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The Effect of Poor Maternal Nutrition on the Growth and Development of Offspring

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ABSTRACT

Poor maternal nutrition can have long lasting detrimental effects on offspring growth and development. Specifically, these offspring will exhibit increased carcass adiposity, reduced muscularity and altered growth rates resulting in animals that are less productive and yield a less desirable product for consumers. However, the mechanisms by which poor maternal nutrition alters offspring growth and development are not well understood. We hypothesized that maternal under- and over-nutrition will alter the expression of key genes and novel pathways involved in the development of muscle and adipose tissue of lambs at two early postnatal time points. To evaluate these hypotheses, pregnant sheep were fed 100 % [control (CON)], 60 % [restricted (RES)] or 140 % [over-fed (OVER)] of National Research Council requirements based on total digestible nutrients. One lamb from each ewe was necropsied either within 24 hour of birth or 3 months of age. RNA was isolated from adipose and muscle tissue respectively and gene expression quantified using real time RT-PCR. Alternately, transcriptome analysis was performed on isolated RNA using the Ion Torrent Proton sequencer and analyzed using the Cufflinks analysis package. Gene expression of *insulin-like growth factor (IGF)-I* was increased in the quadriceps muscle tissue of RES lambs at 1 day of age. Expression of *β-Catenin*, a factor involved in WNT signaling, was greater in the quadriceps muscle tissue of RES and OVER at 1 day compared with CON ($P = 0.07$). At day 1, the semitendinosus muscle tissue of OVER lambs exhibited increased *myostatin* (*MSTN;* $P = 0.06$) and *follistatin* (*FSTN;* $P = 0.04$) gene expression. Expression of factors involved in the somatotropic axis, *IGF-I* and *insulin-like growth factor binding protein (IGFBP)-3*, were also altered in the semitendinosus muscle tissue

of lambs born to RES and OVER ewes respectively ($P \leq 0.08$). No change in the expression of factors involved in adipogenesis were observed at either time point. As determined by RNA-seq analysis performed on semitendinosus muscle tissue collected from lambs at birth, 35 differentially expressed genes were identified using the bovine reference annotation whereas mapping to the ovine reference yielded 10. The identified genes are involved in various processes including the regulation of metabolism, hypertrophy, nutrient uptake, and muscle protein turnover. In summary, poor maternal nutrition can alter muscle development through several potential mechanisms, including the somatotropic axis and wnt signaling. Additionally, these mechanisms affected by maternal nutrition vary between OVER vs CON and RES vs CON born lambs despite similar alterations to muscle phenotype. Future studies are needed to identify additional factors altered by maternal nutrition as well as determine the effects of poor maternal nutrition on epigenetic mechanisms in offspring.

The Effect of Poor Maternal Nutrition on the Growth and Development of Offspring

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APPROVAL PAGE

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The Effect of Poor Maternal Nutrition on the Growth and Development of Offspring

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DEDICATION

This dissertation is dedicated to my husband Gregory Hoffman Jr, my daughter Isabel Grace, and my parents Joseph and Deborah Procopio

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INTRODUCTION

Livestock production is an integral component of the U.S. economy. In 2011, the U.S. livestock industry was responsible for generating $211 billion in revenue from the sale of fluid milk, fiber, and animals for meat (USDA National Agriculture Statistics Service, 2009). The products produced by these animals are a key component of the U.S. food supply. For example, meat and meat products account for 40 and 20 percent of daily protein and fat intakes of US consumers, respectively (Daniel et al., 2011). As a result, consumer demands on the livestock industry calls for meat products that are lean, highly palatable, and affordable. Therefore, livestock producers must raise animals that grow as efficiently as possible and yield a high quality product. Animals that exhibit a reduced growth rate, increased adipose tissue deposition, and increased intramuscular connective tissue content are undesirable to the consumer and can be less profitable for the producer. There are several factors that can reduce productivity of livestock species. One of these factors, maternal programming, impacts offspring growth and development postnatally. This is achieved through changes to the development of various organ systems and tissues of the offspring. Offspring often exhibit increased size of vital organs at the expense of other tissues such as the muscle and are predisposed to developing adiposity (Wu et al., 2004, 2006). By altering the balance between animal growth, muscle development, and carcass adiposity, maternal programming is a threat to livestock operation efficiency and profitability. Therefore, research is needed to better understand how maternal programming is caused in livestock species and its effects on growth regulation and offspring development.

REVIEW OF LITURATURE

Maternal Programming

Maternal programming is defined as changes in the intrauterine environment during gestation that can negatively impact embryonic and fetal development resulting in alterations to organ function, tissue development, and metabolism which can have long-lasting, detrimental effects on the offspring (Barker and Clark, 1997; Petry et al., 2001; Barker, 2004, 2007; Wu et al., 2006). These programming effects are permanent and the adaptations that occur can affect subsequent stages of fetal and postnatal growth and development (Nesterenko and Aly, 2009). There is also evidence that the effects of maternal programming may be passed on to subsequent generations when these offspring reproduce (Nesterenko and Aly, 2009). Several maternal factors can have a "programming" effect on offspring development. These factors include but are not limited to, uterine capacity, exposure to disease, stress, and maternal diet composition (Wu et al., 2006). Of these factors, recent research interests have focused on the effects of poor maternal nutrition on offspring as altered nutritional status during pregnancy frequently occurs in livestock production operations.

Poor Maternal Nutrition during Gestation

During gestation, maternal nutrient partitioning is altered to ensure that the nutritional demands of the developing fetus are met. However, within livestock production systems there are several instances where nutrient availability is altered resulting in changes to the maternal and fetal planes of nutrition. Specifically, poor maternal nutrition can result from under- or over-feeding during gestation which can occur in various animal management systems. Forage based systems are commonly used within the sheep and cattle industry (Greenwood and Thompson, 2007). Animals reared within these

systems can experience periods of under-nutrition due to seasonal variations in forage quality and quantity, which decreases through the fall and winter months. This decrease in feed availability and nutrient content correlates with when these animals are typically bred or are pregnant. Over-feeding during pregnancy is most commonly observed in humans within first world countries (King, 2006). For example, one third of women who are pregnant are classified as obese (King, 2006; Center for Disease Control, 2012). In the livestock industry over-feeding during gestation can result from management practices as well. Flushing is a practice specific to the sheep industry where total dietary nutrients consumed are increased approximately 60 percent for a period of two weeks before and up to four weeks after breeding {National Research Council (NRC), 1992}. This is done to increase ovulation to ensure that the maximum number of twins is conceived in order to achieve maximum profitability during the lambing season.

Both maternal under- and over-feeding can affect fetal growth and development; however, how the effects are manifested will vary depending on the nutrients that have been altered, stage of gestation, and the duration of under- or over-feeding (Wu et al., 2006). The early stages of development are classified as embryonic development. The majority of development that occurs during this stage pertains to the establishment of the placenta and growth of rudimentary structures of the offspring during organogenesis. Therefore, changes to maternal diet during the early stages of gestation can affect the establishment of key organs and tissues. Alternately, fetal development occurs after these basic structures have been established (Wu et al., 2004, 2006). The last six weeks of fetal development are synonymous with the most rapid stage of fetal growth and development with proper nutrition being essential for adequate body weight gains, tissue accretion, and metabolic function (Wu et al., 2004, 2006). Exposure of offspring to poor maternal nutrition during the later stages of development has the potential to effect offspring size, body weight, and body composition (Wu et al., 2004, 2006). Several

studies have indicated that changes to maternal diet throughout and at specific stages of gestation can result in reduced growth rates, reduced muscle development, and increased fat deposition (Wu et al., 2006; Daniel et al., 2007; Ford et al., 2007; Tong et al., 2009; Huang et al., 2010; Yan et al., 2011).

These alterations are problematic from both a livestock production and animal health perspective. Reduced muscle development and increased adipose tissue deposition is synonymous with reduced carcass quality and yield. There is also potential for these changes to muscle and adipose tissue to have a role in altering metabolic regulation, thus affecting animal growth rates and overall animal health (Gardner et al., 2005; King, 2006; De Blasio et al., 2007; Ford et al., 2007; Muhlhausler and Smith, 2008; Pinney and Simmons, 2010; Smith et al., 2013; Hoffman et al., 2014b).

Muscle Growth and Development

Muscle Function and Structure:

Muscle comprises 50% of an animal's body weight and therefore is a major component of animal body composition (Sanchez et al., 2014) and is responsible for locomotion. In livestock species, proper muscle development is critical since muscle is the major product yielded by animals used for meat production. Muscle is also responsive to insulin and capable of utilizing glucose (Brown, 2014). Briefly, the primary function of muscle involves contracture of muscle fibers. Muscle fibers can be divided into two different fiber types; slow twitch and fast twitch fibers which can be further divided into subtypes based on energy substrate preferences (Biressi et al., 2007; Schiaffino et al., 2013). Predominance of these subtypes can alter the role of muscle in metabolism as well as affect palatability and meat quality (Biressi et al., 2007; Daniel et al., 2007; Dayton and White, 2008; Rehfeldt et al., 2011; Schiaffino et al., 2013). Another key

component of muscle is a population of quiescent stem cells known as satellite cells. Satellite cells play a critical role in muscle repair and postnatal hypertrophy of postmytotic myofibers (Pallafacchina et al., 2013; Pallafacchina et al., 2013; Schiaffino et al., 2013). In considering the multifaceted function of muscle, issues pertaining to animal productivity and health can arise if myogenesis is altered. The following sections will discuss the stages of myogenesis and the factors that are involved in regulating these processes.

Embryonic and Fetal Myogenesis:

Prenatal muscle development can be divided into two different stages: primary and secondary myogenesis (Biressi et al., 2007; Messina and Cossu, 2009; Braun and Gautel, 2011). Embryonic myogenesis occurs during the early stages of development shortly after implantation and begins with the migration of mesenchymal stem cells (MSC) from the somites (Braun and Gautel, 2011). Mesenchymal stem cells are a pool of multipotent stems cells that are capable of committing to different cell lineages depending on the regulatory factors present (Braun and Gautel, 2011). During muscle development, MSC will commit to the myogenic cell lineage and become myoblasts with the aid of several wingless-int (Wnt) proteins (Le Grand and Rudnicki, 2007; Messina and Cossu, 2009) and the transcription factors paired box (PAX) 3 and 7. Specifically, Wnt, along with sonic hedgehog, will induce the expression of PAX3 and 7 in MSC cells. Next, PAX3 and 7 will increase expression of the myogenic regulatory factors (MRFs) myogenic factor (Myf) 5 and myogenic differentiation 1 (MyoD), thus establishing the progenitor cells into myoblasts (Messina and Cossu, 2009). The aforementioned MRFs will in turn increase the expression of myogenin and MRF4 resulting in the differentiation of the myoblasts into myotubes (Messina and Cossu, 2009). This establishment and fusion of myoblasts

into myotubes occurs in two waves known as primary and secondary myogenesis (Biressi et al., 2007; Maltin, 2008). Primary myogenesis begins at approximately 32 to 38 d gestation in sheep (Maltin, 2008). This stage of myogenesis involves the establishment of primary muscle fibers from embryonic myoblasts (Biressi et al., 2007). Alternately, secondary myogenesis, which occurs during fetal development, begins at 62 d of gestation (Maltin, 2008). Secondary myogenesis includes fusion of myoblasts with primary myofibers as well as the establishment of smaller secondary myofibers which will surround primary myofibers and eventually increase in size (Biressi et al., 2007). There is also a third class of myofibers known as tertiary myofibers. These fibers develop after primary and secondary myogenesis and differ in myosin composition when compared to primary and secondary muscle fibers (Mascarello et al., 1992). In addition to regulating PAX gene expression in precursor cells the aforementioned Wnt proteins are also involved in other aspects of myogenesis. Binding of Wnt to its receptor will inhibit glycogen synthase kinase (GSK)β/axin/ASP complex formation and allow β-catenin to accumulate within the cell ultimately increasing the gene expression of MyoD (Tong et al., 2009; Du et al., 2010). Binding of Wnt proteins is key as degradation of β-catenin by the GSKβ complex favors commitment of cells to the adipocyte lineage (Tong et al., 2009; Du et al., 2010; Duarte et al., 2014). If the Wnt pathway is not activated, the β-catenin protein will be degraded by GSK-3β which can result in an increased number of adipocytes and a reduction in the number of myocytes (Tong et al., 2009; Du et al., 2010).

In addition to muscle fibers, the establishment of satellite cells also occurs during embryonic myogenesis. Satellite cells lie between the sarcolemma and basal lamina of the muscle fiber (Mauro, 1961; Messina and Cossu, 2009; Pallafacchina et al., 2013; Schiaffino et al., 2013). Populations of satellite cells are greatest in number before and in early postnatal life (Mauro, 1961; Messina and Cossu,

2009; Pallafacchina et al., 2013). As the animal ages, the number of satellite cells is reduced to less than 5% of the total nuclei associated with the muscle fiber (Pallafacchina et al., 2013). Satellite cells are established from PAX3 expressing progenitor cells at the end of secondary myogenesis (Biressi et al., 2007; Le Grand and Rudnicki, 2007). These cells, unlike other muscle cells, will continue to express PAX7 and remain in a quiescent state until activated or when undergoing self-renewal postnatally (Clemmons, 2009; Pallafacchina et al., 2013; Schiaffino et al., 2013).

Postnatal Myogenesis:

In contrast to prenatal myogenesis, muscle fiber hyperplasia is limited during postnatal myogenesis; therefore, postnatal myogenesis primarily involves hypertrophy of the muscle fibers and changes in protein accretion. Hypertrophy of the muscle fiber predominantly occurs through the incorporation of satellite cells rather than establishment of new myoblasts from MSC. In their quiescent state, satellite cells will express PAX7 while maintaining minimal amounts of gene expression and protein synthesis (Le Grand and Rudnicki, 2007; Messina and Cossu, 2009). Activation of satellite cells can result from intrinsic factors such as sphingosine-1-phosphate production or from growth factors such as fibroblast growth factor and IGF-I (Le Grand and Rudnicki, 2007; Clemmons, 2009). Quiescent satellite cells will become activated in response to injury or an increase in physical loading on the muscle tissue (Le Grand and Rudnicki, 2007; Pallafacchina et al., 2013; Schiaffino et al., 2013). As satellite cells become activated, PAX7 will be coexpressed with MyoD, the cells will enter the cell cycle and migrate from the basal lamina inward towards the muscle fiber (Le Grand and Rudnicki, 2007). As a result, the satellite cells will become myoblasts which in turn will down regulate PAX7 and will exhibit increased expression of MRFs such as myogenin (Le Grand and Rudnicki, 2007). This results in the

differentiation and incorporation of these cells into the muscle fiber and therefore will increase the number of fiber-associated nuclei (Le Grand and Rudnicki, 2007). Satellite cell self-renewal can also occur during postnatal myogenesis and will aid in the maintenance of the quiescent satellite cell population. During self-renewal, satellite cells will become activated and proliferate as myoblasts expressing PAX7 in addition to Myf5, MyoD, and PAX3 (Le Grand and Rudnicki, 2007; Clemmons, 2009). After proliferating for several cell cycles, these cells will cease expression of MRFs and return to a quiescent state (Le Grand and Rudnicki, 2007).

Another key aspect of postnatal myogenesis is protein turnover. This involves the accretion of amino acids and the degradation of proteins in muscle tissue (Clemmons, 2009). This process can be altered as a result of postnatal diet as well as various growth factors, such as insulin and insulin-like growth factor-I (IGF-I; Clemmons, 2009), which will be discussed in detail in the following section. Myostatin (MSTN) is another transcriptional regulator, which is responsible for increasing protein degradation in muscle tissue (Huang et al., 2011; Argiles et al., 2012; Schiaffino et al., 2013). Myostatin can regulate both prenatal and postnatal myogenesis by inhibiting muscle growth and differentiation (Huang et al., 2011). Deficiency in MSTN has been observed in mice and cattle, and results in increased muscle development (Huang et al., 2011; Argiles et al., 2012; Schiaffino et al., 2013). Myostatin regulates myogenesis by binding to an activin type two receptor, which results in the phosphorylation of SMAD family member (SMAD) 2/3, which in turn will complex with SMAD4 (Argiles et al., 2012). This complex is capable of translocation into the nucleus, binding to a response element and inhibiting expression of MRFs (Argiles et al., 2012). Myostatin binding also inhibits the canonical Wnt signaling pathway and activation of phosphoinositide 3-kinase (PI3K; Joulia-Ekaza and Cabello, 2006; Argiles et al., 2012). Binding of MSTN to its receptor can be inhibited by the protein follistatin

(FSTN), thus preventing proteolysis and promoting myogenesis. In addition to the aforementioned factors, myogenesis and protein turnover can be altered as a result of factors involved in the somatotropic axis.

