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EFFECTS OF MATERNAL NUTRIENT RESTRICTION DURING EARLY OR MID-GESTATION ON BOVINE FETAL GROWTH, PLACENTOMES, AND miRNA EXPRESSION

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EFFECTS OF MATERNAL NURIENT RESTRICTION DURING EARLY OR MID-GESTATION ON BOVINE FETAL GROWTH, PLACENTOMES, AND miRNA EXPRESSION

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CHAPTER 1

REVIEW OF LITERATURE

Introduction

Developmental programming refers to the phenomenon in which maternal metabolic state, physiological traits, or environmental factors influence fetal growth and development that leads to permanent changes in the physiology of a postnatal animal (Barker et al., 1997). These factors can be gynecological immaturity, nutritional stress, climate, and genetic, among others. Failure of any one maternal system to successfully support the fetus during gestation can lead to poor physical, physiological, and psychological development (Barker et al., 1997).

The placenta is a transient fetal organ that is the most important factor in allowing a fetus to develop to term as well as ensuring proper development. The placenta is responsible for nutrient and gas exchange as well as hormone production; an abnormality in either of those areas can cause detrimental effects on the fetus that can persist later in life (Jansson et al., 2000b).

Developmental programming can be traced through epidemiology, where a strong correlation can be made between low birth weight or maldevelopment with increased risks of physical or mental ailments such as obesity and behavioral problems (Barker 2004). However, developmental programming is not always accompanied by decreased birth weight. For example, dams that undergo stress during the beginning of gestation but not the end are likely to produce a normal birth weight offspring that may still suffer from

poor growth and metabolic issues because of the stress early in pregnancy (Barker 2004; Ford et al., 2007; Vonnahme et al., 2007; Dong et al., 2008).

In 1992, Hales and Barker presented the "Thrifty Phenotype Hypothesis." This hypothesis suggests that Type 2 diabetes mellitus and other metabolic disorders have a strong correlation to the maternal environment. The Thrifty Phenotype hypothesis states that fetal malnutrition can be a result of either maternal malnutrition or placental dysfunction. In response to its poor nutrition status, the fetus adopts several coping mechanisms. Firstly, the fetus partitions nutrients to the development of the brain and heart at the expense of other organs, such as the skeletal system and musculature. Secondly, the fetus alters its metabolic physiology to prepare for malnutrition during postnatal life. Unfortunately, if the postnatal animal has adequate or above adequate nutrition, this can cause the animal to become obese, consequently leading to diabetes (glucose/insulin dysregulation) and hypertension (Ozanne et al., 2002). Ewes fed a 50% global nutrient restriction diet during d 28 – d 78 of gestation had female offspring who showed increased insulin secretion and altered glucose metabolism (George et al., 2012), which is consistent with efficient energy acquisition and storage (Hales et al., 2001) as well as predisposition for metabolic diseases later in life (Hales et al., 2003). The differences in glucose metabolism correspond to a greater capacity for glucose production from the liver and an increased expression of phosphoenolpyruvate carboxykinase (PEPCK) (George et al., 2012), the enzyme that catalyzes gluconeogenesis (Pilkis et al., 1992). Additionally, the lack of change in glucose-6-phosphatase (G6P) expression, the liver enzyme which catalyzes glucose-6-phosphate to glucose, suggests that new glucose-6-phosphate from increased gluconeogenesis could be directed toward

glycogen synthesis and storage rather than converted to glucose, essentially increasing glycogen storage and subsequent weight gain (George et al., 2012). Offspring from nutrient restricted dams gain more weight per unit feed when fed a high quality ad libitum diet, suggesting a higher efficiency for body tissue deposition and the gene expression of PEPCK and G6P could explain this efficiency (George et al., 2012). This higher efficiency and early programming may be the cause of people gaining weight versus remaining lean despite similar lifestyles (George et al., 2012).

In 1995, Barker presented a similar but different theory, "The Barker Hypothesis", which asserts that fetuses that undergo stress during gestation will most likely have physical, physiological, or psychological defects later in life. The best study of this phenomenon is the Dutch Hungry Winter. The population of Nazi occupied Holland was subjected to a severe famine and the offspring of women that were pregnant were tracked and are now in their early 70's. Multiple generations later, observations of the population have revealed long-term detrimental health effects such as heart disease, obesity, and diabetes (Stein et al., 1995). The Barker Hypothesis also suggests that birth weight and cardiovascular disease have an inverse relationship – as birth weight decreases, the incidence of cardiovascular disease increase.

Developmental programming likely has significant effects on livestock production. Breeding of young animals can cause stress, especially if the animal is immature, as metabolic energy is being used for growth and development of the dam as well as the fetus. Furthermore, lactation causes an energy competition between the developing fetus and production (Knight et al., 2001). In beef cattle, animals that experience stress in-utero can have reduced skeletal growth, which can affect carcass

merit and meat production (Greenwood et al., 2005). This could be especially problematic in heifers if they are used as breeding stock. Intrauterine growth retardation (IUGR) is defined as any stressor on an animal that causes reduced fetal growth during gestation; IUGR can be environmental or physiological, occur at any point during gestation, and has permanent negative effects on the offspring's physiology and development (Baker et al., 1969). These negative effects include decreased growth rate, decreased meat and body composition, and reproductive performance in swine and ewes (Baker et al., 1969; Pond et al., 1969; Wallace et al., 2005). Not only is the particular offspring affected, but also IUGR can have transgenerational effects, making the inclusion of an animal affected by IUGR detrimental to future generations (Anderson et al., 2006; Long et al., 2013b; Long et al., 2013c). The objective of this review is to consider aspects of fetal and placental development, both normal and abnormal.

Maternal

Normal adaptations to pregnancy in bovine

After ovulation, the follicular cells reorganize to form the corpus luteum (CL) on the ovary. The corpus luteum produces progesterone (P_4), which is required to maintain pregnancy. After fertilization at the ampullary-isthmus junction in the oviduct at d 0-1 (d 0 = ovulation), the zygote begins cellular division and moves through the oviduct towards the uterus (Senger 2005). Around d 4-5, the embryo is approximately 30 cells and moves into the uterine body. Around d 9-10, the embryo is now considered a blastocyst and

"hatches", releasing the intracellular mass (ICM) and trophectoderm cells, which form the placental membranes (Senger 2005). Around d 16-17, elongation of the embryo provides for the production of interferon-τ (IFN- τ), the pregnancy recognition signal for ruminants (Spencer et al., 1996; Spencer et al., 2004; Spencer et al., 2007; Bazar et al., 2010). IFN- τ is also required for adhesion (Spencer et al., 2007). After adhesion, complete around d 30-35 (Senger 2005), the placenta and placentomes begin to develop (Spencer et al., 2007). Near the end of bovine gestation, progesterone in the corpus luteum declines (Erb et al., 1968), the corpus luteal cells begins to degenerate (Hutchinson, 1962), and surrounding capillaries around the corpus luteum begin to decrease in number and size (Hutchinson 1962). However, systemic progesterone concentrations remain constant, suggesting presence of progesterone production outside the ovary, most likely the placenta (Gomes et al., 1965; Stabenfeldt et al., 1970) during the last 100 days of pregnancy.

Pregnancy is a delicate condition and subjects the dam to metabolic stress. Nutritional demands are increased to meet the demands of the dam as well as the fetus. These demands are increased even more when the dam is young and still growing (Durnin 1987). As nutritional demands are increased, the condition leads to an increased need for gastrointestinal hormones and gastrointestinal mass (Faulkner et al., 1988). Gastric inhibitory polypeptide (GIP) is a key hormone in regulation of glucose metabolism and will therefore change during pregnancy as the pregnant animal's requirements change (Irwin et al., 2010). Pregnancy is associated with increased insulin resistance, generally associated with a decrease in adiponectin (Li et al., 2009), and

increased insulin demand; it is possible that GIP has a role in altered glucose homeostasis (Retnakaran et al., 2010).

In ruminant digestion, starches are fermented into volatile fatty acids (VFAs) and therefore the animal relies mostly on gluconeogenesis in the liver, and occasionally kidneys, to provide glucose. Glucose requirements are quadrupled in high producing dairy cattle and doubled in twin bearing ewes compared with non-lactating, non-pregnant animals (Bell et al., 1997). Propionate (VFA produced by pre-gastric fermentation) is the primary precursor for hepatic gluconeogenesis. The concentration of propionate produced is directly related to dietary intake of starch substrates that can be fermented by amylolytic bacteria in the rumen (Peters et al., 1983; France et al., 1993). Without propionate, the liver uses lactate, certain amino acids, or glycerol as a gluconeogenesis substrate (Brockman 1993). Throughout the ruminant animal, some tissues such as muscle and adipose that require glucose for non-oxidative functions can produce glucose through substitute VFAs and derivative ketoacids without propionate in order to meet increased glucose demands (Petterson et al., 1993). Beyond this, ruminants are also able to utilize acetate as an oxidative fuel (Lindsay 1978) and free fatty acids (FFAs) if the energy requirements of the animal require it to do so (Bird et al., 1981; Pethick et al., 1981). The placenta is a major contributor to increased glucose demands in the pregnant animal (Bell et al., 1986). Glucose is transported from the maternal environment to the placenta by facilitated diffusion (Widas 1952; Simmons et al., 1979) through GLUT 1 and GLUT3 transporters and the expression of these transporters increase between mid and late gestation (Ehrhardt et al., 1997).

Gluconeogenesis in the fetus is controlled by PEPCK (PCK1 gene), glucose-6-phosphatase (G6Pase, G6P gene), and fructose-1, 6-bisphosphatase and is not activated until just prior to parturition (Hanson et al., 1997). Increases in glucagon, cortisol, and catecholamines activate the glycogenolytic and gluconeogenic pathway (Pilkis et al., 1992; Fowden et al., 1993; Hanson et al., 1997). Insulin acts as a gene suppressor that prevents gluconeogenesis from activating too soon (Edgerton et al., 2009). Fetuses with IUGR have a subsequent shift in fetal metabolism, decreasing basal insulin concentrations and increasing regulatory hormones, therefore increasing gluconeogenic gene expression and causing gluconeogenesis to activate sooner than normal (Limesand et al., 2006; Limesand et al., 2007; Thorn et al., 2011). In a well-fed dam, almost 100% of the glucose utilized by the fetus comes from maternal glucose (Hay et al., 1984). In an underfed dam, the fetus then relies on endogenous hepatic gluconeogenesis through use of amino acids of substrates (Dalinghaus et al., 1991). Transport of amino acids from dam to fetus are not impaired by maternal nutrition (Lemons et al., 1983), but the use of amino acids for glucose decreases protein synthesis, impairing muscle development in the fetus (Krishnamurti et al., 1984). The previously mentioned Baggs ewe study (Jobgen et al., 2007) suggests that through selection, it is possible for the dam to compensate during nutrient restriction and increase placental efficiency when considering amino acid transport.

Effects of age

A nutrient restriction (86.7% metabolizable protein, 68.1% NE_m) study in cattle showed marked differences in fetal development when comparing NR IUGR (nutrient restricted intrauterine growth restriction) and NR non-IUGR (Long et al., 2009). The NR IUGR group was, on average, 18 months younger than the NR non-IUGR group. The NR IUGR group showed a decreased fetal weight, decreased empty carcass weight, and decreased abdominal circumference, and an increase in heart ventricle weight and septum thickness. They observed a decrease in liver, lung, heart, and brain weight, but when corrected for decreased fetal weight, the changes were not significantly different than the non-IUGR group. They also reported a decrease in cotyledonary weight, caruncle weight, and plasma glucose (Long et al., 2009). It has been suggested that younger animals are perhaps more susceptible to nutrient restriction because cattle grow until about 4 years of age (Morrow et al., 1978; Johnson et al., 1990; Arango et al., 2002), though the growth rate is very low. Furthermore, with the decreased cotyledonary growth, perhaps the IUGR group was unable to transport as much glucose to the fetus, even if it had been available (Long et al., 2009).

Fetus

Normal fetal development

In a study done by Prior and Laster (1979) on bovine fetal development, it was shown that initial fetal growth rate is 7.4%/day and the rate declines by 0.125% each day of gestation. A previous study by Ferrell et al. (1976) showed a 5.1%/day initial growth

rate with a decrease of 0.007% for each day of gestation. Fetal weight, protein, fat, ash content, DNA, RNA, protein/DNA ratios (cell enlargement: cell division), and RNA/DNA (hypertrophy) were shown to increase exponentially with each day of gestation (Prior et al., 1979). Fetal weight gain reaches its peak at day 232 of gestation while protein growth peaks at 242.5 days of gestation (Prior et al., 1979). Eley et al. (1978) reported that peak growth rate for the bovine fetus was at 230 days of gestation, but the study done by Prior and Laster (1979) showed an increased weight gain of 131g/day. Ash accretion levels peaked at day 249.5, though ash content, like protein, did not maximize until after expected parturition date. DNA reached a maximum at 217.5 days of gestation. Fat deposition and RNA accretion, along with ash and protein content, did not peak until after expected parturition date (Prior et al., 1979).

After embryo elongation and maternal recognition, bovine fetal growth is primarily due to hyperplasia, the increase in number of cells, while each individual cell maintains a consistent size. Later in gestation, fetal development involves hypertrophy (increase individual cell size) as well as hyperplasia, during the last third of gestation, only hypertrophy contributes to fetal development (Winick et al., 1965). However, in the Prior study (1979), it was shown the hyperplasia occurs throughout gestation in the bovine by observing increases in DNA content through the entirety of gestation. Hyperplasia contributes to DNA increases while hypertrophy helps increase fetal weight through increased protein/DNA and RNA/DNA ratios (Winick et al., 1965). Growth retardation at the level of hyperplasia often lead to permanent detrimental effects, while retardation at the level of hypertrophy can often be overcome; therefore, as hyperplasia occurs throughout bovine gestation, any retardation of fetal growth during gestation could

have a long-term detrimental effect on the fetus. However, while hyperplasia does occur during the entire gestation, its affects are less critical as the fetus ages, making growth retardation less of an issue at the end of gestation. If a dam undergoes nutrient restriction during late gestation, then the fetus will most likely be subjected to decreased mechanical growth and possibly a few postnatal issues such as decreased average daily gain, because by late gestation the fetus is already mostly developed and only lacks in physical size (Prior et al., 1979). A dam that undergoes nutrient restriction during the first third or middle third of gestation can subject the fetus to an actual detriment in development, as cells are still dividing and determining into specific organ systems and functions (Prior et al., 1979). Tissues and organs develop at different speeds and time during gestation and as skeletal muscle is one of the last tissue groups to be effected by hyperplasia, growth retardation near the end of gestation is most likely to cause a detrimental effect on skeletal muscle (Prior et al., 1979). However, more recent studies have shown that 50% maternal nutrient restriction during early gestation (d 30 – d 70) in ewes results in lambs with increased fast twitch fibers and increased muscle fiber diameters (Daniel et al., 2007). Furthermore, as skeletal muscle growth is considered less important than other organ systems (i.e., heart or brain); the musculature is especially vulnerable to nutrient restriction. Fetal muscle maturity proceeds through the development of primary myofibers and then secondary myofibers, which arise from previously undifferentiated precursor cells. Limited nutrients can slow the division of precursor cells, leading to overall fewer muscle fibers (Zhu et al., 2006).

Adaptions to IUGR

Fetuses that undergo intrauterine growth restriction (IUGR) often exhibit "symmetrical" or "asymmetrical" growth patterns. Symmetrical growth restriction is often caused by a genetic disorder or infection that affects the fetus during gestation as opposed to a sub-par maternal environment (Anthony et al., 2003). A symmetrical growth restriction is often categorized by a size reduction in all organs and tissues similarly. Conversely, an asymmetrical growth pattern shows a reduction in some organs or structures but not all (Anthony et al., 2003). Asymmetrical growth is linked to maternal and placental factors as opposed to a genetic predisposition. This suggests that the fetus prioritizes nutrients and blood flow to the organs and structures that are most pertinent to development (Anthony et al., 2003).

When exposed to a lack of nutrients, whether from maternal diet or placental inefficiency, the fetus can adjust its endocrine environment (Barker 1998). One of the adjustments that can occur because of a nutrient deficient environment is an increase in glucocorticoids during late gestation. This increase in glucocorticoids can increase the development of some organs (Anthony et al., 2003). However, this excess of glucocorticoids consequently causes decreased fetal weight, decreased placental size, and long-term effects such as hypertension and hyperglycemia (Jobe et al., 1998; Moss et al., 2001; Seckl 2001; Jensen et al., 2002).

Several studies have shown that uterine capacity has an impact on fetal growth and birth weight. When a fetus with a genetic disposition for increased (or large) size is moved to a dam with a smaller uterine capacity, the birth weight is lower than expected, and vice versa for a fetus with a genetic disposition to a decreased (or small) size

(Dickinson et al., 1962; Ferrell 1991; Allen et al., 2002). This suggests some factor in the fetus or placenta that alters fetal size while in utero. In an ovine surgical uterine capacity model by Meyer and colleagues, it was shown that fetuses with a restricted capacity had 51% fewer placentomes and a 31% reduction in placentomal weight when compared to non restricted (2010b). At day 130 of gestation, space restricted fetuses showed a decrease in weight, reduced crown-rump, abdominal and thoracic girth, as well as decreased fetal heart, kidney, liver, spleen, and thymus weights as compared to non space restricted fetuses, while lung and brain weights were unaffected (Meyer et al., 2010a). The placental efficiency of restricted fetuses increased, but elevated levels of fetal arterial creatinine, blood urea nitrogen, and cholesterol suggests that placental transport capacity had decreased (Meyer et al., 2010a).

Placenta

Histomorphology and Ultrastructure

The placenta is a transient organ that is crucial to fetal growth and development. The placenta allows for nutrient and gas exchange to the developing conceptus (Ramsey 1982) and hormone production that can influence both fetal and maternal physiology (Gey et al., 1938; Josimovich and MacLearn, 1962; Kaplan and Grumbach, 1964). The development and function of the placenta can be heavily impacted by environmental factors and nutrition (Arnett et al., 1997; Constancia et al., 2002).

The bovine placentome consists of both maternal and fetal tissues. The maternal tissues of the placentome consist of the caruncular stalk, maintaining the basal plate and septae (Leiser et al., 1997). The fetal tissues form convex cotyledons with the chorionic plate covering the maternal tissues. Villous "trees" radiate out from the chorionic plate towards the maternal tissues, connecting the maternal and fetal tissues like Velcro. Each villous "tree" contains a single artery from the allantochorionic arterial system, which branch off into arterioles and capillaries along the tree. Placentomes cluster together nearest where the fetus adheres at the beginning of gestation. These placentomes are larger and have the most developed vasculature. Placentomes located elsewhere along the placenta are smaller and less developed, called "accessory placentomes" (Leiser et al., 1997). At day 30 of gestation, the bovine placentomes are small (20-40um wide), rounded, and either flat or slightly raised. Within a few days (day 33), the placentomes are all visibly raised and begin to develop visible villi and crypts for association with the fetal tissues. By day 42, the villi 6 times longer than as on day 33 and include secondary branching. This increase in complexity causes a tighter association with fetal tissues to provide adequate exchange between the maternal and fetal environment. Throughout gestation, the placentome continues to increase in size and complexity (King et al., 1979).

Trophoblasts form the outer cell layer of the blastocyst and interaction with the endometrium forms placental connections between the developing embryo and uterine walls. These connections serve as transport of nutrients and gas from the maternal to the fetal circulation (Wimsatt 1950). Two types of these trophoblasts have phagocytic action. One group of the phagocytic trophoblasts is located toward the fetus between the bases of the cotyledons, where they target maternal erythrocytes that are caught between the

endometrial and chorionallantois tissues. The other group of trophoblasts is located near the openings of the endometrial glands in the chorionallantois. These trophoblasts target uterine milk produced by the endometrial glands. Trophoblasts have both an endocrine and paracrine function, producing hormones such as progesterone (Reimers et al., 1985), bovine placental lactogen (bPL) (Bolander et al., 1975), pregnancy associated glycoproteins (bPAG-1, bPAG-2, bPAG-3) (Sasser et al., 1986), and transforming growth factor beta (Munson et al., 1996) required for fetal development and maternal maintenance as well as expressing receptors for specific hormones, specifically progesterone (Schlafer al., 2000). In ruminants, one subpopulation of trophoblasts diverges into villous structures. They are organized in clusters throughout the placenta forming cotyledons (Wooding et al., 1994). Another population of cells in placenta is fetal macrophages, deriving from chorionic mesenchyme or fetal bone marrow derived monocytes. The cells assist in producing cytokines and antigen presentation and are important in fetal development (Schlafer et al., 2000).

Vasculogenesis

Adequate blood flow between the maternal environment and the developing fetus is critically important for successful fetal development as the blood flow has a direct impact on nutrient and waste exchange. Uterine and umbilical blood flow is centered on correct vascularization of the placenta. In the developing fetus, organs and tissues with a higher metabolic activity receive the majority of the blood and therefore the majority of the nutrients available. While the fetus experiences most of its mechanical growth during

the last trimester, the placenta undergoes the majority of its growth and development during the first two-thirds of gestation (Ferrell et al., 1976), though its structure and cellular component change throughout gestation (Kappes et al., 1992). Vonnahme and Ford showed that in some species of pigs, vasculogenesis continues to increase markedly during the end of gestation, increasing placental efficiency (2004). Lack of placental blood flow during the last trimester of human pregnancies has resulted in intrauterine growth restriction. Furthermore, decreased blood flow correlates with a high-risk pregnancy and inhibited fetal growth (Haggarty et al., 2002). During the last half of gestation, vascular growth on the fetal side of the placenta exceeds that of the maternal side in order to support the increased growth of the fetus.

Angiogenesis

Maternal and fetal angiogenesis continues throughout gestation. Angiogenesis is the development of new blood vessels from existing vessels and is crucial for normal growth and development (Barcroft et al., 1946; Meschia 1983; Reynolds et al., 1995). There are two types of angiogenesis – branching and non-branching – both occur throughout placental vascularization (Reynolds et al., 2005). Angiogenesis action is increased during placenta vascularization. Many of the factors regulating angiogenesis are a result of placenta vasculogenesis, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF-2), and angiopoietin protein families (ANG-1 and ANG-2) (Zygmunt et al., 2003). Angiogenic proteins VEGF and FGF-2 have been published as having activity from the placental tissues (Reynolds et al., 1988; Millaway

et al., 1989; Zheng et al., 1998). Vascular endothelial growth factor (VEGF) is critically important for the angiogenesis of brain ventricles, kidney glomeruli, and placental tissue during late pregnancy (Durmont et al., 1995; Breier et al., 1997). Fibroblast growth factor-2, produced throughout gestation, plays a dominant role in the maturity of the uterine arterial and fetal placental arterial cells (Fokman et al., 1987; Klagsbrun et al., 1991; Cale et al., 1997; Zheng et al., 1999).

Branching angiogenesis primarily occurs during the first two-thirds of gestation and transitions primarily to non-branching angiogenesis during the last trimester (Geva et al., 2002). During the first trimester, ANG-2 levels are high relative to ANG-1, which allows fetal blood vessels to undergo changes (Geva et al., 2002). As the fetal blood vessels change and develop, this allows the placenta to increase in size to better provide nutrient and gas exchange. At the end of the second trimester, ANG-2 levels begin to decrease, preventing villous destabilization and permitting the transition from primarily branching to non-branching angiogenesis (Geva et al., 2002).

Blood circulation

The process of vasculogenesis and angiogenesis during gestation allows for increased blood circulation to the uterus during pregnancy (Hard et al., 1982). Placental blood flow has both chronic and acute regulators. The chronic regulators influence the development of the placenta and vasculogenesis to control blood flow to the uterus. Chronic regulators include reactive oxygen species, which in low levels contribute to regulation of vascular smooth muscle and endothelial cell function in blood vessels.

However, in instances of fetal growth restriction or preeclampsia, these reactive oxygen species are upregulated and can lead to impaired vascular function (Tagaki et al., 1994). Acute regulators concentrate on the diameter of the blood vessels nearest to the placenta. Nitric oxide (NO) is an acute regulator, is activated by guanylate cyclase (cGMP), and is a vasodilator, which results in blood vessel relaxation. Exogenous NO antagonist leads to intrauterine growth restriction (IUGR) and preeclampsia in pregnant rats (Maul et al., 2003). Its vasodilation action is stimulated by FGF-2 and VEGF, though NO also regulates FGF-2 and VEGF in turn through intracrine action (Rosenfeld et al., 1996; Babaei et al., 1998; Hood et al., 1998; Sasaki et al., 1998; Vagnoni et al., 1998; Benoit et al., 1999; Frank et al., 1999; Zheng et al., 1999).

