel perfusion (described below); while the second placentome was processed on site. A middle portion of the second placentome was selected and sectioned into a 1- by 1-cm, placed in an embedding mold containing Optimal Cutting
Temperature media (OCT; Fisher Scientific, Pittsburgh, PA) and frozen by submersion in supercooled isobutane and stored at -80 °C for further immunohistochemistry analysis. The remainder of the middle portion of the placentome was separated into the maternal caruncle and cotyledonary villi, placed in cryogenic tubes, snap frozen in liquid nitrogen, and stored at -80 °C for further mRNA expression analysis. Fetal trunk blood was collected during exsanguination.

**Placentome Perfusion and Macroscopic Blood Vessel Density**

Macroscopic blood vessel density was determined according to Lemley et al. (2018) with minor modifications. Briefly, the cotyledonary artery from an intact placentome was isolated and catheterized with a 20-g by 2-inch catheter (Exel Safalet Cath, Exelint International, Los Angeles, CA). After catheterization of the cotyledonary artery, 30 mL of 1 x PBS was perfused to remove blood. The placentomes were then perfused with approximately 35 mL of 100-µg/mL of Concanavalin A, Alexa Fluor 647 conjugate (ThermoFisher Scientific, Waltham, MA) until the fluorophore was drained through the cotyledonary vein. Subsequently, the cotyledonary artery and vein were ligated with silk suture to ensure a closed system prior to imaging. Placentomes were covered with aluminum foil to prevent any light exposure and were immediately photographed with an *in vivo* imaging system, Lumina XRMS Series III (IVIS, PerkinElmer, Waltham, MA). The radiance signal (photon/s/cm²/sr) was determined from each sample using the region of interest function from the IVIS. A negative control placentome was imaged following the PBS perfusion alone.

**Placentome Immunohistochemistry and Microscopic Blood Vessel Density**

The immunofluorescence imaging of blood vessels was performed according to Lemley et al. (2018) with minor modifications. Briefly, the placentomes that were embedded in optimal
cutting temperature molds and were sectioned into 7 μm using a CRYOSTAR NX50 (Thermo Scientific, Waltham, MA), and placed on positively charged microscope slides. Two slides per cow were used and slides were blocked for nonspecific antigen binding using 10% goat serum in PBS containing 0.2% of Tween-20 for 30 min. Subsequently, a 1:50 dilution of Anti-Von Willebrand Factor (ab6994; Abcam, Cambridge, MA) was used as the primary antibody incubated by 1 h; followed by a 1:250 dilution of Goat Anti-Rabbit IgG H&L AlexaFluor 594 (ab150080; Abcam, Cambridge, MA) as the secondary antibody with an incubation time of 30 min. To contrast the caruncular crypts and cotyledonary villi, slides were incubated for 5 min with a 1:500 dilution of Fluorescein labeled Griffonia Simplicifolia Lectin I (FL-1101; Vector Laboratories, Burlingame, CA). The dilutions were made in 3% goat serum in PBS and slides were kept in humidified boxes protected from light sources. Lastly, slides were treated with Fluoroshield mounting medium with DAPI (ab104139; Abcam, Cambridge, MA) for nuclear staining. Six images per slide were captured using 10 × magnification with an EVOS microscope (AMAFD1000; Life Technologies, Carlsbad, CA). Twelve representative images per animal were then analyzed using ImageJ (https://imagej.nih.gov/ij/download.html). Total capillary number per tissue area (vessel number/mm²), percent capillary area (%/mm²), average capillary size (μm²), and average capillary perimeter per tissue area (mm/mm² or mm⁻¹) were determined.

**Blood Sampling Analysis**

Maternal and fetal blood were collected into K² EDTA blood tubes (BD Vacutainer, Franklin Lakes, NJ) and immediately placed on ice. Samples were spun at 2000 x g for 12 min and plasma was collected and stored at -80 °C until melatonin analysis. The melatonin concentrations were determined using a melatonin ELISA kit (RE54021; IBL international, Hamburg, Germany) with an analytical sensitivity of 1.6 pg/mL and inter- and intraassay of
variation of 11.4 and 19.3 %, respectively. The melatonin concentrations were determined following the manufacturer’s recommendations. A total of 0.5 mL of the animal’s plasma was combined with 0.5 mL of distilled water and pass through the provided extraction columns. The yield of extraction was determined by the manufacturer to be approximately 90 to 100 % and the cross-reactivity of other substances were less than 0.01 %.

**Statistical Analysis**

Data normality was tested using the Shapiro-Wilks statistics of the UNIVARIATE procedure in the SAS software version 9.4 (SAS Institute, Cary, NC). Significant differences were found for all the variables between years ($P \leq 0.05$) therefore data were individually analyzed for 2019 and 2020 heifers. The weekly maternal body weights, uteroplacental blood flow, vaginal temperatures, and maternal melatonin concentrations from the day of ultrasound were analyzed using repeated measurements of the MIXED procedure of SAS. The best fit of the variance-covariance structure was determined to be compound symmetry with the lowest Akaike Information Criteria. Moreover, the fetal weights, fetal organs, macroscopic blood vessel density, microscopic capillary density, maternal (collected at the Cesarean section) and fetal melatonin concentrations, and gene expression data were analyzed using ANOVA of the MIXED procedure of SAS. The model included the fixed effect of nutritional plane, treatment, time, and corresponding interactions. Fetal sex was included as covariates in the model and removed from the model if determined to have no influence ($P > 0.25$). Means were separated using the PDIFF option of the LSMEANS statement. Statistical differences were considered significant if $P \leq 0.05$. Data are presented as means ± pooled SE.
Results

Maternal Body Weight

A nutritional plane by week of gestation interaction was observed for maternal body weight in the Fall 2019 ($P < 0.0001$) and Summer 2020 ($P < 0.0001$) heifers (Figure 3.1A and Figure 3.1B, respectively). In Fall 2019, the RES heifers displayed reduced body weight at week 30 of gestation ($P = 0.022$) when compared to the ADQ counterparts (513.21±11.05 vs. 549.94±11.41 kg). Furthermore, the RES dams body weights remained decreased until the completion of data collection at week 33 of gestation compared to ADQ dams (521.66±11.05 vs. 568.64±11.41 kg; $P = 0.003$). No main effect of treatment was found in maternal body weights between control and melatonin supplemented pregnant heifers (512.98±11.21 vs. 515.25±10.85 kg). In Summer 2020, the RES dams displayed reduced body weight compared to ADQ dams (474.95±14.15 vs. 513.67±13.49 kg) at week 29 of gestation ($P = 0.048$). These differences in maternal body weights remained through week 33 of gestation where RES heifers continued to exhibit reduced body weights vs. ADQ heifers (489.37±14.15 vs. 540.04±13.49 kg; $P = 0.010$). Similar to Fall 2019, no treatment differences were observed in Summer 2020 ($P = 0.864$) for CON (484.87±13.92 kg) vs. MEL (481.53±13.27 kg).

Uterine Artery Hemodynamics and Vaginal Temperatures

Fall 2019 and Summer 2020 UBF data are presented in Figure 3.2. In Fall 2019, a nutritional plane by treatment interaction was found ($P = 0.044$) where RES-CON showed decreased ipsilateral UBF compared to ADQ-CON (3.98±0.58 vs. 6.12±0.46 L/min; $P = 0.007$) and no differences were found ($P > 0.05$) between ADQ-MEL and RES-MEL compared to ADQ-CON and RES-CON (Figure 3.2A). No differences were observed between AM and PM ipsilateral UBF (data not shown). The pulsatility index, resistance index and diameter of the
ipsilateral uterine artery were not affected \((P > 0.05)\) by nutritional plane or dietary treatment (Table 3.3). The contralateral UBF was not different \((P > 0.85)\) between nutritional plane or dietary treatment (Figure 3.2B). The diameter of the contralateral uterine artery was not affected by nutritional plane or dietary treatment \((P = 0.713)\). A nutritional plane by treatment by time interaction was observed for the pulsatility and resistance indices of the contralateral uterine artery \((P < 0.05)\) where both parameters increased from morning to afternoon in the ADQ-CON heifers \((P < 0.008; \text{Table } 4)\). The total UBF decreased in RES-CON compared to ADQ-CON (5.67±0.68 vs. 7.97±0.54 L/min; \(P = 0.012\)) whereas the ADQ-MEL and RES-MEL dams total UBF was not different from the aforementioned groups (Figure 3.2C; \(P > 0.08\)). Lastly, the total UBF was not different in the morning (6.42 ±0.48 L/min) vs. afternoon (6.95±0.48 L/min).