Muscle development and the somatotropic axis:

Previous work in ruminant and non-ruminant species has demonstrated that nutritional status can alter several factors involved in the somatotropic axis (Govoni et al., 2002, 2003, 2004). Similarly, it has been observed that maternal nutrient restriction can alter the somatotropic axis in the offspring with effects persisting into adulthood and subsequent generations (Bauer et al., 1995). The somatotropic axis is comprised of a series of hormones, growth factors, and regulatory proteins that are involved in the growth and development of tissues including muscle and adipose (Le Roith et al., 2001). Briefly, the somatotropic axis involves the secretion of GH into circulation from the anterior lobe of the pituitary gland. The release of GH is either facilitated by growth hormone releasing hormone or inhibited by somatostatin, both of which are produced by the hypothalamus (Le Roith et al., 2001). Growth hormone is capable of binding receptors on tissues and eliciting its effects directly or can act via IGF-I (Le Roith et al., 2001; Le Roith, 2003). Insulin like growth factor-I is produced and secreted into circulation from the liver as a result of GH binding to its hepatic receptors (Le Roith et al., 2001). The liver also produces six IGF-I binding proteins (IGFBP) in response to circulating GH and IGF-I concentrations (Le Roith et al., 2001; Le Roith, 2003). These binding proteins can help to facilitate or sequester the actions of IGF-I. For example, IGFBP-3 will bind circulating IGF-I proteins along with an acid liable subunit (ALS) forming a complex that prevents the degradation of IGF-I and aids in IGF-I receptor binding, thus facilitating the effects of IGF-I (Le Roith et al., 2001; Le Roith, 2003).

Insulin-like growth factor is also produced locally within tissues and acts in an autocrine and/or paracrine manner (Le Roith et al., 2001; Le Roith, 2003; Clemmons, 2009). During fetal development there are some alterations to the regulation of the somatotropic axis (Gluckman and Pinal, 2002; Holt, 2002). Specifically, during fetal development, IGF-I and IGF-II production and secretion are mediated by insulin rather than GH (Gluckman and Pinal, 2002; Holt, 2002).

Both GH and IGF-I are capable of binding to receptors present on muscle tissue. The primary role of GH in muscle tissue is to decrease glucose utilization, increase fatty acid utilization, and increase amino acid sparing (Clemmons, 2009). This can be achieved by GH binding directly to its receptor; however, the actions of GH are predominantly mediated by IGF-I in this tissue type (Clemmons, 2009). During prenatal myogenesis IGF-I can aid in the proliferation and differentiation of precursor cells. During postnatal myogenesis, IGF-I regulates DNA synthesis, protein turnover, cell survival, and satellite cell maintenance and incorporation (Clemmons, 2009). The involvement of IGF-I in myogenesis is key as the loss of a functional IGF-I receptor results in decreased muscle size and cell number (Clemmons, 2009). Insulin-like growth factor-I can increase cellular proliferation by inhibiting the expression of the myogenic factor, myogenin. However, at greater concentrations, IGF-I can induce expression of myogenin favoring differentiation (Clemmons, 2009). Protein turnover is affected by the activation of the phosphatidylinositol 3-kinase pathway, which is activated by IGF-I receptor binding (Clemmons, 2009). Activation of this pathway results in phosphorylation of protein kinase B (AKT) which will complex with mammalian target of rapamicin (MTOR). This allows for MTOR to phosphorylate P70S6 kinase and eukaryotic translation initiation factor 4E-binding protein 1(4EBP1). Phosphorylation of these factors results in an increase in translation and prevention of translation inhibition respectively (Clemmons, 2009). Protein kinase B is also responsible for

phosphorylating forkhead box (FOXO) 1. Forkhead box1 is involved in the regulation of IGF-I signaling by translocation to the nucleus and increasing expression of genes involved in proteolysis (Clemmons, 2009). When IGF-I binds its receptor and AKT is activated, FOXO1 will be phosphorylated by AKT preventing the translocation of this protein (Clemmons, 2009).

This is a portion of the IGF-I signaling pathway that can be altered by nutritional status (Clemmons, 2009). Increased energy status results in increased AKT activity, favoring protein accretion (Clemmons, 2009). Alternately, when energy status of an individual is reduced, AKT is reduced leading to an increase in AMP kinase. Adenosine monophosphate (AMP) kinase then phosphorylates tuberous sclerosis factor (TSC) 2 which leads to the degradation of MTOR (Clemmons, 2009). Insulin-like growth factor-I stimulation of AKT also results in the phosphorylation of serine 9 of GSKβ, a protein involved in the WNT signaling pathway. This results in increased predominance of eukaryotic translation initiation factor 4E (eIF4E), which regulates genes needed for increased protein accretion within muscle tissue (Clemmons, 2009).

Overall, the development of muscle tissue can be divided into two distinct stages, prenatal and postnatal myogenesis. Establishment of muscle fibers occurs during prenatal myogenesis with changes in muscle fiber size and protein content occurring both prenatally and postnatally. Muscle tissue development is an essential part of rearing animals for livestock production. Likewise, carcass adiposity and adipose tissue development is another key aspect of livestock production. Therefore, alterations to adipose tissue development can affect muscle composition as well as overall body composition.

Adipose Tissue Function and Structure:

Adipose tissue accretion is a key aspect of the livestock industry and meat production as adiposity can affect meat palatability and carcass quality. Adipose depots that can alter carcass quality are abdominal and subcutaneous fat. Increased adiposity of these depots is synonymous with reduced carcass yield as more of the animal's mass is fat which will be discarded. Physiologically, these depots aid in thermoregulation, protection of vital organs, and nutrient storage (Mohamed-Ali et al., 1998; Ailhaud, 2006). Adipose tissue can be divided into two different types: brown adipose tissue (BAT) and white adipose tissue (WAT; Symonds et al., 2003; Feve, 2005; Ali et al., 2013). Both are capable of storing triglycerides (TG); however, the primary function of BAT is non-shivering thermogenesis (Symonds et al., 2003; Ali et al., 2013). Primarily, BAT is found in neonatal animals and is replaced with WAT as animals mature.

The primary function of WAT is the storage of nutrients which in turn can be used to supply energy as needed (Morrison and Farmer, 2000). Adipocytes are capable of responding to insulin and facilitating the uptake of glucose into the cells where it is stored as TG (Morrison and Farmer, 2000). Likewise adipocytes will also take up and store free fatty acids (FFA; Morrison and Farmer, 2000). The typical morphology of adipocytes are rounded cells with lipid droplets present in the cytoplasm which comprises the majority of the cell. As the cell takes up more lipid, the nucleus will be pushed further to the periphery of the cell and the cell will increase in size (Morrison and Farmer, 2000). Mature adipocytes comprise one-third of the cells present in adipose tissue with vasculature, fibroblasts, and pre-adipocytes also found in this tissue type (Ali et al., 2013).

In addition to facilitating nutrient storage, adipose tissue is capable of producing several growth factors and hormones that contribute to the regulation of metabolism and inflammatory response

(Morrison and Farmer, 2000; Ali et al., 2013). These factors include, but are not limited to, leptin, adiponectin, interleukin (IL)-6 and tumor necrosis factor (TNF)-α (Morrison and Farmer, 2000; Ali et al., 2013). Changes to the production of these factors can occur as a result of increased or decreased adiposity, altering the metabolic regulation of an individual (Ailhaud, 2006). Adipose tissue is also responsive to hormones and growth factors; specifically insulin, IGF-I, and GH (Morrison and Farmer, 2000; Le Roith et al., 2001). These hormones and growth factors help to regulate adipogenesis, as well as, facilitate the uptake of nutrients into the adipocytes (Morrison and Farmer, 2000). In considering the key structural and functional roles of adipose tissue, the establishment of adipocytes from precursor cells via adipogenesis is critical.

Adipogenesis:

The establishment of adipose tissue begins with the commitment of MSC to the adipose cell lineage through the formation of pre-adipocytes (Ali et al., 2013). These pre-adipocytes then undergo a secondary differentiation event resulting in mature adipocytes capable of storing lipid. Adipocytes can increase both in size and number throughout the lifetime of the animal as populations of pre-adipocytes are maintained within adipose tissue (Ali et al., 2013). The differentiation of pre-adipocytes during fetal and postnatal development involves several key transcription factors, specifically CCAAT enhancer binding proteins (CEBP) α and β and peroxisome proliferator-activated receptor (PPAR)-γ ; Ali et al., 2013). These transcription factors are essential for the development and function of adipose tissue as mutations in these factors negatively affect adiposity (Ali et al., 2013). CCAAT enhancer binding protein β is responsible for increasing PPARγ expression during differentiation. Peroxisome proliferator-activated receptor γ will then complex with retinoic acid receptor (RXR), translocate to the nucleus and

bind to the corresponding response element (Morrison and Farmer, 2000; Ali et al., 2013). Binding of this response element ultimately increases the expression of genes involved lipid metabolism, glucose homeostasis, and adipocyte differentiation (Ahmadian et al., 2013). Specific to adipocyte differentiation, PPARγ induces the expression of CEBPα. In turn, CEBPα will increase the expression of PPARγ via a feedback loop mechanism (Ahmadian et al., 2013).

While the involvement of PPAR and CEBP in the differentiation of adipocytes remains consistent between prenatal and postnatal adipogenesis, fetal adipose tissue development largely involves the establishment of BAT. In sheep, 2% of a lamb's birth weight is comprised of BAT and is primarily found surrounding vital organs such as the kidney. Increased deposition of BAT occurs during the last third of gestation when BAT fat mass is increased and up-regulation of the gene uncoupling protein (UCP) 1 occurs (Symonds et al., 2003). During fetal adipogenesis, expression of UCP1, in addition to the aforementioned factors, is essential to the development of BAT. The regulation of UCP1 expression is dependent on the concentrations of prolactin, thyroid hormone, and catecholamines, which the fetus is exposed to during development (Symonds et al., 2003). Unlike rodent species, BAT does not persist into adulthood in livestock animals. Postnatally, the function of adipose tissue shifts to the storage of FFA and TG (Symonds et al., 2003).

Regulation of adipogenesis

Differentiation of pre-adipocytes to mature adipocytes postnatally can be induced by insulin, which increases the expression of PPARγ and activates the PI3K pathway (Morrison and Farmer, 2000; Ahmadian et al., 2013; Ali et al., 2013). Insulin also facilitates adipose tissue accretion by increasing the translocation of glucose transporter (GLUT) 4 receptor to the plasma membrane of the adipocyte resulting

in increased glucose uptake and ultimately increased storage of triglycerides (Morrison and Farmer, 2000). Therefore, insulin sensitivity of adipocytes is critical, as reduced response to insulin results in decreased uptake of triglycerides and can ultimately contribute to the development of metabolic syndrome. Similar to muscle tissue, the somatotropic axis is also involved in the regulation of adipose tissue growth and development. Growth hormone is capable of acting directly on adipose tissue since these receptors are present on both pre- and mature adipocytes (Louveau and Gondret, 2004). However, the presence and predominance of growth hormone receptor (GHR) has been found to vary depending on the depot (Louveau and Gondret, 2004).

Exposure of adipocytes to GH results in a decrease in adipogenesis. Specifically, GH treatment in vitro has been found to decrease pre-adipocyte differentiation in several species, including sheep, potentially by reducing expression of PPARγ (Louveau and Gondret, 2004). In mature adipocytes, GH reduces insulin sensitivity and lipoprotein lipase action and inhibits insulin-stimulated activation of fatty acid synthase (Louveau and Gondret, 2004). This results in a decrease in TG uptake by GLUT4 as well as the synthesis of TG for storage. Regulation of GH actions occurs locally as IGF-I concentrations within the adipocyte can be increased in response to GH binding. As discussed in the section on muscle development, IGF-I can increase cellular proliferation and differentiation via activation of the PI3K pathway. IGF-I, like insulin, can also help to facilitate increased TG deposition within adipocytes (Le Roith, 2003). The functions of adipose tissue are regulated by several hormones and growth factors. However, adipose tissue is capable of producing adipokines, such as leptin and adiponectin that can affect the regulation of metabolic processes and tissues.

Endocrine function of adipose tissue:

In addition to storing energy in the form of fat, adipose tissue also has endocrine functions. Leptin is a 16 kDa protein resulting from expression of the obese (OB) gene and primarily produced by WAT (Houseknecht et al., 1998; Ahima and Osei, 2004; Ailhaud, 2006). The concentrations of circulating leptin are proportional to the amount of WAT present and can be increased in response to insulin, glucocorticoids, and overfeeding (Houseknecht et al., 1998). Alternately, leptin concentrations can decrease in response to fasting, thyroid hormone and cold temperatures (Houseknecht et al., 1998). Leptin mRNA expression can also be reduced in response to PPARγ binding a response elements at the obese gene promoter (Houseknecht et al., 1998). Leptin is capable of regulating nutrient partitioning, energy homeostasis, and appetite (Houseknecht et al., 1998; Ahima and Osei, 2004). One of the ways that leptin regulates nutrient partitioning is by increasing secretion of GH which inhibits fat accretion (Houseknecht et al., 1998; Ahima and Osei, 2004). Leptin is also known as an anorexic agent as it is able to suppress appetite. Leptin accomplishes this by decreasing the expression of the protein neuropeptide Y, a known orexic agent (Houseknecht et al., 1998; Ahima and Osei, 2004). In obese individuals, leptin resistance has been observed when, despite increased concentrations of leptin, the normal physiological effects of the hormone do not occur (Houseknecht et al., 1998; Ahima and Osei, 2004). Potential mechanisms of leptin resistance include alterations in leptin receptor binding and leptin transport across the blood brain barrier (Houseknecht et al., 1998).

In addition to leptin, the protein hormone adiponectin is also produced by adipocytes. The expression of this protein is regulated by several transcription factors including PPARγ, CEBPα, and FOXO1 which can increase the expression of adiponectin by the adipose tissue. Like leptin, adiponectin

plays a critical role in the regulation of metabolism. This 30 kDa protein is responsible for increasing lipid catabolism as well as increasing insulin sensitivity within tissues (Shankar et al., 2010; Nigro et al., 2014). For example, within the muscle tissue, this is accomplished via the activation of adenosine monophosphate activated kinase (AMPK) and PPARα resulting in increased glucose uptake and fatty acid oxidation (Ailhaud, 2006). In contrast to leptin, obesity leads to a reduction in the production of adiponectin, thus contributing to the reduced inulin sensitivity and alterations to metabolism often observed by this condition. Therefore, in considering the role of the aforementioned transcription factors in adipogenesis as well as the regulatory roles of leptin and adiponectin, these are potential mechanisms by which poor maternal nutrition my alter offspring growth and development.

Effect of maternal nutrition on offspring muscle development and adiposity:

Development of muscle and adipose tissue requires the involvement of key transcription factors, growth factors, and hormones. In turn, these tissues have an integral role in animal body composition, metabolism, and ultimately production efficiency. Recent work has identified that poor maternal nutrition can alter muscularity and adiposity of offspring (Wu et al., 2004, 2006; Ford et al., 2009; Long et al., 2010a). In regards to muscle development, it has been reported that lambs born to over-fed ewes exhibit increased connective tissue content, reduced myofiber diameter, and increased space between the myofibers. These animals also exhibit a greater number of intramuscular adipocytes (Tong et al., 2008, 2009; Du et al., 2010; Zhu et al., 2012; Yan et al., 2013). Alterations to cross-sectional area and muscle fiber type have also been observed as a result of poor maternal nutrition (Daniel et al., 2007). Alternately, animals born to both under- and over-fed ewes have exhibited greater adiposity with increased deposition at various fat depots, overall increased body fat percentage, and increased expression of factors involved in adipogenesis (Wu et al., 2006; De Blasio et al., 2007; Long et al.,

2009, 2010a). The aforementioned changes demonstrate that poor maternal nutrition is potentially altering the development of these tissues during gestation resulting in listed phenotypical changes during gestation and postnatally. In an effort the better understand how poor maternal nutrition can cause these changes research has been focusing on how factors involved in the development of this tissues may be altered.

As mentioned in the section on muscle development, myogenic factors are needed for the establishment of myofibers during gestation. In a study conducted by Tong et al. (2009), fetuses from ewes fed an obesogenic diet exhibited a reduction in the expression of key myogenic factors, myogenin and MyoD (Tong et al., 2009). Likewise similar studies observed increased expression of genes involved in connective tissue development and muscle inflammation of offspring (Huang et al., 2010). However, knowledge about how these changes persist beyond this stage of fetal development and into postnatal life is limited.

In regards to adipose tissue development, the expression of factors that regulate adipogenesis as well as adipokines produced by adipose tissue can be altered by maternal diet. Offspring born to under- and over-fed ewes have exhibited altered insulin production and insulin response when evaluated using a glucose tolerance test (Long et al., 2010b; Yan et al., 2011). This can be problematic as response to insulin is needed in order to take up TG and store FFA in adipose tissue. Consequently, these animals may be predisposed to the development of obesity, cardiovascular disease, and insulin resistance (i.e. metabolic syndrome). Likewise, alternations in leptin production have been observed as a result of poor maternal nutrition in both livestock as well as rodent studies (Long et al., 2011; Coupe et al., 2012). The secretory patterns of this hormone differ in offspring who were born to mothers fed an obesogenic diet when compared with those that were born to control fed mothers (Long et al., 2011; Coupe et al., 2012). These

data suggest that poor maternal nutrition can alter leptin production in offspring postnatally and may contribute to alterations observed in offspring metabolism and body composition.

In considering the findings published in recent literature and the regulation of adipose and muscle tissue development, there are several potential mechanisms that may be altered by poor maternal nutrition. However, the persistence of these alterations into postnatal development and impacts on tissue development are not well understood. Therefore, in considering the importance of muscle and adipose tissue in livestock production, we have selected these two tissues as the focal point of our work. In evaluating these tissues, the focus will be on the mechanisms by which poor maternal nutrition alters muscle and adipose tissue growth and development during early postnatal growth.