Maternal obesity or under nutrition can have a profound effect on both chronic and acute regulators influencing placental blood circulation (Bird et al., 2003). The amino acid arginine is a necessary component in NO synthesis as well as polyamines synthesis. As previously mentioned, NO is a vasodilator directly involved in regulating blood flow to the placenta. Arginine is one of the ten essential amino acids (i.e., required in animal's diet and cannot be derived from other amino acids) and therefore a suboptimal diet may cause an arginine deficiency. This deficiency would decrease the amount of NO available to regulate placental blood flow, subsequently decreasing polyamine synthesis (Flynn et al., 2002). Polyamines regulate fetal DNA and protein synthesis, both of which are required for cell differentiation and proliferation. Polyamine reduction also detrimentally affects placental growth as well as fetal growth (Ishida et al., 2002). Citrulline (an arginine precursor) concentration in ovine allantoic fluid increases by 34 fold between day 30 and day 60 of gestation while glutamine (citrulline precursor) concentration

increases by 18 fold during the same interval (Kwon et al., 2003b). The nitrogen in these compounds makes up 60% of the total nitrogen amino acid content in ovine allantoic fluid (Kwon et al., 2003b). When ovine placental growth is rapidly developing (Bell et al., 2002), high rates of arginine precursors are correlated with a high level of NO and polyamines (Kwon et al., 2003a; Kwon et al., 2004b; Wu et al., 2004). This association suggests that arginine-dependent metabolic pathways in the conceptus are fundamental for development (Flynn et al., 2002). Maternal under nutrition of ewes during day 28-78 of gestation led to a decrease in concentrations of arginine, citrulline, and polyamines by nearly one-third by day 78 (Kwon et al., 2004a). These decreases would impair NO synthesis, leading to reduced blood flow between maternal and fetal fluids. Thus, maternal under nutrition impairs NO vasodilation and increases fetal arterial pressure in the ovine (Ozaki et al., 2000).

On the opposite end of the spectrum, over-nutrition can also negatively affect NO synthesis and therefore placental blood flow. In obese animals, high levels of low-density lipoproteins or hypercholesterolemia (increased cholesterol blood levels) impairs NO synthesis through one of several mechanisms: reduced availability of 6R)-5,6,7,8-tetrahydrobiopterin (BH_4), a co-factor for nitric oxide synthase [NOS], the enzyme required to convert arginine to NO), a reduced expression of NOS, or the inactivation of NOS (Maxwell et al., 1998). Ma et al. (2010) showed that from d 75 to d 135 of gestation there was an approximately 44.9% increase in cotyledonary arteriole diameter in control (100% NRC) sheep versus obese (150% NRC) sheep, equating to a 440% increase in blood flow to the fetus. At d 75 of gestation, obese fetuses had an increased cotyledonary arteriole diameter of about 35.5%, equating to a 337% increase in blood flow to the fetus

(Ma et al., 2010). Cotyledonary arterioles develop primarily from branching angiogenesis (Stegeman 1974) and Ma et al. (2010) observed that at d 75 of gestation, obese ewes had decreased expression of hypoxia induced factor 1α (HIF-1α), a compound known to stimulate angiogenesis (Shih et al., 1999; Simon et al., 2008). Likewise, there was an observable decrease in VEGF, ANG-1, and ANG-2 in obese ewes, suggesting that there is a fetal:maternal signal suppressing cotyledonary angiogenic factors when confronted with maternal over nutrition (Ma et al., 2010) in order to prevent fetal overgrowth, which would occur because of the increased amount of blood flow, and therefore nutrients, available to the fetuses of obese ewes (Ma et al., 2010).

Placental role in fetal development

The placenta supports fetal development through metabolic and endocrine functions. Placental lactogen (PL) is a protein hormone that stimulates partitioning of maternal nutrition to the growing fetus (Handwerger et al., 1976). Placental lactogens are involved in fetal growth, as deletions in the human growth hormone and placental lactogen gene cluster has resulted in severe fetal growth retardation (Rygaard et al., 1998). Hormones such as progesterone and estrogen also have a role in placenta development. Estrogen produced by the placenta is metabolized into estrone sulphate (ES) by the cotyledons (Sullivan et al., 2009). Estrone sulphate is an indicator of placental function. Nutrition has been shown to affect placental growth, increasing ES and bPL during late gestation in thin cows (Rasby et al., 1990) and increasing progesterone in heifers fed to sustain a positive energy balance (Villa-Godoy et al.,

1990). With these relationships in mind, a correlation between ES, bPL, bPAG, and progesterone and leptin/insulin-like growth factor (IGF), as leptin (Wathes et al., 1998) and IGF (Gad et al., 2000) are nutritionally sensitive hormones, as their concentrations can be influenced by diet. The placental trophoblasts also produce leptin, which is then secreted into the maternal and fetal circulation. There are several pathological conditions that could lead to an increased leptin production, including preeclampsia and diabetes. In preeclamptic pregnancies, this increase in leptin often stems from placental insufficiency (Lepercq et al., 2003). Women suffering from diabetes during pregnancy may also experience an increase in leptin concentrations, which has a positive correlation with fetal growth (Manderson et al., 2003). Preeclampsia and diabetes are both associated with fetal hypoxia (Hytinantti et al., 2000a; Hytinantti et al., 2000b) and low oxygen levels may cause an increase of leptin production (Lolmede et al., 2003).

Oxygenation

Oxygen transport keeps pace with fetal growth throughout gestation (Meschia 1983; Reynolds et al., 1986; Reynolds et al., 1987). Oxygen exchange across the placenta not only depends on blood flow to the uterus, but also gas permeability, hemoglobin (Hg) content, pressure, and placental permeability. Oxygen diffuses through blood vessels from the maternal tissues to the fetal tissues (Faber et al., 1966).

Maternal Nutrition

Maternal nutrition is arguably the most significant factor in developmental programming. The fetus is highly sensitive to maternal nutrition throughout the entire gestation, despite the fact that fetal nutrient requirements are inconsequential during the earliest stages of gestation (Ferrell et al., 1976). Decreased maternal nutrition can limit fetal growth if inflected at any point in gestation. The most critical window for fetal hypertrophy (increase in cell size) is at the end of gestation, while the most critical window for fetal hyperplasia (cell division) and fetal development is during early gestation (Ferrell et al., 1976).

In work done by Olausson et al. (2003), maternal nutrient restriction decreased maternal IGF-II while IGF-1 remained unaffected. Protein restriction (60% of control diet) in heifers during the first trimester had no effect on fetal birth weight or cotyledon number, but increased dry cotyledon weight, suggesting compensatory gain of the placenta after the protein restriction had ended (Perry et al., 1999). If this is true, it could follow that protein restriction in early gestation could lead to a larger overall placenta because of increased microvilli development, increasing nutrient exchange between the mother and fetus and increased birth weights, though this was not observed because of the small number of animals available (Perry et al., 1999).

In a global restriction model, dams were subjected to a nutrient restricted diet that was also unbalanced in terms of protein (86.7% metabolizable protein) and energy (68.1% NE_m). Early gestation animals were fed either a restricted or control diet d 30-125 of gestation before harvesting. Late gestation animals in a second group were fed either a restricted or control diet d 30-125 of gestation and d 125-245 control animals received the same control diet while restricted animals received a dietary supplement in attempts for

restricted animals to match BCS of control cows before harvesting at d 245. It was shown that with global nutrient restriction during early gestation in cattle (d 30-d 125), some fetuses had intrauterine growth restriction (IUGR) while others did not (Long et al., 2009). The IUGR fetuses did have the expected asynchronous organ development along with restricted growth (Schoknect et al., 1994; McMillen et al., 2001; Vonnahme et al., 2003; Platz et al., 2008). It was reasoned that the nutrient restricted animals did not all exhibit IUGR because the diet lacked severity and other factors (such as animal age) played a larger role in IUGR in this particular instance (Long et al., 2009). Further, NR animals exhibiting IUGR showed decreased cotyledonary tissue at d 125 as well as a decrease in total placentome area when compared with NR non-IUGR and control cows at d 125, which could account for the decrease in fetal growth in NR-IUGR cows (Long et al., 2009). Cotyledonary tissue was also decreased in NR cattle at d 245, but realimentation from d 125-245 allowed overall placentome size and caruncular vasculature to increase, therefore increasing maternal blood flow and nutrient transport, allowing the fetus to realize its full growth potential (Long et al., 2009).

Nutrient Transport (Amino Acids, Glucose, Fatty Acids)

Post embryo adhesion, the most crucial role of the placenta is nutrient transport. Nutrients are transported via either facilitated diffusion or active transport (Hay 1994; Reik et al., 2003). There is mounting evidence that increased levels of growth hormone (GH) can increase the rate of facilitated diffusion (Bauer et al., 1998). Maternal IGF-1 treatments have been shown to increase transcription of facilitated glucose transporters,

specifically Glucose Transporter 1 (GLUT1) and Glucose Transporter 3 (GLUT3) (Bauer et al., 1998). In the mouse model, mice fed a high fat diet (32% energy from fat) increased glucose and amino acid placental transport. Expression of GLUT1 increased by 5 fold and sodium coupled neutral amino acid transport (SNAT2) increased by 9 fold while GLUT3 and SNAT4 were both unchanged in expression as compared with the control (11% energy from fat). In nutrient restricted mice, there is a decrease in GLUT3 transporters and leucine amino acid transporter (LAT) family member 2. However, there was no change in expression of GLUT1, LAT1, SNAT, or SNAT2 transporters. This suggests that GLUT3 and LAT2 transporters are decreased so that GLUT1, LAT, SNAT and SNAT2 can attempt to compensate for the lack of nutrients (Ganguly et al., 2012).

In the sheep model, obese (150% NRC) ewes showed an increased expression of Fatty Acid Transporter (FATP) 1 and FATP 4 during midgestation, the two transporters primarily responsible for placental fatty acid uptake. Additionally, it was discovered that fetuses from obese dams had an increased fatty acid concentration in their circulation (Zhu et al., 2010b). These findings suggest that maternal obesity prior to and during pregnancy increase nutrient transport from dam to fetus, leading to fetal overgrowth and possible metabolic issues (such as metabolic syndrome) in the postnatal animal (Jones et al., 2009). Metabolic syndrome includes multiple metabolic issues including obesity, hypertension, dyslipidemia, and glucose intolerance. Typically metabolic syndrome affects offspring from obese mothers who also suffer from diabetes during pregnancy (Boney et al., 2005).

Transport of materials across the placenta depends on the outer trophoblast surface and the inner vascular endothelial surface (Leiser et al., 1997). Amino acids are

important in fetal development because they are required in the production of fetal neurotransmitters, proteins, and nucleotides (Avagliano et al., 2012). Previously mentioned, the terminal villi are responsible for nutrient/gas exchange in the placenta and this includes amino acids. The amino acids must cross both the microvillus plasma membrane and the basal membrane before reaching the fetus. Amino acids have an active transport across the placenta, from maternal to fetal circulation (Phillips et al., 1978). The placenta contains a higher concentration of amino acids than either the maternal or fetal environment. The placenta not only provides transport of amino acids from maternal to fetus, but also utilizes the amino acids. This utilization helps determine the flow of amino acids into the fetus (Montgomery et al., 1982). In a 2007 study, Baggs ewes were assigned to control and nutrient restricted (50% restriction NRC requirements) dietary treatments and at day 78 of pregnancy, the ewes were euthanized and amino acid concentrations of maternal and fetal plasma were tested. The nutrient restricted Baggs ewes showed a decrease in concentrations of 9 amino acids as compared with control ewes. However, the fetal amino acid concentrations showed no difference between the two groups. This suggests that through indirect selection, it is possible for the dam to overcome nutrient restriction by increased placental efficiency (Jobgen et al., 2007). Two major classes of amino acid transporters have been identified Na^+ dependent transporters and Na^+ independent transporters. These transporters are located at both the microvillus plasma membrane and the basal membrane. The microvillus plasma membrane has Na^+ dependent transporters, because energy is required to work against the concentration gradient. The basal membrane is less studied, but it has been suggested that amino acids pass through via facilitated diffusion (Desforges et al., 2010). Glucose is transported

through GLUT1 and GLUT3 transporters. The GLUT1 transporter is responsible for transport of glucose from the maternal tissues to the placenta and the GLUT2 transporter then moves glucose from the placenta into the fetal tissues (Bauer et al., 1998). Maternal IGF-1 increases the transport of both glucose and amino acids (Bauer et al., 1998).

Fatty acids in the fetus are precursors to cell membranes and other crucial structures, as well as energy and cellular metabolism. The fetal brain and retinas have heavy concentrations of arachidonic acid and docosahexaenoic acid, both long conjugated polyunsaturated fatty acids (LCPUFA), indicating a high need for fatty acids during early gestation. Linoleic and linolenic fatty acids in the maternal diet are metabolized into LCPUFAs, indicating maternal metabolism of linoleic and linolenic acid crucial for fetal development. Fatty acid transport from the maternal circulation to the placenta has limited data, but there have been some studies done. Competition studies with fatty acids led to the conclusion that binding sites on the placenta have the highest affinity for LCPUFAs (Dutta-Roy 2000). However, when trans fatty acids were introduced into the competition, trans fatty acids outstripped the affinity of LCPUFAs (Campbell et al., 1996). Trans fatty acids may have detrimental effects on the fetus by blocking the positive effects of LCPUFAs. Analysis of fatty acid binding protein (FABP) through polyacrylamide gel radio binding assay and Western blot analysis led to the conclusion that FABP (Campbell et al., 1994) is located on the microvillus membrane on the maternal side in the human placenta, suggesting that fatty acids flow only from the maternal to the fetus, with no back flow (Dutta-Roy 1997). Some data suggests that linoleic acid travels to the fetal tissues from the maternal via synchtiotrophoblast membranes (Simneau et al., 1994).

Maternal obesity increases the expression of fatty acid transporters in the placenta, increasing the rate at which the fetus receives fatty acids (Zhu et al., 2010b). The increase of fatty acids in fetal circulation leads to inflammation in fetal tissues (Tong et al., 2009; Yan et al., 2010). Dube et al. (2012) reported that maternal obesity in humans leads to altered expression of fatty acid transport proteins on both sides of the synctiotrophoblast: SLC27A4 and CD36. A study by Larque et al. (2006) suggests that SLC27A4 in the placenta is more involved with fatty acid esterification rather than transport and decreased expression would alter the esterification of fatty acids rather than the uptake. Increased expression of CD36 is likely correlated to the high levels of T3 (Feng et al., 2000) in obese women and increases fatty acid uptake and metabolism (Dube et al., 2012).

Normal and abnormal hormones

Glucocorticoids

Smith et al. (1973) analyzed maternal bovine serum glucocorticoid concentrations d 26 prior to parturition until d 9 post parturition. They concluded that glucocorticoid concentration stays fairly constant (5.1 ng/mL) until approximately 12 h prior to parturition, when glucocorticoid concentration nearly doubles (10.3 ng/mL). At parturition, glucocorticoid concentrations increased ~50% to 16.7 ng/mL. Post parturition, glucocorticoid concentration returned to the levels observes in the days

leading up to parturition (5.1 ng/mL). These findings suggest that glucocorticoids play a major role in initiation of parturition.

Glucocorticoids are generally found in low concentrations in fetal circulation. Glucocorticoids move to the fetal from the maternal environment down a concentration gradient and are regulated by placental 11β-Hydroxysteroid dehydrogenase (11βHSDS), an enzyme that converts maternal glucocorticoids and cortisol into metabolites (Fowden et al., 2004). This enzyme is crucially important to limiting fetal exposure to active maternal glucocorticoids (Fowden et al., 2004). While fetal glucocorticoid levels are low, it appears that they have a limited effect on tissue growth. However, when glucocorticoids are exogenously administered or 11βHSDS activity declines (leading to an increase in endogenous glucocorticoids), fetal growth actually declines. However, this is limited to mechanical growth, because the glucocorticoids cause the maturation of tissues and activation of biochemical processes that are not required in utero (Fowden et al., 2004). The rise in glucocorticoids suggests that stress on the dam causes fetus compensation by reducing mechanical growth and increasing tissue maturation to prepare for post natal life earlier than would otherwise be normal (Fowden et al., 2004). Synthetic glucocorticoids are used as therapy to increase survival of premature neonates by accelerating lung maturation if administered at the appropriate time during gestation (Liggins 1969; Liggins et al., 1972). Some synthetic glucocorticoids such as dexamethasone or betamethasone are unaffected by 11βHSDS and cross the placenta easily without any structural changes. Other substrates such as prenesilone are rapidly inactivated by 11βHSDS (Brown et al., 1996). The type of exogenous glucocorticoid

administered during pregnancy is important dependent on its intended effect (maternal versus fetal) and ability to cross into the placenta.

Glucocorticoids accelerate fetal lung development in sheep (Liggins 1969). Furthermore, when pregnant ewes were administered exogenous synthetic glucocorticoids, lambs were more likely to survive premature birth (Liggins 1969). Despite the increased lung maturation, there is evidence to suggest that synthetic glucocorticoids may have deleterious long-term effects on endocrine, renal, and metabolic function (Long et al, 2013b).

IGFs

During gestation, IGF-1 concentrations in the maternal environment are greater than the concentration of IGF-I in the fetus. IGF-II is more prevalent in prenatal life (Godfredson et al., 1991). Other key hormones associated with fetal development regulate IGF hormones. Human placental lactogen (hPL) shares similar biological traits to growth hormone and it has been suggested that placental lactogen is the fetal equivalent to growth hormone (Underwood et al., 1984), making placental lactogen the primary stimulus for IGFs during embryonic development). Efstratiadis (1998) reported that blocking of IGF genes or IGF-1 receptor led to a severe growth retardation, while overexpression led to overgrowth; furthermore, fetal exposure to differing levels of IGF showed abnormal development of tissues including bone, skin, respiratory, and muscles. Fetal IGF-I has little effect on body weight, but alters growth of individual tissues (Fowden et al., 2004). Insulin-like growth factors affect fetal growth both through

metabolic and non-metabolic action, working in the cell cycle to prevent apoptosis and increasing DNA and protein synthesis (Hill et al., 1998). Insulin-like growth factor-1 is involved in tissue accretion and is more sensitive to nutrition deficiencies, while fetal IGF-II provides a general cell growth stimulus (Fowden et al., 2004). It has been shown that IGF-I infusions causes an increase in fetal blood glucose concentrations but does not change glucose uptake in either the maternal or fetal environment. Furthermore, maternal plasma insulin levels and both maternal and fetal blood amino nitrogen concentrations fell. There were no observed changes in levels of fetal protein oxidation, feto-placental oxygenation, placental blood flow, or placental transfer by either simple or facilitated diffusion (Liu et al., 2013).

Leptin

Adipocytes and the placenta produce leptin and its concentration has a positive correlation with fetal growth (Cinaz et al., 1999). In postnatal life, insulin, GH, and IGF regulate leptin and there is a possibility that this relationship may also occur in utero. Leptin is positively correlated with birth weight (Marchini, et al., 1998). The timing of the initial postnatal leptin peak is crucial to setting the appetite regulation centers (Long et al., 2011). The lack of a leptin peak, as seen in offspring from obese dams can lead to an imperfect set point in the appetite control center, causing the offspring to eat more and put on more adipose tissue when exposed to increased nutrition postnatally (Long et al., 2011).

Epigenetics

"Epigenetics" is the term applied when there has been no change in gene sequencing but there is an observable difference in gene expression (Wilkins 2005). DNA methylation and chromatin organization are the two main contributors to epigenetics (Probst et al., 2009). Changes in gene expression can alter how nutrients are transported across cell membranes and even from the dam to the fetus during gestation.

Genes

There are more than 100 genes involved in placental development and function (Hemberger et al., 2001; Rossant et al., 2001; Simmons et al., 2005; Watson et al., 2005). Several gene families expressed in the ruminant placenta encode the intercellular signaling proteins pregnancy associated glycoproteins, trophoblast Kunitz domain proteins, and IFN-τ (Elsik et al., 2009). These proteins regulate aspects specific to ruminants including fetal growth, maternal immune function, maternal adaptations, endocrine function, and parturition (Larson et al., 2006; Hashizume et al., 2007).

Amino Acids and Micronutrients

During gestation, nutrient requirements of the dam greatly increase, especially protein as the fetus is creating muscle, making the demand for amino acids much higher than in a maintenance requirement for a postnatal animal (Langley-Evans et al., 2006).

However, the protein profile must be well balanced in amino acids, as high circulating levels of particular amino acids can cause toxicity (Langley-Evans et al., 2006).

DNA methylation relies on methyl group donors and cofactors, which it receives from methionine and folate metabolism. Methionine is an amino acid and its levels in the body are dependent on dietary intake and protein degradation (Chmurzynska 2010). There are two main reactions that contest for methionine: protein synthesis and S-adenosylmethionine (Finkelstein 1998). S-adenosylmethionine is a coenzyme and major methyl donor for several cellular processes, including DNA methylation (Brosnan et al., 2006). Demethylated S-adenosylmethionine becomes S-adenosylhomocystein and high levels can actually inhibit methylation (Mason 2003) and therefore must be eliminated for proper flow of the methionine cycle (Finkelstein 1998). The methionine cycle is dependent on the folate cycle. Folate from the diet is converted to methyl-THF, which is the main form of circulating folate in humans. The methyl group from methyl-THF can be used in the remethylation of homocysteine all the way back to methionine via methionine synthase (Chmurzynska 2010). Methionine synthase requires the vitamin cobalamin and enzymes within the reaction require pyridoxal phosphate, a B6 vitamin (Brosnan et al., 2006). While dietary requirements of methionine, folate, and B6 vitamins in the dam are very small, without them it would be impossible to successfully perform DNA methylation in the fetus.

High levels of methionine can be toxic to the developing fetus, as the methylation of SAH requires methyl groups from THF (derived from ingested folate). Furthermore, homocysteine may not be eliminated because the fetus is lacking in the synthases

responsible for homocysteine elimination. The increased methylation of homocysteine depletes methylated folate levels, leading to a folate deficiency (Rees et al., 2006).

DNA methylation/histone modification

It is suggested that poor nutrition during gestation may detrimentally alter postnatal function through changes in DNA methylation (Rakyan et al., 2001). In mammals, DNA methylation occurs at the fifth position of the pyrimidine ring of cytosine base followed by guanine, known as CpG dinucleotides (Rakyan et al., 2001). The 5' end and promoter regions of DNA contain CpG islands, which are clusters of non-methylated CG rich areas (Sulewska et al., 2007). Methylation of these islands decreases transcription (Urnov 2002). It has been suggested that methylation is reversible and that the balance is dependent on the rate of methylation and demethylation (Delcuve et al., 2009; Niehrs 2009). During gestation, methylation is catalyzed by DNA methyltransferse 1 (Dnmt 1) and maintained by both Dnmt 3A and Dnmt 3B (Rakyan et al., 2001). As gestation progresses and the fetus becomes more differentiated, Dnmt 1 decreases (Turek-Plewa et al., 2005). Mutations to any of the DNMTs led to abnormal development or fetal mortality (Gaudet et al., 2003). In mice, a down regulation of Dnmt 1 leads to hypomethylation of all tissues, which led to tumors in the postnatal life (Gaudet et al., 2003). When Dnmt 3A and 3B were blocked to stem cells and the early embryo, there was no effect on maintenance of methylation. However, Dnmt 3A blocked mice were born normal and died 4 weeks later and Dnmt 3B blocked mice were born dead with multiple abnormal defects (Okano et al., 1999).

In the nucleus, DNA exists as a nucleoprotein called chromatin. Chromatin is made up of individual nucleosomes. A nucleosome is comprised of 146 DNA base pairs wrapped around 8 histone proteins – four different proteins in duplicate (Rakyan et al., 2001). Any change to the histone structure affects nucleosome structure and therefore function. Changes in nucleosome structure affect its accessibility to transcription machinery within the cell (Munshi et al., 2009). Histones undergo reversible post translation modification including the methylation and phosphorylation of different amino acids. Other histone modifications also include the regulation of gene expression by transcription activation, transcription repression, repression of specific genes, chromatin remodeling, nucleosome loosening, and chromatin stabilization – all of which can stimulate or repress gene expression (Munshi et al., 2009).

Gene expression

Chromosomes are comprised of two different parts: euchromatin and heterochromatin. Euchromatin is transcriptionally active where heterochromatin is inaccessible to DNA binding and therefore transcriptionally inactive (Grewal et al., 2002). Large blocks of heterochromatin surround centromeres and telomeres while smaller pieces are interspersed throughout the chromosome (Grewal et al., 2002). Heterochromatin plays a crucial role in centromere function (Grewal et al., 2002), as well as sister chromatin cohesion and chromosome segregation (Bernard et al., 2001; Peters et al., 2001; Nonanka et al., 2002). Heterochromatin stabilizes DNA sequences (Grewal et al., 1997) and controls the regulation of gene expression during development and

differentiation through the stable inactivation of developmental regulators (Cavalli et al., 2002). The haploid eukaryote cell DNA content is measured by its "C-value" (Thomas 1971). While initially thought to be correlated to gene number and species complexity, it has since been agreed that there is no significant correlation between species gene number, developmental complexity, and the C-value (Thomas 1971).

Genomic imprinting

Genomic imprinting has a significant effect on human genetic diseases (Nicholls et al., 1989). The key mechanism of imprinting is DNA methylation (Reik et al., 1987); methylation marks imprinted genes differently (Sapiens et al., 1987) and inheritance of epigenetic markers leads to different gene expression (Swain et al., 1987). Imprinted genes are important in the phenotypic effect and control fetal growth and postnatal behavior. There have been no observable differences in protein sequences encoded by imprinted genes, though there are functional relationships between proteins with roles in fetal development. All imprinted genes have two general features in common: They are rich in CpG islands and have clustered/direct repeats near or within the CpG islands (Reik et al., 2001).