In Summer 2020, no interactions were observed for UBF, therefore main effects are presented. No differences were found between ADQ compared to RES (5.76±0.55 vs. 4.53±0.58 L/min; \(P = 0.157\)) in ipsilateral UBF (Figure 3.2D). Additionally, no differences \((P = 0.141)\) were found in the ipsilateral UBF when comparing CON to MEL dams (4.52±0.54 vs. 5.77±0.36 L/min; Figure 3.2D). Similar to the ipsilateral UBF in Fall 2019 animals, no differences \((P = 0.9903)\) were found between AM and PM in the ipsilateral UBF in Summer 2020 animals (data not shown). Nevertheless, a nutrition by treatment by time interaction was observed for the pulsatility \((P = 0.027)\) and resistance indices \((P = 0.010)\) of the ipsilateral uterine artery where the RES-MEL dams showed decreased pulsatility and resistance indices in the afternoon when compared to the morning (Table 3.4). The diameter of the ipsilateral uterine artery was no affected by nutritional plane or dietary treatment \((P > 0.05; \text{Table } 3.4)\). Melatonin supplemented heifers exhibited increased \((P = 0.041)\) contralateral UBF when compared to CON dams (2.49±0.037 vs. 1.38±0.34 L/min), whereas nutrition did not influence \((P = 0.694)\) the
The contralateral UBF (Figure 3.2E). The pulsatility index, resistance index and diameter of the contralateral uterine artery were not affected by nutritional plane or dietary treatment ($P > 0.05$; Table 3.4). Lastly, the total UBF increased ($P = 0.048$) in the MEL dams compared to the CON counterparts (8.15±0.73 vs. 6.00±0.70 L/min; Figure 3.2F). Similar to Fall 2019, the total UBF in the Summer 2020 heifers was not different ($P = 0.969$) in the morning (7.07 ±0.48 L/min) vs. afternoon (7.09±0.60 L/min).

A nutrition by treatment by time interaction in VT was observed for both Fall 2019 and Summer 2020 animals ($P \leq 0.005$). In Fall 2019, all groups had decreased VT in the morning compared to the afternoon ($P < 0.05$), nevertheless, no differences in VT were observed among treatment groups in the afternoon ($P > 0.05$). In the morning of Fall 2019, the RES-MEL heifers had decreased VT compared to ADQ-MEL (38.24±0.07 vs. 38.45±0.07°C; $P = 0.044$), but these values were not different from the ADQ-CON and RES-CON (Figure 3.3A). In contrast, VT increased for ADQ-CON, RES-CON, and ADQ-MEL from morning to afternoon in the Summer 2020 dams ($P < 0.0001$), while the RES-MEL remained constant throughout the day ($P = 0.648$; Figure 3.3B). In the morning of the Summer 2020 the RES-CON animals displayed reduced VT compared to ADQ-CON (38.71±0.10 vs 39.08±0.09°C; $P = 0.039$). Furthermore, in the afternoon, the RES-MEL heifers exhibited decreased VT compared to ADQ-CON animals (38.91±0.09 vs. 39.26±0.09°C, respectively; $P = 0.018$).

**Fetal Measurements at Day 240 of Gestation**

The fetal growth parameters and organ weights collected at the Cesarean section day for Fall 2019 are illustrated in Table 3.5. In Fall 2019, a main effect of nutrition was observed on fetal weights, where the RES dams had fetuses with decreased body weight when compared to ADQ (24.08±0.62 vs. 26.57±0.64 kg; $P = 0.0087$). Dietary melatonin
supplementation did not alter the fetal body weights ($P = 0.2304$). Similar to fetal weights, the exsanguinated weight of fetuses from RES dams was reduced by 10% when compared to their ADQ counterparts ($P = 0.0095$). Fetal curved crown rump length was not different among nutritional planes or treatment groups ($P > 0.15$). The abdominal girth of fetuses from MEL dams was increased when compared to fetuses from CON animals (64.89±0.89 vs 62.022±0.92 cm). A main effect of nutrition was found on heart girth, where fetuses from RES heifers had reduced heart girth when compared to their ADQ counterparts (61.86±0.62 vs 64.23±0.64 cm; $P = 0.0131$). No differences were observed on head circumference or biparietal distance among groups ($P > 0.05$).

Continuing in Fall 2019, the fetal brain weights were not different between nutritional plane or treatment groups ($P > 0.05$), nevertheless the fetal brain weight relative to fetal body weight was increased in the fetuses from RES compared to ADQ dams ($P = 0.024$). Fetal lung weight and lung weight relative to fetal weight were not different between treatment groups ($P > 0.05$). Similarly, the absolute and relative adrenal weights did not differ between treatment groups ($P > 0.05$). A main effect of nutrition was found in the fetal kidney weights which was reduced in the fetuses from RES vs. ADQ dams (101.61±4.42 vs. 116.04±4.79 g; $P = 0.0437$). Additionally, a main effect of treatment was observed in fetal kidney weights where the fetuses from MEL heifers had increased absolute kidney weights when compared to their CON counterparts (115.88±4.45 vs. 101.76±4.83 g; $P \leq 0.05$). The fetal kidney weights relative to fetal weight were also increased in fetuses from MEL dams compared to CON ($P = 0.011$). The absolute and relative perirenal fat was not altered by nutrition or treatment ($P > 0.05$). The fetal absolute and relative liver weights were reduced in the fetuses from RES heifers compared to ADQ ($P < 0.001$). Absolute and relative spleen weights were not different between treatment
groups \((P > 0.05)\). The fetal pancreas weight \((6.93 \pm 0.69 \text{ vs. } 9.8 \pm 0.75 \text{ g}; P = 0.0144)\) and the pancreas weight relative to fetal weight \((290.4 \pm 21 \text{ vs. } 361.1 \pm 23.0 \text{ mg/kg}; P = 0.0392)\) were reduced in fetuses from RES vs. CON dams. A nutritional plane by treatment interaction was found on the fetal stomach weight which was decreased in the ADQ-MEL dams compared to ADQ-CON and RES-MEL \((P = 0.0181)\). The relative stomach weight was increased in fetuses from RES dams when compared to ADQ \((P \leq 0.05)\). The intestines weights were decreased in the fetuses from RES heifers compared to ADQ \((P < 0.001)\), while the relative intestines weight was not different between groups \((P = 0.064)\). The absolute and relative fetal heart weight was not different between treatment groups \((P > 0.05)\). No differences were observed in the left ventricle thickness \((P > 0.05)\), but the right ventricle thickness was reduced in the fetuses from RES dams compared to CON \((P = 0.001)\). No differences were observed in the longissimus dorsi m. measurements neither in the semitendinosus m. between treatment groups \((P > 0.05)\).