CHAPTER I: POOR MATERNAL NUTRITION DURING GESTATION IN SHEEP REDUCES CIRCULATING CONCENTRATIONS OF INSULIN-LIKE GROWTH FACTOR-I AND INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 IN OFFSPRING

Hoffman, M. L., M. A. Rokosa, S. A. Zinn, T. A. Hoagland, and K. E. Govoni. 2014b. Poor maternal nutrition during gestation in sheep reduces circulating concentrations of insulin-like growth factor-I and insulin-like growth factor binding protein-3 in offspring. Domest. Anim. Endocrinol. 49: 39-48.

INTRODUCTION

Fetal development is a period of rapid growth that is tightly regulated by several systemic and local factors. Therefore, alterations to the maternal environment during gestation can negatively impact the normal growth and development patterns of the offspring during postnatal growth. Maternal nutrition can greatly impact fetal development and have persistent effects throughout postnatal growth and into adulthood. In sheep, poor maternal nutrition during gestation due to either under- or over-nutrition can alter growth rate, reduce muscle development and increase fat deposition in offspring (Wu et al., 2004; Daniel et al., 2007; Ford et al., 2007; Tong et al., 2009; Yan et al., 2011). Specifically, poor maternal nutrition reduces myofiber diameter and myofiber number and increases muscle connective tissue content in the offspring (Zhu et al., 2006; Tong et al., 2009; Huang et al., 2010; Yan et al., 2011; Yan et al., 2013). Similarly, increased adipose accumulation within muscle tissue and other adipose tissue depots has been observed in the offspring (Du et al., 2010; McNamara et al., 2011). Consequently, these modifications can affect carcass quality and predispose the offspring to metabolic disorders. While some of the phenotypic changes in offspring that result from poor maternal nutrition have been identified, the mechanisms through which these modifications occur are not fully understood.

The somatotropic axis, including GH, IGF-I and IGFBP, is an important regulator of growth and development of several tissues, including muscle and adipose. Knockout-mouse models in which BW is reduced 25 and 67% in GH- and IGF-I-deficient mice, respectively have demonstrated the critical roles of GH and IGF-I in development (Mohan et al., 2003). In addition, we and others have shown that components of the GH/IGF pathway are altered by nutritional status in cattle and humans (Elsasser et al., 1992; Grinspoon et al., 1996; Rausch et al., 2002). Specifically, in a state of reduced growth and/or restricted nutrition, serum concentrations of IGF-I and IGFBP-3 are reduced and IGFBP-2 is increased (Rausch et al., 2002). Intrauterine growth retardation is associated with reduced circulating IGF-I and IGFBP-3 and increased GH and IGFBP-2 (de Zegher et al., 1997). Similarly, both circulating GH and IGF-I concentrations are reduced in the offspring of mothers who have been undernourished (Gluckman and Pinal, 2002). Poor maternal nutrition can impact circulating concentrations of several other hormones in offspring including leptin, insulin and thyroid hormones (Wu et al., 2006; Ford et al., 2009; Long et al., 2011; Limesand et al., 2012). These data provide potential mechanisms by which poor maternal nutrition alters offspring growth and metabolism.

In addition to changes in circulating hormones and growth factors, poor maternal nutrition may affect local production of key genes involved in tissue development. Specifically, over-feeding during gestation alters expression of factors involved in the canonical Wnt signaling pathway and myogenesis in the fetus (Tong et al., 2009). Therefore, changes in gene expression may be a potential mechanism through which poor maternal nutrition alters tissue development in offspring. However, little is known about how these changes may be reflected during postnatal muscle growth and development. In addition, many studies target fetal time points or only one variation in diet (over- or under-fed). Based on the negative effects of poor

maternal nutrition on offspring development and the potential role of the somatotropic axis, Wnt signaling pathway and metabolic factors, we hypothesized that poor maternal nutrition would alter offspring growth, body composition, circulating growth factors and expression of genes involved in the development of muscle and adipose tissue of lambs at two early postnatal time points. To test our hypothesis, we under- or over-fed ewes during gestation and determined effects on early postnatal growth of offspring at 1 d and 3 mo of age.

MATERIALS AND METHODS

All procedures were approved by the University of Connecticut's Institutional Animal Care and Use Committee (Protocol Number; A10-040).

Animals

Twenty-four multiparous (n = 18) and primiparous (n = 6) Dorset (n = 21) and Shropshire ewes (n = 3), confirmed pregnant with twins by ultrasound, were placed in individual pens at day 95 ± 10 of gestation (average BW: 102 ± 2 kg) and fed a control diet (100% of NRC requirement) for an initial acclimation period of 12 d. Ewes were transitioned to treatment diets over a 9 d period. Beginning at day 116 ± 6 of gestation ewes began treatment diets and were fed either 100% (control-fed), 60% (restricted-fed), or 126% (overfed-fed) of the NRC requirements for TDN for a ewe in late gestation and pregnant with twin lambs {National Research Council (NRC), 1992}. Ewes were balanced across treatments for parity and breed. We chose to start diets at 116 d of gestation to determine the effects of maternal diet on offspring during the last one-third of gestation. Our target diet for over-fed was 140% of NRC, but based on actual feed intake, the over-fed group consumed 126% of control-fed. Diets for ewes were

calculated on a BW basis and adjusted accordingly with changes in BW and stage of gestation. Second cutting hay was fed to control-fed and over-fed ewes (daily average of 2.2 kg per ewe, respectively) and first cutting hay was fed to restricted-fed ewes (daily average of 1.3 kg per ewe). The overfed ewes received 0.45 kg of cracked corn per ewe daily in addition to hay. During the last 6 wk of gestation the amount of corn fed to overfed ewes was increased to 0.66 kg per d, whereas, control-fed and restricted-fed ewes received cracked corn for the last 6 wk of gestation (daily average of 0.17 kg and 0.10 kg per ewe, respectively). All feed was weighed daily and any residual feed was weighed and removed the following morning. Nutrient analysis of feeds was performed by Dairy One (Ithaca, NY; Table 1).

A total of 52 lambs were born to all ewes (Dorset = 47; Shropshire = 5). Contrary to earlier ultrasound information, the actual occurrence of singletons, twins and triplets, respectively, was 2, 5, 1 for control-fed, 1, 5, 2 for restricted-fed and 0, 4, 4 for over-fed ewes. Upon parturition, lambs from control-fed ewes (CON), over-fed ewes (OVER) and restricted-fed ewes (RES) remained with the mother for up to 24 h to allow adequate colostrum intake. A commercially available colostrum supplement was provided to the lamb as needed (Lamb's Choice Total Colostrum; Saskatoon Colostrum Co; Saskatoon, Canada). One lamb born to a restricted-fed ewe had to be supplemented with colostrum. After 24 h, one lamb was selected from each ewe and was euthanized for sample collection at birth (1 d; n = 7 males; n = 2 females) or 3 mo (n = 10 males; n = 3 females). The remaining lambs stayed with the ewe and were removed from the study and returned to the flock. The larger of the two lambs was chosen when lambs were the same gender. If lambs were different genders, the male lamb was chosen for use on the study since males are often slaughtered for market and females kept for breeding. One-half of the lambs from each diet group were slaughtered within 1 d of birth. The remaining

lambs were maintained on a control diet until 3 mo of age. These lambs were fed milk replacer (1.7% of BW; Land O'Lakes Animal Milk Product Company; Shoreview, MN) from a bottle until weaning at 60 d of age and allowed ad libitum access to water, creep feed (Lamb BT, Blue Seal Feeds; Litchfield, CT), and second cutting hay for the entire 3 mo period. The eventual gender distribution of lambs within treatment groups for CON, RES and OVER, respectively, at 1 d of age was 2 males/1 female, 2 males/1 female and 3 males, and 3 males/2 females, 4 males/1 female and 1 male/2 females for 3 mo of age. Each 3 mo group had one Shropshire lamb.

Sample Collection

Animals were euthanized with an intravenous injection of Beuthanasia-D Special followed by exsanguination (0.22 mL/kg; Merck Animal Health, Summit, NJ) within 24 h after birth (1 d) or at 3 mo of age. Organs (heart and liver) were removed and weighed. Loin eye area was determined by evaluating the cross-sectional area (cm^2) of the longissimus dorsi muscle between the twelfth and thirteenth rib. Back fat was also measured at this region for lambs at 3 mo. Muscle tissue (quadriceps and longissimus dorsi), adipose tissue (perirenal fat), and liver were collected, immediately snap frozen in liquid nitrogen, and stored at -80°C for RNA isolation. The muscle samples were collected from the mid-point of the quadriceps to avoid the fascia and connective tissue. A cross section of the longissimus dorsi was obtained and samples collected from the mid-point. These two sites were chosen based on their use as meat products in the sheep industry.

RNA Extraction

Tissue was ground in a mortar cooled with liquid nitrogen and cells lysed with 1 mL TriReagent (Sigma, Aldrich; Valencia, MO). Adipose, muscle, and liver RNA was extracted using a Qiagen Mini Kit according to the manufacturer`s protocol (Qiagen; Valencia, CA). Genomic DNA was removed from samples using a Turbo DNA Free kit (Ambion, Foster City, CA). The quality of RNA was determined using an Agilent analysis system (Agilent Technologies, Santa Clara, CA). Quantity of RNA was determined using a Nanodrop spectrophotometer (Thermoscientific, Lafayette, CO).

Real-time Reverse Transcription (RT)-PCR

Reverse transcription-PCR was performed using 300 ng total RNA with OligodT primer (Ambion) and master mix containing 5.5 µL of 5x buffer (Invitrogen; Carlsbad, CA), 1.0 µL dNTP (Promega; Madison, WI), 2.0 µL DL-dithiothreitol (DTT) and 0.5 µL Superscript II (Invitrogen) for a total reaction volume of 20 µL. Reverse transcription was performed with a standard protocol starting at 70ºC for 10 min, 4ºC for 20 min, 37ºC for 3 min, 42ºC for 1 h, 4ºC for 3 min, 90ºC for 2.5 min. Primers were designed using Primer3 and NCBI BLAST, validated as previously described (Govoni et al., 2006) and synthesized by Integrated DNA Technologies (Coralsville, IA; Table 2). Four endogenous control genes [p*entidylprolyl isomerase (PPIA),* t*ubulin*, *18s* and g*lyceraldehyde 3-phosphatase* (*GAPDH*)] were tested and variation in Ct values between treatment and time points were determined. Expression of *GAPDH* did not vary between treatments or time points and was used as the endogenous control gene for gene expression analysis. Genes of interest for adipose tissue and myogenic factors are listed in Table 2. Primers were designed for genes involved in the somatotropic axis [*GH, GH receptor* (*GHR*),

IGF-I, *IGF-I receptor* (*IGF-IR*), *IGFBP-1*, *IGFBP-2*, and *IGFBP-3*], Wnt signaling pathway [T*SC-2*, *β-catenin*, and *glycogen synthase kinase* (*GSK*)-*3 β*], myogenesis [*paired box protein* (*PAX*)-*7*, *myogenic factor* (*MYF*)-*5*, *myogenin*, and *MYOD*], adipogenesis [*peroxisome proliferator-activated receptor* (*PPAR*)-*γ*, *CCAAT/enhancer binding protein (C/EBP)-α*, and *C/EBP-β*], metabolism [*leptin*, *glucose transporter type 4* (*GLUT4*), *and carbohydrate-responsive element-binding protein* (*CHREBP*)], and cell function [*T-box (TBX)-2* and *-3*]. Real-time RT-PCR was performed using Power SybrGreen Master Mix (Invitrogen) and the ABI 7900 HT Fast Real-time PCR machine (Applied Biosystems, Foster City, CA). The total volume of the reaction mixture was 25 µL containing 5 µL of cDNA, 3 µL of nuclease free water, 1 µL each of 10 nM forward and reverse primer, and 10 µL of Sybrgreen. For the Tbx3 primer, 0.5 µL of each forward and reverse primer was used. Real-time RT-PCR was performed using standard cycling conditions (Stage 1: 50ºC for 2 min and 95ºC for 10 min, Stage 2: 95ºC for 15 sec and 60ºC for 40 cycles). Cycle threshold (Ct) values were used to calculate the $^{\Delta\Delta}$Ct values to determine changes in gene expression (Livak and Schmittgen, 2001). Changes in gene expression are expressed relative to the control.

Statistical Analysis

All statistical analysis was performed using Statistical Analysis Software version 9.2 (SAS Inst. Inc., Cary, NC). Back fat data were analyzed as a percentage of BW of the animal. All other data were analyzed using the mixed models procedure. Where appropriate, mean comparisons were made using least square means (LSMEANS). Statistical significance was considered at $P \leq 0.05$ and a tendency at $P > 0.05$ and ≤ 0.10. Due to the limited number of females and Shropshire lambs in each treatment and time point, we were not able to test for

breed or gender differences. Therefore, all animals were included and the main effect of treatment was tested.

RESULTS

Body composition and body parameters

Over the duration of the study control-fed, restricted-fed or over-fed ewes consumed 1.43, 0.79, or 1.83 kg of TDN (SEM ± 0.09) and 0.30, 0.10, and 0.31 kg of CP (SEM ± 0.02) per day, respectively. Ewes remained on study until parturition (time on treatment = 32 ± 10 d). At parturition, restricted-fed ewes weighed 14% less than control-fed ewes (95 ± 4 kg vs. 111 ± 3 kg; $P = 0.002$). Body weights for over-fed ewes were not different compared with control-fed ewes (118 ± 3 vs 111 ± 3 kg; $P = 0.18$). Overall, for all time points between 1 d and 3 mo of age, RES lambs weighed 16% less compared with CON lambs ($P = 0.01$; Figure 1). A difference in BW was not observed between OVER and CON between 1 d and 3 mo of age ($P = 0.24$). However, between 7 to 10 wk of age, OVER lambs tended to weigh 11% less than CON lambs ($P \leq 0.08$). Data for organ weights, fat depots, and circulating growth factors are presented elsewhere (Rokosa, 2013; Hoffman et al., 2014b)

Gene expression in liver from offspring

Based on changes in circulating IGF-I and IGFBP-3, we determined expression of genes involved in the somatotropic axis in the liver at 1 d and at 3 mo of age. At 1 d, expression of *IGF-I* tended to increase in OVER ($P = 0.06$), but did not change in RES ($P = 0.69$; Table 3) compared with CON lambs. No effects of dietary treatment were observed in expression of *GHR*, *IGFBP-1*, *IGFBP-2*, and *IGFBP-3* at 1 d of age ($P \geq 0.15$; Table 3). At 3 mo of age, no

change was observed in mRNA expression of factors involved in the somatotropic axis (*GHR, IGF-I, IGFBP-1, IGFBP-2* and *IGFBP-3*; $P \geq 0.11$; Table 4). Maternal diet did not alter expression of *GLUT4* or *CHREBP* in offspring at 1 d or 3 mo of age ($P \geq 0.13$; Table 3 and 4).

Gene expression in adipose tissue from offspring

Maternal diet did not alter expression of markers of adipogenesis (*PPARγ, C/EBPα, and C/EBPβ*) at 1 d or at 3 mo of age ($P \geq 0.34$; Tables 3 and 4). Similarly, maternal diet did not alter expression of *leptin* at 1 d or 3 mo of age compared with CON ($P \geq 0.20$; Tables 3 and 4). Maternal diet did not alter expression of *adiponectin*, *CHREBP* or *TSC2* at 1 d or at 3 mo of age ($P \geq 0.12$; Tables 3 and 4).

Gene expression in muscle from offspring

In quadriceps muscle, expression of *β-Catenin* tended to be 1.9-fold greater in RES and OVER at 1 d compared with CON ($P = 0.07$; Table 3), but no differences were observed at 3 mo of age ($P = 0.89$; Table 3). Maternal diet did not affect expression of markers of myogenesis (*PAX7, Myf5, and myogenin*), *IGF-I*, *IGF-IR*, *GSKβ*, *GLUT4*, *TSC2*, *Tbx2 and Tbx3* at 1 d or 3 mo of age ($P \geq 0.31$; Tables 3 and 4). We did not detect a change in expression of any genes in longissimus dorsi at 1 d or 3 mo of age.

Table 1. Ewe diet feed composition[1]

Feed	% CP	% ADF	% NDF	% NFC	% TDN
Mixed grass hay; first cutting	8.2	43.7	66.4	18.9	55.0
Mixed grass hay; second cutting	14.2	37.5	56.8	23.0	58.0
Corn, cracked	9.0	4.0	9.0	75.0	90.0

[1]Diets for ewes were based on NRC requirements for TDN for a ewe pregnant with twins during late gestation (National Research Council (NRC), 1992).Diets for each ewe were adjusted weekly for changes in BW. ADF = acid detergent fiber; NFC = Non-fiber carbohydrates.