The majority of imprinted genes show different DNA methylation than parental alleles, but differentially methylated regions have different properties. Imprinted genes can differ in chromatin structure and histone acetylation (Ferguson-Smith et al., 1993). Lastly, imprinted genes may have two large effects: DNA in imprinted regions replicate asynchronously in S phase of cell cycle and have different frequencies of meiotic

recombination found in or near imprinted clusters, though the effect on DNA methylation is unclear (Reik et al., 2001).

In a non-human primate study, animals from dams receiving a 70% restricted diet (MNR) showed little external phenotypic differences than animals from dams receiving the control diet (CTR). Despite this, there were significant differences between the MNR and CTR groups when comparing structure, gene, and protein abundance in placenta, fetal liver and kidney (Nijland et al., 2010). This study suggest that fetal weight and the weight of individual fetal organs is an inadequate measure of compromised fetal development and analysis of cellular structure and function at the molecular level is required to determine the nature and mechanisms responsible for fetal adaptations resulting from maternal challenges during pregnancy (Nijland et al., 2010), a conclusion previously put forth by Armitage et al. (2004) and Zambrano et al. (2005b). The fetal renal system contains mammalian target rapamycin (mTOR), which is a sensor of nutrient stress and has been shown to decrease in the primate fetal kidney with a 30% nutrient restriction in the dam, suggesting the fetus has the ability to sense a decrease in nutrients (Nijland et al., 2007). Furthermore, an increase in fetal liver glycogen occurs in nutrient challenged dams with a 30% decrease in dam nutrition (Li et al., 2007), suggesting that the fetus can initiate mechanisms to conserve energy by decreasing glycolysis or increasing glucose availability through gluconeogenesis (Nijland et al., 2010).

In the pregnant rat model, a low protein and high carbohydrate diet lead to an increase of enzyme activity of fetal liver PEPCK (Franko et al., 2009), which indicates that there are multiple mechanisms of activation for an increase in hepatic PEPCK other

than a direct stimulation from mature fetal systems (Kwong et al., 2007). However, the duration and severity of the challenge may be significant, as fetal hepatic PCK1 mRNA does not increase in sheep after d110 if challenged (Hyatt et al, 2008), though there may be some species differences (Nijland et al., 2010).

Cortisol is accepted as a key regulator of PEPCK, mediated by glucocorticoid response element (GRE) in the PCK1 gene promoter (Cassuto et al., 2005). In the sheep model, intravenous cortisol in fetal sheep showed an increase in fetal liver glycogen deposition as well as glucose-6-phosphatase and PEPCK activity (Fowden et al., 1993). Maternally administered dexamethasone had no effect on PEPCK activity, but showed an increase in glucose-6-phosphatase activity. The differences in fetal versus maternal dexamethasone administration suggests that dexamethasone primarily affects glycogenolysis in the fetal liver (Franko et al., 2007).

MicroRNA and Placental Development

MicroRNAs are small, noncoding RNAs that are 20-25 nucleotides long. MicroRNAs regulate posttranscriptional gene expression by binding to the 3' untranslated region of their target RNAs, generally messenger RNA (Bartel et al., 2004). MicroRNAs (miRNA) have recently been discovered to have a significant role in trophoblast function including regulation of maternal-fetal immunology, trophoblast differentiation in placental development, angiogenesis, vasculogenesis, oxygen sensing, and links between miRNA and imprinted genes (Varrault et al, 2006; Doridot et al., 2013; Renfree et al., 2013). Angiogenesis, as previously discussed, is key to early placentation

and involves the remodeling of uterine arteries and blood vessels. Impaired remodeling of the uterine arteries leads to reduced blood flow to the placenta and subsequent decreased oxygen delivery as well as increased cell injury, inflammation, and debris ending up in maternal circulation (Doridot et al., 2013). The relationship of miRNAs and angiogenesis is still unclear, but a recently published study has found an increased expression of miR-17 miRNAs in preeclampsia (PE) versus normotensive human placentas (Wang et al., 2012). Two ephrin receptors involved in angiogenesis, B4 (EPHB4) and B2 (EPHB2) (Wang et al., 1998), have been identified as potential targets of the miR-17 family (Wang et al., 2012), as the coupling of EPHB4/EPHB2 drives extravillous trophoblasts (EVTs) away from the placenta and towards uterine arterioles to facilitate branching (Red-Horse et al., 2005).

The Notch pathway has been previously proven to play a role in vascular patterning and specification of arterial identity (Roca et al., 2007; Swift et al., 2009) and it is now thought to have a part in artery remodeling (Hunkapiller et al., 2011). The components of this pathway include four transmembrane receptors (Notch1-4) and five ligands (Delta1/3/4 and Jagged1/2) (Hunkapiller et al., 2011). MiRNAs are involved in Notch signaling, including miR-210, which is the most up-regulated miRNA in PE (Pineles et al., 2007). While the underlying mechanisms for preeclampsia are largely unknown, placenta and trophoblast dysfunction are requisites for development of PE (Goldman-Wohl et al., 2002; Rampersad et al., 2007). Increased expression of miRNA in the preclampsic placenta suggests that miR-210 is involved in trophoblast migration and invasion (Zhang et al, 2012).

Increased expression of miR-210 leads to an increased expression of Notch1, which induces migration and tube formation in Matrigel (gelatinous protein mixture secreted by mouse sarcoma cells) (Lou et al., 2012). Vascular smooth muscle cells have an increased expression of contractile proteins through Notch signaling and Jagged1 pathway, facilitated through the transcription of miR-143/145 (Boucher et al., 2011). Several miRNAs, including miR-9, miR-21, miR-27, miR-124, miR-130, miR-148, and miR-181 are predicted to target the Notch pathway and are thought to be involved in vascular development and extracellular signaling, though the full interactions are not completely understood (Anand 2013).

Trophoblast cells express human leukocyte antigen (HLA)-G (Hunt et al., 2006) and HLA-C (Hiby et al., 2004). Human leukocyte antigen G is considered a general immune tolerance molecule (Wiendl et al., 2005; Carosella, 2011; Gonzalez et al., 2012). It is expressed by extravillous trophoblasts and its expression is regulated by mir-152 (Tan et al., 2007) and mir-148 (Manaster et al., 2012). Human leukocyte antigen C regulation has not been fully elucidated, but is still strongly expressed in cells from trophoblast origin (Manaester et al., 2012).

Postnatal disease susceptibility

The phenotype of an animal results from the interaction of genetics and environment. Two genetically identical animals may exhibit a different phenotype if raised in a different environment and two animals with different genetics in the same environment will likewise develop differently and may exhibit a different level of

production. The environment during prenatal and early postnatal life has the greatest affect on the adult animal (Yajnik 2004). These environmental factors can increase the risk of adult chronic diseases (St Clair et al., 2005), including mental diseases such as schizophrenia and an altered ability to respond to stress (van Os et al., 1998). Alterations to epigenetic marks play a central role in determining the expression of genes stored in the animal's genome (Jirtle et al., 2007).

Changes in epigenetic status can be because of either molecular modification of DNA or the chromatin via DNA methylation (Li 2002; Klose et al., 2006; Talbert et al., 2006; Richards 2006) or can be a change of non-coding RNAs, such as micro RNAs (Thorvaldsen et al., 2006). Changes can be inherited mitotically through effects of nutritional supplements (Wolff et al., 1998), chemicals (Li et al., 2003), behavior cues (Weaver et al., 2004), reproduction factors (Niemitz et al., 2004), and radiation (Koturbash et al., 2006). Changes can also have a long-term effect on multiple generations (Anyway et al., 2006).

Environmental changes can be nutritional, chemical, or physical factors. There are 3 genomic targets for environmental changes: promoter regions of housekeeping genes, which are unmethylated; transposable elements that lie adjacent to genes with metastable epialleles, which are methylated; regulatory elements of imprinted genes, which are differentiated methylated. Methylation status determines level of gene expression and a change in level of methylation can alter animal function and survivability (Jirtle et al., 2007).

Postnatal

Average Daily Gain (ADG)

In production systems, ADG is a rate of weight gain per day over a specific period of time. Factors decreasing ADG can be detrimental to a production system. In a porcine study, no alterations on maternal diet or diet in piglets post weaning through finishing, led to a decreased ADG in piglets with a lower birth weight (Gondret et al., 2005), suggesting that lighter animals at birth are poorly prepared for adequate levels of ADG on a normal plane of nutrition. A nutritional study using cattle during early gestation showed that limited nutrition or moderate nutrition during the first third of gestation had no effect on calf birth weight or subsequent ADG during any of the production phases (weaning, finishing, etc.) (Long et al., 2010), suggesting that postnatal growth of bovine fetuses are not particularly sensitive to early gestation nutrient restriction.

In calves from dams who were subjected to nutrient restriction during the last half and last third of gestation, calf birth weights were lower and this caused a significant decrease in ADG at every production stage (weaning, back grounding, finishing) up to 30 months of age, compared with non-restricted animals (Greenwood et al., 2005). Heifers showed a lower calf birth weight than cows if subjected to nutrient restriction during mid- and late gestation, but cows and heifers showed similar decreases in calf birth weight when nutrient restriction was limited to the last one third of gestation (Greenwood et al., 2005).

Insulin Resistance

Undernutrition in ewes (fed 1-2 % of body weight versus 3-4% body weight for control ewes) from 61 days before breeding to 30 days post conception showed an increased fetal insulin response to glucose during late gestation (Oliver et al., 2001). In contrast, prolonged undernutrition during gestation showed decreased fetal insulin response to glucose in sheep (Limesand et al., 2003). Another study showed that undernutrition during late gestation resulted in glucose intolerance in yearling lambs (Gardner et al., 2005), which was associated with a decreased expression of GLUT4 receptors in adipose, but showed no changes in expression in muscle tissue (Poore et al., 2002). Ford et al. (2007) subjected 2 groups of wether lambs to two glucose tolerance tests. Control lambs were from ewes fed a diet at 100% of NRC requirements throughout gestation. Nutrient restricted lambs were from ewes fed the same control diet, but at 50% of NRC requirements d 28 – 79 of gestation, after which they were fed the same ration as the control group. The glucose tolerance tests were conducted at d 63 and d 250 of age as to occur pre- and post-weaning. In both glucose tolerance tests, both groups showed a similar baseline. Prior to weaning, nutrient restricted lambs had increased insulin concentrations as compared with control lambs. Post-weaning, nutrient restriction wethers had decreased insulin concentrations as compared with control lambs. This dynamic change in insulin concentration in nutrient restriction lambs is indicative of pancreatic β-cell dysfunction, leading to an overproduction during early life and rapidly degrading as the animal ages (Simmons et al., 2001). This evidence suggests that undernutrition during any point in gestation has a long-term effect on insulin and glucose resistance in the postnatal animal.

Maturation of the hypothalamic pituitary axis and its effects on cognition

The hypothalamic pituitary adrenal axis (HPAA) is responsible for final fetal maturation during late gestation through production of glucocorticoids (Liggins 1994). The glucocorticoids enter fetal circulation and act as a maturation catalyst for organs and metabolic pathways to prepare the fetus for postnatal life (Liggins 1968). The surge of cortisol levels is also a maternal signal to begin labor (Liggins et al., 1973). Exposure to maternal glucocorticoids or synthetic glucocorticoids during critical windows is a mechanism in fetal programming and can lead to altered development of the HPAA and hypertension (Benediktsson et al., 1993; Welberg et al., 2001). Exposure to maternal glucocorticoids most often stems from maternal undernutrition. Lack of nutrients puts stress on the animal, causing her to produce higher levels of cortisol hormones, increasing fetal exposure (Edwards et al., 2001).

Insults on the HPAA are permanent as exposure to glucocorticoids during critical windows alters the development and therefore function during postnatal life. High cortisol levels are associated with atherosclerosis, immunosuppression, depression, cognitive impairment, and increased cholesterol levels (Sapolsky 2000). Depressed cognitive impairment is most likely because the insults on the HPAA have likewise compromised the development of the hippocampus. This upset on the hippocampus is not noticed until later in life, when cognitive ability begins to decline before expected in the normal aging process. The hippocampus is most often associated with memory and with

the decreased operating capacity of the hippocampus comes a loss of memory and therefore cognitive function (Lupien et al., 2001).

Increase adiposity

It has been suggested that offspring from overweight mothers are more prone to becoming overweight (Plagemann et al., 1997; Silverman et al., 1998). Studies in the sheep model have shown that this is most likely because of a gestational programming affecting the appetite regulation center in the brain. Pregnant sheep were fed either a control diet or a well-fed diet (~100%, ~160% of metabolizable energy requirements, respectively) (Muhlhausler et al., 2006). Thirty days post lambing, lambs were sacrificed and fat samples were taken. Lambs from well-fed dams had an increased amount of subcutaneous fat when compared with control lambs. Furthermore, expression of appetite regulating genes in lambs from well-fed dams was altered to allow for increased adiposity (Muhlhausler et al., 2006). Further studies with obese ewes showed lambs with an increased fetal adiposity without a change in fetal body weight, suggesting that fetal adipose mass is independent of body weight (Ford et al., 2009). Later in life, this increase in neonatal adiposity can lead to early childhood obesity and even obesity into adult life (Catalano et al., 2003).

Decrease skeletal development

Skeletal muscle development and bone development often reflect each other. Animals with a higher amount of muscle are more likely to have a stronger skeleton and vice versa (Frost 2001). Skeletal muscle development begins before bone development and it has been suggested that proper skeletal muscle development is crucial for adequate bone development, as heavier muscles would initiate an increase in bone strength (Schoenau et al., 2005; Pludowski et al., 2006). A study in maintenance fed (fed 100 % maintenance requirements based on NRC) and well-fed sheep (given *ad libitum* access to pasture) showed that lambs from well-fed sheep had stronger bones (as measured by bone mineral content) than lambs from maintenance diet dams (Firth et al., 2008). Maternal under nutrition leads to a decrease in hyperplasia of muscle fibers (Dwyer et al., 1994) and it could therefore be argued that a decrease in skeletal muscle development would subsequently lead to a decrease in bone development. Furthermore, skeletal muscle growth is crucial in utero, because muscle fiber numbers do not increase after birth. During gestation, skeletal muscle has low priority for nutrition partitioning compared with heart and brain development, making it particularly vulnerable to nutrient insults (Bauman et al., 1982; Close et al., 1990). In nutrient restricted ewes during early to mid gestation (Zhu et al., 2006), skeletal muscle was decreased in developing lambs. In nutrient restricted (50 %) cattle during early gestation (d 30 – d 125), fetuses were found to have increased levels of calpastatin (Du et al., 2004). Calpastatin inhibits calpains, which are proteases responsible for muscle degradation (Barnoy et al., 2000). The increased levels of calpastatin in nutrient restricted fetuses suggests that nutrition levels play a role in fetal muscle maintenance (Du et al., 2004). In nutrient restricted (60 %) cattle during early gestation (d 30 – d 85) showed decreased myofiber diameter

(Gonzalez et al., 2013). As skeletal muscle is the primary site for insulin resistance in obese and type 2 diabetes patients (Kemp et al., 2003; Lowell et al., 2005), it is suggested that the decrease in skeletal muscle predisposes the animal to obesity and glucose/insulin dysregulation later in life (Stannard et al., 2004; Zambrano et al., 2005a). Further, while nutrient restricted ewes had significantly increased muscle fiber diameter, the distribution of myosin was altered from that of the control fed ewes. Nutrient restricted ewes had a significantly increased distribution of myosin IIb fibers, which are the least insulin sensitive, and significantly decreased myosin Ia type fibers, which are the most insulin sensitive. The altered muscle fiber distribution may contribute to glucose intolerance in the postnatal lambs (Zhu et al., 2006).

Thermogenesis

IUGR can affect the development of skeletal muscles and adiposity, particular visceral adipose tissue (Bagley et al., 2013), both of which are key factors in thermogenesis. A decrease in skeletal muscle decreases the animal's ability for respiration, posture, and thermogenesis (Rossdale et al., 2002).

Furthermore, maternal under nutrition decreases the adipose tissue mass in newborn animals. As most newborn animals receive 50% of their heat production through thermogenesis in brown fat, the decrease in adipose tissue mass could increase mortality of newborn animals (Satterfield et al., 2013). Restriction of prepartum protein (Carstens et al., 1987) or energy (Ridder et al., 1991) of heifers decreased the

thermoneutral metabolism capacity in newborn calves. Carstens et al. (1987) showed that the decrease in metabolic capacity was independent of birth weight.

Glucose and insulin levels

IUGR can lead to adult diseases, including non-insulin dependent diabetes mellitus (NIDDM) as a result of increased insulin resistance, decreased insulin secretion, or a combination of both. Maternal under nutrition often leads to an increased resistance to both glucose and insulin. Studies in prepubertal children have shown that plasma glucose levels are inversely related to birth weight. These levels are indicative of an increased chance of NIDDM later in life (Veening et al., 2002).

Transgenerational effects

In a multi generation study, it was shown that an F2 generation can be affected by the diet fed to the F0 generation during gestation without changing the diet of the F1 generation. In a study by Pineherio et al. (2008), virgin female rats (F0) were blocked into one of 4 treatments: CC (control diet during gestation and lactation), CR (control diet during gestation, restricted during lactation), RC (restricted diet during gestation, control diet during lactation), and RR (restricted diet during gestation and lactation). The restricted diet consisted of a decreased protein intake while remaining isoenergetic for all groups. Once weaned, the F1 generation was kept on a control diet throughout gestation and lactation.

In the F1 generation, there were no significant differences between the treatment groups for litter size, pup mortality, or gender. Body mass and naso-anal length were decreased for offspring of both RC and RR rats. RC1 rats (offspring of RC rats; F1 generation2) had a "catch up" growth when compared with CC1, while both CR1 and RR1 had decreased growth. Male RC1 rats had increased fasting glucose and showed evidence of insulin resistance, though there were no significant differences in circulating plasma insulin. Male RC1 rats also had increased circulating leptin versus CC1, CR1, and RR1 treatment groups.

Data collected on the F2 generation was consistent in showing that F0 diet had an impact on hormone levels, though all F1 rats were fed a control diet throughout gestation and lactation. RC2 (offspring of RC1 rats; F2 generation) rats were heavier and had a longer naso-anal length versus CC2 offspring, but this change was gone at weaning. CR2, RC2, and RR2 offspring were all heavier than their F1 counterparts. Overall, there was no significant change in growth rate between the F2 offspring. Fasting glucose was increased for groups CR2, RC2, and RR2 when compared with CC2 and plasma glucose was increased in the CR2 group versus RC2 and RR2 and treatment groups CR2 and RR2 had increased glucose levels when compared with their F1 counterparts. Fasting insulin was increased in CR2 and RC2 versus CC2 and plasma insulin was increased for CR2 and RC2 versus RR2. Plasma insulin was also significant increased in CR2 and RC2 when compared with their F1 counterparts. CR2 and RC2 male offspring showed an increased insulin resistance when compared with RR2 and CC2, and insulin resistance was increased in the F2 generations versus the F1 generation. Lastly, plasma leptin was increased in CR2 versus other F2 offspring as well as its F1 counterpart. The data

collected from this study shows that a restricted maternal diet can have effects far beyond their offspring.

In the guinea pig, maternal under-nutrition modifies the HPA (hypothalamic pituitary adrenal) and cardiovascular function in the F2 generation (Dobbing et al., 1970; Dobbing et al., 1979; Owen et al., 2003; Owen et al., 2005). The F0 generation was fed 70% of the control diet during either the first half or second half of gestation. The F1 generation was fed ad libitum during production of the F2 generation. Birth weight and growth were most affected in restricted animals versus control, but showed greater changes in the F2 generation than the F1 generation. Both generations had increased cortisol levels and changes in HPA response from the control group, though the changes were more marked from late gestation restricted F0 dams. F1 offspring from early gestation restriction had increased blood pressure and left ventricular wall thickness from the control group; effects that were also passed to the F2 generation (Bertram et al., 2008).

In multigenerational sheep studies, it was shown that glucocorticoid treatment (dexamethasone) in solely the F0 generation during pregnancy led to growth restriction in subsequent F1 and F2 generations (Long et al., 2013b). While glucocorticoids are successful in preventing preterm delivery in humans, it is important to consider potential consequences not only to the F1 generation but also to the generations following (Long et al., 2013b). Also, synthetic glucocorticoid treatment to the F0 generation during pregnancy led to postnatal changes in the F2 generation including a decreased postnatal leptin peak, increased appetite, increased adiposity, increased plasma leptin, glucose, and cortisol, and decreased plasma insulin in response to an ad libitum feeding challenge

(Long et al., 2013b). Lastly, diet-induced obesity of the F0 generation had effects into the F2 generation when the F1 generation was fed a control diet. F1 ewes from the obese mothers had greater glucose and insulin baseline concentrations and increased insulin resistance when compared with their F1 control counterparts (Shasa et al., 2015). Lambs in the F2 generation did not differ in birth weights between the two treatment groups but F2 lambs from obese grandmothers had increased adiposity, hyperglycemia, and hyperinsulinemia than their control counterparts (Shasa et al., 2015).

While there are not many studies on transgenerational effects in the human model, there is ongoing data collection on the population subjected to the Dutch Winter Famine (1944-45), which is now in its 6^{th} generation and offspring are still showing some detrimental signs, including diabetes, cardiovascular disease, and a change in metabolism (Painter et al., 2008). The mechanisms involved in transgenerational effects are still in question. Insults are most likely carried across generations through either an altered maternal endocrine adaptation to pregnancy or transgenerational transmission of epigenetic modification (Matthews et al., 2010).

Conclusion

The fetus relies completely on the dam during gestation; everything from nutrition, oxygen, and a safe environment are provided from the time of conception until parturition. However, maternal physiological or environmental factors may have detrimental effects on the fetus (Barker et al., 1997).

Arguably, the placenta is the most important factor in ensuring that the fetus develops properly from conception to parturition. The placenta allows for nutrient and gas exchange to the developing conceptus (Ramsey 1982) and hormone production that can influence both fetal and maternal physiology (Gey et al., 1938; Josimovich and MacLearn, 1962; Kaplan and Grumbach, 1964). The placenta needs an adequate blood supply in order to provide nutrient and gas exchange; decreased blood flow is correlated with high-risk pregnancy and inhibited fetal growth (Haggarty et al., 2002). While vasculogenesis typically occurs primarily during the first two-thirds of gestation, vasculogenesis can continue to increase until the end of gestation if the fetus is experiencing limited growth (Vonnahme et al., 2004). In conjunction with vasculogenesis, angiogenesis is fundamental to ensuring sufficient blood supply to the placenta. Most of the factors regulating angiogenesis occur because of vasculogenesis, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF-2), and angiopoietin protein families (ANG-1 and ANG-2) (Reynolds et al., 1988; Millaway et al., 1989; Zheng et al., 1998).

As the fetus grows, it grows through both hyperplasia (increase in cell number) and hypertrophy (increase in cell size). It was initially suggested that hyperplasia occurred only in early gestation and hypertrophy occurred only in late gestation (Winick et al., 1965). However, it has now been shown that while hyperplasia is more prevalent during early gestation and hypertrophy is more prevalent during late gestation, both types of cell growth exist from conception to parturition (Prior et al., 1979). The fetus can alter its growth and endocrine environment when faced with nutrient insufficiency in order to better prepare for postnatal life, known as the Thrifty Phenotype (Hales et al., 1992). The

fetus can alter its growth by prioritizing nutrients to organ systems of more importance, such as the brain and heart, at the impairment of other organ systems. Further, the fetus can alter its endocrine environment by increasing glucocorticoids to escalate the maturation of organs, readying them for postnatal life (Anthony et al., 2003).

Fetal growth restriction can have multi generational effects, in which the F2 generation can be affected by the F0 generation's diet, even without a change to the F1 generation during gestation and has been shown to affect hormone levels (Pineherio et al., 2008), increased blood pressure (Bertram et al., 2008), growth restriction caused by synthetic glucocorticoid treatment (Long et al., 2013c), and increased adiposity, hyperglycemia, and hyperinsulimia (Shasa et al., 2015).

Nutrition is most certainly an important factor in ensuring a fetus survives and thrives from conception to parturition and beyond. Undernutrition and over nutrition are both culprits in altering fetal growth and endocrine environment and the far reaching effects of poor nutrition during gestation are still being elucidated.