The fetal growth parameters and organ weights collected at the Cesarean section day for Summer 2020 are illustrated in Table 3.6. In Summer 2020, a nutrition by treatment interaction was observed on fetal weights, fetal exsanguinated weight, and fetal eviscerated weight where the RES-CON dams had fetuses with reduced weight when compared to ADQ-CON and RES-MEL \((P < 0.05)\). The fetal curved crown rump length and the abdominal girth were not different among nutritional planes or treatment groups \((P > 0.05)\). The heart girth of fetuses from MEL dams was increased \((P = 0.025)\) when compared to fetuses from CON animals \((62.82 \pm 0.53 \text{ vs } 60.65 \pm 0.56 \text{ cm})\). No differences were observed on head circumference or biparietal distance among groups \((P > 0.05)\).

In Summer 2020, the absolute and relative fetal brain and lung weights were not different between nutritional plane or treatment groups \((P > 0.05)\). In the contrary, the absolute adrenal
weights were decreased in RES vs. ADQ fetuses (1.27±0.05 vs. 1.62±0.04 g; \(P = 0.001\)). Additionally, a nutrition by treatment interaction was found in the relative adrenal weights which was increased in fetuses from ADQ-MEL compared to ADQ-CON (\(P = 0.044\)). A main effect of nutrition was found in the fetal kidney weights which was reduced in the fetuses from RES vs. ADQ dams (99.63±3.27 vs. 113.20±3.21 g; \(P = 0.009\)). The fetal kidney weights relative to fetal weight were not affected by nutritional plane or dietary treatment (\(P > 0.05\)). A main effect of treatment was found on the absolute perirenal fat where fetuses from MEL dams exhibited decrease perirenal fat compared to their CON counterparts (86.61±5.14 vs. 107.95±5.09 g; \(P = 0.010\)). Similarly, the relative perirenal fat was decreased in fetuses from MEL heifers compared to CON (\(P = 0.010\)). A nutrition by treatment interaction was observed on fetal liver weights which resulted in fetuses from RES-CON dams with reduced weights compared to ADQ-CON, ADQ-MEL, and RES-MEL (\(P < 0.05\)). No differences were found in the relative fetal liver weights (\(P > 0.05\)). Absolute and relative spleen weights were increased in MEL fetuses compared to CON (\(P < 0.05\)). Fetal absolute and relative pancreas weight was not different between groups (\(P > 0.05\)). The fetal stomach weight was decreased in the fetuses from MEL dams compared to CON (\(P \leq 0.05\)). The relative stomach weight was not affected by nutritional plane or treatment (\(P > 0.05\)). The absolute and relative fetal intestines and heart weights were not different between treatment groups (\(P > 0.05\)). No differences were observed in the left or right ventricle thickness (\(P > 0.05\)). A nutrition by treatment interaction was observed for the left and right longissimus dorsi m. (\(P < 0.05\)) which the fetuses from RES-MEL dams displayed an increase in the muscle weight compared to the RES-CON. The relative weights of the aforementioned muscles were not different between treatments (\(P > 0.05\)). Lastly, no main
effects were observed for the absolute and relative weights of the semitendinosus muscle ($P > 0.05$).

**Maternal and Fetal Blood Analysis**

In Fall 2019, a treatment by time interaction was found for maternal plasma melatonin at day 220 of gestation ($P < 0.0001$; **Figure 3.4A**). During the morning, the MEL heifers exhibited increased melatonin concentrations when compared to CON (43.31±10.14 vs. 25.09±5.47 pg/mL; $P = 0.016$). The CON heifers exhibited a reduction in melatonin concentrations from morning to afternoon ($P = 0.0017$), whereas the melatonin concentrations increased in the MEL heifers ($P < 0.0001$). In the afternoon, the MEL dams exhibited greater melatonin concentrations compared to the CON dams (151.38±16.96 vs. 11.44±2.36 pg/mL; $P < 0.0001$). Similarly, at day 240 of gestation, a treatment by time interaction was observed where in the morning, the melatonin concentrations were greater ($P = 0.0046$) in the MEL heifers compared to the CON (**Figure 3.4B**). The maternal melatonin concentrations increased from morning to afternoon in the MEL dams compared to the CON ($P < 0.0001$). In the afternoon, the MEL dams had increased melatonin concentration compared to the CON (101.46±22.17 vs. 3.40±0.29 pg/mL; $P < 0.0001$). A main effect of treatment was observed in the fetal melatonin concentrations ($P < 0.0001$) where the fetuses from the MEL dams had increased melatonin concentrations compared to the CON (83.59±20.68 vs. 22.94±8.76 pg/mL; **Figure 3.4C**). Additionally, a main effect of nutrition was observed where the fetuses from RES dams had increased melatonin concentrations compared to the control (72.91±22.09 vs. 34.37±9.73 pg/mL; $P = 0.0417$).

In Summer 2020, a treatment by time interaction was observed for maternal melatonin concentrations at day 220 of gestation ($P < 0.0001$; **Figure 3.4D**). No differences were observed on melatonin concentrations between CON and MEL heifers in the morning ($P = 0.7899$), but...
the melatonin concentrations in the CON dams decreased from morning to afternoon \((P = 0.0276)\), while the melatonin concentrations in MEL dams increased from morning to afternoon \((P < 0.0001)\). In the afternoon, the MEL dams exhibited increased melatonin concentrations compared to CON animals \((179.69\pm20.41 \text{ vs. } 14.73\pm2.41 \text{ pg/mL}; P < 0.0001)\). At day 240 of gestation main effects of treatment and time were found \((\text{Figure 3.4E})\) where MEL heifers had increased maternal melatonin concentrations compared to the CON dams \((P = 0.0001)\) and the melatonin concentrations were increased in the afternoon compared to the morning \((87.17 \pm25.16 \text{ vs. } 6.35\pm5.14 \text{ pg/mL}; P = 0.0026)\). Lastly, melatonin concentration was increased \((P = 0.0007)\) in the fetuses from MEL dams vs. CON \((61.78\pm14.90 \text{ vs. } 5.43\pm3.10 \text{ pg/mL}; \text{Figure 3.4F})\).

**Placentome Vascularity**

In Fall 2019, there were no significant interactions or main effects between nutritional plane or dietary melatonin treatment groups for macroscopic blood vessel density analysis \((P > 0.05)\). However, the time of placentome collection significantly impacted the fluorescent signal radiance. Representative images of cotyledonary macroscopic blood vessel density for morning and afternoon are illustrated in \text{Figure 3.5A}. The absolute fluorescent signal radiance increased \((P = 0.0137)\) in the placentomes collected in the afternoon compared to the morning \((21.2\pm2.05\text{E}12 \text{ vs. } 13.7\pm1.97\text{E}12 \text{ p/s}; \text{Figure 3.5B})\). Similarly, the total fluorescent signal radiance relative to placentome weight was increased \((P = 0.0124)\) in the placentomes collected in the afternoon vs. the ones collected in the morning \((28.8\pm3.63\text{E}10 \text{ vs. } 14.5\pm3.70\text{E}10 \text{ [p/s]/g}; \text{Figure 3.5C})\).

Representative images of microscopic blood vessel density are illustrated in \text{Figure 3.6A-C}. In Fall 2019, the capillary number per mm\(^2\) was not different between nutritional plane or
treatment group ($P > 0.05$; **Figure 3.7A**). A nutrition by treatment by time interaction was found for average capillary size ($P = 0.052$; **Figure 3.7B**). The average capillary size ($\mu m^2$) of the placentomes from RES-CON dams decreased from morning to afternoon ($P = 0.0325$).