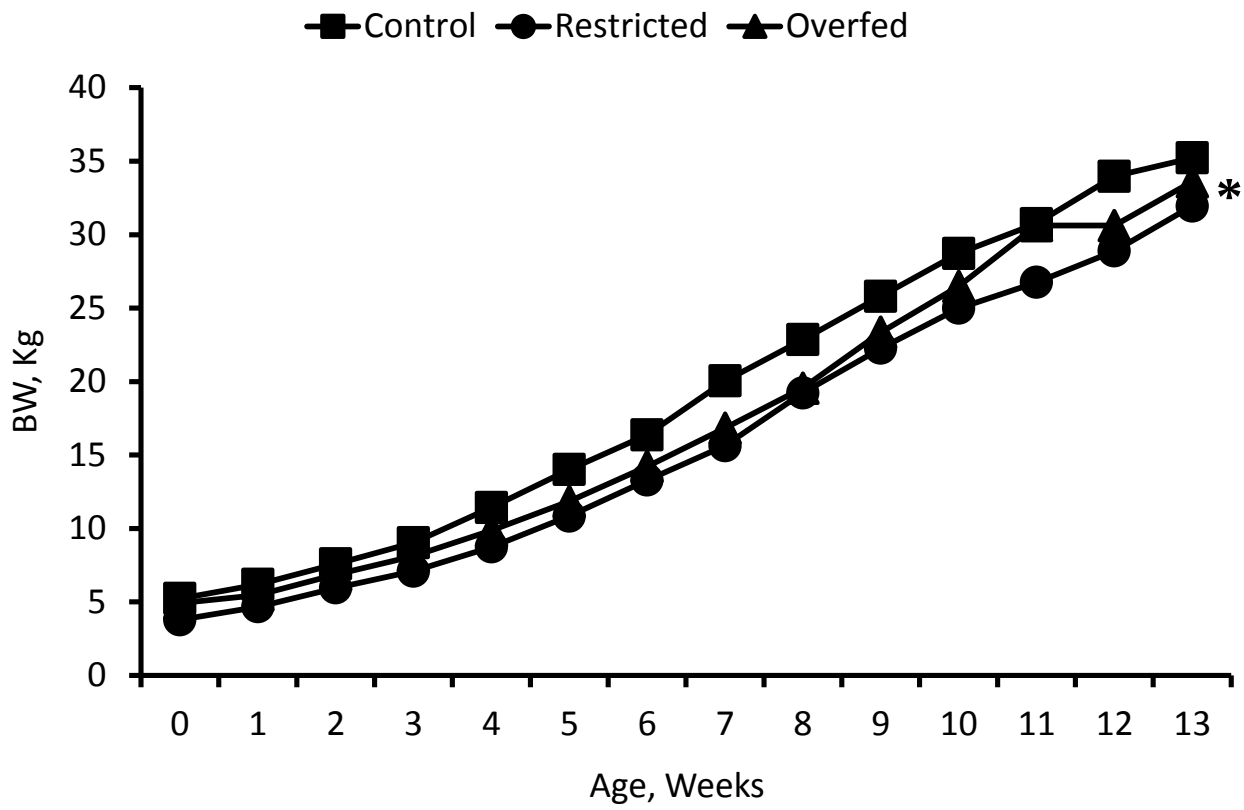
Table 2. Primer sequences for real-time RT-PCR

Gene	Primer Sequences (5' to 3')	Reference
ADIPOQ		
Forward	ATCAAACTCTGGAACCTCCTATCTAC	(Muhlhausler et al., 2007)
Reverse	TTGCATTGCAGGCTCAAG	
β-Catenin		
Forward	GGATGTGGATACCACCCAAG	(Tong et al., 2009)
Reverse	CCCTCATCTAGCGTCTCAGG	
CEBPα		
Forward	AGTCCGTGGACAAGAACAGC	XM_004015623.1
Reverse	TTGTCACTGGTCAGCTCCAG	
CEBPβ		
Forward	GACAAGCACAGCGACGAGT	(Dervishi et al., 2011)
Reverse	GTGCTGCGTCTCCAGGTG	
CHREBP		
Forward	CTCCGCTCCACATACTGGAT	NM_001205408.1
Reverse	GTTGTTGAGGCGGATCTTGT	
GAPDH		
Forward	GGCGTGAACCACGAGAAGTATAA	(Buza et al., 2003)
Reverse	CCTCCACGATGCCAAAGTG	
GHR		
Forward	AAATTCACCAAGTGCCGTTC	NM_00100323.2
Reverse	TGTTTTCACCAGCAGAGACG	
GLUT-4		
Forward	AGGACGTTTGACCAGATCTCA	AB_005283.1
Reverse	CAGTTCTGTGCTGGGTTTCA	
GSK-3β		
Forward	TCCGACCCCGAACTCCACCC	NM_001129740.1
Reverse	GTGCAGGTGTGTCTCGCCCA	
IGFBP1		
Forward	TGATGACCGAGTCCAGTGAG	NM_001145177.1
Reverse	GCTCCTTCCACTTCTTGACG	
IGFBP2		
Forward	CCCTACACATCCCCAACTGT	NM_0010009436.1
Reverse	CAGTGTTGGGGTTCACACAC	
IGFBP3		
Forward	CAGAGCACAGACACCCAGAA	NM_001159276.1
Reverse	CACAGTTGGGAATGTGGATG	
IGF-I		
Forward	CCAGACAGGAATCGTGGATG	NM_001009774.3
Reverse	ACTTGGCGGGCTTGAGAG	
IGF-IR		
Forward	ACCTACACAGCCCGGATCCA	XM_004018023.1

Reverse	ACACAGGCTCCGTCCATGAC	
Leptin		
Forward	TGACACCAAAACCCTCATCA	U84247.1
Reverse	CCAAACCAGTGACCCTCTGT	
MYF-5		
Forward	AGACGCCTGAAGAAGGTGAA	XM_004006219.1
Reverse	AGCAGCTCCTGCAGACTCTC	
MYOD		
Forward	CCCTGGTGACTTCAGCTGTT	(Tong et al., 2009)
Reverse	CCTGCCTGCCGTATAAACAT	
Myogenin		
Forward	TGGGCGTGTAAGGTGTGTAA	(Tong et al., 2009)
Reverse	TGCACAGGATCTCCACTTTG	
PAX-7		
Forward	GAGACCGACTGCTGAAGGAC	XM_002685738
Reverse	ATGCTGTGCTTGGCTTTCTT	
PPAR-γ		
Forward	CTTGCTGTGGGGATGTCT	NM_001100921.1
Reverse	GGTCAGGAGACTCTGGGTC	
TBX2		
Forward	CTT GCA GTG CTC CTC CTA	NM_001191443.1
Reverse	CAC GCA GCT TAA GAT CGA CA	
TBX3		
Forward	ATC GCT GTG ACT GCA TAC CA	XM_002694588.3
Reverse	TCT CTC CTG CCA TTT CCA GT	
TSC-2		
Forward	TGCAAGCTGTCTTCCACATC	XM_003587808.2
Reverse	AACTTGAAGTCCTCGCCAGA	

Adiponectin (*ADIPOQ*), *Carbohydrate-responsive element-binding protein* (*ChREBP*), Follistatin (FSTN), Forkhead box (FOXO)-1 and 3a, *Glyceraldehyde 3-Phosphatase* (*GAPDH*), *Growth hormone* (*GH*), *Growth hormone receptor* (*GHR*), *Glucose transporter type 4* (*GLUT4*), *Glycogen synthase kinase* (*GSK*)*-3β*, *IGF-I receptor* (*IGF-IR*), *Tuberous sclerosis-2* (*TSC2*), *Myogenic factor-5* (*Myf5*), *Myogenic differentiation-1* (*MyoD*), Myostatin (MSTN), *Paired box protein* (*PAX*)*-7*, *T-box* (*Tbx*)*-2* and *-3*.

Figure 1. Lamb body weights over time



Ewes were fed 100% (CON), 60% (RES), or 126% (OVER) of NRC requirements for ewes pregnant with twins. One lamb from each ewe remained on study and was necropsied at birth (n = 3/treatment) or 3 mo (n = 3 to 5/treatment) of age. *indicates $P = 0.01$ vs. control. Average BW over time were 19 ± 3 kg, 16 ± 3 kg, 17 ± 3 kg for lambs born to CON, RES, and OVER ewes, respectively.

Table 3. mRNA expression at 1 d in liver, adipose and muscle of lambs born to ewes fed 100% (CON), 60% (RES), or 126% (OVER) of NRC requirements for ewes in late gestation and pregnant with twins

	Treatment[a]				P-Value[b]
Gene	CON	RES	OVER	SEM	TRT
Liver					
GHR	1.03	0.78	0.84	0.06	0.27
IGF-I	1.01[x]	0.95[x]	1.22[y]	0.06	0.07
IGFBP-1	1.05	1.97	0.60	0.23	0.15
IGFBP-2	1.11	0.73	1.16	0.25	0.81
IGFBP-3	1.03[x]	0.68[y]	0.93[x]	0.08	0.19
CHREBP	1.10	0.89	1.05	0.21	0.95
GLUT4	1.87	1.54	1.21	0.39	0.93
Adipose					
PPAR-γ	1.05	1.21	0.93	0.07	0.58
CEBPα	1.43	1.91	1.82	0.33	0.78
CEBPβ	1.02	0.86	0.80	0.22	0.47
Leptin	2.34	4.82	7.25	0.30	0.30
CHREBP	1.31	1.41	1.41	0.30	0.86
GLUT4	1.35	1.23	2.06	0.19	0.77
ADIPOQ	1.09	1.49	0.97	0.16	0.55
Quadriceps					
β-Catenin	1.12	1.90	1.91	0.18	0.11
GSKβ	1.32	1.43	1.67	0.21	0.63
PAX7	1.66	1.18	0.91	0.41	0.73
Myf5	1.29	2.08	1.77	0.23	0.38
Myogenin	1.92	1.34	1.73	0.55	0.98
IGF-I	1.27	2.92	1.94	0.24	0.16
IGF-IR	1.23	1.56	1.42	0.30	0.64
GLUT4	1.40	1.25	1.33	0.24	0.80
TSC2	1.20	0.97	1.19	0.25	0.61
TBX2	1.23	1.56	1.54	0.21	0.63
TBX3	1.08	1.12	1.01	0.11	0.94

Ewes were fed 100% (CON), 60% (RES), or 126% (OVER) of NRC requirements for ewes in late gestation and pregnant with twins. Tissue samples were collected from lambs at 3 mo (3 to 5/trt) necropsies. *ADIPOQ = Adiponectin*, *GHR = Growth hormone receptor*, *IGFBP = Insulin like growth factor binding protein*, *Myf-5 = Myogenic factor-5*, *PAX-7 = Paired box protein-7*, TRT = treatment. SEM = Standard error of the mean. Shaded values demonstrate a significant difference ($P \leq 0.05$ or trend $P \leq 0.10$ and ≥ 0.05). [a]Values given as mean ± SEM. [b]Values provided for treatment effect. [x,y] Means within a row with different superscripts differ ($P \leq 0.10$).

Table 4. mRNA expression at 3 mo in liver, adipose and muscle of lambs born to ewes fed 100% (CON), 60% (RES), or 126% (OVER) of NRC requirements for ewes in late gestation and pregnant with twins

	Treatment[a]				P-Value[b]
Gene	CON	RES	OVER	SEM	TRT
Liver					
GHR	1.37	0.80	1.10	0.24	0.80
IGF-I	1.17	0.85	0.77	0.14	0.68
IGFBP-1	2.45	1.68	0.92	0.62	0.92
IGFBP-2	1.76	2.03	1.55	0.37	0.83
IGFBP-3	1.04	0.80	1.29	0.10	0.25
CHREBP	1.06	1.51	0.74	0.25	0.24
GLUT4	1.07	1.80	1.90	3.97	0.13
Adipose					
PPAR-γ	1.02	0.91	1.20	0.07	0.37
CEBPα	1.13	0.67	1.74	0.28	0.34
CEBPβ	1.16	1.28	0.65	0.22	0.42
Leptin	1.21	0.92	2.59	0.30	0.19
CHREBP	1.60	0.88	1.82	0.30	0.54
GLUT4	1.33	1.18	1.54	0.19	0.66
ADIOPQ	1.00	0.69	0.92	0.07	0.12
Quadriceps					
β-Catenin	1.06	1.19	1.19	0.09	0.89
GSKβ	1.02	0.99	1.1	0.08	0.74
PAX7	1.36	1.29	2.00	0.50	0.86
Myf5	1.13	0.98	1.43	0.23	0.59
Myogenin	1.04	1.46	0.90	0.26	0.49
IGF-I	1.06	1.22	1.23	0.16	0.95
IGF-IR	1.46	1.80	1.12	0.47	0.90
GLUT4	1.28	1.55	1.05	0.21	0.65
TSC2	1.02	0.74	1.18	0.16	0.52
TBX2	1.29	1.09	0.83	0.30	0.57
TBX3	1.09	1.39	1.33	0.45	0.85

Ewes were fed 100% (CON), 60% (RES), or 126% (OVER) of NRC requirements for ewes in late gestation and pregnant with twins. Tissue samples were collected from lambs at 3 mo (3 to 5/trt) necropsies. *ADIPOQ = Adiponectin*, *GHR = Growth hormone receptor*, *IGFBP = Insulin like growth factor binding protein*, *Myf-5 = Myogenic factor-5*, *PAX-7 = Paired box protein-7*, TRT = treatment. SEM = Standard error of the mean. Shaded values demonstrate a significant

different ($P \leq 0.05$ or trend $P \leq 0.10$ and ≥ 0.06). [a]Values given as mean ± SEM. [b]Values provided for treatment effect.

DISCUSSION

Proper maternal nutrition is critical for fetal development and there is increasing evidence that negative effects of poor maternal diet can persist into adulthood contributing to poor growth and metabolic disorders in offspring. Poor maternal nutrition can result from many factors including, inadequate intake of calories, protein, or specific vitamins and minerals. In addition to impaired growth due to inadequate nutrients, over-feeding of mothers during gestation also negatively impacts growth and health of the offspring leading to increased fat deposition (Long et al., 2010a; Nielsen et al., 2013; Parlee and Macdougald, 2013; Yan et al., 2013). We used both under- and over-feeding models in the same study to allow for these direct comparisons.

Under feeding ewes during gestation reduced BW of offspring at birth. These findings are similar to George et al., in which nutrient restriction occurred during the first half of gestation (d 28 to 78; George et al., 2012). Whereas, in the current study, ewes were restricted for the last 32 d of gestation. Therefore, regardless of timing of maternal nutrient restriction, it leads to reduced BW and early postnatal growth. Typically, offspring born small for gestational age undergo compensatory growth, which is due to increased adipose tissue accretion (Mericq et al., 2005; De Blasio et al., 2007; Neville et al., 2010). However, in the current study, the reduced BW persisted until 3 mo of age. In fact, similar to reduced BW, there was a reduction in back fat in the RES offspring at 3 mo suggesting that, although they were fed ad libitum postnatally, we did not observe a compensatory weight gain in these lambs during early postnatal growth. Alternatively, previous studies (Sebert et al., 2001; Neville et al., 2010) quantified BW and body composition of the offspring for longer periods of time (e.g., 6 to 16 mo of age). Therefore, compensatory growth may occur during later stages of postnatal growth. Similar to previous studies (George et al., 2010; Long et al., 2010a), a difference in BW between CON and OVER

was not observed. This is not surprising based on the 26% increase in feed intake in mothers during the last third of gestation. Although a difference in BW was not observed, there was a tendency for increased heart size in OVER at birth.

Lambs born to RES ewes exhibited reduced circulation IGF-I and IGFBP concentrations (Rokosa, 2013; Hoffman et al., 2014b) As a result, gene expression of factors involved in the somatotropic axis was evaluated in the liver of lambs at 1 d and at 3 mo of age. Consistent with reduced circulating IGF-I and IGFBP-3, mRNA expression of IGFBP-3 tended to be reduced at birth in RES suggesting that reduced production of IGFBP-3 from liver may have contributed to reduce BW at birth. It is not clear why liver IGF-I mRNA did not parallel circulating concentrations since this is the primary source of IGF-I in circulation. The lack of change in expression of other genes involved in the somatotropic axis could be due to the limited number of animals. Additionally, it could also be possible that changes in circulating factors, IGF-I and IGFBP-3, were not affected similarly at the local level in response to maternal nutrition. Future studies with increased animal numbers and global gene expression analysis are needed to fully elucidate the mechanisms by which poor maternal nutrition impair offspring growth.

Although a difference in loin eye area in RES and OVER lambs, when adjusted for BW, was not detected in the current study (Hoffman et al., 2014b), previous studies have demonstrated that poor maternal nutrition, due to both under- and over-feeding, reduced muscle development in offspring (Wu et al., 2006; Zhu et al., 2006; Daniel et al., 2007; Tong et al., 2009; Yan et al., 2011). The canonical Wnt signaling pathway has been shown to enhance bone and muscle formation and inhibit adipogenesis (Shang et al., 2007; Du et al., 2010). Expression of *β-catenin* tended to increase in muscle of RES and OVER lambs at 1 d of age. This is in contrast to a previous study in which expression was reduced (Tong et al., 2009). The previous

study evaluated effects of maternal obesity and also observed reduced muscle fiber diameter and expression of markers of myogenesis (Tong et al., 2009). It is not clear why the increased expression was observed at 1 d; however it was not present at 3 mo suggesting that this is not a primary mechanism by which poor maternal nutrition alters postnatal muscle growth. Since these are early time points, it is possible that these changes in expression may contribute to changes that would occur later in life as the animal continues to grow.