CHAPTER II

Effects of maternal nutrient restriction during early or mid-gestation on bovine fetal growth

ABSTRACT

Primiparous Angus-cross cows (*n* = 38) were synchronized using a Co-Synch+CIDR protocol and inseminated with semen from one of two Angus sires. Animals were fed at 1.3 x (Control [CON]) or 0.55 x (Nutrient Restricted [NR]) of maintenance energy and protein requirements based on BW (NRC 2000). Diets were fed at either 1.3 x (Control [CON]) or 0.55 x (Nutrient restricted [NR]) of maintenance energy and crude protein values based on BW (NRC 1996). A subset of animals (*n* = 16) was fed either NR (*n* = 8) or C (*n* = 8) from d 30-110 of gestation. The remaining animals (*n* = 22) were fed CON (*n* = 8) d 30-190; NR (*n* = 7) d 30-110 followed by CON d 110-190; or CON (*n* = 7) d 30-110 followed by NR d 110-190. Cows were slaughtered on d 110 or d 190 of gestation, when fetal measurements and samples were taken for analysis. Fetal weights and empty fetal weights were also reduced (*P* = 0.0027, *P* = 0.0023, respectively) in d 110 NR animals. Fetal weights tended to be reduced (*P* = 0.07) in NR/CON and CON/NR versus CON/CON cattle. Empty fetal weights were reduced (*P* = 0.03) in NR/CON and CON/NR versus CON/CON cattle. Abdominal and thoracic circumferences were reduced (*P* = 0.0265, *P* = 0.0002, respectively) in d 110 NR animals and were also reduced (*P* = 0.01 and *P* = 0.03, respectively) in NR/CON and CON/NR versus CON/CON cattle. Brain weight as a percent of empty fetal weight (*P* < 0.01) was increased in d 110 NR animals compared with d 110 CON animals. Brain weight as a

percent of empty fetal weight was increased ($P < 0.001$) in NR/CON and CON/NR versus CON/CON cattle. Fetal pancreas weight as a percent of empty fetal weight was reduced ($P = 0.06$) in NR d 110 cattle. Fetal pancreas weight as a percent of empty fetal weight was reduced ($P = 0.04$) in CON/NR versus CON/CON cattle (0.062 ± 0.004 versus. 0.079 ± 0.004 %) while NR/CON values (0.069 ± 0.004 %) were intermediate. Fetal perirenal adipose as a percent of empty fetal weight was increased ($P = 0.01$) in NR d 110 female fetuses. Fetal perirenal adipose as a percent of empty fetal weight was increased ($P = 0.003$) in NR/CON and CON/NR versus CON/CON cattle. The data show that maternal nutrient restriction during early or mid gestation causes asymmetrical fetal growth restriction, regardless if the restriction is preceded or followed by a period of non-restriction.

Keywords: fetal growth, undernutrition, fetal programming

INTRODUCTION

Fetal programming has significant effects on livestock production. In beef cattle, animals that experience stress in-utero can have reduced skeletal growth, which can affect carcass merit and meat production (Greenwood et al., 2005). This could be especially problematic in heifers if they are used as breeding stock. Intrauterine growth retardation (IUGR) is defined as any stressor on an animal that causes reduced fetal growth during gestation; IUGR can be environmental or physiological, occur at any point during gestation, and has permanent negative effects on the offspring's physiology and development (Baker et al., 1969). Specifically, environmental stress (transportation) of the dam can lead to alteration of the hypothalamic-pituitary axis in the fetus, inhibiting

the postnatal animal from accurate regulation of hormones involved in growth and reproduction (Lay et al., 1997). Fetuses that undergo IUGR often exhibit asymmetrical growth patterns, which suggests that the fetus compartmentalizes nutrients and blood flow to the organs and structures that are most pertinent to development (Anthony et al., 2003). Early gestation is a critical period for fetal hyperplasia (Ferrell et al., 1976), fetal organ and tissue development, and maternal development of the placenta (Ford 1995; Reynolds et al., 1995). The objective of this study was to determine the severity of asymmetrical fetal growth caused by maternal nutrient restriction during early (d 30 – d 110) or midgestation (d 110 – d 190) and to what extent, if any, the effects of fetal growth restriction could be alleviated with a trimester of non-restriction either preceding or following the trimester of restriction.

MATERIALS AND METHODS

All animal procedures were approved by Clemson University Animal Care and Use Committee (AUP #2013-062).

Animals and Sample Collection

Primiparous Angus-cross cows (n = 38) were synchronized using a 7 d Co-Synch+CIDR protocol. On d 0, cows received an EAZI-Breed CIDR insert (Zoetis Animal Health, New York, NY) and an injection of GnRH (100µg [IM]; Zoetis Animal Health, New York, NY). On d 7, CIDRs were removed and cows received an injection of $PGF_{2\alpha}$ (25mg [IM]; Zoetis Animal Health, New York, NY). Cows were observed for estrus at 0700 and 1700 h for 30 min and were inseminated by one of two AI technicians

with sexed (d 190 fetuses) or conventional (d 110 fetuses) semen from one of two Angus sires 12 h after first observed estrus. Any animal not observed in estrus was timed inseminated 60 h post $PGF_{2\alpha}$ injection and a GnRH injection administered. At 30 d post breeding, pregnancy was determined via transrectal ultrasonography (Aloka 500-V with 7.5-MHz probe, Corometrics Medical Systems, Wallingford, CT). Blood samples were collected from pregnant animals via caudal venipuncture into a 9 mL Lithium-Heparin plasma syringe (SARSTEDT, Nümbrecht, Germany) and placed immediately on ice. Samples were centrifuged within 2 h of collection at 2000 x *g* for 20 min at 4° C. After centrifuging, the plasma was collected and stored at -20° C until analysis of metabolites.

Animals were blocked into treatment groups by BCS (Wagner et al., 1988), shown in Figure 1**.** Throughout the experiment, all animals were fed a TMR (30% soybean hulls, 20% cottonseed hulls, 17.5% peanut hulls, 17% corn screening, 12.5% corn gluten feed, 1.5% salt, 0.5% calcium, 0.5% trace mineral, 0.5% vitamin premix: 0.65 Mcal NEm/lb, 8.9% CP) at one of two levels. Diets were fed at either 1.3 x (Control [CON]) or 0.55 x (Nutrient restricted [NR]) of maintenance energy and crude protein values based on BW (NRC 2000). A subset of animals (*n* = 16) was fed either NR (*n* = 8) or C (*n* = 8) from d 30-110 of gestation. The remaining animals (*n* = 22) were fed CON (*n* = 8) d 30-190; NR (*n* = 7) d 30-110 followed by CON d 110-190; or CON (*n* = 7) d 30-110 followed by NR d 110-190.

Cattle were maintained together on a dry lot with free access to water and were fed individually once daily in the morning. Animal BW was taken weekly and used to adjust feed allowances throughout the experiment; BCS and blood samples were taken every 2 wk. All blood samples were collected and processed as previously described.

All animals were harvested at the same commercial plant. Final BW, BCS, and blood samples were collected ~24 h prior to harvest. Blood samples were collected via jugular venipuncture into 10 mL sodium heparin, serum, and EDTA BD Vacutainers (Becton, Dickinson and Company, Franklin Lakes, NJ). The sodium heparin and EDTA tubes were treated exactly as previous blood samples. Serum samples were allowed to clot for approximately 60 min at room temperature and then refrigerated overnight before centrifuging at 2000 x *g* for 20 min at 4° C. After centrifuging, plasma and serum was collected and stored at -20° C until analysis of metabolites. Cows were slaughtered and the gravid uterus was removed and weighed. Fetal blood samples were collected from the umbilical cord into 10 mL sodium heparin, serum, and EDTA BD Vacutainers, treated as previously described for maternal blood, and stored at -80° C until analysis. Fetal weight, crown rump length, abdominal circumference, and thoracic circumference were recorded. Fetal brain, heart, liver, lungs, pancreas, kidneys, adrenals, perirenal adipose, viscera, and longissimus dorsi muscles were collected and weights recorded at time of harvest. The fetal heart was dissected into right and left atria, right and left ventricle, and septum; each atria, ventricle, and septum was individually weighed and thickness measured in 3 locations and measurements averaged. The eviscerated fetus was weighed to obtain an empty fetal weight.

Samples of caruncle, cotyledon, placentomal artery, perirenal adipose, mesenteric adipose, large intestine, small intestine, liver, longissimus dorsi (LD) muscle, left heart ventricle, right heart ventricle, pancreas, kidney, and adrenal tissue were collected into 2 mL CryoELITE tubes (Wheaton, Millville, NJ) and flash frozen in liquid Nitrogen. The samples were stored at -80° C for further analysis. Identical samples were collected into

Embedding Cassettes (EconoLab, Chambly, Quebec) and stored in 10 % Formalin for Histology. A complete placentome was randomly collected from the uterus (cut into eighths) and stored in 50 mL tubes with 10 % neutral buffered Formalin for histology.

All placentomes from each cow were counted and greatest and least diameter of each at the fetal-maternal interface was recorded. The average diameter of each placentome was used to calculate surface area of each individual placentome, total placentome surface area, and average surface area per placentome. Placentomes were then divided into cotyledonary and caruncular tissue, separated by tissue type, and total caruncular and total cotyledonary tissue weights were recorded. The tissue weights were used to calculate the ratio of fetal and maternal contribution to placentome.

Histology

All cassette samples were transferred from neutral 10 % Formalin buffer to paraffin wax using the Leica ASP 300 Advanced Smart Processor (Leica Biosystems, Buffalo Grove, IL). Samples were embedded in paraffin using Tissue-Tek Base Molds (Electron Microscopy Sciences, Hatfield, PA) and stored at room temperature. The placentome samples were kept in 50mL tubes and were transferred from neutral 10 % Formalin to 70 % Ethanol by hand. Prior to embedding, placentomes were sliced into approximately 3 mm thick samples and set into Embedding Cassettes. The cassettes were then placed in the Leica ASP 300 Advanced Smart Processor transferred from 70 % Ethanol to paraffin. Samples were then embedded in paraffin using Tissue-Tek Base Molds and stored at room temperature.

Perirenal adipose tissue embedded in paraffin was sectioned (5 μm) using a Leica RM 2165 microtome (Leico Biosystems, Buffalo Grove, Illinois). Four 5 μm sections, 5 sections apart were collected for evaluation. Tissues were deparaffinized with 100% xylene and rehydrated in graded ethanol solutions (100% – 95%). Sections were then placed in Gill's hematoxylin (Sigma-Aldrich, St. Louis, Missouri) for 5 minutes, rinsed rapidly with distilled water for 2 minutes, then transferred to Ammonium water (1 mL NH_4OH in 1L H_20) for 30 seconds, and then rinsed rapidly with distilled water for another 30 seconds. Slides were then stained in Eosin Y (Sigma-Aldrich, St. Louis, Missouri) for 3 minutes. Finally, slides were dehydrated in graded ethanol (100%), cleared with 100% xylene, and cover slipped with Permount (Fisher Scientific). Images of adipocytes were captured digitally at 10x magnification using iSolution Lite Version 9.4 software (Image & Microscope Technology Inc., Vancouver, British Columbia, Canada). Two randomly chosen fields were captured per section for a total of 8 pictures per animal. Cell diameter was evaluated on fifty randomly chosen cells per field for a total of 400 adipocytes per animal using iSolution Lite Version 9.4 software. The histological measure of cell diameter has been previously validated using DNA quantification (Long et al., 2012).

Colorimetric Assays

Animals were blocked by treatment (NR or CON) and gestation group (d 110 or d 190) into assay plates. All d 190 samples (n = 22) were run separately from d 110 samples (n = 16), but within the gestation group, all treatments were represented on the assay block. All colorimetric reagents were obtained from Pointe Scientific (Canton, MI).

Glucose concentrations were determined by previously colorimetric assay previously validated in our lab (Long et al., 2013a) on both maternal and fetal plasma for early and midgestation animals. Intrassay and interassay CV of the assays were 3.5% and 3.1%, respectively. Triglyceride concentrations were determined by colorimetric assay previously validated in our lab (Long et al., 2013a) on both maternal and fetal serum for early and midgestation animals. Intrassay and interassay CV for late gestation animals were 2.4% and 3.1%, respectively. Cholesterol concentrations were determined by colorimetric assay previously validated in our lab (Long et al., 2013a) on both maternal and fetal serum for early and midgestation animals. Intrassay and interassay CV were 2.7% and 3.7%, respectively.

Statistics

For d 110 animals, BW and BCS change was analyzed as an ANOVA analysis using the GLM procedure of SAS. Fetal weights and measurements, uterine weights, placentome measures, plasma glucose in dams and fetuses at harvest, serum triglycerides in dams and fetuses at harvest, and serum cholesterol in dams and fetuses at harvest were analyzed using the MIXED procedure of SAS with treatment, sex, and their interaction in the model statement. Maternal plasma glucose for d 110 animals was analyzed as a repeated measure analysis using the MIXED procedure of SAS with treatment, day, and their interaction in the model statement. For d 190 animals, BW and BCS changes, fetal weights and measurements, uterine weights, placentome measures, plasma glucose in dams and fetuses at harvest, serum triglycerides in dams and fetuses at harvest, and serum cholesterol in dams and fetuses at harvest and adipocyte diameter were analyzed as an

ANOVA analysis using the GLM procedure of SAS. Maternal plasma glucose was analyzed as a repeated measure analysis using the MIXED procedure of SAS with treatment, day, and their interaction in the model statement. A tendency was defined as $P < 0.10$ and a significance difference was defined as $P < 0.05$

RESULTS

Maternal BW and BCS change (Table 1) showed that d 110 NR cattle had a decrease in both BW ($P < 0.0001$) and BCS ($P < 0.0001$) from d 30 – d 110 when compared with d 110 CON cattle. In d 190 cows, NR/CON cattle had a decrease in both BW ($P < 0.0001$) and BCS ($P = 0.0008$) from d 30 – d 114 when compared with CON/NR and CON cattle. A decrease in BW ($P < 0.0001$) and BCS ($P < 0.0001$) occurred in CON/NR cows from d 114 – d 190 when compared with NR/CON and CON cattle.

Monthly plasma glucose concentrations of d 110 cows (Figure 2) exhibited a treatment x day effect ($P = 0.0018$), with the plasma concentration of the NR group decreased ($P < 0.05$) compared with CON cows at d 56 and this decrease was maintained until harvest. Maternal plasma glucose concentrations at harvest of d 110 cows (Figure 3) were decreased ($P = 0.0267$) in dams on the NR diet versus dams on the CON diet at time of harvest. Fetal plasma glucose concentrations at harvest of d 110 cows (Figure 3) were decreased ($P = 0.0137$) in fetuses from dams on the NR diet versus fetuses from dams on the CON diet. Monthly plasma glucose concentrations of d 190 cows (Figure 4) exhibited a treatment x day effect ($P = 0.0001$). Cows that were nutritionally restricted on d 30 – d 110 had decreased ($P < 0.05$) plasma glucose compared with animals on the control diet

during the same time period on d 58 and d 86. On d 114, both nutrient restricted groups had decreased ($P < 0.05$) plasma glucose compared with the control cows. Cows that were nutritionally restricted on d 110 – d 190 had decreased ($P < 0.05$) plasma glucose compared with animals on the control diet during the same time period on d 142 and 170. Maternal plasma glucose concentrations at harvest of d 190 cows (Figure 5) were decreased ($P = 0.0009$) in dams on the NR diet, while plasma glucose concentrations were similar between animals on the CON diet at harvest. Fetal plasma glucose concentrations at harvest of d 190 cows (Figure 5) were decreased ($P = 0.049$) in fetuses from dams on a NR diet, while plasma glucose concentrations were similar between fetuses from dams on CON diets prior to harvest.

Maternal serum triglyceride concentrations at harvest of d 110 cows (Figure 3) was decreased ($P = 0.0029$) in NR dams compared with CON dams at time of harvest. Fetal serum triglyceride concentrations at harvest of d 110 cows (Figure 3) was decreased ($P = 0.0331$) in fetuses from CON dams compared with NR dams. Maternal serum triglyceride concentrations at harvest of d 190 cows (Figure 5) was decreased ($P = 0.0078$) in dams on the NR diet compared with CON diet. Fetal serum triglyceride concentrations at harvest of d 190 fetuses (Figure 5) was decreased ($P = 0.0005$) in CON/NR versus CON fetuses, however NR/CON fetuses had an increased ($P < 0.05$) serum triglyceride concentration.

Maternal serum cholesterol concentrations at harvest of d 110 cows (Figure 3) were similar to each other ($P = 0.9836$) regardless of treatment. Fetal serum cholesterol concentrations at harvest of d 110 cows (Figure 3) were similar to each other ($P = 0.2845$) regardless of treatment. Maternal serum cholesterol concentrations at harvest of d

190 cows (Figure 5) had a tendency to be decreased ($P = 0.0976$) in dams on the NR diet compared with CON diet. Fetal serum concentrations at harvest of d 190 cows (Figure 5) were decreased ($P = 0.0194$) in fetuses from dams on the NR diet compared with fetuses from dams on the CON diet.

Fetal size and weights at d 110 of gestation are shown in Table 2. Fetal weights and empty fetal weights were reduced ($P = 0.0027$, $P = 0.0023$, respectively) in the NR fetuses versus CON fetuses. Crown rump length and abdominal circumference was unaffected ($P = 0.0110$, $P = 0.0265$, respectively), while thoracic circumference was reduced in NR fetuses versus CON fetuses ($P = 0.0002$). Fetal wt was increased ($P = 0.0095$), in male CON fetuses versus male NR fetuses and female NR and CON fetuses. Empty fetal weight was ($P = 0.01$) reduced due to treatment in the NR fetuses versus the CON fetuses. Crown rump length and thoracic circumference were ($P = 0.0462$, $P = 0.0026$, respectively) reduced due to treatment in the NR fetuses versus CON fetuses while abdominal circumference only had a tendency ($P = 0.0795$) to be reduced due to treatment in NR fetuses versus CON fetuses. Liver weights had a tendency to be decreased ($P = 0.0928$) because of treatment in NR fetuses versus CON fetuses. Pancreas weights were reduced ($P = 0.0058$) due to treatment in NR fetuses versus CON fetuses. Left LD muscle weights had a tendency to be decreased ($P = 0.0764$) in NR fetuses versus CON fetuses, while the right LD muscle had a tendency to be deceased in female NR fetuses ($P = 0.0668$) versus female CON fetuses and male NR and CON fetuses. Brain weight had a tendency ($P = 0.0989$) to be increased in male CON fetuses versus male NR fetuses and female NR and CON fetuses. The heart, left atrium, right ventricle, and septum were unaffected while the right atrium weight had a tendency ($P = 0.086$) to

be reduced in NR fetuses versus CON fetuses and the left ventricle weight had a tendency ($P = 0.0525$) to be decreased in female CON fetuses versus female NR fetuses and male CON and NR fetuses. Ventricle wall thickness was unaffected but septum wall thickness was increased ($P = 0.0239$) in NR fetuses versus CON fetuses. The right and left kidneys ($P = 0.43$, $P = 0.63$, respectively), lungs ($P = 0.14$), and adrenal glands ($P = 0.12$) were unaffected.

Fetal organ weights as a percent of fetal weight for d 110 fetuses are shown in Table 3. Brain weight as a percent of fetal weight was increased ($P = 0.0006$) in NR male fetuses versus CON male fetuses, with no differences seen in female fetuses. Total lung weight as a percent of fetal weight was increased ($P = 0.0463$) in female fetuses versus male fetuses. Right atrium weight as a percent of fetal weight had a tendency ($P = 0.073$) to be increased in CON fetuses versus NR fetuses. Pancreas weight as a percent of fetal weight had a tendency ($P = 0.0521$) to be increased in CON fetuses versus NR fetuses. Right and left kidney weight as a percent of fetal weight had a tendency ($P = 0.083$ and $P = 0.0907$, respectively) to be increased in female fetuses versus male fetuses. Fetal total heart, left and right ventricles, left atrium, septum, liver, adrenal, LD muscles, viscera, and perirenal adipose tissue weight as a percent of fetal weight were unaffected by either maternal diet or fetal sex.

Fetal organ weights as a percent of empty fetal weight for d 110 fetuses are shown in Table 4. Total lung weight and total heart weight as a percent of empty fetal weight was increased ($P = 0.027$ and $P = 0.0603$) in female fetuses versus male fetuses. Pancreas weight as a percent of empty fetal weight had a tendency ($P = 0.058$) to be increased in CON fetuses versus NR fetuses. Right and left kidneys as a percent of empty fetal weight

were increased ($P = 0.044$ and $P = 0.0402$, respectively) in female fetuses versus male fetuses. Perirenal adipose as a percent of empty fetal weight was increased ($P = 0.0066$) in female NR fetuses versus female CON fetuses, with no differences between male fetuses. Brain weight, left and right ventricle, left and right atrium, septum, liver, adrenal, LD muscles, and viscera weight as a percent of empty fetal weight were unaffected by either maternal diet or fetal sex.

Fetal size and weights, as well as fetal organ weights at d 190 of gestation, are shown in Table 5. Fetal weights tended to be reduced ($P = 0.07$) in NR/CON and CON/NR versus. CON/CON cattle and empty fetal weights were reduced ($P = 0.03$) in NR/CON and CON/NR versus. CON/CON cattle. Abdominal and thoracic circumference was reduced ($P = 0.01$, $P = 0.03$, respectively) in NR/CON and CON/NR fetuses versus. CON/CON fetuses, but crown rump length was unaffected ($P = 0.32$). Pancreas weight was reduced ($P = 0.02$) in NR/CON and CON/NR compared with CON/CON fetuses. Fetal liver weight was also reduced ($P = 0.04$) in NR/CON and CON/NR versus. CON/CON. Right LD muscle was decreased ($P = 0.006$) in NR/CON and CON/NR versus. CON/CON fetuses as well as left LD muscle ($P = 0.045$) in NR/CON and CON/NR versus. CON/CON fetuses. Perirenal adipose weight tended to be reduced ($P = 0.08$) in NR/CON and CON/NR compared with CON/CON. Total heart weight tended to be reduced ($P = 0.07$) in NR/CON and CON/NR fetuses versus. CON/CON fetuses and left ventricular weights were reduced ($P = 0.03$) in NR/CON and CON/NR fetuses versus. CON/CON fetuses but atria, the right ventricle, and septum weights were similar between groups. Left ventricle thickness tended to be increased ($P = 0.07$) in NR/CON

and CON/NR fetuses versus. CON/CON fetuses but right ventricles thickness ($P = 0.36$) and septum thickness ($P = 0.44$) were both unaffected.

Fetal organs weights as a percent of fetal weight for d 190 fetuses are shown in Table 6. Brain weight as a percent of fetal weight was increased ($P = 0.001$) in CON/NR and NR/CON fetuses versus. CON/CON fetuses. Total heart weight as a percent of fetal weight was increased ($P = 0.01$) in NR/CON and CON/NR fetuses versus. CON/CON fetuses. Left ventricle weight as a percent of fetal weight ($P = 0.004$) was increased in NR/CON and CON/NR fetuses versus. CON/CON fetuses as well as right ventricle weight ($P = 0.05$) in NR/CON and CON/NR fetuses versus. CON/CON fetuses while the left atria ($P = 0.26$), right atria ($P = 0.35$) and septum ($P = 0.11$) percentages were unaffected. Pancreas weight as a percent of fetal weight tended to be decreased ($P = 0.06$) in NR/CON and CON/NR fetuses compared with CON/CON fetuses. Perirenal adipose weight as a percent of fetal weight had a tendency to be increased ($P = 0.06$) in NR/CON and CON/NR fetuses compared with CON/CON fetuses. Fetal lungs, kidneys, adrenal, liver, and LD muscle as a percentage of fetal weight were unaffected by maternal diet.

Fetal organ weights as a percent of empty fetal weight for d 190 fetuses are shown in Table 7. Brain weight as a percent of empty fetal weight was increased ($P = 0.0002$) in NR/CON and CON/NR fetuses versus. CON/CON fetuses. Total heart weight as a percent of empty fetal weight was increased ($P = 0.04$) in NR/CON and CON/NR fetuses versus. CON/CON fetuses. Left ventricle weight as a percent of empty fetal weight was increased ($P < 0.0001$) in NR/CON and CON/NR fetuses versus. CON/CON as well as right ventricle weight ($P = 0.001$) in NR/CON and CON/NR fetuses versus. CON/CON fetuses. Septum weight as a percent of empty fetal weight was also increased ($P = 0.04$)

in NR/CON and CON/NR fetuses versus. CON/CON. Fetal pancreas weight as a percent of empty fetal weight was reduced ($P = 0.04$) in NR/CON and CON/NR fetuses versus. CON/CON fetuses and fetal perirenal adipose as a percent of empty fetal weight was increased ($P = 0.003$) in NR/CON and CON/NR fetuses versus. CON/CON fetuses. Average adipocyte diameter in perirenal adipose tissue from d 190 fetuses was unaffected between treatment groups ($P < 0.15$)

Uterus weights and placenta characteristics for d 110 and d 190 fetuses are shown in Table 8. In d 190 and d 110 fetuses, no differences were found between gravid uterus weights ($P = 0.4$, $P = 0.13$, respectively) and while there was no difference found between empty uterus weights of d 190 fetuses ($P = 0.732$), d 110 fetuses showed a tendency for decreased empty uterus weight in NR fetuses versus CON fetuses ($P = 0.0887$). In d 110 fetuses, total placentome weight and total caruncle weight was unaffected, while total cotyledonary weight was increased ($P = 0.0069$) in NR fetuses versus CON fetuses. Ratio of cotyledonary weight to caruncle weight had a tendency ($P = 0.0879$) to be increased in NR fetuses versus CON fetuses. Number of placentomes was increased ($P = 0.0282$) in NR fetuses versus CON fetuses. Average surface area per placentome did not differ between treatment groups, but total placentome surface area was increased ($P = 0.0281$) in NR fetuses versus CON fetuses. In d 190 fetuses, total placentome weight ($P = 0.05$) and total caruncle weight ($P = 0.07$) was decreased in NR/CON animals versus. CON/CON and CON/NR animals. Total cotyledonary weight was increased ($P = 0.01$) in CON/NR fetuses versus. CON/CON and NR/CON. Ratio of cotyledonary weight to caruncle weight was increased ($P = 0.01$) in NR/CON and CON/NR animals versus. CON/CON. Number of placentomes did not differ between

treatment groups. Average surface area per placentome did not differ, but total placentome surface area had a tendency to be increased ($P = 0.09$) in CON/NR animals versus CON and NR/CON.