Moreover, in the afternoon, the collected placentomes from RES-CON dams exhibited decreased average capillary size compared to the RES-MEL (48.57±10.34 vs. 81.44±10.34 $\mu m^2$; $P = 0.0353$), while no differences were observed between ADQ-CON and RES-MEL for this variable ($P = 0.9017$). A nutrition by treatment by time interaction was observed for percent capillary area per $mm^2$ ($P = 0.045$; **Figure 3.7C**). The percent capillary area of the RES-CON placentomes were decreased from morning to afternoon ($P \leq 0.05$), whereas in the afternoon the placentomes from RES-CON dams (2.76±1.07 $mm^2$) displayed reduced ($P = 0.0325$) percent capillary area compared to ADQ-CON (6.48±1.13 $mm^2$) and RES-MEL (6.20±1.07 $mm^2$). No differences were observed between ADQ-CON and RES-MEL for the aforementioned variable ($P = 0.8619$). Lastly, there were no significant interactions or main effects between nutritional plane or dietary melatonin treatment groups for capillary perimeter (**Figure 3.7D**).

For Summer 2020, representative images for each treatment group are illustrated in **Figure 3.8A**. A nutrition by treatment by time interaction was observed for macroscopic blood vessel density on the absolute and relative fluorescent signal radiance ($P < 0.05$; **Figure 3.8B and 3.8C**). The absolute fluorescent signal radiance increased ($P = 0.0104$) in placentomes of RES-CON animals from morning to afternoon (17.34±2.24E12 vs. 42.63±7.05E12 p/s).

Furthermore, the placentomes collected in the afternoon from RES-CON dams displayed increased absolute and relative fluorescent signal radiance when compared to ADQ-CON and RES-MEL placentomes ($P < 0.05$), while no differences were observed between ADQ-CON and RES-MEL ($P > 0.05$) for both variables. The capillary number per $mm^2$ was not different
between nutritional plane or treatment ($P > 0.10$; **Figure 3.9A**). No main effect of treatment was observed in average capillary size ($P = 0.78$; **Figure 3.9B**), nevertheless the placentomes from RES dams had increased average capillary size compared to ADQ ($60.0 \pm 4.02$ vs. $48.0 \pm 3.99 \mu m^2$; $P \leq 0.05$). Lastly, there were no significant interactions or main effects between nutritional plane or dietary melatonin treatment for percent capillary area per mm$^2$ or capillary perimeter in the placentomes from the Summer 2020 ($P > 0.05$; **Figure 3.9C** and 3.9D, respectively).

**Discussion**

Spring calving heifers received their treatments during Fall 2019 and the Fall calving heifers received their treatments during Summer 2020. Cain et al. (2017) showed that Spring calving heifers had increased ipsilateral and total uterine blood flow at day 210 of gestation compared to Fall calving heifers and authors suggested that this difference could be related to photoperiod. Furthermore, the spring born calves were heavier than the fall born counterparts (Cain et al., 2017). In the present study, the maternal nutrient restriction was more severe in the Spring calving heifers where the ipsilateral and total UBF at day 220 of gestation decreased in the RES-CON heifers compared to the ADQ-CON. The average ambient temperature during ultrasound collection was 9.5°C with the minimum at -4.4°C and the maximum at 24.1°C for Fall 2019. Sheep and cattle respond to cold exposure by increasing appetite and feed intake to increase metabolic rate and thermal insulation (Young, 1975). Our Spring calving heifers were exposed to cold temperatures in the fall and most likely had increased metabolism to maintain thermoregulation while the imposed nutrient restriction seemed to be detrimental to the UBF causing intrauterine growth restriction. In this season, the ipsilateral and total UBF from the ADQ-MEL and RES-MEL dams were not different from the ADQ-CON or RES-CON. This lack of difference in UBF among the melatonin supplemented dams and the control groups could be
explained by the melatonin seasonal rhythms. Endogenous melatonin is known to be increased during short-days compared to long-days; therefore, supplementing melatonin during fall may not be beneficial to the Spring calving heifers since they have naturally more endogenous melatonin during this season.

Nevertheless, melatonin supplementation during summer has shown to simulate winter photoperiod in ruminants increasing melatonin concentration in these animals and altering their physiology (Arendt, 1998). Therefore, this can explain why the melatonin supplemented pregnant heifers from the Fall calving group exhibited increased contralateral and total UBF after 60 days of supplementation compared to the control counterparts. In the Fall calving heifers, nutrient restriction did not influence ipsilateral, contralateral, or total UBF. The Fall calving heifers received their treatments during the summer where elevated ambient temperatures were expected. During ultrasonography in Summer 2020 the average ambient temperature was 27.3°C, with the minimum temperature at 20.7°C and the maximum temperature at 34.1°C. Increased ambient temperatures have been associated with reduced UBF in sheep and cattle (Oakes et al., 1976; Roman-Ponce et al., 1978). Thus, the UBF in ADQ and RES pregnant heifers may have been affected by thermal stress limiting the observance of differences between nutritional planes. In both seasons, maternal and fetal body weights were reduced in our IUGR model.

Interestingly, melatonin supplementation to nutrient restricted dams in the summer, but not in the fall, rescued fetal body weights at day 240 of gestation. This is different from previous work in sheep and cattle where melatonin supplementation has shown to increase umbilical or uterine blood flow during late pregnancy, but alterations in fetal body weights have not been reported (Lemley et al., 2012; McCarty et al., 2018). Contrary to our hypothesis, no temporal changes were observed in UBF for any of the seasons. Nonetheless, the resistance and pulsatility
indices of the ipsilateral uterine artery decreased from morning to afternoon in the nutrient restricted dams supplemented with melatonin during the summer. The resistance index is not influenced by the angle of inclination and is inversely proportional to blood flow. This decrease resistance index indicates increased arterial blood perfusion downstream and potentially greater placental transport capacity. It is important to note that the animals on the current research were fed once daily at 0900 h and the ultrasound examinations were performed 4 h before and after feeding time. The reduction in resistance and pulsatility index in the gravid uterine artery observed in the afternoon appear to be a mechanism by which melatonin prevented IUGR in nutrient restricted dams facilitating more nutrients to the fetus right after feeding. Clinically, an increase in uterine or umbilical vascular resistance along with reduced uterine and umbilical blood flow are common signs of high-risks pregnancies negatively affecting fetal growth (Reynolds and Redmer, 2001).

Melatonin is not the only therapeutic proposed to ameliorate the negative consequences of IUGR. Underfed pregnant ewes that received i.v. arginine infusions three times daily from day 60 of gestation until parturition showed to have lambs with 21% increased birth weights compared to control underfed ewes (Lassala et al., 2010). However, when comparing the methods of administering melatonin or arginine as therapeutics, the top-dress melatonin seems to be more feasible and economic viable to producers compared to i.v. infusions or even subdermal ear implants (Lassala et al., 2010; McCarty et al., 2018). Previous research has shown that melatonin is capable to cross the placenta unaltered (Tamura et al., 2008) and in the current study it was shown that melatonin concentration increased in fetuses from dams that received dietary melatonin supplementation. Besides fetal and organ growth, melatonin effects on developmental programming should be further examined.
Melatonin is a circadian rhythm modulator, and it is coupled with core body temperature since the peak of nocturnal melatonin secretion corresponds to the lowest point in body temperatures (Arendt, 1998). Moreover, Cagnacci et al. (1992) showed that exogenous melatonin reduced core body temperature in women attributing hypothermic properties to this hormone. Cattle also exhibit a body temperature circadian rhythm with a minimum in the morning and a maximum in the afternoon since ambient temperature and seasons influence the body temperature circadian rhythms in cows (Kendall and Webster, 2009). Similarly, in the present study, vaginal temperatures during fall increased from morning to afternoon in all groups independent of nutritional plane or dietary treatment. Additionally, no differences in vaginal temperatures were observed between groups in the afternoon for the aforementioned season. Environmental temperatures during the fall might be comfortable for these heifers, therefore the antioxidant or hypothermic properties of melatonin may not be of benefit during this season regarding vaginal temperatures. Nevertheless, cattle in tropical and subtropical conditions are known to undergo through thermal stress during summer. Maternal hyperthermia in pregnant mice has shown to increase oxidative stress, while melatonin injections alleviated this effect by maintaining a neutral redox status (Matsuzuka et al., 2005). The vaginal temperatures from the ADQ-CON and RES-CON heifers enrolled in the summer trial increased from morning to afternoon, while the ADQ-MEL and RES-MEL remained constant throughout the day. Moreover, when comparing vaginal temperatures in the afternoon, the RES-MEL had decreased vaginal temperature compared to the ADQ-CON heifers. The differences between ADQ and RES vaginal temperatures, can be related to the amount of feed received since the adequately fed heifers will be generating more heat of digestion and metabolism. Nonetheless, the dietary
Melatonin treatment showed to prevent the drastic increase in vaginal temperatures in the afternoon.