As expected, there is very little subcutaneous fat at birth. Therefore, for consistency, we evaluated expression of genes in renal fat at both time points. Although changes in gene expression are not observed at 1 d of age, consistent with reduced backfat in RES lambs at 3 mo (Hoffman et al., 2014b), expression of adiponectin tended to be reduced in renal fat of RES lambs at 3 mo of age. Previous studies also demonstrate that reduced expression and circulating concentrations of adiponectin are associated with obesity and metabolic disorders (Kern et al., 2003; Shankar et al., 2010). Although the RES offspring were not growing as well as CON and have reduced fat mass during early postnatal growth, the reduced expression of adiponectin could be indicative of programmed altered metabolism which could contribute to increased fat later in life. Future studies determining circulating adiponectin and long-term body condition of offspring are needed to determine if adiponectin is a key factor in increased obesity of offspring. Leptin is an important factor in regulating feed intake, metabolism and development of key tissues (Chilliard et al., 2005; Forhead et al., 2008). Previous studies have demonstrated that poor maternal nutrition can alter circulating leptin in offspring as early as the first week of life (Long et al., 2011). These effects can persist into adulthood and may contribute to altered metabolism later in life due to hyperleptinemia (Stocker et al., 2005; Long et al., 2011). Similar to previous studies, circulating leptin increased in both OVER offspring (Hoffman et al., 2014b).

Consistent with these findings, expression of leptin in adipose tissue was numerically greater in RES and OVER at 3 mo. At these early time points we were not able to determine if the increased leptin would lead to altered metabolism later in life, but based on previous reports (Long et al., 2010a; Coupe et al., 2012) this is a likely scenario.

IMPLICATIONS

These data indicate that poor maternal nutrition alters the expression of several genes involved in the somatotropic axis, WNT signaling, and adipokine production. Additional research needs to be conducted to better understand the mechanisms that these genes may be involved in with regards to the effects of poor maternal nutrition. In order for these gene expression data to be applied into production systems or be used to develop a study to comprise a realimentation program/solution for these animals, more research needs to be conducted. These data serve as a starting point to identify specific pathways or factors that require additional evaluation to better understand the effects of poor maternal nutrition postnatally. Specifically results from this study have resulted in an increased focus on muscle tissue development. A second study was conducted beginning at an earlier point during gestation in an effort to target earlier stages of tissue development such as secondary myogenesis in its entirety.

CHAPTER II: POOR MATERNAL NUTRITION INHIBITS MUSCLE DEVELOPMENT IN OVINE OFFSPRING

Reed, S. A., J. S. Raja, M. L. Hoffman, S. A. Zinn, and K. E. Govoni. 2014. Poor maternal nutrition inhibits muscle development in ovine offspring. J. Anim. Sci. Biotechnol. 5(1): 43-1891-5-43. eCollection 2014.

INTRODUCTION

Proper growth and development of muscle tissue in production animals is critical to maintain maximum productivity and profitability. However, the development of muscle tissue has been found to be susceptible to the effects of maternal programming as a result of poor maternal nutrition with offspring exhibiting reductions in myofiber number, increased connective tissue content, and reductions in muscle mass (Wu et al., 2004; Zhu et al., 2004; Daniel et al., 2007; Tong et al., 2009; Du et al., 2010; Huang et al., 2010). Alternately, little is known about the mechanisms that mediate these phenotypic changes, thus limiting our understanding of the problem and the development of methods to realiment these offspring. To identify and understand potential mechanisms involved in altering muscle development, current research has focused on how poor maternal nutrition may affect factors involved in myogenesis (Tong et al., 2008; Huang et al., 2010). Establishment of muscle tissue prenatally and its development postnatally relies on the involvement of various growth factors, cell signals, and transcription factors (Adams, 2002; Clemmons, 2009; Du et al., 2010). One potential mechanism is the involvement of myogenic factors such as myogenin and MyoD. These MRFs are required for differentiation of precursor myocytes and have been found to be reduced in fetuses from over-nourished ewes that in turn exhibited reduced myofiber number and diameter (Tong et al., 2009). Changes in factors involved in Wnt signaling, inflammatory markers, and factors involved in protein degradation have also been observed during similar time points (Du et al., 2010).

However, knowledge is limited in regards to what factors and pathways may be involved in mediating the effects of poor maternal nutrition in muscle postnatally. A possible mechanism

by which poor maternal nutrition could alter postnatal muscle development is satellite cells. Satellite cells are a population of quiescent stem that express the transcription factor PAX7 during their quiescent state and will express MRFs upon activation in response to injury or load bearing (Le Grand and Rudnicki, 2007). Satellite cell populations are established during late gestation and therefore are vulnerable to the effects of maternal programming (Le Grand and Rudnicki, 2007). Local involvement of the somatotropic axis may also be involved in mediating the effects of poor maternal nutrition postnatally. In a previous study, we demonstrated that poor maternal nutrition during late gestation resulted in alterations to local expression of IGF-I and β-catenin mRNA in the quadriceps muscle tissue of lambs at birth (Hoffman et al., 2014b). In considering the critical role of myogenesis in establishing muscle tissue, our previous work and the current literature we hypothesized that over and restricted nutrition (total nutrient intake) in gestating ewes would negatively affect muscle growth in the offspring by reducing muscle fiber size, and altering gene and protein expression via the Akt signaling pathway.

MATERIALS AND METHODS

Animals

All animal experiments were reviewed and approved by the University of Connecticut Institutional Animal Care and Use Committee.

Thirty-six multiparous ewes (25 Dorsets, 7 Shropshires, and 4 Southdowns) were selected from the University of Connecticut flock followed by estrus synchronization with CIDRs (Pfizer Animal Health, New York, NY) and Luteylase (Pfizer Animal Health). Ewes were bred by a ram of like breeding and considered bred on the day that they were marked by the ram. For Dorset ewes one of three different rams was used with the number of ewes evenly distributed across rams. At approximately d 30 of gestation, ewes were moved into individual pens and ultrasounded to confirm pregnancy and determine fetal number. Ewes confirmed as pregnant were assigned to one of three treatment diets that were 100% (CON), 60% (RES) and 140% (OVER) of NRC requirements. Ewes were transitioned onto diets at d 31 ± 1.3 of gestation and remained on their respective diets until parturition. At parturition, lambs from control-fed ewes (CON), lambs from over-fed ewes (OVER) and lambs from restricted-fed ewes (RES) remained with their dam for up to 24 h in order to ensure adequate colostrum intake. One lamb from each ewe was taken by d 1 to use on study at either the birth or 3 mo time point. The larger of the two lambs was chosen when lambs were the same gender. If lambs were different genders, the male lamb was chosen for use on the study since males are often slaughtered for market and females kept for breeding. One-half of the lambs from each diet group were slaughtered within 1 d of birth. The remaining lambs were maintained on a control diet until 3 mo of age. These lambs were fed milk replacer (1.7% of BW; Land O'Lakes Animal Milk Product Company; Shoreview, MN) from a bottle until weaning at 60 d of age and allowed ad libitum access to water, creep feed (Lamb BT, Blue Seal Feeds; Litchfield, CT), and second cutting hay for the entire 3 mo period.

Sample Collection

Animals were euthanized with an intravenous injection of Beuthanasia-D Special (Merck Animal Health; Summit, NJ) followed by exsanguination. Muscle samples were collected from the left semitendinosus immediately after euthanasia. This was accomplished by removing the surrounding fascia and removing muscle tissue from the central most aspect of the muscle. Samples were immediately snap frozen in liquid nitrogen and then stored at -80 °C until utilized for RNA isolation.

RNA Extraction

Tissue was homogenized using the Qiagen Tissuelyser system with 1 mL TriReagent (Sigma Aldrich). A Qiagen Mini Kit was used to extract RNA according to the manufacturer's protocol (Qiagen; Valencia, CA). Genomic DNA was removed from samples using a Turbo DNA Free kit (Ambion, Foster City, CA). The quality of RNA was determined using a Bioanalyzer analysis system (Agilent Technologies, Santa Clara, CA).

Real-time RT-PCR

Reverse Transcription (RT)-PCR was performed using 300 ng total RNA with OligodT primer (Ambion) and master mix containing 5.5 µL of 5X Buffer (Invitrogen), 1.0 µL dNTP (Promega, Madison, WI), 2.0 µL DTT and 0.5 µL Superscript II (Invitrogen) for a total reaction volume of 20 µL. The samples and master mix underwent a standard RT protocol starting at 70ºC for 10 min, 4ºC for 20 min, 37ºC for 3 min, 42ºC for 1 h, 4ºC for 3 min, 90ºC for 2.5 min. Real-time RT-PCR primers were designed using Primer3 and NCBI BLAST, validated as

previously described (Hoffman et al., 2014a) and synthesized by Integrated DNA Technologies (Coralville, IA). Primer sequences are presented in Table 5. Real-time RT-PCR was performed using Power SybrGreen Master Mix (Invitrogen) and the ABI 7900 HT Fast Real-time PCR machine (Applied Biosystems, Foster City, CA) as previously described (Hoffman et al., 2014a). The total volume of the reaction mixture was 25 µL (5 µL cDNA, 3 µL nuclease free water, 1 µL each 10 nM forward and reverse primer, and 10µL SybrGreen). Real-time RT-PCR was performed using standard cycling conditions (Stage 1: 50 ºC for 2 min and 95 ºC for 10 min, Stage 2: 95 ºC for 15 sec and 60 ºC for 1 min for 40 cycles, Stage 3: 95 ºC for 15 sec and 60ºC for 15 sec with a 2% ramp to 95 ºC for 5 min). $^{\Delta}$Ct values were obtained and used to calculate the $^{\Delta\Delta}$Ct values to determine relative gene expression (Livak and Schmittgen, 2001). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as the internal control and did not differ between treatment groups.

Statistics

Data were sorted by age (1 d or 3 mo of age) and analyzed using the PROC MIXED procedure (SAS Institute, Inc, Cary, NC) using dietary treatment as the fixed effect. Treatment mean comparisons were performed using LSMEANS statement and PDIFF option. Differences were determined to be significant at $P \leq 0.05$ or a tendency at $P > 0.05$ and ≤ 0.10. Gender and breed differences were not evaluated due to the limited number of females, Shropshire and Southdown lambs in each treatment at each time point. For gene expression analysis, 15 lambs were used at 1 d (CON: n = 4, RES: n = 6, and OVER: n = 5) and 12 lambs were used at 3 mo (CON: n = 5, RES: n = 4, and OVER: n = 3).

RESULTS

Body Variables:

By the end of gestation, restricted and over-fed diets effectively reduced (-18.9 %; 18.3 ± 3.6 kg; $P < 0.01$) or increased (6.6 %; 119.7 ± 3.6 kg; $P < 0.10$) ewe BW, respectively compared with control-fed ewes (112.9 ± 3.6 kg). Overall, OVER lambs weighed 13% more than CON lambs [$P \leq 0.05$ (Peck et al., 2013)]. However, there were no differences in BW between RES and CON lambs ($P = 0.70$). Additional data for cross sectional area of muscle, muscle fiber type composition and body parameters are previously reported (Reed et al., 2014) .

Gene Expression Analysis:

In considering the role MRFs in regulating muscle development, we evaluated the expression of *PAX7*, *MyoD*, and *myogenin*. We did not observe an effect of poor maternal nutrition on the mRNA expression of *PAX7*, *MyoD*, or *myogenin* in whole muscle tissue of lambs at 1 d ($P \geq 0.57$) or 3 mo of age ($P \geq 0.24$; Table 6). Alternately, at d 1, OVER lambs exhibited a tendency toward increased *myostatin* gene expression ($P = 0.06$; Table 6); however no change was observed in RES lambs ($P = 0.78$). At d 1, *follistatin* gene expression was greater in OVER lambs ($P = 0.04$) and tended to be increased in RES lambs ($P = 0.06$, Table 6).At 3 mo of age, reduced *FoxO1* mRNA expression was observed in RES lambs ($P = 0.04$, Table 6). No change was observed in *FoxO1* mRNA expression at d 1 in OVER lambs ($P = 0.37$) or *FoxO3a* mRNA expression at d 1 or 3 mo for either treatment group ($P \geq 0.20$, Table 6). Factors involved in the somatotropic axis were also evaluated. Increased IGF-I mRNA expression was

observed in RES lambs at 1d of age ($P = 0.08$) but not in OVER lambs ($P > 0.05$). No change was observed in IGF-I receptor mRNA at 1 d or 3 months of age (P = 0.20). Increased expression of IGFBP-3 mRNA was reduced 2.82 fold in OVER lambs when compared to CON lambs at 1d ($P = 0.01$)

Table 5. Primer Sequences

Gene	Primer Sequences (5' – 3')	Amplicon Length	Reference
PAX7			
Forward	GAGACCGACTGCTGAAGGAC	167	XM_002685738
Reverse	ATGCTGTGCTTGGCTTTCTT		
MYF5			
Forward	AGACGCCTGAAGAAGGTGAA	134	XM_004006219.1
Reverse	AGCAGCTCCTGCAGACTCTC		

Myogenin			
Forward	TGGGCGTGTAAGGTGTGTAA	169	Tong et al., 2009
Reverse	TGCACAGGATCTCCACTTTG		
MYOD			
Forward	CCCTGGTGACTTCAGCTGTT	239	Tong et al., 2009
Reverse	CCTGCCTGCCGTATAAACAT		
MSTN			
Forward	CACAGAAGGTCTTCCCCTCA	147	NM_001001525.2
Reverse	GGTTAAATGCCAACCATTGC		
FSTN			
Forward	AAAACCTACCGCAACGAATG	120	NM_001257093.1
Reverse	GAGCTGCCTGGACAGAAAAC		
FOXO1			
Forward	TGACTTGGACGGCATGTTTA	157	XM_004012275.1
Reverse	CCAGCTGTGTGTTGTCGTCT		
FOXO3a			
Forward	GGGGAGTTTGGTCAATCAGA	170	NM_001267889.1
Reverse	TTTGCATAGACTGGCTGACG		
GAPDH[1]			
Forward	GGCGTGAACCACGAGAAGTATAA	118	Buza et al., 2009
Reverse	CCCTCCACGATGCCAAAGT		

Paired box protein (PAX)-7, Myogenic factor-5 (Myf5), Myogenic differentiation-1 (MyoD), Myostatin (MSTN), Follistatin (FSTN), Forkhead box (FOXO)-1 and 3a, Glyceraldehyde 3-Phosphatase (GAPDH)

Table 6. Gene expression[1] in semitendinosus muscle of CON[2], OVER or RES lambs at d 1 and 3 mo of age.

	Day 1				3 Months			
Gene	CON	OVER	RES	*P*-value	CON	OVER	RES	*P*-value
PAX7	1.38 ± 0.67	1.20 ± 0.27	1.86 ± 0.51	0.70	1.14 ± 0.31	1.42 ± 0.24	1.94 ± 0.77	0.46
MYOD	2.16 ± 1.36	3.51 ± 0.90	2.70 ± 0.98	0.88	1.11 ± 0.29	1.01 ± 0.01	1.27 ± 0.29	0.83
Myogenin	1.53 ± 0.74	2.13 ± 0.74	2.65 ± 0.71	0.57	1.21 ± 0.35	1.24 ± 0.47	2.41 ± 0.66	0.24
MSTN	1.17 ± 0.36	2.94 ± 0.50†	1.63 ± 0.51	0.11	1.02 ± 0.10	1.89 ± 0.26	1.51 ± 0.58	0.36
FSTN	1.03 ± 0.16	1.92 ± 0.39*	1.73 ± 0.28†	0.09	1.10 ± 0.21	1.56 ± 0.21	1.51 ± 0.34	0.36
FOXO1	1.12 ± 0.30	0.64 ± 0.29	0.74 ± 0.29	0.37	1.06 ± 0.16	0.91 ± 0.36	0.32 ± 0.07*	0.04
FOXO3a	1.60 ± 0.73	0.72 ± 0.26	1.12 ± 0.56	0.70	1.09 ± 0.24	1.42 ± 0.43	0.77 ± 0.14	0.21

[1] Relative to CON, mean ± SEM

[2] CON = lambs from control-fed ewes (d 1, n = 4; 3 mo, n = 5), OVER = lambs from overfed ewes (d 1, n = 5; 3 mo, n = 3), RES = lambs from restricted-fed ewes (d 1, n = 6; 3 mo, n = 4)

* $P < 0.05$ compared with CON

† $P < 0.10$ compared with CON

DISCUSSION:

Poor maternal nutrition during gestation has been identified as a way that muscle development can be negatively impacted, thus affecting animal productivity. However, information pertaining to the mechanisms that are involved in these changes is limited. In an effort to better understand how poor maternal nutrition alters postnatal muscle development, we evaluated gene expression of specific factors in the semitendinosus muscle of offspring. Phenotypic differences in the muscle tissue of lambs were observed with altered cross sectional area at 1 d and three mo of age in RES and OVER lambs as presented in a separate manuscript (Raja, 2013; Reed et al., 2014). In observing these phenotypic differences, changes in the expression of myogenic factors were evaluated. However, no difference in the expression of PAX7, MYOD, and myogenin was observed. This is in contrast to observations by Tong et al (2008); where 75 d old fetuses exhibited reduced MYOD and myogenin expression when ewes were fed 160% of NRC requirements. The observed differences between the literature and the current study could be due to differences in the time points at which these samples were taken. Sampling was performed during the early postnatal period when muscle fibers are postmytotic and undergo hyperplasia as a result of satellite cell incorporation (Clemmons, 2009). Satellite cells will express the factors that were qualified, however changes in gene expression of these cells would be difficult to identify in whole tissue.