Adipocyte diameter did not differ (P = 0.15) regardless of treatment in d 190 fetuses (Figure 6).

DISCUSSION

To the authors' knowledge, this is the first study with maternal nutrient restriction during early or mid gestation in cattle without subsequent realimentation with fetal endpoints. Previous studies have shown in multiple mammal species that maternal nutrient restriction during early and midgestation can lead to intrauterine growth restriction (IUGR) exhibited by asynchronous organ growth (i.e., enlarged hearts and brains) (Schoknecth et al., 1994; McMillen et al., 2001; Vonnahme et al., 2003; Platz et al., 2008; Long et al., 2009), even with realimentation following the period of nutrient restriction. This study revealed asynchronous organ growth in fetuses from nutrient restricted dams regardless of which trimester the nutrient restriction took place. Further, IUGR fetuses are more likely to develop metabolic issues such as obesity, glucose/insulin dysregulation, and cardiovascular disease in postnatal life (Poore et al., 2002; Gardner et al., 2005; Gilbert et al., 2005). Other studies with maternal nutrient restriction (Vonnahme et al., 2007; Camacho et al., 2013) have shown similar maternal BW and BCS loss in restricted cows as seen in this study. Body weight and BCS loss was more significant in this study than other early gestation maternal nutrient restriction

(Vonnahme et al., 2007; Camacho et al., 2013), despite similar restriction levels because our diet did not incorporate nutrition requirements for pregnancy.

Plasma glucose was decreased in NR dams and fetuses at both d 110 and d 190 of gestation. This data is consistent with that of Ford et al. (2007) who observed decreased plasma glucose concentrations in ewes on 50% nutrient restricted diet during the first half (d 28 – 78) of gestation. A similar trend was seen in a nutrition study utilizing obese pregnant ewes. Obese ewes and their fetuses had the higher plasma glucose levels, reflecting their increased nutritional status. Control ewes and their fetuses had the lower plasma glucose levels of the study, reflecting their comparatively lower nutritional status (Tuersunjiang et al., 2013). In contrast, a study from that of Long et al. (2009), showed that not all NR cattle and fetuses would exhibit decreased plasma glucose concentrations, though this was speculated to result from increased age of the cattle and therefore an increased ability to adequately partition nutrients to maintain normal fetal growth.

Serum triglycerides were decreased in NR dams and fetuses at d 110 and d 190 of gestation. Ma et al. (2011) also showed significant decreases of triglyceride concentrations in fetuses from nutrient restricted ewes versus control ewes during the first half of gestation (d 78). In a study by Zhu et al. (2010b), obese ewes and their fetuses had increased triglyceride concentrations relative to their control counterparts as a reflection of their increased nutritional status. In contrast, pregnant rats fed a high fat diet and/or supplemented with lipopolysaccharides (Hao et al., 2014) showed no changes in maternal serum triglyceride levels from the control group.

While the fetus can synthesize cholesterol on its own, there is still transport of maternal cholesterol to the fetal environment (Woollett 2011). During early gestation,

when demand for growth is lower, cholesterol is not as compulsory as is indicated by the similarities in serum cholesterol concentration at harvest in either maternal or fetal serum, regardless of treatment in d 110 animals. This trend was similar to a sheep study by Ma et al. (2011), where fetal cholesterol concentrations from nutrient restricted or control ewes was unaffected. By midgestation (d 110 – d 190) as demand for cholesterol increases, nutrient restricted animals are less likely to be able to meet the demand, as seen in the significant decrease of serum cholesterol in nutrient restricted d 190 dams and fetuses. With decreased maternal cholesterol, there is decreased birth weight and a possibility of microcephaly in children (Edison et al., 2007). Other studies have found that mice exhibited reduced growth rates in nutrient restricted dams (McConihay et al., 2000) and in humans; there is a positive correlation between intrauterine growth restriction and low serum cholesterol (Sattar et al., 1999; Wadsack et al., 2007).

Placentome number was increased in d 110 nutrient restricted animals, which is in contrast to data in Long et al. (2009) where nutrient status did not affect placentome number in early gestation cattle. Increased placentome number also contrasts with data presented by Vonnahme et al. (2006), in which Baggs ewes restricted during the first half of gestation (d 28 – 78) had similar placentome number to control ewes. However the same study showed an increased placentome number in twin bearing, nutrient restricted Baggs ewes (sheep selected based on nomadic lifestyle). Placentome number was unaffected in d 190 cattle, which is in contrast to data in Long et al. (2009), where nutrient restricted animals had decreased placentome numbers, but is in agreement to data presented by Ma et al. (2011) in which nutrient restriction during early gestation had no effect on placentome numbers in sheep. Total placentome surface area was increased for

d 110 nutrient restricted cattle and d 190 cattle that were restricted from d 110 – d 190, which contrasts with data presented in Long et al. (2009). In the present study we presented a decreased placentome weight in d 190 animals that were restricted d 30 – 110 of gestation, which is in contrast to data presented by Ma et al. (2011) in which ewes that were nutrient restricted during early gestation had similar placentome weights after a period of realimentation. In contrast to both the present study and Ma et al. (2011), work done on the sheep model by Vonnahme et al. (2006) showed an increase in placentome weight in twin-bearing, nutrient restricted, and twin-bearing nutrient restricted ewes during early gestation, except the Baggs ewe model showed a decrease in placentome weight in nutrient restricted single bearing ewes compared with single bearing control fed ewes.

Caruncle weights for d 110 cows were unchanged between nutrient restricted and control treatments, consistent with data reported by Long et al., (2009). Caruncle weights were decreased in d 190 animals that were restricted d 30 – 110 of gestation, which contrasts with the data of Long et al. (2009) and Rasby et al. (1990), in which caruncular weight was unaffected based on diet. However, decreased caruncular weight was reported in ewes carrying twin fetuses versus ewes carrying singleton fetuses (van der Linden et al., 2012), because twin-bearing ewes were at a higher nutrient disadvantage. Cotyledonary weights were increased for nutrient restricted d 110 cows, which is in contrast to the data of Long et al. (2009). Cotyledonary weights were increased for d 190 cows when restricted from d 110 – 190, which contrasts with data produced by van der Linden et al. (2010), in which twin-bearing ewes (higher nutrient disadvantage) had

decreased cotyledonary weights. However, Rasby et al. (1990) did show an increase in cotyledonary weight for nutrient restricted cattle versus control fed cattle.

The placenta, and therefore nutrient transport, can respond in one of two ways when exposed to maternal nutrient restriction. The placental nutrient sensing model and the fetal nutrient demand model work through entirely different mechanisms, result in two different placental responses, but can also occur simultaneously. In the placental nutrient sensing model (Jansson et al., 2006a; Jansson et al., 2006b; Rosario et al., 2011), the placenta responds to decreased maternal nutrition by downregulation of placental nutrient transporters. In the fetal demand model (Constancia et al., 2005; Sibley et al., 2010; Angiolini et al., 2011), the fetus signals to the placenta to upregulate placenta nutrient transporters. The opposing action of these two models and its subsequent results can differ based on the species as well as the time, duration, and severity of the nutrient restriction.

There was a slight sex effect in d 110 animals, with the kidneys and lungs as a percent of fetal weight of male fetuses being decreased compared with female fetuses. While we can only speculate on the cellular composition of the kidneys, previous studies have shown decreased glomerular number and glomeruli per gram of tissue in nutrient restricted fetuses (Long et al., 2009) and that inefficiency of nutrient transport across the placenta can decrease nephron numbers by as much as 30% in rats, rabbits, pigs, and sheep. This decrease in nephron number correlates with a decrease in filtration efficiency of the kidneys (Merlet-Benichou et al., 1994; Bassan et al., 2000; Bauer et al., 2002). Furthermore, nutrient restriction of ewes during the first half of pregnancy showed a reduction in glomeruli at d 135 gestation in male fetuses (Gilbert et al., 2007). Lastly,

male fetuses from 50% nutrient restricted ewes during the first half of gestation showed reduced nephron numbers, which correlated with increased blood pressure (Gilbert et al., 2005). Again, while we can only conjecture as to the molecular composition of the lungs, previous studies in rats have shown that maternal nutrient restriction have led to altered lung structure of the offspring (Rehan et al., 2014) including decreased alveolar numbers and increased septal thickening in response to increased glucocorticoids caused by the stress of the dam's nutrient restriction.

In d 110 animals, fetal weight, brain weight, left ventricle weight, brain weight as a percent empty fetal weight, and perirenal adipose tissue as a percent of empty fetal weight exhibited a treatment x sex effect, in which fetuses that were both male and nutrient restricted showed increased evidence of growth restriction. Typically, male fetuses grow quicker and heavier than female fetuses (Pederson 1980) and are therefore more susceptible to nutrient restriction. This variance in growth rates could mean that nutrient restriction may have differing levels of severity depending on the sex of the fetus, or that nutrient restriction during a particular time of gestation may have increased effects on one of the two sexes.

Increased percentage of perirenal adipose tissue in nutrient restricted fetuses has been indicated by the "thrifty phenotype", with offspring from undernourished dams being programmed for decreased nutrients in postnatal life if subjected to undernutrition during gestation (Hales and Barker, 2001). This increase in relative perirenal adipose tissue depots has been reported in twin bearing ewes on a 70% restricted diet during gestation (Edwards et al., 2005). Twin fetuses from restricted ewes were potentially more severely restricted than singleton fetuses from restricted ewes because of increased

nutrient partitioning in utero. This increase in adipose percentage may alter appetite regulation in the postnatal animal, caused by changes in endocrine secretions from the adipose tissue (Wang et al., 2008). This alteration of appetite and increased adiposity leads to further metabolic disorders such as insulin and glucose dysregulation, also observed in sheep that were restricted during early (d 28 - 78) gestation (George et al., 2012).

Average adipocyte diameter of d 190 fetuses was unaffected, regardless of treatment group, which is in contrast to Long et al. (2012), in which nutrient restricted fetuses had slightly increased average adipocyte diameter and suggested that this could be because of a depot specific effect. However, the fetuses in this project were sampled at d 190 instead of sampling from the postnatal animal as done in Long et al. (2012). Furthermore, the cattle in this study were not realimented as the cattle in the previously reported study, which could be why no changes in adipocyte diameter were observed.

In conclusion, while it has been previously illuminated that nutrient restriction during early gestation causes IUGR and decreased fetal development, midgestation nutrient restriction has now been shown to cause the same damaging effects to the same extent, even with no nutritional stress during early gestation. Realimentation of animals after a period of nutrient restriction alleviates some of the damaging results of nutrient restriction, but without realimentation this study shows that the effects of nutrient restriction cannot be overcome. Nutrient restriction without subsequent realimentation has a higher level of detriment on uterine characteristics, further limiting the nutrient transport from dam to fetus.

Table 1: Maternal BW (kg) and BCS change throughout gestation for d 110 and d 190 animals[1]

d 110 animals

Item	CON	NR	*P* value
n	8	8	
Initial BW	573.8 ± 47.0	553.9 ± 61.3	
d 30 - 110 BW	11.88 ± 3.33	-53.52 ± 3.14	< 0.0001
Initial BCS	5.81 ± 0.49	5.72 ± 0.63	
d 30 - 110	0.125 ± 0.169	-1.222 ± 0.159	< 0.0001

d 190 animals

Item	CON	NR/CON	CON/NR	*P* value
n	8	7	7	
Initial BW	537.4 ± 21.8	537.1 ± 23.3	522.0 ± 23.3	0.866
Initial BCS	5.31 ± 0.16	5.57 ± 0.18	5.21 ± 0.18	0.351
d 30 – 114 BW	11.70 ± 4.36	-50.19 ± 4.66	12.91 ± 4.66	< 0.0001
d 30 – 114 BCS	0.12 ± 0.20	-1.14 ± 0.21	0.00 ± 0.21	0.0008
d 114 – 190 BW	14.48 ± 4.46	19.28 ± 4.77	-77.33 ± 4.77	< 0.0001
d 114 – 190 BCS	-0.06 ± 0.14	0.28 ± 0.15	-1.28 ± 0.15	< 0.0001

[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NEm and CP recommendations

Table 2: Fetal measurements and selected organ weights of male and female fetuses on d 110 of gestation from CON and NR cattle[1]

Item	CON		NR		*P* value		
	Male	Female	Male	Female	trt	sex	trt*sex
n	6	2	6	2			
Fetal wt kg	0.71 ± 0.01	0.62 ± 0.01	0.59 ± 0.01	0.63 ± 0.02	0.03	0.20	0.01
Empty Fetal Weight	0.54 ± 0.01	0.49 ± 0.02	0.47 ± 0.01	0.46 ± 0.02	0.01	0.10	0.31
Crown rump length, cm	26.6 ± 0.3	26.2 ± 0.6	25.3 ± 0.3	25.2 ± 0.6	0.05	0.64	0.76
Abdominal circumference, cm	20.3 ± 0.9	19.5 ± 1.6	17.4 ± 0.9	17.1 ± 1.6	0.08	0.68	0.86
Thoracic circumference, cm	18.8 ± 0.2	18.5 ± 0.4	17.2 ± 0.2	17.5 ± 0.4	0.003	0.91	0.41
Viscera wt, g	53.8 ± 6.0	53.5 ± 10.5	45.5 ± 6.0	49.8 ± 10.5	0.50	0.82	0.79
Liver wt, g	24.6 ± 0.8	21.5 ± 1.4	20.9 ± 0.8	21.0 ± 1.4	0.09	0.22	0.19
Pancreas, g	0.61 ± 0.07	0.91 ± 0.12	0.41 ± 0.07	0.44 ± 0.12	0.01	0.14	0.22
Total lung wt, g	20.1 ± 1.1	17.9 ± 2.0	18.3 ± 1.1	21.3 ± 2.0	0.65	0.80	0.14
Right kidney wt, g	2.8 ± 0.1	2.8 ± 0.3	2.4 ± 0.1	2.9 ± 0.3	0.60	0.40	0.43
Left kidney wt, g	2.82 ± 0.23	2.92 ± 0.40	2.5 ± 0.2	3.00 ± 0.40	0.78	0.43	0.63
Adrenal wt, g	0.24 ± 0.21	0.99 ± 0.36	0.56 ± 0.21	0.32 ± 0.36	0.57	0.42	0.12
Brain wt, g	12.4 ± 0.3	12.6 ± 0.6	12.9 ± 0.3	11.1 ± 0.6	0.34	0.15	0.07
Right LD muscle, g	6.3 ± 0.2	5.4 ± 0.4	4.7 ± 0.2	5.0 ± 0.4	0.02	0.39	0.10
Left LD muscle, g	6.4 ± 0.3	5.2 ± 0.5	4.7 ± 0.3	5.1 ± 0.5	0.08	0.41	0.10
Heart wt, g	5.5 ± 0.3	5.2 ± 0.6	4.7 ± 0.3	5.2 ± 0.6	0.45	0.81	0.50
Left atrium wt, g	0.55 ± 0.06	0.26 ± 0.11	0.32 ± 0.06	0.32 ± 0.11	0.40	0.16	0.16
Right atrium wt, g	0.45 ± 0.04	0.38 ± 0.07	0.27 ± 0.04	0.33 ± 0.07	0.09	0.87	0.32
Left ventricle wt, g	1.36 ± 0.1	0.82 ± 0.2	1.05 ± 0.1	1.41 ± 0.2	0.50	0.65	0.05
Right ventricle wt, g	0.94 ± 0.15	0.09 ± 0.26	1.03 ± 0.15	1.04 ± 0.26	0.59	0.96	0.91
Septum wt, g	1.34 ± 0.08	0.8 ± 0.1	1.22 ± 0.08	1.1 ± 0.1	0.52	0.05	0.13
Avg left venticle thickness, mm	4.1 ± 0.2	3.1 ± 0.5	4.5 ± 0.2	4.9 ± 0.4	0.02	0.41	0.11
Avg right venticle thickness, mm	3.0 ± 0.1	3.0 ± 0.4	3.9 ± 0.1	3.4 ± 0.3	0.05	0.40	0.43
Avg septum thickness, mm	4.0 ± 0.1	3.7 ± 0.4	4.7 ± 0.1	4.7 ± 0.3	0.02	0.72	0.57
Perirenal adipose wt, g	1.69 ± 0.20	1.57 ± 0.34	1.47 ± 0.20	1.57 ± 0.34	0.72	0.98	0.70

[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NEm and CP recommendations

Table 3: Measurements and selected organ weights of male and female fetuses on d 110 of gestation from CON and NR cattle[1]

Item	Female		Male		*P* value		
	CON	NR	CON	NR	trt	sex	trt*sex
n	2	2	6	6			
Fetal wt kg	0.62 ± 0.01	0.63 ± 0.02	0.71 ± 0.01	0.59 ± 0.01	0.03	0.20	0.01
CRL, cm	26.2 ± 0.6	25.2 ± 0.6	26.6 ± 0.3	25.3 ± 0.3	0.05	0.64	0.75
Abdominal circ, cm	19.5 ± 1.6	17.1 ± 1.6	20.3 ± 0.9	17.4 ± 0.9	0.08	0.68	0.86
Thoracic circ, cm	18.5 ± 0.4	17.5 ± 0.4	18.8 ± 0.2	17.2 ± 0.2	0.003	0.91	0.41
Viscera wt, g	53.5 ± 10.5	49.8 ± 10.5	53.8 ± 6.0	45.5 ± 6.0	0.50	0.82	0.79
Liver wt, g	21.5 ± 1.4	21.0 ± 1.4	24.6 ± 0.8	20.9 ± 0.8	0.09	0.22	0.19
Pancreas, g	0.91 ± 0.12	0.44 ± 0.12	0.61 ± 0.07	0.41 ± 0.07	0.01	0.14	0.22
Total lung wt, g	17.9 ± 2.0	21.3 ± 2.0	20.1 ± 1.1	18.3 ± 1.1	0.65	0.80	0.14
Rt kidney wt, g	2.8 ± 0.3	2.9 ± 0.3	2.8 ± 0.1	2.4 ± 0.1	0.60	0.40	0.43
Lt kidney wt, g	2.9 ± 0.4	3.0 ± 0.4	2.8 ± 0.2	2.5 ± 0.2	0.78	0.43	0.63
Adrenal wt, g	0.99 ± 0.36	0.32 ± 0.36	0.24 ± 0.21	0.56 ± 0.21	0.57	0.42	0.12
Brain wt, g	12.6 ± 0.6	11.1 ± 0.6	12.4 ± 0.3	12.9 ± 0.3	0.34	0.15	0.07
Rt LD muscle, g	5.4 ± 0.4	5.0 ± 0.4	6.3 ± 0.2	4.7 ± 0.2	0.02	0.39	0.01
Lt LD muscle, g	5.2 ± 0.5	5.1 ± 0.5	6.4 ± 0.3	4.7 ± 0.3	0.08	0.41	0.10
Heart wt, g	5.2 ± 0.6	5.2 ± 0.6	5.5 ± 0.3	4.7 ± 0.3	0.45	0.81	0.51
Lt atrium wt, g	0.26 ± 0.11	0.32 ± 0.11	0.55 ± 0.06	0.32 ± 0.06	0.40	0.16	0.16
Rt atrium wt, g	0.38 ± 0.07	0.33 ± 0.07	0.45 ± 0.04	0.27 ± 0.04	0.09	0.87	0.32
Lt ventricle wt, g	0.82 ± 0.2	1.41 ± 0.2	1.36 ± 0.1	1.05 ± 0.1	0.50	0.65	0.05
Rt ventricle wt, g	0.09 ± 0.26	1.04 ± 0.26	0.94 ± 0.15	1.03 ± 0.15	0.59	0.96	0.91
Septum wt, g	0.8 ± 0.1	1.1 ± 0.1	1.34 ± 0.08	1.22 ± 0.08	0.52	0.05	0.13
Avg lt venticle thickness, mm	3.1 ± 0.5	4.9 ± 0.4	4.1 ± 0.2	4.5 ± 0.2	0.02	0.41	0.11
Avg rt venticle thickness, mm	3.0 ± 0.4	3.4 ± 0.3	3.0 ± 0.1	3.9 ± 0.1	0.05	0.40	0.43
Avg septum thickness, mm	3.7 ± 0.4	4.7 ± 0.3	4.0 ± 0.1	4.7 ± 0.1	0.02	0.72	0.57
Perirenal adipose wt, g	1.57 ± 0.34	1.57 ± 0.34	1.69 ± 0.20	1.47 ± 0.20	0.72	0.98	0.70

[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations
Rt: right
Lt: left
circ: circumference

Table 4: Organ weights divided by weight of fetus (FW) at d 110 of gestation from CON and NR cattle[1]

Item	Female		Male		P value		
	CON	NR	CON	NR	trt	sex	trt* sex
n	2	2	6	6			
Brain wt, g/FW, g	0.020 ± 0.001	0.017 ± 0.001	0.017 ± 0.001	0.022 ± 0.001	0.29	0.52	<0.01
Lung wt, g/FW, g	0.035 ± 0.003	0.033 ± 0.003	0.026 ± 0.001	0.031 ± 0.001	0.59	0.05	0.22
Heart wt, g/FW, g	0.009 ± 0.001	0.008 ± 0.001	0.007 ± 0.001	0.008 ± 0.001	0.60	0.10	0.14
Lt ventricle wt, g/FW, g	0.0015 ± 0.0004	0.0022 ± 0.0004	0.0018 ± 0.0002	0.0017 ± 0.0002	0.42	0.85	0.35
Rt venticle wt, g/FW, g	0.0018 ± 0.0004	0.0016 ± 0.0004	0.0011 ± 0.0002	0.0017 ± 0.0002	0.60	0.48	0.30
Lt atrium wt, g/fetal wt, g	0.0008 ± 0.0002	0.0005 ± 0.0002	0.0006 ± 0.0001	0.0005 ± 0.0001	0.28	0.90	0.63
Right atrium wt, g/FW, g	0.0007 ± 0.0001	0.0005 ± 0.0001	0.00061 ± 0.00006	0.00050 ± 0.00006	0.07	0.49	0.49
Septum wt, g/FW, g	0.0017 ± 0.0002	0.0018 ± 0.0002	0.0017 ± 0.0001	0.0020 ± 0.0001	0.44	0.48	0.58
Liver wt, g/FW, g	0.034 ± 0.002	0.033 ± 0.002	0.034 ± 0.001	0.035 ± 0.001	0.94	0.62	0.62
Pancreas wt, g/FW, g	0.0013 ± 0.0002	0.0007 ± 0.0002	0.0009 ± 0.0001	0.0007 ± 0.0001	0.05	0.31	0.27
Rt kidney wt, g/FW, g	0.0051 ± 0.0006	0.0047 ± 0.0006	0.0038 ± 0.0003	0.0042 ± 0.0003	1.00	0.08	0.43
Lt kidney wt, g/FW, g	0.0054 ± 0.0006	0.0048 ± 0.0006	0.0037 ± 0.0004	0.0043 ± 0.0004	1.00	0.09	0.31
Adrenal wt, g/FW, g	0.0014 ± 0.0005	0.0005 ± 0.0005	0.0003 ± 0.0003	0.0009 ± 0.0003	0.68	0.52	0.14
Lt LD muscle, g/FW, g	0.010 ± 0.001	0.008 ± 0.001	0.0085 ± 0.0006	0.0080 ± 0.0006	0.23	0.41	0.52
Rt LD muscle, g/FW, g	0.010 ± 0.001	0.008 ± 0.001	0.009 ± 0.001	0.008 ± 0.001	0.13	0.31	0.31
Viscera wt, g/FW, g	0.074 ± 0.015	0.078 ± 0.015	0.078 ± 0.009	0.077 ± 0.015	0.92	0.90	0.82
Perirenal adipose wt, g/FW, g	0.927 ± 0.082	0.788 ± 0.082	0.919 ± 0.047	0.706 ± 0.047	0.02	0.52	0.59

[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations
CRL: crown rump length
Rt: right
Lt: left

Table 5: Fetal measurements and selected organ weights on d 190 of gestation from CON, NR/CON, and CON/NR cows[1]