Elevated maternal body temperatures difficult the fetal heat exchange which is largely through the fetal-placental circulation and the uterine wall (Tao and Dahl, 2013), creating an unfavorable environment to the fetus. Furthermore, researchers have found that cows in the last third of pregnancy have increased VT compared to cows in other reproductive status and it has been associated with increased luteal and placental progesterone during late pregnancy (Wrenn et al., 1961; Kendall and Webster, 2009). Even though this research did not measure maternal progesterone concentrations, previous research from our laboratory has shown that dietary melatonin supplementation decreased serum progesterone and increased total antioxidant capacity in dairy heifers during late gestation (Brockus et al., 2016). Therefore, melatonin supplementation during the summer may be aiding compromised pregnancies through these pathways and future research should evaluate the hypothermic effect of melatonin during heat stress exposure.

Compromised pregnancies models such as overfed, underfed, heat stress, and multiple pregnancies have shown to decreased placentome vascularity in sheep (Reynolds et al. 2010). Placentome vascularity is vital for the maternal system to support the exponential fetal growth observed in the last third of gestation (Reynolds and Redmer, 2001). In the current study, two approaches were used to determine temporal changes in placentome vascularity from dams that were nutrient restricted and/or melatonin supplemented. The inclusion of the novel technique that employs fluorescent signaling to determine to measure macroscopic placentome vascularity in the current research is beneficial since it is a better indicator of in vivo blood perfusion profiles (Lemley et al., 2018). Compared to placentomes obtained in the morning, placentomes collected
after feeding displayed greater cotyledonary macroscopic blood vessel density, but neither melatonin nor nutritional plane influenced the vascularity. These results do not mean a rapid angiogenesis formation from morning to afternoon, rather an increase in blood vessel size to facilitate the nutrient transport across the placenta after feeding time. Moreover, the significant changes in fluorescent signal within a day could directly inform the development of guidelines for the proper temporal administration of placental blood flow therapeutics. Using immunofluorescent techniques similar to Lemley et al. (2018) and McCarty et al. (2018), microscopic blood vessel density was assessed. The placentome capillary number and capillary perimeter were not different among treatments or time of the day in any of the seasons. However, for Spring calving heifers, capillary size and capillary area from RES-CON dams were reduced in the afternoon compared to the morning. This may be a nutrient partitioning mechanism exerted by the underfed dams to retain more nutrients to maintain their central nervous system functioning along with their own growth and metabolism leading to fetal IUGR. Moreover, in the afternoon, the capillary size and capillary area from the RES-CON dams were reduced when compared to ADQ-CON and RES-MEL. An increase in capillary size and area may be reasonably interpreted as an increase in blood flow to the placenta (Vonnahme et al., 2008). Therefore, our microscopic blood vessel results along with the UBF results presented earlier in this study provide an insight on how nutrient restriction leads to placental insufficiency and impaired fetal growth during fall affecting the Spring calving heifers. In contrast, the placentomes from RES-CON dams in the summer exhibited increased macroscopic blood vessel perfusion in the afternoon compared to the morning. In the afternoon, the fluorescent signal was increased in the placentomes from RES-CON heifers compared to the ADQ-CON. Additionally, the average capillary size was increased in RES placentomes compared to ADQ. These results
are similar to Lemley et al. (2018) where placentomes from Angus and Brahman nutrient restricted dams collected at day 180 of gestation showed an increased in macroscopic fluorescent perfusion and average capillary size. This increase in the aforementioned variables at the placentome level could be a compensatory response to allow blood redistribution to the placenta and it is possible that this mechanism prevented us to observe differences in UBF between nutritional planes in the Fall calving heifers.

In conclusion, nutrient restriction during the fall when animals are exposed to cold weather seemed to be more detrimental to the dam UBF since the metabolism is increased for thermoregulation and less nutrients are available for fetal growth. Additionally, dietary melatonin supplementation is not as beneficial in the fall compared to the summer and this could be related to greater endogenous melatonin concentrations during fall. Interestingly, melatonin supplementation during the summer increased UBF at day 220 of gestation and at day 240 of gestation showed to rescue the fetal weights from dams that were nutrient restricted. Moreover, melatonin supplementation to nutrient restricted dams from Fall calving showed to improve the fetus economically important tissues such as the longissimus dorsi muscle. On the other hand, the cotyledonary macroscopic blood vessel perfusion is a novel technique that shows blood perfusion in specific areas of the placentome and provides a better perspective of in vivo blood perfusion profiles. Lastly, the hypothermic effects of melatonin during the summer are promising and future research should consider dietary melatonin supplementation during heat stress exposure; while future research should also evaluate epigenetic modifications or gene expression alterations in fetal organs from dams that were supplemented with melatonin.
Figure 3.1  Maternal body weights through weeks of gestation.

Maternal body weight changes in adequately fed versus nutrient restricted pregnant heifers through week of gestation for (A) Fall 2019 (ADQ, n=14; RES, n=15) and (B) Summer 2020 (ADQ, n=12; RES, n=11). Treatments initiated at week 22 of gestation and initial maternal body weights were not different among groups for both seasons. Asterisks (*) represents significance difference (P ≤ 0.05) for the interaction between nutritional plane by week of gestation.
Figure 3.2  Uterine blood flow at day 220 of gestation.

Ipsilateral and contralateral to the conceptus uterine blood flow, as well as the summation of the ipsilateral and contralateral presented as total UBF at day 220 of gestation. (A) Nutrition by treatment interaction for ipsilateral UBF, (B) main effect of nutrition and treatment in contralateral UBF, and (C) the nutrition by treatment interaction in the total UBF for Fall 2019 animals. The main effects of nutritional plane and treatment for (D) ipsilateral UBF, (E) contralateral UBF, (F) total UBF for heifers enrolled in Summer 2020. Heifers were assigned to treatments at day 160 of gestation. Least squares means with different letters represent significance difference ($P \leq 0.05$).
Figure 3.3  Averaged vaginal temperatures recorded at day 220 of gestation.

Nutrition by treatment by time interaction for Fall 2019 (A) and Summer 2020 (B). Vaginal temperatures were recorded every 5 minutes using thermometers data loggers and temperatures were averaged from 0400 to 0600 h (AM) and from 1200 to 1300 h (PM). Least squares mean with different letters represent a difference ($P \leq 0.05$).
Figure 3.4  Maternal and fetal melatonin concentrations in blood.

A treatment by time interaction was found for maternal melatonin concentrations (A) at day 220 of gestation and (B) at day 240 of gestation for Fall 2019 dams. (C) Main effect of treatment for melatonin concentrations in fetal blood during Fall 2019. (D) Treatment by time interaction in maternal melatonin concentrations at day 220 of gestation during Summer 2020. (E) Main effect of treatment and time at day 240 of gestation. (F) Main effect of treatment in fetal blood melatonin concentration at day 240 in Summer 2020. AM represents in average 0500h and PM represents in average 1300 h. Treatments were provided daily at 0900 h. Melatonin supplemented heifers received 20 mg/d. Least squares mean with different letters represent a difference ($P \leq 0.05$).
Figure 3.5  Fluorescent signaling for cotyledonary macroscopic blood vessel density.