Satellite cells also express the myogenic factors that were evaluated when activated or undergoing self-renewal (Le Grand and Rudnicki, 2007). Therefore differences in these factors were anticipated as differences in cross-sectional area were noted at both 1 d and 3 mo of age (Raja, 2013; Reed et al., 2014). Satellite cells only comprise less than 10% of the cell types

present within muscle tissue (Le Grand and Rudnicki, 2007) and therefore differences in expression could be masked by expression from other cell types within the whole muscle tissue. It is possible that satellite cell gene expression may be altered but would require looking at this cell type specifically. As a result the expression of select myogenic factors within satellite cell was evaluated. However, the relatively due to low animal number and increased variation in expression these results were inconclusive. In order to strengthen confidence in these observations, increased animal number would be needed.

Another subset of factors that can be involved in regulating muscle growth is MSTN and FSTN. Expression of both MSTN and FSTN were altered in response to poor maternal nutrition at birth. Myostatin, a member of the TGFβ family, is a negative regulator of muscle development by inhibiting the expression of myogenic factors by inducing Smad complex formation (Argiles et al., 2012). Mutations of this gene have been identified in mice, sheep, and cattle where these animals exhibit a significant increase in muscle growth. Alternately, follistatin is involved in the inhibition of MSTN. Follistatin can increase muscle development by inhibiting the binding of myostatin to its receptor and by increasing MTOR activity via the PI3K pathway (Argiles et al., 2012). Follistatin is essential for proper development as knockdown of this protein is lethal and results in retardation of muscle growth. Interestingly, myostatin protein expression was unaltered by maternal diet in OVER lambs as presented by Reed et al (2014). It is possible that the disparity between mRNA and protein expression in these animals could be due to the prevalence of specific microRNAs which regulate expression of myogenic factors like MSTN (Hitachi and Tsuchida, 2014). Specifically MiR-206, MiR-27, MiR-209, and MiR-499 are responsible for regulating the expression of myostatin in skeletal muscle tissue (Hitachi and Tsuchida, 2014). Studies have also found that the prevalence of these microRNAs can be affected by nutritional

status (Yan et al., 2013). Based on these observations evaluating the prevalence of specific micoRNAs within muscle tissue of lambs born to poorly nourished mothers may be needed. In future studies plans are to increase the animal number in order to reduce variation and increased statistical power. We also plan on conducting a transcriptome analysis to identify novel genes and pathways that are altered as a result of poor maternal nutrition.

Reduced FOXO1 mRNA was observed in lambs born to RES ewes at three mo of age. As presented by Reed et al. (2014) phosphorylated Akt, which is considered to be the active from of Akt, is increased in RES lambs at 3 mo. Akt is responsible for binding FOXO and preventing the activating of 4EBP1 resulting this preventing processes that favor reduced protein accretion and increased protein breakdown (Clemmons, 2009; Sanchez et al., 2014). Therefore, it is possible that the regulation of this mechanism is altered as a result of maternal nutrient restriction. Additional work evaluating additional factors involved in this mechanism needs to be conducted.

Another mechanism that is involved in regulating protein accretion in muscle postnatally is the somatotropic axis. Restricted born lambs exhibited increased IGF-I mRNA expression was in response to maternal diet at 1 d of age. This is in agreement with previous observations from Chapter 1 (Hoffman et al., 2014b). Insulin-like growth factor-I can be produced locally by muscle tissue and satellite cells. Although no difference in circulating IGF-I concentrations at this time point was observed (Peck et al., 2013), it is possible that local production of IGF-I may contribute to changes in muscle development postnatally. In OVER lambs, a significant reduction in IGFBP-3 gene expression was also observed. A reduction of IGFBP-3 locally in muscle could affect the availability and binding of IGF-I. In order to better understand how these alterations may be involved in muscle tissue development, western blot analysis would need to be performed on these and like factors, as well as additional gene expression analysis.

IMPLICATIONS

From this second study, we observed changes in the physical characteristics of muscle. However, our understanding of the mechanisms that may be involved is still limited. The changes that were observed in gene expression were in only a few of the selected genes thus suggesting more research is needed to better understand the changes in gene expression that were observed and identify other potential mechanisms. In order for these data to be utilized in other applications and to better answer the overall problem we have elected to utilize RNA-seq analysis to gain a wider perspective of the genes that may be altered by poor maternal nutrition.

CHAPTER III: THE USAGE OF RNA-SEQ ANALYSIS TO DETERMINE DIFFERENTIAL GENE EXPRESSION IN MUSCLE OF LAMBS BORN TO UNDER- AND OVER-FED EWES

INTRODUCTION

Muscle is one of the key tissue types in offspring affected by maternal nutrition during gestation (Wu et al., 2004, 2006). Offspring born to under-and over-fed dams exhibit reduced myofiber number, alterations to cross-sectional area and increased connective tissue and adipose tissue content (Tong et al., 2009; Huang et al., 2010; Zhu et al., 2012; Yan et al., 2013), thus reducing animal productivity. Our previous findings, as presented in Chapters 1 and 2, have demonstrated that poor maternal nutrition can alter offspring body weights, body composition, and concentrations of circulating growth factors (Hoffman et al., 2014b; Reed et al., 2014). Cross-sectional area and muscle fiber composition were also altered in lambs as a result of poor maternal nutrition (Reed et al., 2014). Analysis using real-time RT-PCR identified changes in expression of factors that regulate muscle development and the somatotropic axis (Hoffman et al., 2014b; Reed et al., 2014). However, limited changes have been identified as we have targeted key pathways known to be involved in muscle development. To gain pan-genomic information about the genes and pathways that are altered as a result of poor maternal nutrition, we used RNA-seq analysis. We hypothesized that poor maternal nutrition would alter the expression of key pathways and novel genes involved in the growth, development and maintenance of the semitendinosus muscle tissue of lambs.

MATERIALS AND METHODS

Animals

Information pertaining to the animal portion of this study can be found in Chapter Two: Materials and Methods section. For the RNA-seq analysis, only samples taken from lambs at 1 d were used.

Sample Collection and Processing

Animals were euthanized with an intravenous injection of Beuthanasia-D Special (Merck Animal Health; Summit, NJ) based on BW, followed by exsanguination. Muscle samples were collected from the midpoint of the left semitendinosus immediately after euthanasia. Samples for RNA extraction were immediately snap frozen in liquid nitrogen.

*RNA Extractio*n

Tissue samples for three Dorset lambs from each treatment group were selected for this analysis. Gender distribution across treatment groups was 2 rams and 1 ewe for CON, 3 rams for RES and 2 ewes and 1 ram for OVER. Tissue was homogenized using the Qiagen Tissuelyser system with 1 mL TriReagent (Sigma Aldrich). A Qiagen Mini Kit was used to extract RNA according to the manufacturer's protocol (Qiagen; Valencia, CA). Genomic DNA was removed from samples using a Turbo DNA Free kit (Ambion, Foster City, CA). The quality of RNA was determined using a Bioanalyzer analysis system (Agilent Technologies, Santa Clara, CA).

Removal of Ribosomal RNA

RNA quality was assessed before ribodepletion and cDNA library preparation via bioanalyzer (Agilent Technologies). All samples selected for RNAseq analysis had an RNA integrity number ≥ 8. RNA quantity was determined via a Qubit flourometer (Invitrogen). For ribosomal RNA depletion, 4.5 to 5 µg of total RNA was treated using the Ribo-Zero Gold ribodepletion kit for humans and mice (Epicenter, Madison, WI) according to manufacturer's protocol. Yield of ribodepleted RNA ranged between 30 to 60 ng total after purification, which was performed using a Qiagen RNAeasy Mini Kit (Qiagen). To assess RNA quality post-ribodepletion and to ensure that ribosomal RNA had been removed, a bioanalyzer analysis was performed using a Total RNA Pico Chip (Agilent Technologies).

Preparation of cDNA libraries

cDNA libraries were prepared using ribodepleted total RNA with the Ion Total RNA-seqV2 kit (Life Technologies) as per the manufacturer's instructions. Briefly, total RNA was fragmented using RNAse III and then samples were purified in to remove any small fragments. After initial purification steps, RNA fragment size and yield were evaluated using an Agilent Bioanalyzer and Total RNA Pico Chip kit (Agilent Technologies). Adaptors were conjugated to samples followed by reverse transcription and addition of barcoded adapters to samples. cDNA quality, size, and concentration were determined using the Agilent Bioanalyzer and High Sensitivity DNA chip (Agilent Technologies). Samples were then pooled and conjugated to Ionsphere particles (ISPs) using the Ion P1 Template OT2 Solution 200 V3 Kit and the Ion OneTouch 2 instrument (Life Technologies). Template positive ion sphere particles were then enriched using the Ion One Touch ES instrument, melt off solution and Dynabeads (Life

Technologies). The percent template ISPs was determined using a Qubit flurometer (Life Technologies) to determine relative fluorescence unit values. The optimal range for template ISPs is between 10 to 25%.

Sequencing

Templated and enriched ISPs were loaded onto an Ion P1 chip and run on an Ion Torrent Proton DNA sequencer (Life Technologies). Data were downloaded from the server in FASTA format.

Data Analysis

Quality control analysis was performed to eliminate any sequences that were less than 35 base pairs in length or reads with a Phred score less than 20. PCR duplicates (i.e. sequence reads that start and end at the same genome positions) were also removed before analysis. Sequences obtained were mapped to either the *Ovis arie*s (Oar_V.3.1/OviAri3) or *Bos taurus* (Btau_4.61/BosTau7) reference genome sequence using the Tophat aligner software package (Trapnell et al., 2009). Mapped reads were then assembled and then merged using the Cufflinks and Cuffmerge packages, respectively (Trapnell et al., 2010). The resulting assembly was then used to evaluate differential gene expression along with the previously mapped reads using the Cuffdiff package (Trapnell et al., 2010). Genes were considered to be differentially expressed using a false-discovery rate-corrected *q*-value threshold ≤ 0.05. False discovery rates were determined using the Benjamini-Hochberg multiple testing correction (Benjamini and Hochberg, 1995). A secondary analysis using R was performed to further evaluate differential gene expression. For this analysis, BAM files from the Cuffdiff analysis were converted to non-

normalized count data using HT-seq counts (Anders et al., 2014). These files were then utilized in the respective edgeR analysis. Filters utilized include removal of ribosomal genes and genes with low expression. These data are presented in Appendix IX.

RESULTS

Sequencing Output

The average number of raw reads yielded from sequencing were 13,036,643 for CON, 11,806,935 for RES and 10,065,059 for OVER lambs (Tables 7 and 8). These raw reads were then mapped to either the *Bos taurus* or *Ovis aries* reference genome. Of the initial reads 59%, 56%, and 55% of the reads mapped to the Bovine reference for CON, RES, and OVER lambs, respectively (Table 7). Mapped reads for the analysis using the Ovine genome were 33%, 51%, and 52% for CON, RES, and OVER lambs (Table 8). In total, 16,470 and 931 genes were identified for data mapped to the Bovine and Ovine references, respectively.

Differential Gene Expression

For the differential gene expression analysis using the bovine reference, 35 unique differentially expressed genes were identified whereas mapping to the ovine reference yielded 10 (Table 9 and 10). Genes that were unique to the ovine dataset but not identified in the bovine analysis included the myogenic factor 6 (herculin) [*MYF6*] gene; the BTG family, member 2 (*BTG2*) gene, *LOC443255*, and the zinc finger, AN1-type domain 5 (*ZFAND5*) gene (Table 10). Specifically, expression of *MYF6, LOC443255*, and *ZFAND5* were reduced 1.72-, 1.88, - and

2.03-fold in CON when compared to RES animals ($q \leq 0.05$; Table 11b). A 1.48-fold increase in *BTG2* expression was observed in CON lambs when compared with OVER ($q = 0.01$; Table 11b). Analysis of the bovine genome mapped transcripts revealed that the majority of differentially expressed genes were identified when comparing OVER vs. CON and OVER vs. RES animals, respectively. Expression of the arrestin domain containing 2 (*ARRDC2*) gene and AT rich interactive domain 5B (*ARID5B*) gene was greater in OVER lambs relative to CON (Table 11a; $q \leq 0.05$). Interestingly, CON animals expressed increased quantity of PPARγ coactivator 1 alpha (*PPARGC1A*), the regulator of G-protein signaling 16 (*RGS16*), the thrombomodulin (*THBD*) gene, and the jun B proto-oncogene (*JUNB*) relative to OVER lambs with fold-change differences ≥ 2 ($q \leq 0.05$; Table 11a). Three genes, Tripartit motif containing 63 (*TRIM63*), FHJ murine osteosarcoma viral oncogene homolog (*FOS*) and suppressor of cytokine signal 3 (*SOCS*), displayed increased relative expression in RES animals compared with CON animals ($q \leq 0.03$; Table 12). In comparing the differentially expressed genes that were identified in the bovine analysis with genes that we had previously evaluated (Reed et al., 2014), *MSTN* was up-regulated in OVER lambs. Genes that were identified by both analysis included *MSTN*, *Phosphoserine Phosphatase (PSPH*), *Ankyrin repeat domain 1 (ANKRD1*) for CON relative to OVER and *RAS, Dexamethasone-Induced 1 (RASD1*), *solute carrier family 25, member 33 (SLC25A33*), and *ANKRD1* for RES relative to OVER (Table 13a and 13b). FHJ murine osteosarcoma viral oncogene homolog was the only differentially expressed gene identified in CON versus RES animals when comparing the Bovine and Ovine analysis. Fold change of these genes was similar across analysis types. In an effort to identify potential pathways altered by poor maternal nutrition, the Database for Annotation, Visualization and Integrated Discovery (DAVID) knowledgebase (Huang da et al., 2007) was used. However, due

to the limited number of differentially expressed genes identified from our analysis, we were not able to identify any notable enriched canonical pathways.

Table 7. Number of reads for data mapped to *Bos taurus* reference

TREATMENT	REPLICATE	INPUT	MAPPED	UNMAPPED
CON	1	13,161,614	9,245,342	3,916,272
CON	2	20,174,934	11,197,413	8,977,521
CON	3	5,773,382	2,969,725	2,803,657
RES	1	13,782,363	8,727,793	5,054,570
RES	2	14,378,203	7,743,945	6,634,258
RES	3	7,260,240	3,708,684	3,551,556
OVER	1	11,479,977	5,256,230	6,223,747
OVER	2	11,245,656	7,803,404	3,442,252
OVER	3	7,469,543	3,668,086	3,801,457

Ewes were fed 100% (CON) or 140% (OVER) of NRC requirements for ewes in late gestation and pregnant with twins. Tissue samples were collected from lambs at 1d necropsies. Input represents the number of raw reads obtained from sequencing. Mapped refers to the number of input reads that mapped to the reference genome. Unmapped is the number of reads that did not map to the reference genome.

Table 8. Number of reads for data mapped to *Ovis aries* reference

TREATMENT	REPLICATE	INPUT	MAPPED	UNMAPPED
CON	1	13161614	7401249	12421365
CON	2	20,174,934	9,256,069	10,918,865
CON	3	5,773,382	2,971,277	2,802,105
RES	1	13,782,363	7,048,003	6,734,360
RES	2	14,378,203	7,468,385	6,909,818
RES	3	7,260,240	3,594,983	3,665,257
OVER	1	11,479,977	5,357,075	6,122,902
OVER	2	11,245,656	6,953,984	4,291,672
OVER	3	7,469,543	3,620,310	3,849,233

Ewes were fed 100% (CON) or 140% (OVER) of NRC requirements for ewes in late gestation and pregnant with twins. Tissue samples were collected from lambs at 1d necropsies. Input represents the number of raw reads obtained from sequencing. Mapped refers to the number of input reads that mapped to the reference genome. Unmapped is the number of reads that did not map to the reference genome.