Item	CON	NR/CON	CON/NR	P value
n	8	7	7	
Fetal wt, kg	10.3 ± 0.4	9.1 ± 0.5	8.9 ± 0.5	0.08
Fetal empty carcass wt, kg	8.5 ± 0.4	7.2 ± 0.4	7.2 ± 0.4	0.03
CRL, cm	65.6 ± 1.4	62.4 ± 1.5	63.5 ± 1.5	0.32
Abdominal circ, cm	47.3 ± 0.9	43.5 ± 0.9	43.9 ± 0.9	0.02
Thoracic circ, cm	45.6 ± 0.8	42.7 ± 0.9	42.5 ± 0.9	0.03
Brain wt, g	106.5 ± 4.4	97.3 ± 4.7	100.9 ± 4.7	0.37
Heart wt, g	77.2 ± 5.0	65.2 ± 5.4	59.9 ± 5.4	0.08
Lt atrium wt, g	4.9 ± 0.6	5.1 ± 0.6	5.7 ± 0.6	0.65
Rt atrium wt, g	5.9 ± 0.8	3.9 ± 0.8	4.8 ± 0.8	0.23
Lt ventricle wt, g	21.4 ± 0.9	17.9 ± 0.9	18.9 ± 0.9	0.04
Rt ventricle wt, g	19.7 ± 1.4	16.7 ± 1.5	16.9 ± 1.5	0.28
Septum wt, g	18.2 ± 1.1	18.2 ± 1.2	17.2 ± 1.2	0.80
Avg lt venticle thickness, mm	10.3 ± 0.3	11.2 ± 0.4	11.4 ± 0.4	0.08
Avg rt venticle thickness, mm	8.4 ± 0.5	7.4 ± 0.5	7.8 ± 0.5	0.37
Avg septum thickness, mm	10.3 ± 0.4	11.1 ± 0.4	10.7 ± 0.4	0.44
Liver wt, g	315.2 ± 13.6	267.0 ± 14.6	271.0 ± 14.6	0.05
Lung wt, g	238.3 ± 19.25	218.2 ± 20.6	226.1 ± 20.6	0.78
Pancreas wt, g	6.3 ± 0.4	5.6 ± 0.4	4.5 ± 0.4	0.03
Rt kidney wt, g	39.2 ± 2.5	38.4 ± 2.6	35.7 ± 2.6	0.61
Lt kidney wt, g	42.3 ± 2.6	38.8 ± 2.8	35.8 ± 2.8	0.26
Adrenal wt, g	1.6 ± 0.2	1.7 ± 0.2	1.2 ± 0.2	0.21
Lt LD muscle, g	110.5 ± 7.0	93.9 ± 7.5	83.29 ± 7.5	0.05
Rt LD muscle, g	113.8 ± 6.7	91.6 ± 7.2	78.9 ± 7.2	0.01
Viscera wt, g	595.1 ± 38.6	557.8 ± 41.3	503.3 ± 41.3	0.29
Perirenal adipose wt, g	34.3 ± 1.7	28.3 ± 1.9	30.8 ± 1.9	0.09

[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations
CRL: crown rump length
circ: circumference
Rt: right
Lt: left

Table 6: Fetal organ weights as a percent of fetal weight (FW) on d 190 of gestation from CON, NR/CON, and CON/NR cows[1]

Item	CON	NR/CON	CON/NR	P value
n	8	7	7	
Brain wt, g/FW, g	1.00 ± 0.02	1.08 ± 0.02	1.15 ± 0.02	0.002
Total lung wt, g/FW, g	2.3 ± 0.1	2.3 ± 0.1	2.4 ± 0.1	0.76
Heart wt, g/fetal wt, g	0.69 ± 0.01	0.75 ± 0.01	0.76 ± 0.01	0.01
Left ventricle wt, g/FW, g	0.201 ± 0.004	0.209 ± 0.005	0.228 ± 0.005	0.01
Right venticle wt, g/FW, g	0.178 ± 0.005	0.194 ± 0.006	0.198 ± 0.006	0.06
Left atrium wt, g/FW, g	0.047 ± 0.006	0.056 ± 0.006	0.062 ± 0.006	0.27
Right atrium wt, g/FW, g	0.055 ± 0.005	0.044 ± 0.005	0.052 ± 0.005	0.35
Septum wt, g/FW, g	0.172 ± 0.008	0.198 ± 0.008	0.191 ± 0.008	0.12
Liver wt, g/FW, g	2.99 ± 0.08	2.95 ± 0.08	3.05 ± 0.08	0.75
Pancreas wt, g/FW, g	0.065 ± 0.004	0.057 ± 0.004	0.049 ± 0.004	0.07
Right kidney wt, g/FW, g	0.37 ± 0.02	0.42 ± 0.02	0.39 ± 0.02	0.37
Left kidney wt, g/FW, g	0.40 ± 0.02	0.42 ± 0.02	0.40 ± 0.02	0.72
Adrenal wt, g/FW, g	0.015 ± 0.002	0.019 ± 0.002	0.013 ± 0.002	0.18
Left LD muscle, g/FW, g	1.1 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.52
Right LD muscle, g/FW, g	1.1 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.19
Viscera wt, g/FW, g	5.7 ± 0.3	6.1 ± 0.3	5.7 ± 0.3	0.56
Perirenal adipose wt, g/FW, g	0.31 ± 0.01	0.35 ± 0.01	0.34 ± 0.01	0.06

[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations

Table 7: Fetal organ weights as a percent of empty fetal weight (EFW) on d 190 of gestation from CON, NR/CON, and CON/NR cows[1]

Item	CON	NR/CON	CON/NR	P value
n	8	7	7	
Brain wt, g/EFW, g	1.21 ± 0.03	1.38 ± 0.03	1.44 ± 0.03	< 0.01
Lung wt, g/EFW, g	2.83 ± 0.20	2.98 ± 0.21	3.08 ± 0.21	0.69
Heart wt, g/EFW, g	0.82 ± 0.03	0.92 ± 0.03	0.93 ± 0.03	0.04
Lt ventricle wt, g/EFW, g	0.24 ± 0.01	0.28 ± 0.01	0.28 ± 0.01	<0.01
Rt venticle wt, g/EFW, g	0.21 ± 0.01	0.25 ± 0.01	0.24 ± 0.01	0.002
Lt atrium wt, g/EFW, g	0.06 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.16
Rt atrium wt, g/EFW, g	0.07 ± 0.04	0.13 ± 0.04	0.07 ± 0.04	0.45
Septum wt, g/EFW, g	0.21 ± 0.01	0.25 ± 0.01	0.24 ± 0.01	0.04
Liver wt, g/EFW, g	3.7 ± 0.1	3.7 ± 0.1	3.8 ± 0.1	0.79
Pancreas wt, g/EFW, g	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.05
Rt kidney wt, g/EFW, g	0.46 ± 0.03	0.53 ± 0.03	0.49 ± 0.03	0.25
Lt kidney wt, g/EFW, g	0.50 ± 0.03	0.54 ± 0.03	0.50 ± 0.030	0.58
Adrenal wt, g/EFW, g	0.019 ± 0.002	0.023 ± 0.002	0.016 ± 0.002	0.13
Lt LD muscle, g/EFW, g	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	0.52
Rt LD muscle, g/EFW, g	1.34 ± 0.09	1.33 ± 0.09	1.13 ± 0.09	0.22
Viscera wt, g/EFW, g	7.04 ± 0.41	7.76 ± 0.44	7.08 ± 0.44	0.43
Perirenal adipose wt, g/EFW, g	0.38 ± 0.01	0.43 ± 0.01	0.44 ± 0.01	< 0.01

[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations
EFW: empty fetal weight
Rt: right
Lt: left

Table 8: Uterus weights and placenta characteristics on d 190 and d 110 of gestation CON or NR cows[1]

d 110

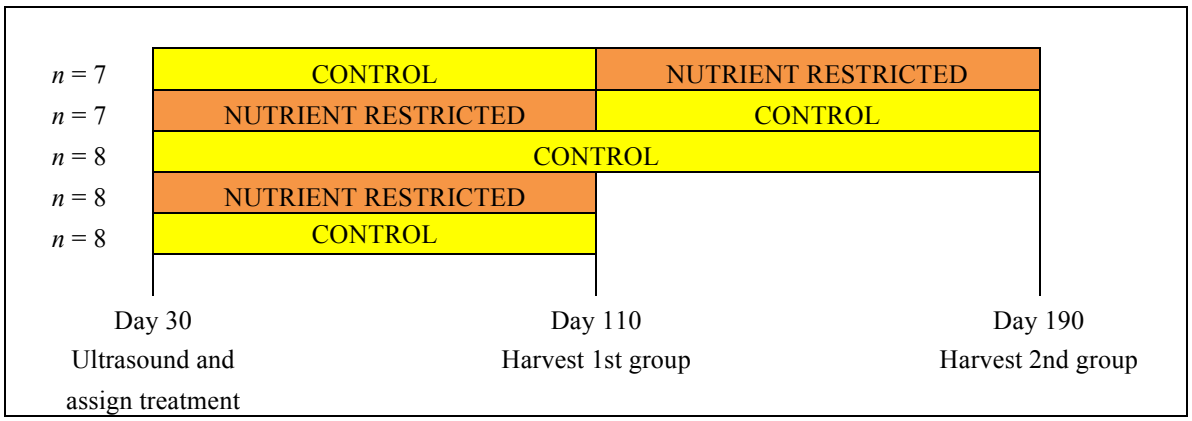
Item	CON	NR	*P* value
n	8	8	
Full uterus wt, kg	4.7 ± 0.2	5.3 ± 0.2	0.133
Empty uterus wt, kg	1.5 ± 0.1	1.2 ± 0.1	0.176
Total placentome wt, kg	0.53 ± 0.06	0.66 ± 0.06	0.205
Total caruncle wt, kg	0.31 ± 0.05	0.32 ± 0.05	0.928
Total cotyledonary wt, kg	0.22 ± 0.02	0.33 ± 0.02	0.007
Cot wt, kg/Car wt, kg	0.81 ± 0.14	1.194 ± 0.143	0.087
Placentomes, n	54.5 ± 6.5	77.8 ± 6.5	0.028
Average placentome SA, mm^2	526.9 ± 63.5	676.3 ± 63.5	0.123
Total placentome SA, mm^2	34952 ± 4925	52343 ± 4925	0.028

d 190

Item	CON	NR/CON	CON/NR	*P* value
n	8	7	7	
Full uterus wt, kg	23.2 ± 1.2	20.6 ± 1.3	21.6 ± 1.3	0.401
Empty uterus wt, kg	2.6 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	0.732
Total placentome wt, kg	3.1 ± 0.1	2.7 ± 0.1	3.3 ± 0.1	0.056
Total caruncle wt, kg	1.8 ± 0.1	1.4 ± 0.1	1.7 ± 0.1	0.07
Total cotyledonary wt, kg	1.29 ± 0.07	1.31 ± 0.07	1.60 ± 0.07	0.017
Cot wt, kg/Car wt, kg	0.72 ± 0.04	0.91 ± 0.05	0.92 ± 0.05	0.02
Placentomes, n	103.3 ± 5.2	102.1 ± 5.5	106.5 ± 5.5	0.846
Avg placentome SA mm^2	1724.3 ± 105.2	1816.3 ± 112.50	1916.7 ± 73.000	0.473
Total placentome SA, mm^2	177311 ± 612100	18422 ± 654400	197913 ± 654400	0.093

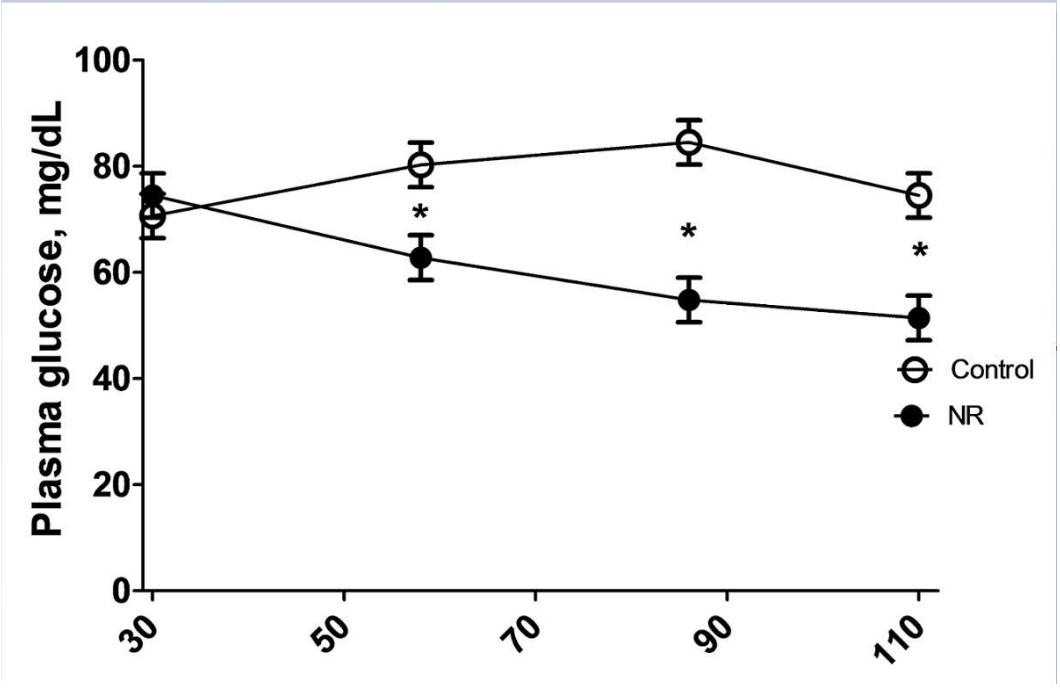
[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations
SA: Surface area

Figure 1: Experimental layout



[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations

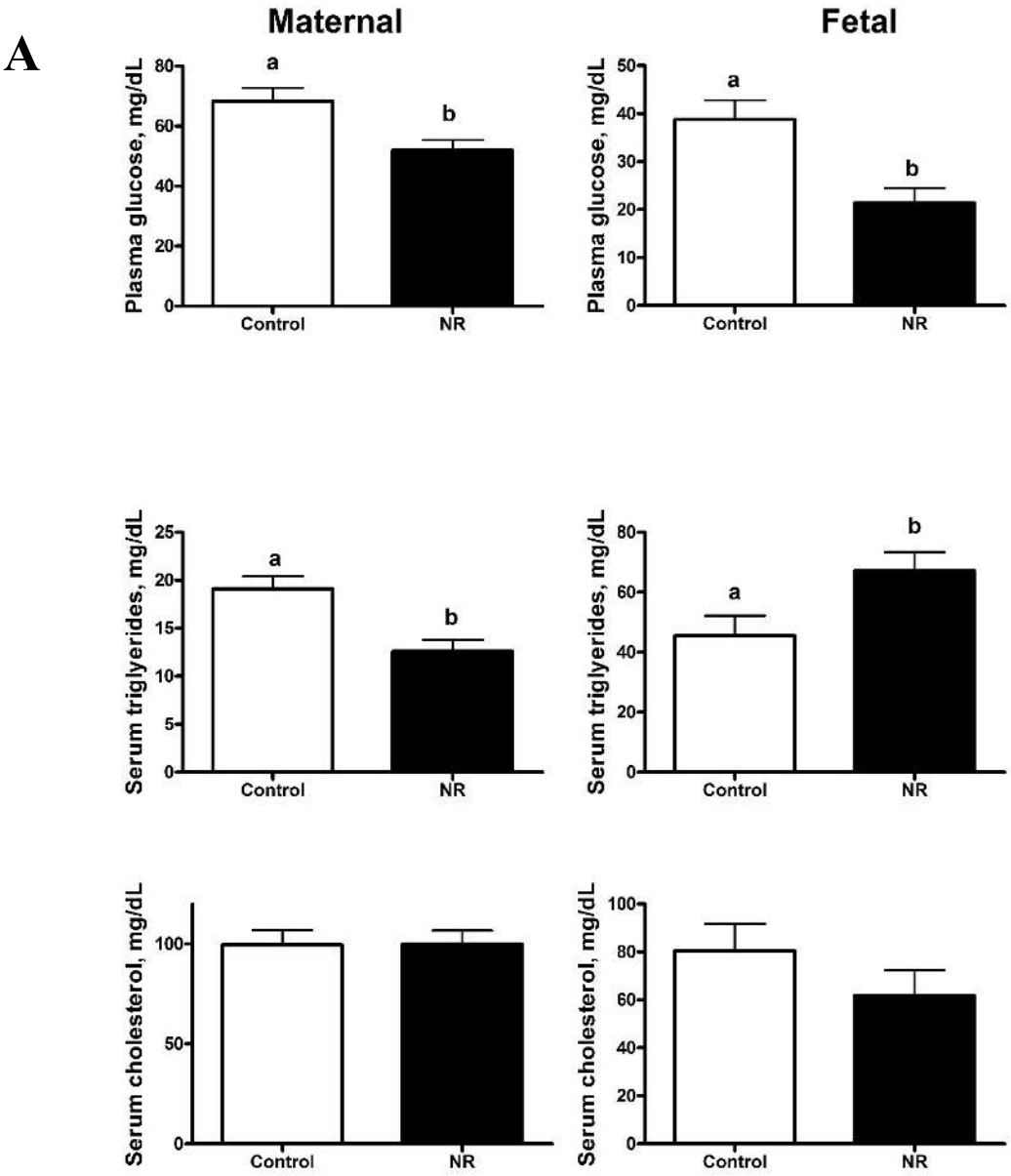
Figure 2: Monthly plasma glucose concentrations of CON and NR[1] cows from d 30 – d 110 of gestation (trt*day, $P = 0.0018$)



*Means differ $P < 0.05$

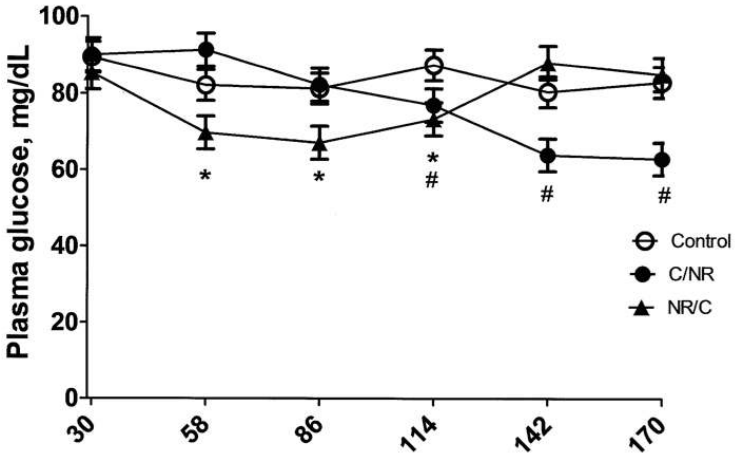
[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations

Figure 3: (A) Plasma glucose, (B) serum triglycerides, and (C) serum cholesterol concentrations from control and nutrient restricted fetuses and dams[1] at d 110 of gestation
[a,b] Means differ $P < 0.05$



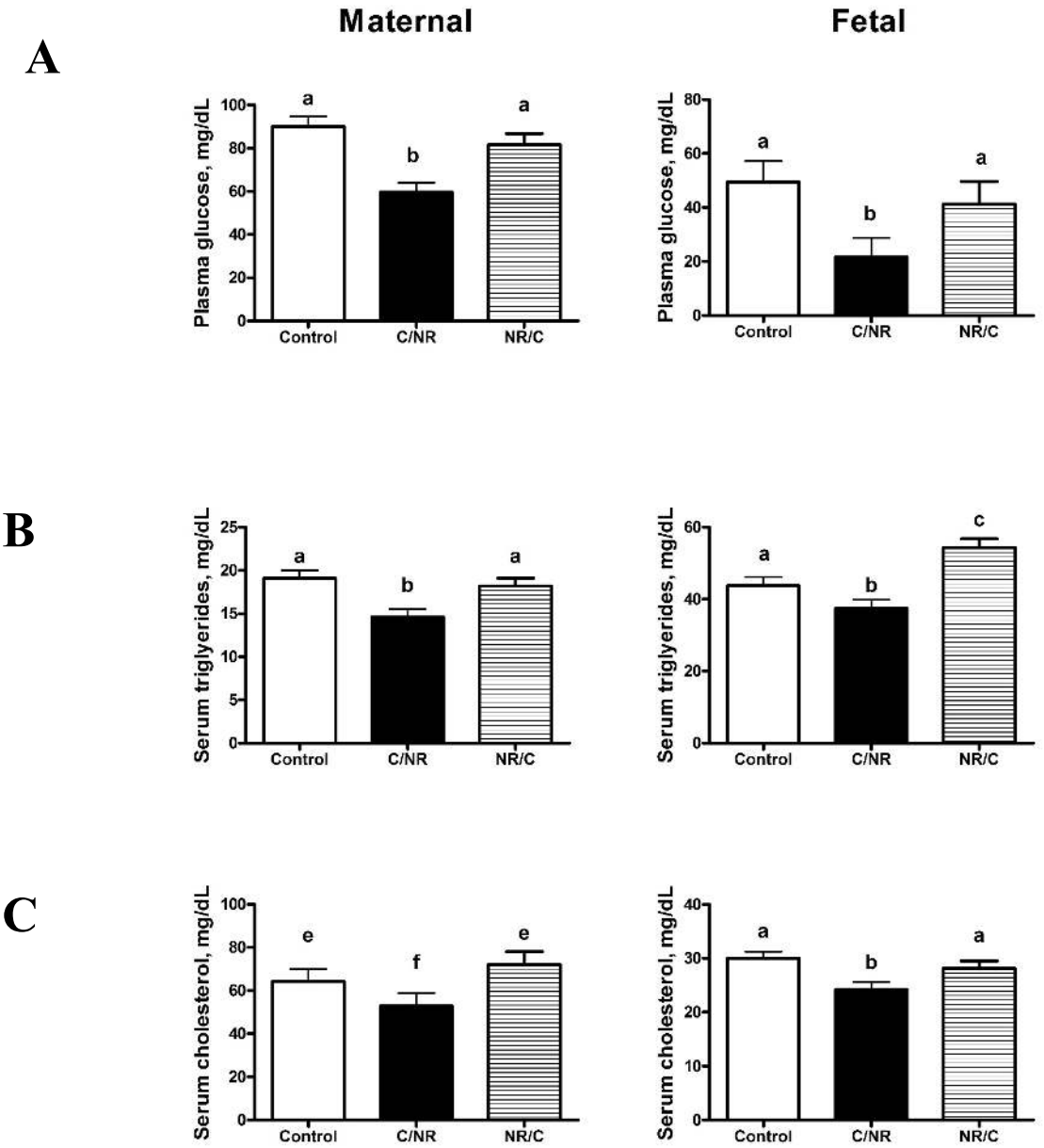
[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations

Figure 4: Monthly plasma glucose concentrations of CON and NR[1] cows from d 30 – d 190 of gestation (trt*day, $P = 0.0001$)



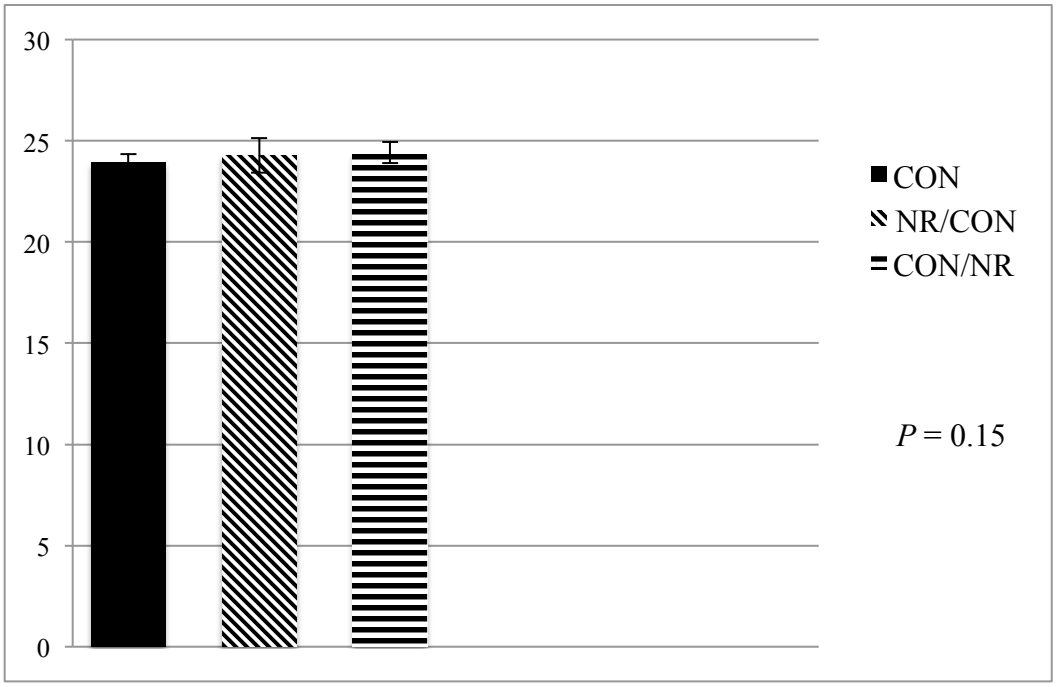
[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations
*Means differ between CON and NR/CON; $P < 0.05$
#Means differ between CON and CON/NR; $P < 0.05$

Figure 5: (A) Plasma glucose, (B) serum triglycerides, and (C) serum cholesterol concentrations from control and nutrient restricted fetuses and dams[1] at d 190 of gestation
[a,b,c] Means differ $P < 0.05$
[e,f] Means differ $P < 0.10$



[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations

Figure 6: Adipocyte Diameter (um) of d 190 control and nutrient restricted fetuses[1]



[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations

CHAPTER III

Effects of maternal nutrient restriction during early or mid-gestation on miRNA expression in the bovine cotyledon

ABSTRACT

Primiparous Angus-cross cows (*n* = 38) were synchronized and artificially inseminated with semen from a single Angus sire. Animals were fed at 1.3 x (Control [CON]) or 0.55 x (Nutrient Restricted [NR]) of maintenance energy and crude protein requirements based on BW (NRC 1996). Animals were blocked by BCS and BW and assigned to treatments. A subset of animals (*n* = 16) was fed either NR (*n* = 8) or C (*n* = 8) from d 30-110 of gestation. The remaining animals (*n* = 22) were fed CON (*n* = 8) d 30-190; NR (*n* = 7) d 30-110 followed by CON d 110-190; or CON (*n* = 7) d 30-110 followed by NR d 110-190. Cows were sacrificed on d 110 or d 190 of gestation and the fetus and placenta collected. RNA was isolated from flash frozen cotyledon samples (3 subsamples/treatment) using the *mir*Vana microRNA Isolation kit and analyzed using a previously validated microarray. MicroRNA expression was analyzed as an ANOVA analysis using appropriate procedures correcting for false discovery rate of microarray data. Of the sequences chosen for microarray, 88 showed significant ($P < 0.05$) differential expression between d 190 treatment groups. A total of 40 miRNAs were upregulated in the control group compared with both nutrient restricted groups and a total of 26 miRNAs were upregulated in the nutrient restricted groups compared with the control group. In d 110 animals, 142 showed significant ($P < 0.05$) differential expression between control and nutrient restricted groups. A total of 51 were upregulated in the

control group versus the nutrient restricted group and a total of 91 miRNAs were upregulated in the nutrient restricted group versus the control group. In a comparison of d 110 and d 190 control animals, 139 sequences were ($P < 0.05$) differentially. A total of 57 miRNAs were upregulated for d 110 control fetuses and 82 miRNAs were upregulated for d 190 control fetuses. Of the 157 individual miRNAs, only 33 were available in TargetScan or picTar for predicted target analysis. Top KEGG pathway analysis included: Axon guidance, endocytosis, neuroactive ligand receptor interaction, MAPK signaling pathway, and spliceosomes. The data show that maternal nutrient restriction during early or mid gestation causes asymmetrical fetal growth restriction and affects miRNA regulation differently depending on whether or not the restriction is preceded or followed by a non-restriction period.