Placentomes were collected in Fall 2019 at day 240 of gestation. (A) Visual representation of negative control perfused with PBS, and placentomes collected in the morning (AM) or afternoon (PM) perfused with Concanavalin A, Alexa Fluor 647 conjugate (ThermoFisher Scientific, Waltham, MA). Main effect of nutrition, treatment, and time are presented for the (B) absolute fluorescent signal radiance and (C) the fluorescent signal radiance relative to placentome weight (g). The AM represents in average 0500h and PM represents in average 1300h. Least squares mean with different letters represent a difference ($P \leq 0.05$).
Figure 3.6  Representation of immunofluorescent images used to determine microscopic blood vessel density in bovine placentomes collected at day 240 of gestation.

The sectioned placentomes were stained for capillaries with Anti-Von Willebrand Factor Alexa Fluor 594 (red), FITC for caruncular and chorionic epithelium (green), and Dapi for the nuclei (blue). (A) A represents a negative controlled stained without the Anti-Von Willebrand Factor, (B) represents a fully stained imaged with the 3 fluorescent channels overlaid, and (C) represents only the Texas red channel used for analysis. The white scale bars correspond to 400µm.
Figure 3.7  Microscopic blood vessel density for heifers enrolled in Fall 2019.

(A) Main effects of nutritional plane and treatments for capillary number. Nutrition by treatment by time interactions for (B) average capillary size, (C) percent capillary area, and (D) capillary perimeter for animals enrolled in the Fall 2019 trial. Least squares mean with different letters represent a difference ($P \leq 0.05$).
Figure 3.8 Fluorescent signaling for cotyledonary macroscopic blood vessel density in placentomes collected at day 240 of gestation from Summer 2020 heifers.

(A) Representation of negative control perfused with PBS, and placentomes from all treatment groups collected in the morning (AM) or afternoon (PM) perfused with Concanavalin A, Alexa Fluor 647 conjugate (ThermoFisher Scientific, Waltham, MA). Nutrition by treatment by time interaction for (B) absolute fluorescent signal radiance and (C) the fluorescent signal radiance relative to the placentome weight (g). The AM represents in average 0500 h and PM represents in average 1300 h. Least squares mean with different letters represent a difference ($P \leq 0.05$).
Figure 3.9  Microscopic blood vessel density determination for Heifers enrolled in Summer 2020.

Main effects of nutritional plane and treatments for (A) capillary number, (B) average capillary size, (C) percent capillary area, and (D) capillary perimeter for animals enrolled in the Summer 2020 trial. Asterisks (*) represents significant differences ($P \leq 0.05$) between nutritional plane or treatment groups.
Table 3.1  Number of animals allocated into maternal diets and treatments.

<table>
<thead>
<tr>
<th>Diets and Treatments</th>
<th>Spring Calving (Fall 2019)</th>
<th>Fall Calving (Summer 2020)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adequately Fed + Control (ADQ-CON)</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Nutrient Restricted + Control (RES-CON)</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Adequately Fed + Melatonin (ADQ-MEL)</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Nutrient Restricted + Melatonin (RES-MEL)</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

Spring Calving heifers were inseminated between March 27 through April 12, 2019; started treatment between September 3 through 17, 2019; and their expected calving date was January 5 through 21, 2019. Fall Calving heifers were inseminated between January 15 through 30, 2020; started treatments between June 23 through July 8, 2020; and their expected calving date was October 25 through November 9, 2020. All diets and treatments were imposed at day 160 of gestation and finalized with the Cesarean sections at day 240 of gestation. ADQ-CON: 100% nutritional recommendations; RES-CON: 60% nutritional recommendations; ADQ-MEL: 100% nutritional recommendations with 20 mg of melatonin supplementation; RES-MEL: 60% nutritional recommendations with 20 mg of melatonin supplementation.

Table 3.2  Animal number distribution for Cesarean section timepoints.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Morning (0500 h) 2019</th>
<th>Morning (0500 h) 2020</th>
<th>Afternoon (1300 h) 2019</th>
<th>Afternoon (1300 h) 2020</th>
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</thead>
<tbody>
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<td>ADQ-CON</td>
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<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>RES-CON</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>ADQ-MEL</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>RES-MEL</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

All diets and treatments were imposed at day 160 of gestation and finalized with the Cesarean sections at day 240 of gestation. ADQ-CON: 100% nutritional recommendations; RES-CON: 60% nutritional recommendations; ADQ-MEL: 100% nutritional recommendations with 20 mg of melatonin supplementation; RES-MEL: 60% nutritional recommendations with 20 mg of melatonin supplementation. Pregnant heifers were staggered in groups of 3 to 4 animals per Cesarean section day to average 0500 h (Morning; AM) or 1300 h (Afternoon; PM).
Table 3.3  Ipsilateral (IPSI) and contralateral (CON) to the conceptus uterine artery pulsatility index (PI), resistance index (RI), and diameter at day 220 of gestation for animals enrolled in Fall 2019.

<table>
<thead>
<tr>
<th></th>
<th>AM</th>
<th>PM</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADQ-CON</td>
<td>RES-CON</td>
<td>ADQ-MEL</td>
</tr>
<tr>
<td>PI_IPSI</td>
<td>0.60</td>
<td>0.69</td>
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<td>0.80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.79&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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</table>

ADQ-CON: 100% nutritional recommendations; RES-CON: 60% nutritional recommendations; ADQ-MEL: 100% nutritional recommendations with 20 mg of melatonin supplementation; RES-MEL: 60% nutritional recommendations with 20 mg of melatonin supplementation. Pregnant heifers were staggered in groups of 5 to 6 animals per ultrasound day to average 0500 h (Morning; AM) and 1300 h (Afternoon; PM). Means with different letter subscripts represent significant differences ($P \leq 0.05$).
Table 3.4  Ipsilateral (IPSI) and contralateral (CON) to the conceptus uterine artery pulsatility index (PI), resistance index (RI), and diameter at day 220 of gestation for animals enrolled in Summer 2020.

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</table>

Means with different letter subscripts represent significant differences ($P \leq 0.05$).

ADQ-CON: 100% nutritional recommendations; RES-CON: 60 % nutritional recommendations; ADQ-MEL: 100% nutritional recommendations with 20 mg of melatonin supplementation; RES-MEL: 60% nutritional recommendations with 20 mg of melatonin supplementation. Pregnant heifers were staggered in groups of 5 to 6 animals per ultrasound day to average 0500 h (Morning; AM) and 1300 h (Afternoon; PM). Means with different letter subscripts represent significant differences ($P \leq 0.05$).
Table 3.5  Fetal organs and growth parameters at day 240 of gestation for animals in the Fall 2019 trial.

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<td>Maternal weight, kg</td>
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<td>0.2304</td>
<td>0.6166</td>
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<td>Fetal exsanguinated weight, kg</td>
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<td>1.0</td>
<td>0.0095</td>
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<td>Fetal eviscerated weight, kg</td>
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<td>Abdominal girth, cm</td>
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<td>64.2</td>
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<td>Head circumference, cm</td>
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<tr>
<td>Kidney weight/FW, g/kg</td>
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<td>4.1</td>
<td>4.6</td>
<td>4.3</td>
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<td>109.1</td>
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<tr>
<td>Perirenal fat weight/FW, g/kg</td>
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<td>3.8</td>
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<td>1.9</td>
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<td>302.6a</td>
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Table 3.5 (continued)

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<td>Longissimus dorsi m. (left), g</td>
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<td>0.5736 0.3226 0.7920</td>
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<td>Longissimus dorsi m. (left)/FW, g/kg</td>
<td>10.4</td>
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<td>Longissimus dorsi m. (right), g</td>
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<td>271.9</td>
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<tr>
<td>Longissimus dorsi m. (right)/FW, g/kg</td>
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<td>10.0</td>
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<td>11.1</td>
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<td>Semitendinosus m., g</td>
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<td>82.9</td>
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<td>97.0</td>
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<td>3.6</td>
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<td>0.1773 0.5836 0.2116</td>
</tr>
</tbody>
</table>

ADQ-CON: 100% nutritional recommendations; RES-CON: 60 % nutritional recommendations; ADQ-MEL: 100% nutritional recommendations with 20 mg of melatonin supplementation; RES-MEL: 60% nutritional recommendations with 20 mg of melatonin supplementation. Curved crown rump (CCR). Means with different letter subscripts represent significant differences ($P \leq 0.05$).
Table 3.6  Fetal organs and growth parameters at day 240 of gestation for animals in the Summer 2020 trial.