Table 9. Differentially expressed genes as determined by mapping to *Bos taurus* reference

GENE	FUNCTION
ANKRD1	Involved in the myofibrillar stretch-sensor system
ARID5B	Involved in adipogenesis and liver development
ARRDC2	Protein coding gene
BTG1	Regulates cell growth and differentiation
DDIT4L	Inhibits cell growth
FBXO32	Functions in phosphorylation-dependent ubiquitination
FOS	Role in signal transduction, cell proliferation, and differentiation
GADD45G	Regulation of growth and apoptosis
GFPT2	Controls the flux of glucose into the hexosamine pathway
JUNB	Regulating gene activity following the primary growth factor response
KIAA0408	Protein coding gene
KLHL38	Protein coding gene
MGC165715	Protein coding gene
MSTN	Negative regulator of skeletal muscle growth
MT1E	Bind heavy metals
PFKFB3	Synthesis and degradation of fructose 2,6-bisphosphate
PPARGC1A	Transcriptional coactivator for steroid receptors and nuclear receptors
PSAT1	Phosphoserine aminotransferase
PSPH	Catalyzes biosynthesis of serine from carbohydrates
RASD1	GTPase
RGS16	Inhibits signal transduction
SESN1	Participates in dendritic cell endocytosis
SLC25A33	RNA gene
SNORA70	RNA gene
SNORA72	RNA gene
SNORD113	Belongs to the cytospin-A family
SOCS3	Regulates cytokine signal transduction
SPECC1	Endothelial cell receptor
THBD	Atrophy of skeletal and cardiac muscle
TRIM63	Small nuclear RNA
U1	Novel miscellaneous RNA
Y_RNA	Proliferation and apoptosis
YPEL3	Regulates cellular proliferation and apoptosis

Ankyrin repeat domain 1 (ANKRD1), arrestin domain containing 2 (ARRDC2), AT rich interactive domain 5B (ARID5B), B-cell translocation gene 1 (BTG1), DNA-damage-inducible transcript 4-like (DDIT4L), F-box protein 32 (FBXO32), FHJ murine osteosarcoma viral oncogene homolog (FOS), Glutamine-fructose-6-phosphate transaminase 2 (GFPT2), Kelch-like family member 38 (KLHL38) ,Uncharacterized protein C4orf29 homolog (MGC165715), Metallothionein 1E (MT1E), myostatin (MSTN), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), Peroxisome proliferator-activated receptor gamma coactivator 1

alpha gene (PPARGC1A), Phosphoserine aminotransferase 1 (PSAT1),Regulator of G-protein signaling 16 (RGS16),Thrombomodulin (THBD), jun B proto-oncogene (JUNB), Tripartit motif containing 63 (TRIM63), Phosphoserine Phosphatase (PSPH), RAS, Dexamethasone-Induced 1 (RASD1), sestrin 1 (SESN1), solute carrier family 25, member 33 (SLC25A33), small nucleolar RNA H/ACA box 70, (SNORA72) small nucleolar RNA H/ACA box 72 (SNORA72), Sperm antigen with calponin homology and coiled-coil domains 1 (SPECC1), Suppressor of cytokine signal 3 (SOCS), U1 spliceosomal RNA (U1), Yippee-like 3 (YPEL3)

Table 10. Differentially expressed genes as determined by mapping to *Ovis aries* reference

GENE	FUNCTION
ANKRD1	Involved in the myofibrillar stretch-sensor system
BTG2	Anti-proliferative protein
FOS	Role in signal transduction, cell proliferation and differentiation
LOC443255	Oxytocin and Thyroid Hormone signaling
MSTN	Inhibits myogenesis
MYF6	Muscle differentiation
PSPH	Catalyzes biosynthesis of serine from carbohydrates
RASD1	Biosynthesis of serine from carbohydrates
SLC25A33	Participates in dendritic cell endocytosis
ZFAND5	Involved in protein degradation

Ankyrin repeat domain 1 (ANKRD1), FHJ murine osteosarcoma viral oncogene homolog (FOS), myostatin (MSTN), myogenic factor 6 (herculin) [*MYF6*], *Phosphoserine Phosphatase (PSPH), RAS, Dexamethasone-Induced 1 (RASD1), solute carrier family 25, member 33 (SLC25A33), BTG family, member 2 (BTG2), zinc finger, AN1-type domain 5 (ZFAND5)*

Table 11a. Differential expression analysis results for OVER vs CON lambs mapped to *Bos taurus* reference.

GENE	TREATMENT A	TREATMENT B	FOLD CHANGE
ARRDC2	OVER	CON	-3.46
ARID5B	OVER	CON	-2.87
MSTN	OVER	CON	-1.96
SPECC1	OVER	CON	1.74
GFPT2	OVER	CON	1.84
JUNB	OVER	CON	2.02
U1	OVER	CON	2.05
SNORA70	OVER	CON	2.05
THBD	OVER	CON	2.07
RGS16	OVER	CON	2.11
PPARGC1A	OVER	CON	2.31
PSAT1	OVER	CON	2.50
SNORD113	OVER	CON	2.55
PSPH	OVER	CON	2.70
U1	OVER	CON	2.77
ANKRD1	OVER	CON	3.1
MT1E	OVER	CON	2.20

Ewes were fed 100% (CON) or 140% (OVER) of NRC requirements for ewes in late gestation and pregnant with twins. Tissue samples were collected from lambs at 1d necropsies. Data are presented as fold change relative of OVER lambs. *Bos taurus* reference was used. *Ankyrin repeat domain 1 (ANKRD1), arrestin domain containing 2 (ARRDC2), AT rich interactive domain 5B (ARID5B), Glutamine-fructose-6-phosphate transaminase 2 (GFPT2), Metallothionein 1E (MT1E), myostatin (MSTN), Peroxisome proliferator-activated receptor gamma coactivator 1 alpha gene (PPARGC1A), Phosphoserine aminotransferase 1 (PSAT1),Regulator of G-protein signaling 16 (RGS16),Thrombomodulin (THBD), jun B proto-oncogene (JUNB), Phosphoserine Phosphatase (PSPH), RAS, Dexamethasone-Induced 1 (RASD1), Sperm antigen with calponin homology and coiled-coil domains 1 (SPECC1), U1 spliceosomal RNA (U1)*

Table 11b. Differential expression analysis results for OVER vs CON lambs mapped to *Ovis aries* reference.

GENE	TREATMENT A	TREATMENT B	FOLD CHANGE
MSTN	OVER	CON	-1.95
MYF6	OVER	CON	-1.67
BTG2	OVER	CON	1.48
PSPH	OVER	CON	2.54
ANKRD1	OVER	CON	3.16

Ewes were fed 100% (CON) or 140% (OVER) of NRC requirements for ewes in late gestation and pregnant with twins. Tissue samples were collected from lambs at 1d necropsies. Data are presented as fold change relative of OVER lambs. *Ovis aries* reference was used. Shading indicates genes that were similar to the bovine analysis. *Ankyrin repeat domain 1 (ANKRD1), myostatin (MSTN),* myogenic factor 6 (herculin) [*MYF6*], *Phosphoserine Phosphatase (PSPH), RAS, Dexamethasone-Induced 1 (RASD1), BTG family, member 2 (BTG2).*

Table 12. Differential expression analysis results for RES vs CON lambs mapped to *Bos taurus* reference.

GENE	TREATMENT A	TREATMENT B	FOLD CHANGE
TRIM63	RES	CON	1.41
FOS	RES	CON	1.59
SOCS3	RES	CON	2.83

Ewes were fed 100% (CON) or 60% (RES) of NRC requirements for ewes in late gestation and pregnant with twins. Tissue samples were collected from lambs at 1d necropsies. Data are presented as fold change relative of RES lambs. *Bos taurus* reference was used. *FHJ murine osteosarcoma viral oncogene homolog (FOS), Tripartit motif containing 63 (TRIM63), Suppressor of cytokine signal 3 (SOCS).*

Table 13a. Differential expression analysis results for OVER vs RES lambs mapped to *Bos taurus* reference.

GENE	TREATMENT A	TREATMENT B	FOLD CHANGE
SNORA72	OVER	RES	-7.46
ARRDC2	OVER	RES	-3.85
FBXO32	OVER	RES	-3.72
TRIM63	OVER	RES	-3.47
PFKFB3	OVER	RES	-3.24
RASD1	OVER	RES	-3.13
SESN1	OVER	RES	-3.07
YPEL3	OVER	RES	-2.88
KLHL38	OVER	RES	-2.73
SLC25A33	OVER	RES	-2.57
GADD45G	OVER	RES	-2.56
BTG1	OVER	RES	-1.94
MGC165715	OVER	RES	-1.65
KIAA0408	OVER	RES	-1.62
DDIT4L	OVER	RES	-1.48
ANKRD1	OVER	RES	2.11
SNORD113	OVER	RES	2.40
U1	OVER	RES	3.47
Y_RNA	OVER	RES	8.47

Ewes were fed 140% (OVER) or 60% (RES) of NRC requirements for ewes in late gestation and pregnant with twins. Tissue samples were collected from lambs at 1d necropsies. Sequencing results were mapped against the *Bos taurus* reference. Data are presented as fold change relative of OVER lambs. *Ankyrin repeat domain 1 (ANKRD1), arrestin domain containing 2 (ARRDC2), AT rich interactive domain 5B (ARID5B), B-cell translocation gene 1 (BTG1), DNA-damage-inducible transcript 4-like (DDIT4L), F-box protein 32 (FBXO32), Kelch-like family member 38 (KLHL38) ,Uncharacterized protein C4orf29 homolog (MGC165715), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), Tripartit motif containing 63 (TRIM63), Phosphoserine Phosphatase (PSPH), sestrin 1 (SESN1), solute carrier family 25, member 33 (SLC25A33), small nucleolar RNA H/ACA box 70, (SNORA72) small nucleolar RNA H/ACA box 72 (SNORA72), U1 spliceosomal RNA (U1), Yippee-like 3 (YPEL3).*

Table 13b. Differential expression analysis results for OVER vs RES lambs mapped to *Ovis aris* reference.

GENE	TREATMENT A	TREATMENT B	FOLD CHANGE
RASD1	OVER	RES	-2.76
SLC25A33	OVER	RES	-2.43
ZFAND5	OVER	RES	-2.03
LOC443255	OVER	RES	-1.88
MYF6	OVER	RES	-1.72
PSPH	OVER	RES	2.04
ANKRD1	OVER	RES	2.17

Ewes were fed 140% (OVER) or 60% (RES) of NRC requirements for ewes in late gestation and pregnant with twins. Tissue samples were collected from lambs at 1d necropsies. Sequencing results were mapped against the *Ovis aries* reference. Data are presented as fold change relative of OVER lambs. Shading indicates genes that were similar to the bovine analysis. *RAS, Dexamethasone-Induced 1 (RASD1), Phosphoserine Phosphatase (PSPH), solute carrier family 25, member 33 (SLC25A33), zinc finger, AN1-type domain 5 (ZFAND5),* myogenic factor 6 (herculin) [*MYF6*], *Phosphoserine Phosphatase (PSPH), Ankyrin repeat domain 1 (ANKRD1).*

DISCUSSION

Fetal muscle development during gestation can be negatively impacted by poor maternal nutrition with these effects persisting into adulthood (Wu et al., 2006; Nesterenko and Aly, 2009). Several studies have identified the phenotypic changes that occur as a result of poor maternal nutrition (Wu et al., 2006; Hoffman et al., 2014b; Reed et al., 2014); however, knowledge of the mechanisms involved is limited. Identifying these mechanisms is critical in order to better understand how these changes are manifested and maintained into postnatal life. This information could then be used to develop management systems or alimentation plans to maximize the production of these offspring. In an effort to identify potential pathways and novel genes that may be altered by poor maternal nutrition, RNA-seq analysis was performed on the semitendinosus tissue samples taken from lambs at 1 d of age. From this analysis we were successfully able to identify several genes that are involved in the regulation of metabolism, hypertrophy, nutrient uptake, and muscle protein turnover.

Targeted gene expression of these tissue samples had been previously evaluated using real-time RT-PCR. Similar to the current analysis, the expression of *MSTN* was greater in OVER lambs. Genes identified using RNA-seq are determined by mapping sequencing reads back to a reference. This differs from real time-RT-PCR where the expression of genes is determined based on amount of PCR product produced using primers for a gene of interest (Marioni et al., 2008). Real-time RT-PCR is commonly used to validate differential gene expression results from sequencing (Marioni et al., 2008) and therefore, adds confidence to our initial observation of *MSTN* being differentially expressed.

In addition to *MSTN*, several of the differentially expressed genes identified are involved in regulating muscle development, including muscle protein turnover, nutrient uptake and

metabolism. These genes include, *JUNB*, *RGS16*, *ARID5B*, and *GFPT2*. The transcription factor, *JUNB*, is responsible for maintaining muscle mass resulting in muscle hypertrophy (Raffaello et al., 2010). This is accomplished when *JUNB* blocks the actions of *TRIM63* and *FOXO* proteins that are involved in increasing proteolysis and muscle atrophy (Raffaello et al., 2010). A decrease in *JUNB* expression was observed in OVER lambs. Interestingly, a reduction *TRIM63* gene expression was observed in RES lambs at d 1. Therefore, these data suggest that protein accretion may be altered in OVER and RES offspring.

Genes that are involved in regulating muscle nutrient uptake have been altered in these offspring as well. Notably, *RGS16* and *GFPT2* were reduced in the muscle of OVER lambs relative to CON. *RGS16* encodes a G protein regulator that is involved in the regulation of fatty acid oxidation in response to glucose production (Rudkowska et al., 2013). *GFPT2* is also responsible for regulating flux of glucose into the hexoseamine biosynthetic pathway. *ARID5B*, also known as *MRF2*, is involved in the differentiation of several cell types including smooth muscle and fat. It is also needed for the accumulation of triglycerides in adipose tissue (Whitson et al., 2003; Wang et al., 2012). Changes to these factors suggest alterations to the regulation of nutrient accretion within muscle tissue. For example, it is possible that *ARID5B* may be involved in the development of increased intramuscular adiposity that was observed in these animals at three mo of age (Reed et al., 2014). Western blot and additional gene expression analysis is needed in order to better understand these changes.

Expression of some of these genes could be due to differences in fiber type. *PPARGC1A* expression in muscle tissue will vary depending on muscle fiber type composition (Handschin, 2010). Specifically, Type I and Type IIa muscle fibers are classified as oxidative fibers and exhibit greater expression of *PPARGC1A* than Type IIa fibers, which are glycolytic (Handschin,

2010). As we previously presented (Reed et al., 2014), tissue collected from both RES and OVER lambs exhibited a reduction in Type I fibers with an increase in Type IIb fibers at birth. However, this difference in expression was only observed in tissue from OVER lambs. A potential reason for this difference could also involve alterations to the metabolic regulation within the muscle tissue of OVER offspring. Expression of *PPARG1A* is regulated by AMP-activated protein kinase. AMP-activated protein kinase is activated in response to increased AMP concentrations and in turn will increase expression of *PPARGC1A* in muscle thus increasing mitochondrial biogenesis (Handschin, 2010). It is possible that the exposure of the offspring to overfeeding during gestation resulted in a decrease in expression of this gene within the muscle tissue. Several other genes that were identified, specifically, JUNB and THBD, in OVER lambs can be regulated by insulin. Typically the expression of these genes is increased in response to insulin (Coletta et al., 2008; Olesen et al., 2010; Raffaello et al., 2010; Broholm et al., 2012). At this time point, altered circulating insulin concentrations were not observed in OVER animals (Peck et al., 2013); however, it is possible that changes to insulin sensitivity in the muscle tissue could affect the expression of these genes. Studies have indicated that maternal obesity is capable of altering the insulin response of the offspring as determined by muscle gene expression analysis of factors involved in insulin signaling as well as glucose tolerance tests (Long et al., 2010a; Yan et al., 2011)

In RES lambs, *SOCS3* expression was reduced as well as the expression of *FOS*. *SOCS3* is responsible for regulating the activation of the janus kinase (JAK)/signal transducer and activator of transcription(STAT) pathway by preventing activation and thus inhibiting the myogenesis (Broholm et al., 2012). Therefore, these data suggest that there may be alterations to

the regulation of these pathways. To better understand these changes, other factors involved in the JAK/STAT pathway and targets of *SOCS3* and *FOS* need to be evaluated.

As stated previously, to our knowledge we are the first to conduct an RNA-seq analysis to evaluate differences in gene expression as a result of poor maternal nutrition in the semitendinosus of lambs. However, considering the general results from the Cuffdiff analysis, the number of differentially expressed genes was low compared with the number of genes that were identified. An additional analysis type, edgeR, was also used in an effort to try and determine additional differentially expressed genes. However, using this analysis, no differentially expressed genes were identified when taking into consideration the *q* values (Appendix IV). A reason for this could be the differences in these analysis types. Data normalization, statistical modeling, and tests for differential gene expression for Cuffdiff are different compared to edgeR (Rapaport et al., 2013). Specifically, edgeR uses negative binomial distribution and fisher exact test to determine differential gene expression. Cuffdiff utilizes test statistics (Rapaport et al., 2013). It is also possible that by increasing the number of biological replicates more genes would be identified using both of these analysis types. Decreased replicate number can be associated with increase false discovery rate values thus ruling genes out as potential candidates for analysis. Liu et al. (2014) compared the number of differentially expressed genes versus increasing number of biological replicates or increasing sequencing depth for samples (i.e., the number of reads per sample). This study indicated the increasing the number of replicates resulted in a significant increase in differentially expressed genes identified. A study by Rapaport et al. (2014) also identified a reduction in the number of false positives and increased sensitivity rates of the analysis when increasing replication and sequencing depth. In considering this, additional animal replicates may be needed to identify more differentially

expressed genes. Overall, however, the length of reads was short. Due to the short read length, a significant number of reads were lost during initial quality control measures, which may have affected overall coverage. The Ion Torrent Proton is reported to produce reads that are approximately 200 bp in length (Quail et al., 2012). However, there is no information available on what read lengths have been observed for sheep muscle samples. It is plausible that a different sequencing platform may result in reads that were longer in length.