Keywords: Undernutrition, miRNA, cotyledons

INTRODUCTION

MicroRNAs are small, noncoding RNAs that are 20-25 nucleotides long. MicroRNAs regulate posttranscriptional gene expression by binding to the 3' untranslated region of their target RNAs, generally messenger RNA (Bartel et al., 2004). Changes in gene expression can alter how nutrients are transported across cell membranes and even from the dam to the fetus during gestation (Constancia et al., 2002; Reik et al*.,* 2003). It is suggested that poor nutrition during gestation may detrimentally alter postnatal function through changes in DNA methylation (Rakyan et al., 2001). mRNAs are regulated by miRNAs, which regulate cellular events including: stem cell differentiation (Houbaviy et al., 2003; Chen et al., 2010;), organ development/formation

(Boettger et al., 2012; , Cochella et al., 2012), aging (Inukai et al., 2012), cancer cell growth and metastasis (Schickel et al., 2008; Lages et al., 2012), genetic disease, cardiovascular diseases (Latronico et al., 2009; Henrion-Caude et al., 2012; Santovito et al., 2012), and metabolic disorders (Rottiers et al., 2012; Rayner et al., 2014). The objective of this study was to determine the alteration of miRNA expression in the cotyledon caused by maternal nutrient restriction during early (d 30 – d 110) or midgestation (d 110 – d 190) and which molecular pathways would subsequently be affected in the fetus.

MATERIALS AND METHODS

All animal procedures were approved by Clemson University Animal Care and Use Committee (AUP #2013-062).

Animals and Sample Collection

Primiparous Angus-cross cows (*n* = 38) were used to produce the fetuses studied here. The protocol for the nutrient restriction of these dams was identical to that described by Taylor et al. (2015). Briefly, cattle were synchronized, artificially inseminated, and blocked into treatment group by BCS on d 30 of pregnancy. Throughout the experiment, all animals were fed a TMR (30% soybean hulls, 20% cottonseed hulls, 17.5% peanut hulls, 17% corn screening, 12.5% corn gluten feed, 1.5% salt, 0.5% calcium, 0.5% trace mineral, 0.5% vitamin premix: 0.65 Mcal NE_m/lb, 8.9% CP) at one of two levels. Diets were fed at either 1.3 x (Control [CON]) or 0.55 x (Nutrient restricted [NR]) of maintenance energy and crude protein values based on BW (NRC 2000). A subset of animals (*n* = 16) was fed either NR (*n* = 8) or CON (*n* = 8) from d 30-110 of

gestation. The remaining animals (n = 22) were fed CON (n = 8) d 30-190; NR (n = 7) d 30-110 followed by CON d 110-190; or CON (n = 7) d 30-110 followed by NR d 110-190. Cattle were maintained together on a dry lot with free access to water and were fed individually once daily in the morning. Animal BW was taken every wk and used to reformulate rations throughout the experiment and BCS was collected every other week.

All animals were harvested at the same commercial plant. Fetal weight, empty fetal weight, crown rump length, abdominal and thoracic circumference were measured and recorded for each fetus. All placentomes from each cow were counted and greatest and least diameter of each at the fetal-maternal interface was recorded. The average diameter of each placentome was used to calculate placentomal surface area of each individual placentome, total placentomal surface area, and average surface area per placentome. Placentomes were then divided into cotyledonary and caruncular tissue, separated by tissue type, and total caruncular and total cotyledonary tissue weights were recorded. The tissue weights were used to calculate the ratio of fetal and maternal contribution to placentome. Samples of cotyledonary and caruncular tissue were collected into 2 mL CryoELITE tubes (Wheaton, Millville, NJ) and flash frozen in liquid Nitrogen. The samples were stored at -80° C for further analysis.

RNA Isolation

Isolation of RNA was performed from randomly selected animals representing three animals per treatment group using flash frozen cotyledon samples of both d 190 and d 110 fetuses. Total RNA fractions were isolated using the *mir*Vana microRNA Isolation kit (ThermoScientific, Waltham, MA) following manufacturer's recommendations. RNA

quantity as well as 260/280 nm ratios were determined on a Nanodrop 1000 spectrophotometer (ThermoScientific, Waltham, MA). Additionally, the integrity of the total RNA samples were measured using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA) in a 2100 Bioanalyzer (Agilent Technolgies, Santa Clara, CA) where RNA quality was evaluated by RNA integrity number calculated with the Agilent 2100 expert software.

Microarray

Three RNA samples per treatment group were submitted for sequencing to LC Science's Microarray services (Houston, TX). To compare miRNA expression between treatment groups, a microarray was custom designed and conducted by LC Science's Microarray services (Houston, TX). The custom array was designed based on sequencing results and also included all reported *Bos taurus* miRNA (Stowe et al., 2014; Hagen et al., 2015, in press).

Statistics and Analysis

For d 110 animals, BW and BCS change was analyzed as an ANOVA analysis using the GLM procedure of SAS. Fetal weights and measurements were analyzed using the MIXED procedure of SAS with treatment, sex, and their interaction in the model statement. For d 190 animals, Maternal BW and BCS changes and fetal weights/measurements were, analyzed as an ANOVA analysis using the GLM procedure of SAS. A tendency was defined as $P < 0.10$ and a significance difference was defined as $P < 0.05$

An ANOVA test was conducted to compare normalized expression levels between treatment groups. In order to determine functional differences in expressed miRNAs among CON and NR cotyledon tissue, their target genes were extracted using two independent target prediction tools: TargetScan (Lewis et al., 2005) and picTar (Krek et al., 2005). The Database for Annotation, Visualization and Integrated Discovery (DAVID v. 6.7; http://david.abcc.ncifcrf.gov; (Dennis et al., 2003; Huang da et al., 2009)) is a free online bioinformatics resource that provides interpretation of biological themes associated with large gene lists. DAVID was used to annotate predicted target genes of differentially expressed miRNAs and to identify significant functional enrichment in the miRNA gene targets relative to the whole genome background. After identifying function enrichment, gene targets were analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG), a database used for identifying functions and utilities based on molecular –level information (Kanehisa et al., 2000).

RESULTS

Maternal BW and BCS change (Table 1) showed that d 110 NR cows had decreased BW ($P < 0.0001$) and BCS ($P < 0.0001$) from d 30 – d 110 compared with d 110 CON cattle. In d 190 animals, NR/CON cattle had a decrease in both BW ($P < 0.0001$) and BCS ($P = 0.0008$) from d 30 – d 114 when compared with CON/NR and CON cattle. A decrease in BW ($P < 0.0001$) and BCS ($P < 0.0001$) was observed in CON/NR animals from d 114 – d 190 compared with NR/CON and CON cattle. Fetal size and weights at d 110 of gestation are shown in Table 2. Fetal weights and empty fetal weights were reduced ($P = 0.0027$, $P = 0.0023$, respectively) in the NR fetuses versus

CON fetuses. Crown rump length and abdominal circumference was unaffected (P = 0.0110, P = 0.0265, respectively), while thoracic circumference was reduced in NR fetuses versus CON fetuses (P = 0.0002). Fetal size and weights are shown in Table 2. Fetal weights tended to be reduced (P = 0.07) in NR/CON and CON/NR versus. CON/CON cattle and empty fetal weights were reduced (P = 0.03) in NR/CON and CON/NR versus. CON/CON cattle. Abdominal and thoracic circumference was reduced (P = 0.01, P = 0.03, respectively) in NR/CON and CON/NR fetuses versus. CON/CON fetuses, but crown rump length was unaffected (P = 0.32) (Taylor et al., 2015).

A total of 2,931 miRNA sequences were probed for based on sequencing results and including all reported bovine miRNA. The probes were custom designed and have been previously used in bovine studies (Stowe et al., 2014). Of the sequences chosen for microarray, 88 showed differential expression ($P < 0.05$) between d 190 treatments (Table 3), 39 of which were at low signal (detected signal intensity <500, based on a range from 1 to over 65,000) (Supplemental Table 1). A total of 40 miRNAs were upregulated (17 at low expression) in the control group compared with both nutrient restricted groups and a total of 26 miRNAs were upregulated (22 at low expression) in the nutrient restricted treatments compared with the control group. In d 110 animals, 142 showed differential expression ($P < 0.05$) between control and nutrient restricted groups (Table 4), 52 of which were at low signal (Supplemental Table 2). A total of 51 were upregulated (20 at low expression) in the control group versus the nutrient restricted group and a total of 91 miRNAs (22 at low expression) were upregulated in the nutrient restricted group versus the control group. In a comparison of d 110 and d 190 control animals, 139 sequences were differentially expressed ($P < 0.05$; Table 5), 50 of which

were at low signal (Supplemental Table 3). A total of 57 miRNAs were upregulated (21 at low expression) for d 110 control fetuses and 82 miRNAs were upregulated (29 at low signal) for d 190 control fetuses. After eliminating all duplicates, there were 157 miRNA sequences remaining that were differentially expressed. Of the 157 miRNAs, 62 did not map to miRbase (miRbase.org) or the *Bos taurus* genome, which are most likely not miRNA and given the number of probes included in the microarray, some may have been false positives. Of the 62 miRNAs, only 33 were available in TargetScan or picTar for predicted target analysis. The remaining 33 miRNA were predicted to target a total of 23,988 genes, 5959 of which were duplicated across the target lists (targeted by more than one miRNA). All duplicated genes were subjected to gene ontology (GO) to determine which pathways were represented. KEGG pathway analysis of the cotyledon revealed 116 KEGG pathways. Top ranking pathways included: Axon guidance, endocytosis, neuroactive ligand receptor interaction, MAPK signaling pathway, and spliceosomes (Table 6).

DISCUSSION

To our knowledge, this is the first study to evaluate the affect of miRNA expression within the bovine cotyledon. Previous studies have elucidated miRNA of bull sperm (Stowe et al., 2014) and bovine fetal back fat (Sun et al., 2014). Despite these studies, the bovine genome lacks the same level of annotation as the human genome.

While the bovine genome is still lacking literature on elucidating miRNA expression, work has been done to rectify this gap. In a study aimed at characterizing bovine miRNA (Jin et al., 2009) samples of 11 different tissues types were collected from

adult beef cattle, including three subsamples of both the brain and adipose tissue. Of all the miRNAs identified, it was discovered that very few were tissue specific (miR-9, -124 in brain, miR-122 in liver, miR-1, miR-133a and -206 in muscle) while numerous miRNAs were only highly expressed in particular tissues (miR-204, -218, and -129-2-3p in brain tissues, miR-30a, -30e, -30d, -200a and -200b in kidney, miR-192 in liver, miR-451 in spleen, miR-21 in spleen and thymus, miR-193b, -378 in LDM muscle). About 15 miRNAs were highly expressed in all 11 tissues sampled and 31 miRNAs were found to be highly expressed in 8 or more tissues sampled. This broad range of miRNA expression throughout tissue types suggests that miRNAs play important roles in multiple biological pathways in the body. Further, the miRNAs expressed from the tissues also were expressed in three other species (human, mouse, rat), confirming that miRNAs are highly conserved and suggesting that they have similar biological roles in all species.

There has been some miRNA work done in the human placenta, where miR-141 was involved in trophoblast proliferation (Bentwich et al., 2005) and also has a 3.4x higher expression rate when the fetuses exhibits IUGR (Tang et al., 2013), which is supported by the increased expression of miR-141 in nutrient restricted d 110 cotyledons. Also in the human placenta, Pineles et al. (2007) reported the miR-182 inhibits trophoblast apoptosis, which works in tandem with miR-141 and its action on trophoblast proliferation. The upregulation of miR-182 and miR-141 in d 110 nutrient restricted cotyledons would suggest a coping mechanism from the fetus whereby as maternal nutrient stress is detected, upregulation of particular genes can possibly decrease the chances of fetal mortality. However, miR-20b may be involved in inhibition of angiogenesis (Wang et al., 2012) and this particular miRNA was upregulated in d 110

nutrient restricted cotyledons. Therefore, even with a possible increase in capability to form the trophoblast layer of the placenta, nutrient restriction may potentially decrease the amount of blood flow available for maternal:fetal exchange. Mir-195 has been reported to enhance trophoblast migration, invasion, proliferation, and cell survival (Bai et al., 2012; Xu et al., 2014); this particular miRNA has been significantly downregulated in women with preeclampsia (Xu et al., 2014). Mir-195 was upregulated in both control d 190 cotyledons verses control d 110 cotyledons, as well as both d 190 nutrient restricted cotyledons. Preeclampsia is generally caused by abnormal formation of blood vessels into the placenta (Clark et al., 1998); the upregulation of this miRNA not only suggests a role in the proper development of the placenta, as well as angiogenesis and vascularization. Gestational diabetes is correlated with a low expression of miR-29a (Zhao et al., 2011) and is considered a biomarker for the disease. Cotyledons from d 190 animals restricted during the second trimester (d 110 – 190) showed an upregulation of miR-29a, which could indicate that nutrient restriction earlier in gestation may be more susceptible to gestational diabetes. Mir-335 has been implemented in lipid metabolism and adipocyte differentiation (Nakanishi et al., 2009) and it was found to be upregulated in d 110 nutrient restricted fetuses compared with d 110 control fetuses. This is in conjunction with previous studies (Hales and Barker, 2001), that a fetus under nutritional stress during gestation will alter its endocrine status in order to prepare for postnatal life (known as the “thrifty phenotype”). Mir-335 was also found to be upregulated in d 190 control fetuses compared with d 110 fetuses, which may indicate higher levels of lipid metabolism at d 190 of gestation (Nakanishi et al., 2009).

Undernutrition of ewes during the preconception period and preimplantation period plays a role in altering miRNA expression in insulin signaling pathways and hepatic gluconeogenesis in the fetus (Lie et al., 2014a). This study suggested that even if animals are fed an adequate diet from as early as 7 days of gestation, poor nutrition leading into the breeding season can have long term detrimental effects on the post natal animal. Results showed that miRNAs may indirectly alter translation or degradation of the target transcript or protein (Lie et al., 2014a). Out of the same lab and utilizing a similar nutrient restriction model, Lie et al. (2014b) showed that undernutrition could also have a detrimental effect on myogenesis and subsequent glucose metabolism. In the obese sheep model, altered expression of miRNAs were suggested to influence myogenesis and adipogenesis in the fetus. Particularly, overexpression of let-7g seemed to decrease cell proliferation (Kumar et al., 2007; Kumar et al., 2008; Chen et al., 2010). Fetuses from obese mothers were heavier than control fetuses and had decreased expression of let-7g, confirming let-7g's effect on cell proliferation. A sheep study (Torley et al., 2011) suggests that miRNA are important regulators of gonad development, including hormone signaling. At time of sex differentiation, increases in multiple miRNAs suggest that altered expression of miRNAs could lead to incorrect gonad development.

In conclusion, while there is still work to be done in order to fully extrapolate the bovine genome, altered nutrition of the dam during early and mid gestation can modify miRNA expression, subsequently affecting maternal:fetal nutrient transport by decreasing anigogeneiss and vasculogenesis. However, there does seem to be an enhancing effect of placenta development by an increase in trophoblast proliferation and a decrease in

apoptosis, possibly serving as a coping mechanism for those fetuses facing nutrient restriction. Increased expression of particular miRNA may also be the underlying cause of the thrifty phenotype, in which the fetus alters its endocrine status in order to better survive postnatal life.

Table 1: Maternal BW and BCS change throughout gestation for d 110 and d 190 animals[1]

d 110 animals

Item	CON	NR	*P* value
n	8	9	
d 30 - 110 BW, kg	11.88 ± 3.33	-53.52 ± 3.14	< 0.0001
d 30 – 110 BCS	0.125 ± 0.169	-1.222 ±0.159	< 0.0001

d 190 animals

Item	CON	NR/CON	CON/NR	*P* value
n	8	7	7	
d 30 – 114 BW, kg	11.70 ± 4.36	-50.19 ± 4.66	12.91 ± 4.66	< 0.0001
d 30 – 114 BCS	0.12 ± 0.20	-1.14 ± 0.21	0.00 ± 0.21	0.0008
d 114 – 190 BW, kg	14.48 ± 4.46	19.28 ± 4.77	-77.33 ± 4.77	< 0.0001
d 114 – 190 BCS	-0.06 ± 0.14	0.28 ± 0.15	-1.28 ± 0.15	< 0.0001

[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations

Table 2: Fetal measurements and selected organ weights on d 110 and d 190 of gestation from CON and NR cows[1]

Item	CON	NR	*P* value
d 110			
n	8	8	
Fetal wt, kg	0.693 ± 0.017	0.600 ± 0.017	0.003
Fetal empty carcass wt, kg	0.531 ± 0.011	0.471 ± 0.011	0.002
Crown rump length, cm	26.56 ± 0.30	25.31 ± 0.30	0.011
Abdominal circumference, cm	20.12 ± 0.78	17.38 ± 0.78	0.027
Thoracic circumference, cm	18.75 ± 0.19	17.31 ± 0.19	0.0002

Item	CON	NR/CON	CON/NR	*P* value
d 190				
n	8	7	7	
Fetal wt, kg	10.34 ± 0.44	9.05 ± 0.47	8.91 ± 0.47	0.08
Fetal empty carcass wt, kg	8.50 ± 0.36	7.21 ± 0.39	7.18 ± 0.39	0.03
Crown rump length, cm	65.56 ± 1.44	62.35 ± 1.54	63.5 ± 1.54	0.32
Abdominal circumference, cm	47.25 ± 0.89	43.50 ± 0.96	43.92 ± 0.96	0.02
Thoracic circumference, cm	45.56 ± 0.81	42.71 ± 0.86	42.50 ± 0.86	0.03

Table 3: Significant differentially expression of miRNA in d 190 CON versus CON/NR versus NR/CON[1]

	d 190			
mRNA	CON	NR/CON	CON/NR	*P* value
n	8	7	7	
bta-miR-10b	2640	2023	1553	0.03490
bta-miR-148b	1628	1086	1250	0.01660
bta-miR-15a	949	610	743	0.01920
bta-miR-16a	5367	3916	4662	0.04950
bta-miR-16b	6791	5083	5474	0.04580
bta-miR-181a	1699	1340	983	0.01510
bta-miR-195	2787	1762	1680	0.02720
bta-miR-21-5p	4169	3958	7512	0.00169
bta-miR-22-3p	1023	1113	1367	0.04410
bta-miR-2440	326	541	391	0.02990
bta-miR-2484	283	369	518	0.00939
bta-mir-2904-3-p3	1018	1948	2059	0.03120
bta-mir-2904-3-p5	2778	4635	4941	0.01260
bta-miR-29a	1274	1075	1598	0.00498
bta-miR-30e-5p	10085	9113	7536	0.04540
bta-miR-376d	5294	3210	3424	0.04250
bta-miR-409a	605	480	344	0.01060
bta-miR-410	556	476	392	0.03980
bta-miR-487b	2570	1970	1595	0.02590
bta-mir-658-p3_1ss1TG	422	630	758	0.03860
bta-miR-99b	815	994	748	0.00526
hsa-miR-1260a_R+1_1ss9TG	821	992	569	0.02170
hsa-miR-335-3p	752	469	495	0.02080
hsa-miR-4497_1ss17CA	2434	3302	2285	0.02230
mdo-mir-195	2310	1696	1409	0.00596
mdo-miR-21_L+3	4283	4374	7360	0.00736
mdo-miR-22_1ss21GA	1075	1229	1460	0.01910
mml-miR-1260b_R+1+1ss9AG	1059	1255	716	0.01940
mmu-miR-28c	2347	1641	1638	0.02940
mmu-mir-5105-p3	2744	4533	4969	0.00033
mmu-mir-5105-p3_1ss21TC	2743	4533	4931	0.00050
mmu-mir-5105-p3_1ss24TC	2747	4653	4750	0.00189
mmu-mir-5105-p5_1ss21TC	2793	4432	4957	0.00077
mmu-mir-5105-p5_1ss6GC	285	536	900	0.01400
mmu-mir-5117-p3_1ss19TA	1378	2431	1903	0.00154
mmu-mir-6236-p3_4ss6AG23AG25GC27GA	262	537	456	0.02560
mmu-miR-6243_R-5	1155	1884	1593	0.01890

oar-miR-382-3p	641	366	351	0.01650
oar-mir-409-3p	1208	1077	837	0.04550
oar-miR-411a-3p	3043	2100	1998	0.02770
oar-miR-411b-3p	466	278	289	0.01500
PC-5p-4028_62	2592	1822	2326	0.02010
sha-miR-181a-5p_L-1R+7	1615	1279	976	0.03100
sha-miR-181a-5p_R+7	1605	1269	1014	0.03900
sha-mir-716a-p3	2075	5198	5981	0.00252
sha-miR-716b	2042	3423	3134	0.04330
sha-mir-716b-p3_1ss4TC	2425	4416	4156	0.02300
sha-mir-716b-p5_1ss3GC	2366	3966	3633	0.04210
ssc-miR-30e-3p	732	618	432	0.03540

[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NE_m and CP recommendations