<table>
<thead>
<tr>
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<th>ADQ-CON</th>
<th>RES-CON</th>
<th>ADQ-MEL</th>
<th>RES-MEL</th>
<th>SE</th>
<th>Nut</th>
<th>Trt</th>
<th>Nut*Trt</th>
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<td>5</td>
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<tr>
<td>Maternal weight, kg</td>
<td>536.57</td>
<td>505.76</td>
<td>548.93</td>
<td>491.26</td>
<td>18.04</td>
<td>0.014</td>
<td>0.890</td>
<td>0.407</td>
</tr>
<tr>
<td>Fetal weight, kg</td>
<td>24.40(^b)</td>
<td>21.00(^a)</td>
<td>22.92(^ab)</td>
<td>24.28(^a)</td>
<td>0.93</td>
<td>0.199</td>
<td>0.310</td>
<td>0.029</td>
</tr>
<tr>
<td>Fetal exsanguinated weight, kg</td>
<td>23.29(^b)</td>
<td>19.85(^a)</td>
<td>21.83(^ab)</td>
<td>23.19(^b)</td>
<td>0.71</td>
<td>0.100</td>
<td>0.177</td>
<td>0.008</td>
</tr>
<tr>
<td>Fetal eviscerated weight, kg</td>
<td>17.00(^b)</td>
<td>14.77(^a)</td>
<td>15.84(^ab)</td>
<td>16.86(^b)</td>
<td>0.69</td>
<td>0.298</td>
<td>0.473</td>
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<tr>
<td>Fetal hot carcass weight, kg</td>
<td>13.34(^b)</td>
<td>11.17(^a)</td>
<td>12.51(^ab)</td>
<td>13.16(^b)</td>
<td>0.61</td>
<td>0.147</td>
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<tr>
<td>Fetal hide weight, kg</td>
<td>3.43</td>
<td>3.04</td>
<td>3.12</td>
<td>3.44</td>
<td>0.19</td>
<td>0.821</td>
<td>0.754</td>
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<td>CCR length, cm</td>
<td>76.12</td>
<td>73.33</td>
<td>76.29</td>
<td>76.49</td>
<td>2.13</td>
<td>0.455</td>
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<td>0.165</td>
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<tr>
<td>Abdominal girth, cm</td>
<td>62.62</td>
<td>59.55</td>
<td>61.94</td>
<td>63.51</td>
<td>1.61</td>
<td>0.720</td>
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<td>Heart girth, cm</td>
<td>61.58</td>
<td>59.45</td>
<td>62.61</td>
<td>62.67</td>
<td>0.88</td>
<td>0.178</td>
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<tr>
<td>Head circumference, cm</td>
<td>44.56</td>
<td>42.71</td>
<td>44.01</td>
<td>44.01</td>
<td>0.56</td>
<td>0.129</td>
<td>0.537</td>
<td>0.125</td>
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<tr>
<td>Head length, cm</td>
<td>12.36</td>
<td>13.98</td>
<td>13.21</td>
<td>12.74</td>
<td>0.54</td>
<td>0.369</td>
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<td>Biparietal distance, cm</td>
<td>2.18</td>
<td>1.99</td>
<td>2.10</td>
<td>2.17</td>
<td>0.07</td>
<td>0.363</td>
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<tr>
<td>Brain weight, g</td>
<td>66.27</td>
<td>63.32</td>
<td>67.94</td>
<td>70.63</td>
<td>55.75</td>
<td>0.664</td>
<td>0.577</td>
<td>0.491</td>
</tr>
<tr>
<td>Lung weight, g</td>
<td>694.72</td>
<td>634.32</td>
<td>687.94</td>
<td>702.63</td>
<td>55.75</td>
<td>0.664</td>
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<td>0.491</td>
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<tr>
<td>Kidney weight, g</td>
<td>114.32</td>
<td>98.15</td>
<td>111.69</td>
<td>100.68</td>
<td>5.16</td>
<td>0.099</td>
<td>0.987</td>
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<tr>
<td>Pancreas weight, g</td>
<td>4.66</td>
<td>4.61</td>
<td>4.94</td>
<td>4.34</td>
<td>0.23</td>
<td>0.154</td>
<td>0.906</td>
<td>0.249</td>
</tr>
<tr>
<td>Pancreas weight/FW, g/kg</td>
<td>4.66</td>
<td>4.61</td>
<td>4.94</td>
<td>4.34</td>
<td>0.23</td>
<td>0.154</td>
<td>0.906</td>
<td>0.249</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>494.97(^a)</td>
<td>291.05(^b)</td>
<td>421.84(^a)</td>
<td>435.87(^b)</td>
<td>44.62</td>
<td>0.027</td>
<td>0.372</td>
<td>0.032</td>
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<tr>
<td>Liver weight/FW, g/kg</td>
<td>20.38</td>
<td>17.90</td>
<td>19.14</td>
<td>18.75</td>
<td>1.15</td>
<td>0.206</td>
<td>0.860</td>
<td>0.359</td>
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<tr>
<td>Spleen weight, g</td>
<td>38.57</td>
<td>41.64</td>
<td>49.51</td>
<td>49.98</td>
<td>3.92</td>
<td>0.658</td>
<td>0.020</td>
<td>0.732</td>
</tr>
<tr>
<td>Spleen weight/FW, g/kg</td>
<td>1.59</td>
<td>1.81</td>
<td>2.05</td>
<td>2.15</td>
<td>0.16</td>
<td>0.297</td>
<td>0.014</td>
<td>0.695</td>
</tr>
<tr>
<td>Pancreas weight, g</td>
<td>8.57</td>
<td>7.98</td>
<td>8.07</td>
<td>8.36</td>
<td>0.48</td>
<td>0.630</td>
<td>0.816</td>
<td>0.349</td>
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<tr>
<td>Pancreas weight/FW, mg/kg</td>
<td>348.42</td>
<td>364.09</td>
<td>355.02</td>
<td>336.08</td>
<td>20.38</td>
<td>0.969</td>
<td>0.648</td>
<td>0.398</td>
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Table 3.6 (continued)