Table 4: Significant differentially expressed miRNA; d 110 CON versus d 110 NR[1]

	d 110		
mRNA	CON	NR	*P* value
n	8	8	
bta-miR-99a-5p	1819	1273	0.03460
bta-let-7i	2923	4363	0.01110
bta-miR-106a	1408	2653	0.02260
bta-miR-106a_R+1_1ss1AC	1106	1887	0.03730
bta-miR-1246	3226	1511	0.04050
bta-miR-125b	2678	1747	0.01810
bta-miR-141	869	1917	0.01450
bta-miR-146a	1673	2821	0.01670
bta-miR-146b	1996	3108	0.01680
bta-miR-148b	812	1915	0.02450
bta-miR-151-3p	502	770	0.04370
bta-miR-151-5p	2426	4603	0.01470
bta-miR-15a	563	854	0.04480
bta-miR-15b	2254	5204	0.00246
bta-miR-16a	3647	5745	0.01940
bta-miR-17-5p	1067	1893	0.02410
bta-miR-1777a	6228	3770	0.04230
bta-miR-181c	571	807	0.04990
bta-miR-182	500	783	0.00732
bta-miR-183	245	456	0.00915
bta-miR-185	354	640	0.01730
bta-miR-186	2193	3423	0.03680
bta-miR-191	4637	7077	0.02430
bta-miR-20a	980	1765	0.03190
bta-miR-2284x	256	489	0.00694
bta-miR-2391	3173	199	0.00652
bta-miR-23a	7896	10641	0.02820
bta-miR-23b-3p	8779	11978	0.02890
bta-miR-2440	538	226	0.00377
bta-miR-2487_L-2R-3_1ss15CT	2348	686	0.00840
bta-mir-2887-2-p3	2411	970	0.02380
bta-mir-2904-3-p3	3033	1330	0.03510
bta-miR-335	5988	8450	0.04370
bta-miR-369-3p	1426	904	0.03970
bta-miR-377	303	661	0.02820
bta-miR-378	1736	2846	0.04120
bta-miR-378b	700	1297	0.04490

bta-miR-378c	700	1310	0.00248
bta-miR-378c_R+1+1ss21TG	800	1445	0.00035
bta-miR-411c-5p	412	720	0.03460
bta-miR-421	671	1145	0.01260
bta-miR-424-3p	583	764	0.03170
bta-miR-425-5p	584	1243	0.02970
bta-miR-432	2054	2742	0.02840
bta-miR-450b	2084	3199	0.02950
bta-miR-451	1532	3015	0.04740
bta-miR-495	1504	3680	0.04040
bta-miR-503-5p	2349	4314	0.03080
bta-miR-532	215	428	0.02510
bta-miR-574	647	358	0.04510
bta-miR-6119-5p	1086	1788	0.02530
cfa-miR-203	2123	4060	0.02090
cgr-miR-1973_L+5R+6	3116	1511	0.01020
ggo-miR-574_1ss22AT	3244	1054	0.02910
hsa-miR-151b_R+5	1670	3221	0.01060
hsa-miR-378d	751	1319	0.00272
hsa-miR-151b_R+3	2403	4338	0.02670
hsa-miR-4324_L-1R-1_1ss16CT	2265	1487	0.04010
hsa-mir-4485-p5_1ss18CT	750	348	0.00114
mdo-miR-22_1ss21GT	957	1331	0.03680
mdo-miR-26_R+3	12343	17646	0.01890
mml-miR-378d_1ss21CA	679	1244	0.00251
mml-miR-378d_R-1_1ss21CA	717	1335	0.00209
mmu-let-7j	2376	3586	0.02790
mmu-miR-146a-5p_L+1R+1_1ss19GA	1546	2584	0.02850
mmu-miR-1983	886	1577	0.01890
mmu-miR-378c	781	1436	0.01040
mmu-mir-5105-p3	6651	3139	0.02260
mmu-mir-5105-p3_1ss21TC	6547	3082	0.02150
mmu-mir-5105-p3_1ss24TC	6848	3013	0.02810
mmu-mir-5105-p5_1ss21TC	6322	3153	0.01760
mmu-mir-5109-p3	4360	1651	0.00320
mmu-mir-5109-p3_1ss5TG	3352	816	0.00084
mmu-mir-5109-p5_1ss11TG	1866	533	0.00204
mmu-mir-6236-p3_4ss6AG23AG25GC27GA	615	289	0.01740
mmu-mir-6236-p5	1828	436	0.04890
oar-miR-411a-3p	1882	2490	0.02610
PC-3p-21250_6	1742	3078	0.02460
PC-5p-12650_12	1909	1071	0.04310
PC-5p-80741_1	2351	995	0.04760

PC-5p-93007_1	473	890	0.01800
sha-miR-125a_L-6R+2	808	260	0.03670
sha-mir-716a-p3	7859	2609	0.03530
sha-miR-716b	5540	2597	0.02820
sha-mir-716b-p3_1ss4TC	6912	3143	0.01530
sha-mir-716b-p5_1ss3GC	6440	3003	0.00523
ssc-mir-1285-p5	14613	7018	0.02980
ssc-miR-340	649	1032	0.03020
sssc-miR-378_L-2R+3	797	1466	0.04070

[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations

Table 5: Significant differentially expressed miRNA for d 190 CON versus d 110 CON[1]

mRNA	d 190 CON	d 110 CON	*P* value
n	8	8	
hsa-miR-4324_L-1R-1_1ss16CT	1107	2265	0.01860
sha-miR-181a-5p_L-1R+7	1615	849	0.00039
mmu-mir-5109-p3_1ss5TG	957	3352	0.00087
bta-miR-146b_1ss24TG	3274	2028	0.00096
bta-miR-146b	3046	1996	0.00102
cgr-miR-199b_2ss1-TC22TC	554	979	0.00131
sha-mir-125b-p5_1ss23GA	1064	2434	0.00166
sha-mir-716b-p3_1ss4TC	2425	6912	0.02250
mmu-mir-5109-p3	1270	4360	0.00226
oar-miR-411b-3p	466	289	0.00233
sha-mir-716b-p5_1ss3GC	2366	6440	0.00235
bta-miR-99a-5p	604	1819	0.00325
mmu-miR-28c	2347	1310	0.00365
sha-miR-181a-5p_R+7	1605	911	0.00370
bta-miR-2487_L-2R-3_1ss15CT	757	2348	0.00423
mmu-mir-5109-p5_1ss11TG	471	1866	0.00466
ssc-mir-1285-p5	6004	14613	0.00707
bta-mir-2904-3-p3	1018	3033	0.00742
bta-miR-181a	1699	934	0.00749
mmu-let-7j	3207	2376	0.00779
mdo-miR-195	2310	900	0.00822
mmu-mir-5105-p5_1ss21TC	2793	6322	0.00839
bta-miR-574	383	647	0.00848
bta-miR-151-5p	4531	2426	0.00875
hsa-miR-151b_R+5	3178	1670	0.01000
bta-miR-377	693	303	0.01020
bta-miR-146a	2755	1673	0.01040
bta-let-7g	5353	4236	0.01050
cgr-miR-1973_L+5R+6	1910	3116	0.01090
bta-miR-15b	3522	2254	0.01120
ssc-miR-340	1226	649	0.01120
mmu-miR-146a-5p_L+1R+1_1ss19GA	2943	1546	0.01190
oar-miR-382-3p	641	346	0.01260
hsa-mir-4485-p5_1ss18CT	442	750	0.01340
oar-miR-411a-3p	3043	1882	0.01340
oar-miR-379-3p	2839	1891	0.01370
mmu-mir-5105-p5_1ss6GC	285	858	0.01410
PC-5p-80741_1	1436	2351	0.01410

bta-miR-450b	4096	2084	0.01470
cgr-miR-100-5p_R+5	477	1696	0.01540
bta-miR-125b	1161	2678	0.01610
bta-miR-23b-3p	11702	8779	0.01610
mmu-mir-5117-p3_1ss19TA	1378	2311	0.01630
sha-miR-716b	2042	5540	0.01700
PC-5p-54837_2	287	506	0.01710
hsa-miR-151b_R+3	4374	2403	0.01880
bta-miR-376a	3792	2302	0.01930
bta-miR-2440	326	538	0.02010
bta-miR-411c-5p	783	412	0.02030
PC-3p-21250_6	3128	1742	0.02060
PC-3p-29341_4	2214	4006	0.02210
bta-miR-195	2287	694	0.02270
bta-miR-2404	902	1861	0.02270
sha-mir-716a-p3	2075	7859	0.02330
bta-miR-214	2762	4361	0.02460
bta-mir-2887-2-p3	999	2411	0.02530
mmu-miR-1983	1411	886	0.02560
bta-miR-23a	10914	7896	0.02580
bta-miR-378c	1235	700	0.02600
bta-miR-15a	949	563	0.02640
mmu-mir-5105-p3_1ss21TC	2743	6547	0.02770
mmu-mir-5105-p3	2744	6651	0.02810
mmu-mir-5105-p3-_1ss24TC	2747	6848	0.02950
bta-miR-425-5p	898	584	0.02980
sha-mir-199a-p5_1ss23CA	698	1166	0.03230
bta-miR-451	3149	1532	0.03330
bta-miR-335	8903	5988	0.03340
bta-let-7i	3874	2923	0.03380
bta-miR-181c	1015	571	0.03430
mmu-mir-6240-p5_1ss20GT	1633	3378	0.03450
bta-mir-2904-3-p5	2778	5869	0.03460
bta-miR-30a-5p	11883	9888	0.03560
bta-miR-378c_R+1+1ss21TG	1300	800	0.03600
bta-miR-141	1846	869	0.03600
bta-miR-376d	5294	3202	0.03610
ggo-miR-574_1ss22AT	1148	3244	0.03700
bta-miR-432	2784	2054	0.03720
sha-miR-199a_1ss8TC	2177	3165	0.03790
bta-miR-16b	6791	4485	0.03950
bta-miR-100	563	1450	0.03990
bta-miR-199a-5p	801	1350	4.08E-02

mmu-miR-6243_R-5	1155	2238	0.04090
mmu-miR-6243_R+3	729	1605	0.04090
PC-5p-4028_62	2592	1352	0.04230
mml-miR-378d_1ss21CA	1037	679	0.04440
cfa-miR-203	3200	2123	0.04530
bta-miR-450a	1482	833	0.04780
bta-miR-17-5p	1470	1067	0.04820
PC-5p-12650_12	1129	1909	0.04880

[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations

Table 6: KEGG pathways for 33 significant differentially expressed miRNA

KEGG pathway	Gene Count
ABC transporters,	7
Adherens junction,	2
Adipocytokine signaling pathway,	2
Alzheimer's disease,	5
Amino sugar and nucleotide sugar metabolism,	5
Amyotrophic lateral sclerosis (ALS),	3
Antigen processing and presentation,	2
Apoptosis, Insulin signaling pathway,	2
Apoptosis, Toll-like receptor signaling pathway, Neurotrophin signaling pathway,	2
Axon guidance,	45
Axon guidance, Fc gamma R-mediated phagocytosis, Regulation of actin cytoskeleton,	2
B cell receptor signaling pathway,	2
Basal transcription factors,	4
Basal transcription factors, Huntington's disease,	4
Base excision repair,	4
Biosynthesis of unsaturated fatty acids,	2
Calcium signaling pathway,	6
Cardiac muscle contraction, Aldosterone-regulated sodium reabsorption,	4
Cell adhesion molecules (CAMs),	16
Cell adhesion molecules (CAMs), Adherens junction,	3
Cell adhesion molecules (CAMs), Tight junction, Leukocyte transendothelial migration,	2
Cell cycle,	7
Cell cycle, TGF-beta signaling pathway,	3
Chemokine signaling pathway,	6
Chondroitin sulfate biosynthesis,	4
Chondroitin sulfate biosynthesis, Heparan sulfate biosynthesis,	3
Circadian rhythm,	8
Complement and coagulation cascades,	4
Cysteine and methionine metabolism,	3
Cysteine and methionine metabolism, Selenoamino acid metabolism,	4
Cytokine-cytokine receptor interaction,	9
Cytokine-cytokine receptor interaction, Chemokine signaling pathway,	5
Cytokine-cytokine receptor interaction, Jak-STAT signaling pathway,	7
Cytokine-cytokine receptor interaction, TGF-beta signaling pathway,	7
Dorso-ventral axis formation,	3
ECM-receptor interaction,	3
ECM-receptor interaction, Cell adhesion molecules (CAMs),	2
Endocytosis,	39

Endocytosis, Fc gamma R-mediated phagocytosis,	5
Endocytosis, Huntington's disease,	2
Endocytosis, Tight junction,	2
ErbB signaling pathway, Calcium signaling pathway, Endocytosis,	2
Ether lipid metabolism,	3
Fatty acid metabolism, PPAR signaling pathway, Adipocytokine signaling pathway,	4
Fc gamma R-mediated phagocytosis,	3
Focal adhesion,	3
Focal adhesion, ECM-receptor interaction,	9
Focal adhesion, ECM-receptor interaction, Pathways in cancer, Small cell lung cancer,	5
Fructose and mannose metabolism,	2
Gap junction, Pathogenic Escherichia coli infection,	3
Glycerolipid metabolism,	2
Glycerolipid metabolism, Glycerophospholipid metabolism, Ether lipid metabolism,	3
Glycerophospholipid metabolism,	7
Glycosphingolipid biosynthesis,	5
Glycosylphosphatidylinositol(GPI)-anchor biosynthesis,	2
Hedgehog signaling pathway,	5
Hedgehog signaling pathway, Pathways in cancer, Basal cell carcinoma,	4
Heparan sulfate biosynthesis,	10
Huntington's disease,	3
Inositol phosphate metabolism, Phosphatidylinositol signaling system,	8
Insulin signaling pathway,	5
Jak-STAT signaling pathway,	7
Keratan sulfate biosynthesis,	2
Lysine degradation,	12
Lysosome,	21
MAPK signaling pathway,	25
Maturity onset diabetes of the young,	3
mTOR signaling pathway,	3
mTOR signaling pathway, Insulin signaling pathway,	3
N-Glycan biosynthesis,	6
Neuroactive ligand-receptor interaction,	26
Neurotrophin signaling pathway,	5
Nicotinate and nicotinamide metabolism,	3
Nitrogen metabolism,	3
NOD-like receptor signaling pathway,	3
Notch signaling pathway,	10
Nucleotide excision repair,	4
O-Glycan biosynthesis,	10
Oocyte meiosis, Wnt signaling pathway,	2
p53 signaling pathway,	9
Pantothenate and CoA biosynthesis,	2

Pathways in cancer, Acute myeloid leukemia,	2
Pathways in cancer, Pancreatic cancer,	3
Pathways in cancer, Renal cell carcinoma,	6
Pathways in cancer, Small cell lung cancer,	2
Pathways in cancer, Thyroid cancer,	2
PPAR signaling pathway,	2
Proteasome,	4
Purine metabolism,	5
Pyrimidine metabolism,	3
Regulation of actin cytoskeleton,	12
Regulation of autophagy,	3
Regulation of autophagy, mTOR signaling pathway,	2
Renin-angiotensin system,	2
Retinol metabolism,	3
RIG-I-like receptor signaling pathway,	5
RNA degradation,	11
Selenoamino acid metabolism, Aminoacyl-tRNA biosynthesis,	2
SNARE interactions in vesicular transport,	16
Sphingolipid metabolism,	10
Sphingolipid metabolism, Lysosome,	2
Spliceosome,	24
Systemic lupus erythematosus,	2
Terpenoid backbone biosynthesis,	2
TGF-beta signaling pathway,	10
Tight junction,	12
Tight junction, Regulation of actin cytoskeleton, Viral myocarditis,	2
Toll-like receptor signaling pathway, Pathogenic Escherichia coli infection,	2
Ubiquitin mediated proteolysis,	23
Ubiquitin mediated proteolysis, Endocytosis,	3
Ubiquitin mediated proteolysis, Parkinson's disease,	2
Ubiquitin mediated proteolysis, Pathways in cancer, Renal cell carcinoma,	2
Vascular smooth muscle contraction,	6
Vibrio cholerae infection,	4
Viral myocarditis,	2
Wnt signaling pathway,	13

Supplemental Table 1: Significant differentially expressed miRNA in d 190 CON versus CON/NR versus NR/CON[1] (low signal)[2]

	d 190			
mRNA	CON	NR/CON	CON/NR	*P* value
n	8	7	7	
mmu-let-7f-1-3p_1ss22CT	139	208	89	0.00050
bta-mir-2284b-p3_1ss10TA	34	19	26	0.00171
hsa-miR-3653_L+14	21	48	48	0.00215
bta-miR-4286	60	82	51	0.00296
bta-miR-210	46	66	91	0.00442
bta-miR-655	162	109	142	0.00512
ssc-let-7d-3p	296	328	192	0.00760
hsa-miR-4286_R+4	60	75	47	0.00769
bta-miR-2332	88	153	175	0.00850
oar-miR-494-5p_R-1_2ss12GA17AC	54	119	34	0.00882
hsa-miR-3676-5p_L+5R-1	12	13	31	0.00973
bta-mir-3431-p3	257	144	153	0.01050
cgr-miR-93-3p_1ss11TC	89	85	60	0.01500
bta-miR-1247-5p	38	67	29	0.01530
bta-miR-142-5p	94	73	143	0.01730
mmu-miR-3096b-3p_L_13R-1	6	14	26	0.01740
hsa-mir-5100-p3_1ss16TC	138	252	358	0.01770
bta-mir-2284aa-3-p3_1ss5TC	41	22	27	0.01800
bta-miR-3596	22	56	20	0.01930
bta-let-7a-3p	67	32	51	0.02060
bta-miR-2284z	153	92	133	0.02380
bta-miR-2484_R+8	205	274	342	0.02390
bta-miR-105b	52	42	25	0.02430
bta-miR-6120-3p	74	60	49	0.02540
oar-miR-539-3p_L-4R+2	136	68	87	0.02550
bta-mir-342-p5_1ss23GA	29	36	21	0.02720
bta-mir-6529-p3	733	65	45	0.02940
bta-miR-105a	43	36	21	0.03100
bta-miR-149-5p	30	17	18	0.03140
bta-mir-2284-p3_1ss16AT	62	28	32	0.03150
PC-5p-53837_2	287	442	299	0.03240
bta-miR-34a	111	113	181	0.03350
bta-miR-1185	51	32	44	0.03960
mmu-miR-136-3p	230	161	211	0.04080
PC-5p-43323_2	215	244	160	0.04190
bta-miR-362-3p	82	81	150	0.04260
bta-miR-452	361	258	245	0.04650

bta-miR-669	124	249	226	0.04710
bta-miR-500)1ss23AT	213	291	375	0.04940

[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations

[2]Detected signal intensity <500, based on a range from 1 to over 65,000

Supplemental Table 2: Significant differentially expressed miRNA; d 110 CON versus 110 NR[1] (lowly expressed)[2]

	d 110		
mRNA	CON	NR	*P* value
bta-miR-2285k	7	27	0.00961
bta-miR-2455	56	15	0.01180
bta-miR-128	229	358	0.01190
bta-miR-3578	80	22	0.01310
PC-3p-6894_28	23	76	0.01450
bta-miR-2430	35	11	0.01550
PC-3p-8217_21	59	118	0.01640
ssc-miR-196b-3p_L+7R-1	50	13	0.01680
bta-miR-499	16	32	0.01680
bta-mir-6529-p3	37	84	0.01690
bta-miR-192	111	172	0.01880
PC-3p-9715_17	49	101	0.01900
bta-miR-6525	40	18	0.02090
PC-5p-38342_3	20	44	0.02100
PC-5p-18625_8	74	159	0.02110
ssc-miR-450c-3p_1ss21GA	142	301	0.02150
bta-miR-2284z	87	181	0.02220
bta-miR-2285g	8	27	0.02230
bta-miR-1271	39	70	0.02320
sha-miR-7	154	309	0.02390
bta-miR-2487	341	72	0.02390
bta-miR-2285c	33	69	0.02450
hsa-miR-10b-3p	17	35	0.02450
bta-miR-2411-3p	313	181	0.02520
bta-miR-2440_R-4	369	134	0.02550
ssc-miR-24-1-5p	20	30	0.02710
bta-miR-130b	80	104	0.02730
bta-miR-1343-3p	37	25	0.02840
bta-miR-6528	261	70	0.02890
ssc-miR-450b-3p	104	191	0.02980
ssc-miR-331-5p	32	40	0.03000
bta-miR-6119-3p_L-1_1ss23AT	53	86	0.03380
bta-miR-2332	218	127	0.03430
mmu-mir-6236-p3	348	68	0.03440
bta-miR-6120-3p	45	82	0.03450
bta-miR-2284aa-4	98	166	0.03910
ssc-miR-28-3p_1ss11TA	272	377	0.03910
bta-miR-2311	147	33	0.04010

bta-miR-6119-3p	49	79	0.04020
ssc-miR-574_1ss2AG	228	81	0.04040
bta-miR-671	83	25	0.04170
bta-miR-2284y	244	461	0.04180
bta-miR-502b	108	139	0.04240
bta-miR-2374	228	54	0.04280
bta-miR-24	24	33	0.04310
bta-miR-2284h-5p	203	365	0.04340
bta-miR-190a	83	18	0.04380
bta-mir-3431-p3	184	266	0.04620
ssc-miR-148b-5p	19	37	0.04650
PC-5p-25581_4	32	11	0.04650
bta-miR-2449	76	19	0.04680
bta-miR-2904	274	113	0.04700

[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations

[2]Detected signal intensity <500, based on a range from 1 to over 65,000

Supplemental Table 3: Significant differentially expressed miRNA for d 190 CON versus d 110 CON[1] (low signal)[2]

mRNA	d 190 CON	d 110 CON	*P* value
bta-miR-2285e	51	22	0.00061
bta-miR-6525	15	40	0.00208
oan-miR-148-5p_2ss10TA18AC	121	67	0.00389
bta-miR-3596	22	80	0.00445
ssc-miR-450b-3p	255	104	0.00531
hsa-miR-3064-3p	21	47	0.00662
bta-miR-184	179	32	0.00706
ssc-miR-450c-3p_1ss21GA	312	142	0.00826
bta-miR-455-3p	39	111	0.00887
bta-miR-6520_R+3	12	37	0.01150
bta-miR-503-3p	218	271	0.01160
bta-miR-181b	371	231	0.01230
PC-3p-9715_17	79	49	0.01290
bta-miR-2284h-5p	369	203	0.01510
hsa-miR-10b-3p	43	17	0.01530
bta-miR-2332	88	218	0.01590
PC-5p-18625_8	152	74	0.01650
bta-miR-455-3p_1ss22TC	40	107	0.01700
bta-mir-6529-p3	73	37	0.01870
bta-miR-2411-3p	169	313	0.01880
eca-miR-338-5p	49	15	0.01930
PC-3p-6894_28	49	23	0.02010
bta-miR-96	126	86	0.02030
bta-miR-2284x	394	256	0.02060
bta-miR-144_R-3	59	15	0.02190
bta-miR-1343-3p	24	37	0.02210
bta-miR-2284z	153	87	0.02350
bta-miR-499	43	16	0.02560
bta-miR-6123	57	101	0.02600
bta-miR-183	418	245	0.02620
bta-mir-3431-p3	257	184	0.02840
rno-mir-136-p5	61	45	0.03070
hsa-miR-3653_L+14	21	49	0.03140
sha-miR-7	255	154	0.03240
bta-miR-222_R+4	96	203	0.03290
oar-miR-539-3p_L-4R+2	136	62	0.03300
bta-miR-411c-3p	399	218	0.03420
bta-miR-6528	84	261	0.03590

bta-miR-194	222	164	0.03620
bta-miR-2284aa-4	166	98	0.03640
bta-miR-1247-5p	38	72	0.03760
bta-mir-2284e-p3_1ss16AT	62	28	0.04100
bta-miR-2374	64	228	0.04210
bta-miR-2487	90	341	0.04250
bta-miR-331	309	430	0.04250
bta-miR-6120-3p	74	45	0.04320
cgr-miR-125b-3p	11	51	0.04360
hsa-mir-5100-p3_1ss16TC	138	233	0.04480
bta-miR-568	26	139	0.04620
bta-miR-199b	105	137	0.04930

[1]CON = Control cows fed 1.3 x of NRC NE_m and CP recommendations. NR = nutrient restricted cows fed 0.55 x of NRC NE_m and CP recommendations

[2]Detected signal intensity <500, based on a range from 1 to over 65,000

CHAPTER IV

CONCLUSION

Maternal nutrient restriction (0.55x of NRC recommendations for NE_m and CP) caused intrauterine growth restriction (IUGR) and altered expression of miRNA. While early gestation nutrient restriction has been shown to cause IUGR and subsequent decreased growth and higher incidence of metabolic disorders, we were able to show that nutrient restriction during midgestation caused just as severe IUGR. Further, realimentation has been shown to alleviate some of the effects of IUGR. However, with adequate nutrition but no realimentation, it does not seem as though the growth restriction can be completely overcome and may persist until parturition. Plasma glucose, serum triglycerides, and serum cholesterol concentrations were all affected by nutrient status, reflecting both the decreased nutrients available to the dam as well as the decreased nutrient transport due to decreased placentome growth. The placentomes and its components are the primary site for nutrient transport and poor nutrition decreases both the dam and fetus's ability to fully form placental connections, thereby altering placental efficiency. The increase of cotyledons, placentome number, and total placentome surface area in d 110 nutrient restricted cattle are a combination of fetal and maternal response to poor nutrition. The dam attempts to compensate by increasing the number of placentomes and the fetus puts more energy into developing cotyledon structures, overall leading to more surface area available for nutrient transport. However, as the fetus and dam allocate nutrient resources into building placentome structures, this decreases how many nutrients are left over for fetal growth and development. The increase of cotyledons and surface

area of d 110 – d 190 restricted animals again shows how the fetus is attempting to compensate for decreased nutrients by increasing the area available for nutrient transport. The decrease of placentome weight and caruncular weight in d 30 – d 110 restricted fetuses shows that the control diet from d 110 – 190, while not a full realimentation, was adequate enough for the dam and fetus to relax the allocation of nutrients to placental connections. Because there are two different models for nutrient transport, the fetal demand and maternal sensing models, and these models work in different directions, further work on the placentomes is required to fully elucidate precisely which mechanisms are causing tissue accretion.

There is little work done in the bovine model for miRNA and particularly placental expression of miRNA. There have been human placenta studies and as miRNAs are highly conserved across species, it is possible that their functions are also similar across species. The particular miRNA expression suggests that, while nutrient restricted animals are at a disadvantage, there are some coping mechanisms to help placenta development by increasing expression of miR-141 and miR-182. However, other miRNAs were responsible for increased adipogenesis in nutrient a restricted fetus, which works in conjunction with the thrifty phenotype, and also decreases vasculogenesis and angiogenesis during placental development, decreasing the routes available for maternal delivery of nutrients to the fetus.

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