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<th>ADQ-CON</th>
<th>RES-CON</th>
<th>ADQ-MEL</th>
<th>RES-MEL</th>
<th>SE</th>
<th>Nut</th>
<th>Trt</th>
<th>Nut*Trt</th>
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<tr>
<td>Intestine weight, g</td>
<td>804.55</td>
<td>671.88</td>
<td>791.16</td>
<td>834.77</td>
<td>58.66</td>
<td>0.334</td>
<td>0.468</td>
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<td>Intestines weight/FW, g/kg</td>
<td>32.97</td>
<td>29.60</td>
<td>34.43</td>
<td>33.17</td>
<td>2.04</td>
<td>0.183</td>
<td>0.218</td>
<td>0.590</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>177.17</td>
<td>162.19</td>
<td>156.02</td>
<td>162.97</td>
<td>10.25</td>
<td>0.682</td>
<td>0.265</td>
<td>0.296</td>
</tr>
<tr>
<td>Heart weight/FW, g/kg</td>
<td>7.23</td>
<td>7.25</td>
<td>6.73</td>
<td>6.81</td>
<td>0.31</td>
<td>0.874</td>
<td>0.120</td>
<td>0.911</td>
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<tr>
<td>Left ventricle thickness, mm</td>
<td>11.67</td>
<td>11.21</td>
<td>10.52</td>
<td>10.69</td>
<td>0.62</td>
<td>0.745</td>
<td>0.166</td>
<td>0.604</td>
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<tr>
<td>Right ventricle thickness, mm</td>
<td>8.51</td>
<td>7.43</td>
<td>7.41</td>
<td>7.78</td>
<td>0.61</td>
<td>0.568</td>
<td>0.525</td>
<td>0.245</td>
</tr>
<tr>
<td>Longissimus dorsi m. (left), g</td>
<td>240.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>221.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>229.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>261.79&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.76</td>
<td>0.497</td>
<td>0.192</td>
<td>0.034</td>
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<tr>
<td>Longissimus dorsi m. (left)/FW, g/kg</td>
<td>9.77</td>
<td>10.06</td>
<td>10.13</td>
<td>10.68</td>
<td>0.46</td>
<td>0.293</td>
<td>0.283</td>
<td>0.765</td>
</tr>
<tr>
<td>Longissimus dorsi m. (left) length, cm</td>
<td>36.09</td>
<td>36.01</td>
<td>33.46</td>
<td>36.50</td>
<td>1.03</td>
<td>0.347</td>
<td>0.547</td>
<td>0.135</td>
</tr>
<tr>
<td>Longissimus dorsi m. (left) width, cm</td>
<td>11.09</td>
<td>9.89</td>
<td>10.81</td>
<td>10.52</td>
<td>0.42</td>
<td>0.160</td>
<td>0.250</td>
<td>0.282</td>
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<tr>
<td>Longissimus dorsi m. (right), g</td>
<td>237.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>206.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>202.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>251.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.76</td>
<td>0.394</td>
<td>0.665</td>
<td>0.016</td>
</tr>
<tr>
<td>Longissimus dorsi m. (right)/FW, g/kg</td>
<td>9.53</td>
<td>9.74</td>
<td>9.32</td>
<td>10.22</td>
<td>0.41</td>
<td>0.152</td>
<td>0.683</td>
<td>0.414</td>
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<tr>
<td>Semitendinosus m., g</td>
<td>53.58</td>
<td>58.90</td>
<td>58.71</td>
<td>61.83</td>
<td>5.34</td>
<td>0.392</td>
<td>0.464</td>
<td>0.834</td>
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<tr>
<td>Semitendinosus muscle/FW, g/kg</td>
<td>2.18</td>
<td>2.66</td>
<td>2.67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.13</td>
<td>0.499</td>
<td>0.456</td>
<td>0.007</td>
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</tbody>
</table>

ADQ-CON: 100% nutritional recommendations; RES-CON: 60% nutritional recommendations; ADQ-MEL: 100% nutritional recommendations with 20 mg of melatonin supplementation; RES-MEL: 60% nutritional recommendations with 20 mg of melatonin supplementation. Curved crown rump (CCR). Means with different letter subscripts represent significant differences ($P \leq 0.05$).
REFERENCES


CHAPTER IV
GENERAL DISCUSSION

The circadian rhythm controls physiological and behavioral processes based on a 24 h cycle. Furthermore, the circadian rhythms help to regulate the body homeostasis, while anticipating each individual physiological need. Circadian rhythms are controlled mainly by the clock gene network and the neuroendocrine hormone melatonin. For many years, it was thought that the regulation of this biological clock was only by the suprachiasmatic nuclei of the hypothalamus, but more recently researchers have found the expression of the clock gene network in multiple peripheral tissues showing an autonomous behavior independent of the suprachiasmatic nuclei. Some examples of circadian rhythms disruptors are changes in the light and dark cycle, stress, temperature, and what is relevant to this dissertation, feeding regimen.

Unfortunately, many of our livestock species undergo through periods of underfeeding due to severe environmental conditions that causes drought and lack of good quality forages. Moreover, if pregnant animals face this period of nutrient restriction, it compromises the pregnancy and is detrimental for the developing fetus since the lack of nutrients will cause intrauterine growth restriction. It is known that about of the 60 % of IUGR cases observed in humans are due to placental insufficiency. The placenta is the organ responsible for oxygen and nutrient delivery from the dam to the fetus and its proper function is essential for the feto-maternal communication establishing a healthy pregnancy. Therefore, this dissertation aimed to
determine temporal dependent changes in the placenta that would allow us to dictate the appropriate time to provide therapeutics to pregnant animals allowing to prevent IUGR during compromised pregnancies such as maternal nutrient restriction.

Utilizing placental explants, we were able to determine the expression of the clock gene network in the bovine placenta, although, limited temporal variations were observed in the caruncular and cotyledonary tissue. Nevertheless, clock genes are known to regulate around 3,000 other genes in the body, therefore they are capable of regulating other genes of relative importance in the placenta. In this study, it was shown that angiogenic factors and nutrient sensing genes exhibited temporal oscillations in the cotyledonary tissue, but not in the caruncular tissue. Additionally, contrary to our hypothesis, the early to mid-gestational nutrient restriction (days 50 to 180 of gestation) did not alter the amplitude or expression of the genes measured in the study. Lastly, this research bolds the important need to collect multiple samples due to a natural existence of temporal oscillations and it is possible that much of the variability observed in the literature is due to different timings when collecting data.

The bovine fetus growth exponentially in the last third of pregnancy and this drastically fetal growth is supported by the placental nutrient transporter, angiogenesis and uterine blood flow. Previous research has shown that maternal nutrient restriction during late gestation causes a reduction in uterine blood flow limiting the survival rates of the fetus and increasing morbidity and mortality of the surviving offspring. On the other hand, dietary melatonin supplementation has shown to act as an antioxidant and has also shown to have vasoconstriction and vasodilation properties that control blood flow. In the current study, we used a maternal nutrient restriction as a compromised pregnancy model and evaluate if melatonin supplementation had any positive
effects in uteroplacental rhythms. When treatments were administered during the fall, the nutrient restriction was shown to be more severe reducing the total uterine blood flow and causing IUGR. Furthermore, a potential mechanism of how this happened was unveiled when analyzing the microscopic placentome blood vessel density and finding that the restricted-control animals had reduced capillary size and area which is known to be a blood flow determinant.

Melatonin supplementation during this season, did not alter the uterine blood flow or rescued the fetal weight. It is possible that since during the fall we encounter short days and long nights, the animals have already an increased endogenous melatonin production and additional melatonin is not beneficial. However, when supplemented our animals with melatonin during the summer, vascular and hyperthermic benefits were found. Pregnant heifers supplemented with melatonin during the summer exhibited increased uterine blood flow and reduced vaginal temperatures when compared to their control counterparts. Additionally, fetuses from dams that were nutrient restricted and supplemented with melatonin showed increased body weights compared to the fetuses from the nutrient restricted dams. These results are of great relevance since melatonin was shown to decrease body temperature and rescue fetal weights during compromised pregnancies preventing IUGR during the summer.

In conclusion, melatonin supplementation during late gestation, showed to increase uterine blood flow, reduce vaginal temperatures, and rescue fetal weights during compromised pregnancies in a season dependent manner. Additionally, based on mRNA transcript abundance and melatonin plasm concentrations, it appears that the bovine maternal and fetal circadian rhythms are not synchronized rather they are 2 independent systems. Future research should evaluate melatonin therapeutics benefit during the summer in a commercial setting. Lastly, future
research should also evaluate any potential performance improvements in offspring born to melatonin supplemented dams.