In summary, we were able to identify several genes that may be involved in the regulation of muscle metabolism, protein accretion, and growth postnatally. These data have provided additional information to our initial analysis and aided in the identification of potential mechanisms that need to be explored further. Future studies with increase animal replication could aid in the identification of additional genes.

IMPLICATIONS

The objective of this analysis was to identify candidate genes and pathways to identify targets for additional analysis as well as to gain a better knowledge of the ways that poor maternal nutrition alters muscle development in the offspring. A limited number of genes were identified within this analysis as indicated by Cuffdiff and edgeR. These analysis have proved valuable to us however, in understanding how to better our RNA-seq analysis. Increased biological replication and increased read length may be needed in order to derive additional data from this tissue type. This should be taken into consideration in preparation for the analysis that will be performed in subsequent studies using this model.

GENERAL DISCUSSION

Current demands on the livestock industry require animals to be produced as quickly and efficiently as possible to maximize profit for the producer while maintaining an affordable product for the consumer. Meat products produced by livestock need to be high quality and lean cuts of meat appeal to customers. However, it is possible that the productivity of an animal can be negatively affected based on exposure to certain factors during embryonic and fetal development in a process known as maternal programming (Nesterenko and Aly, 2009). There are several factors that can result in maternal programming with the effects of poor maternal nutrition on offspring becoming of increasing interest in the livestock industry.

Overall, the objective of the research presented was to determine what factors and mechanisms were involved in mediating the effects of poor maternal nutrition during postnatal development. It was determined that offspring born to under- and over-fed ewes exhibited persistent alterations to body weight as well as organ mass, back fat measurements, and loin eye area as determined at necropsy. These finding are in agreement with current literature where alterations in body mass and composition are observed as a result of poor maternal nutrition (Wu et al., 2006; Ford et al., 2009; Long et al., 2010a). In the studies from Chapter 1 and 2, differences in circulating factors that are involved in the somatotropic axis, specifically IGF-I and IGFBP-3, were observed (Peck et al., 2013; Hoffman et al., 2014b). Until this point, the understanding of how poor maternal nutrition may affect the somatotropic axis in the offspring postnatally was limited (Bauer et al., 1995). In addition to changes in circulating growth factors and body variables, changes in muscle cross-sectional area and muscle fiber type were identified at these early postnatal time points which contributed to the body of evidence supporting the

effects of maternal under- and over-feeding on growth and development of muscle tissue in offspring.

Gene expression analysis was performed to gain information in regards to the molecular mechanisms that were involved in the phenotypic changes that were being observed. As presented in Chapter 1 and 2, specific genes were targeted for evaluation in liver, and adipose tissue with a central focus on muscle. In performing these analyses, we were able to determine that poor maternal nutrition can locally alter factors involved in the somatotropic axis muscle of offspring (Hoffman et al., 2014b; Reed et al., 2014). Interestingly, expression of IGF-I mRNA was found to be affected by poor maternal nutrition and was opposite to what was observed in circulating concentrations of IGF-I (Peck et al., 2013; Rokosa, 2013; Hoffman et al., 2014b). IGF-I can be produced locally within several tissues, including the muscle (Clemmons, 2009). Therefore, it is possible that poor maternal nutrition can affect both local production and circulating concentrations of factors involved in the somatotropic axis.

In all three Chapters, significant differences in myogenic factor gene expression were not observed. However, the expression of these genes were evaluated at a two specific time points during early postnatal development. It is possible that if the samples were taken during fetal development or later in adult life, changes in the expression of these factors or others may be identified. Currently, a third study is being conducted to evaluate the changes of poor maternal nutrition at three distinct time points during gestation and at 1 d of age. Analysis of these samples will provide information in regards the alterations and mechanisms that may be involved leading up to this early postnatal time point.

Regulatory mechanisms involved in the degradation and/or prevention of translation of mRNA transcripts must also be taken into consideration when discussing these data. It is

possible for mRNAs to be transcribed in increased abundance but degradation or microRNAs could prevent differences in overall mRNA or protein expression from being observed, as may have been the case with *MSTN*. The involvement of microRNAs in regulating transcript abundance could also be another mechanism by which poor maternal nutrition alters gene expression. In a recent study, maternal over-feeding decreased the expression of microRNAs in fetal muscle tissue resulting in increased expression of the associated target genes (Yan et al., 2013). Therefore, it is possible that by evaluating changes in microRNA expression that additional gene targets and pathways could be elucidated.

In contrast to current literature, our model allowed for the evaluation of the effects of maternal under- and over-feeding within the same study. This allowed for the identification of differences in the mechanisms involved between RES and OVER offspring. For example, circulating leptin and local leptin mRNA expression were increased in OVER lambs but not in RES lambs (Hoffman et al., 2014b). Therefore, this may be a differential mechanism that may alter the metabolism of these lambs at this timepoint as a result of maternal overnutrtion but not nutrient restriction. Similarly, in comparing the differentially expressed genes identified by RNA-seq, there are differences in the genes identified depending on if the mother was under- or over-fed. These data suggest that while similar effects on CSA and muscle development are being observed the mechanisms that are involved in mediating these effects may be different. Additional work needs to be conducted in order to evaluate what other factors may be involved in these changes and how despite differences in the mechanisms involved similar phenotypes can result in RES and OVER offspring.

In order to gain a more comprehensive knowledge of the effects of poor maternal nutrition on offspring growth and development, research needs to be conducted that evaluates the

effects of poor maternal nutrition on protein and gene expression as well as the involvement of other mechanisms such as epigenetics. Histone modifications and DNA methylation are two other mechanisms that are evaluated in various tissue types of offspring born to poorly nourished animals (Ford and Long, 2012; Yang et al., 2012; O'Neill et al., 2014). A recent study conducted in rats demonstrated that maternal over feeding can alter the acetylation and methylation of histones resulting in a reduction in the expression of WNT genes in the liver of offspring (Yang et al., 2012). Research is also being conducted to determine the effect of methyl donor consumption during gestation on offspring growth and development (O'Neill et al., 2014). Likewise, epigenetic mechanisms may contribute to the transgenerational effects of poor maternal nutrition that are observed in both human and livestock species (Ford and Long, 2012). As a result, evaluation of this mechanism can help understand how poor maternal nutrition effects offspring development and how to reduce its effects pre- and post-natally. Therefore, studies similar to those presented in Chapters 1, 2,3 need to be conducted and include evaluation of changes in phenotypic markers, protein and gene expression, and well as epigenetic modifications. This will allow for a more encompassing and complete analysis of the mechanisms involved and a better understanding of how these mechanisms can differ as a result of under or overfeeding.

In summary, poor maternal nutrition can alter several aspects of offspring growth and development including adiposity and muscle development. In two separate studies, we have identified both changes to offspring phenotype and gene expression suggesting that poor maternal nutrition may alter postnatal mechanisms involved in muscle growth, metabolism, and protein accretion through altered gene expression. Additional work needs to be conducted to better understand the changes in gene expression observed as well as consider other mechanism,

such as epigenetic modifications, in order to better understand the effects of poor maternal nutrition.

APPENDIX I: RNA Extraction

1. Use the Qiagen tissuelyser to homogenize samples prior to RNA extraction. Cut a 50-100 mg piece of tissue from sample while on dry ice. Add sample to tube containing 1 ml Qiazol and add one bead. Homogenize for 5-6 minutes.

2. Add 200 µL chloroform to each tube. Mix by inverting 5 -7 times and allow for it to incubate at room temperature for 2-3 min. *Centrifuge these tubes at 12000 x g for 15 min

3. Transfer the aqueous phase into a new eppendorf tube taking care not to disturb the other layers. The total volume transferred will be close to 500 µL. Next add one volume (500 µL) of 70% EtOH to each tube and vortex

4. Transfer 700 µL of the sample into the RNA east Spin Column and centrifuge 8,000 X , for 30s. Discard the resulting flow through and add 700 µL RWI wash buffer. Take care to avoid adding any liquid directly onto the filter as this could cause a decrease in RNA yield. Centrifuge spin column again at 8,000 X g for 30s

5. Discard the flow through and collection tube put the filer on the new collection tube. Add 500 µL of Buffer RPE to spin column ensuring that 100% Ethanol has been added to the buffer prior to use. Centrifuge 8,000 x g, for 30s and discard the flow through.

6. Add 500 µL of Buffer RPE and centrifuge at 8,000 X g for 2 min. Discard the flow through and centrifuge at 8,000 X g for 1 min. Discard the flow through and collection tube, put the filter on the new collection capped tube

7. Add 50 µL of Rnase free water to the spin column and centrifuge at 8,000 X g for 1 min. Take 35-40 µL from the collection tube and put back on the filter centrifuge at 8000 X g for 1 min

8. Collect the eluted RNA, in the capped collection tube, keep on ice, and spec it in the Nanodrop. Aliquot it and store in the -80 freezer

APPENDIX II: Turbo DNA-Free Protocol

1. Thaw the reagents (10x Turbo DNase Buffer, Turbo DNase, DNase inactivation reagent) on ice.

2. Add 0.1 volume of 10x turbo Dnase Buffer (i.e.: 5 uL of 50 uL RNA sample) and 1 µL Turbo Dnase to the RNA sample and mix gently. This is for routine DNase treatment with RNA concentration of 10ug/50uL of sample. If it is more than this con. Dilute your sample to have 10 µg/50 µL using C1V1=C2V2

3. Incubate at 37 °C for 20-30 min

4. Vortex the Dnase Inactivation reagent well as this mixture is very turbid. If performing DNA-Free treatment on a significant number of samples be sure to vortex frequently to prevent settling. Add 0.1 volume of resuspended DNase Inactivation reagent.

5. Incubate for 5 min at room temp while mixing occasionally by flicking or tapping

6. Centrifuge at 10000 x g for 1.5 min

7. Transfer the supernatant to a fresh tube Note: Avoid disturbing the pellet

8. The final DNA-free RNA con. Is determined by Nano drop 1000.

   *When using this protocol on samples that will be Ribodepleted. DO NOT dilute samples. Rather add 3 µL Dnase to each reaction and add 0.2 volumes of Dnase Inactivation reagent.

APPENDIX III: Reverse Transcriptase PCR

1. Thaw the reagents on ice (Ambion OligoDT, Invitrogen Superscript kit II containing DTT, 5X buffer and reverse transcriptase)

2. Add 1uL of OligoDT to each 10uL of normalized RNA sample (300ng/10µl) in 8 tube strips. This makes it a total of 11uL.NOTE: Always include a positive and negative control

3. Place these tubes into the thermocycler and state the desired RT PCR program (see below) and start step 1, 70˚C for 10min
   70˚C for 10min
   4˚C for 20min
   37˚C for 3min
   42˚C for 1hr
   4 ˚C for 3min
   90˚C for 2.5 min
   10˚C forever

4. During the time of step 1, make the master mix. Do not add superscript until you are ready to add the master mix to the samples.

   Master Mix
   5X Buffer- 5.5 µL
   dNTP- 1 µL
   DTT- 2 µL
   SuperscriptII 0.5 µL
   (Reverse transcriptase)

   NOTE: The above calculations are per sample, multiply it by the no. of samples+2 for pipetting errors.

5. Pause the thermocycler when the temperature is dropping to 4˚C for the next step (step 2). Take the samples with RNA and Oligo DT out of the machine and keep it on ice.

6. Add SSII to the master mix, mix gently. Add 9uL of master mix to each sample, making it a total of 20uL ensuring that samples are kept on ice while adding the master mix. Mix contents of tube with master mix by pipetting up and down several times.

7. Place the samples back into the machine hit proceed and hit proceed again to move to step# 3. When RT is complete, add 80uL of Nuclease free water to samples and store in -20˚C freezer.

APPENDIX IV: Designing Primers for PCR

1. Go to www.pubmed.com. Ensure that you search "nucleotide" and then type in your gene of interest. Include the species

2. Find your gene of interest and click on FASTA. This will present you with your gene of interest's sequence. Copy only **CDS or CODING REGION** of the sequence. If you do not use this your primers will not bind the correct sequence.

3. Blast this sequence using the link entitled " RUN BLAST" along the upper right of the webpage. This will compare your gene sequence to other sequences to ensure that you will be amplifying the right gene.

4. Once the blast has been run, check the results to ensure that there are no matches for other genes in the species that you will be working with.

5. Go to: http://frodo.wi.mit.edu/primer3/. Paste your sequence of interest in the large box. This program will construct your primers for you .Change the product size range. Usually you want your product to be between 80-300bp in size. Click "Pick Primers"

6. Once you have the primers return to BLAST on the pubmed website and run a BLAST on these primers to ensure that they will amplify only the gene you want to look at in your species.

7. Go to: http://genome.ucsc.edu/. Click on the tab entitled PCR located at the top of the webpage. Change the Genome tab to match the species you are working in. Enter your Forward and Reverse primer as determined by Primer3 (you can copy and paste it)

8. Observe the temperatures at which these primers anneal. If they are close together for both the forward and reverse primer then you are all set

9. Order your primers through Integrated DNA Technologies (IDT) at the following website: http://www.idtdna.com/Home/Home.aspx

*Dilute and optimize your primers once you receive them by running both PCR and RT-PCR with an efficiency curve.

APPENDIX V: Polymerase Chain Reaction

1. Thaw reagents on ice (Promega Green Master mix, Tbx2 114 forward and reverse primer working aliquots, cDNA sample)

2. Add 5 µL of the cDNA to 8 tube strips once the master mix is added there will be a total volume of 25 µL

3. Make Master mix of 20uL for one reaction

Nuclease free water	5.5 µL
Green master mix	12.5 µL
Forward Primer	1.0 µL
Reverse Primer	1.0 µL

   NOTE: This is for one sample, multiply it by the number of samples plus two for pipetting errors. Be sure to include a negative and positive control. Transfer 20uL of the master mix to 8 tube strips

4. Place the tubes into PCR machine and run the following program:

   94˚C for 3min

   94˚C for 30s

   60˚C for 1min

   72˚C for1min

   X 40cycles

   72˚C for 5min

   10˚C forever

5. Once the PCR is complete (about 2.5 hr), it is stored at 4˚C.

APPENDIX VI: Real-time PCR

1. Thaw the Reagents on ice.(Syber green wrapped in foil, forward and reverse primers, cDNA)

2. Add 5uL of cDNA (diluted 1:5, 20 µL of cDNA + 80 µL of nuclease free water) to the tubes. Make sure to pipette to the bottom of the tube.

3. Make Master Mix. Amount will vary depending on the number of samples and number of genes. Turn off lights when using the SYBR green as it is light sensitive. Always add plus two for pipetting error and include negative and positive controls.

Master Mix	
2X Syber Green	10uL
Forward Primer	1uL
Reverse primer	1uL
Nuclease free water	3uL

4. Add 15uL of master mix to each well in 96 PCR optical plate. Gently pipette contents of well with master mix to facilitate mixing. Avoid forming bubbles as this can result in incorrect values.

5. Seal it with a PCR adhesive film tightly to prevent evaporation and centrifuge the plate at 1000RPM for 1-2 minutes

6. Run plate in the ABI 7900 HT Fast real time machine using the following protocol:

   Stage 1: 50 °C for 2 min
   95°C for 10 min

   Stage 2: 95°C for 15 sec
   60°C for 1 min
   *Repeat for 40 cycles

   Stage 3: 95°C for 15 s
   60 °C for 15 s
   95 °C with a 2% ramp for 2 min

APPENDIX VII: Ribodepletion of Total RNA
Catalogue number: MRZG12324 Ribozero Magnetic Kit for Human/Mouse/Rat

*Samples need to be DNAse treated prior to ribodepletion
*Ensure that RNA entering this process is the highest quality. DO NOT use RNA that has an RNA Integrity Number (RIN) less than 8.

Part I. Prepare Magnetic Beads
*equilibrate magnetic beads and corresponding buffers to room temperature before use
*NEVER FREEZE MAGNETIC BEADS

1. Vortex beads well and add 225 µL magnetic beads to an RNAse free microcentrifuge tube. Allow to stand until solution on magnetic stand for approximately one minute or until solution clears. Remove supernatant and discard.

2. Remove tube from stand and add 225 µL nuclease free water. Vortex and place back on the magnetic stand.

3. Repeat steps 2 and 3 two times. Then add 60 µL magenetic bead resuspension solution.

4. Add 1 µL Riboguard Rnase Inhibitor to each tube of prepared beads *This step is not optional!*

Part II. Treat RNA with rRNA removal solution

If using 1-2.5 µg RNA the maximum volume can be 28 µL plus the addition of 8 µL rRNA removal solution. If using greater than 2.5-5 µg RNA the maximum volume can be 26 µL plus the addition of 10 µL of rRNA removal solution should be used

1. In a 0.5 µL RNase free microcentrifuge tube combine the following:
X µL Rnase free water
4 µL Ribozero Reaction Buffer
Y µL RNA sample
8-10 µL Ribozero rRNA Removal Solution

2. Pipette up and down fifteen times to mix and incubate at 68 ^{o}C for 10 minutes. Quick spin the tubes after to remove condensation.

3. Incubate at room temperature for 5 minutes. Then transfer RNA with probes to washed magnetic beads that have been prepared. Pipette up and down immediately ten to fifteen times.

5. Cap tubes and vortex for ten seconds and incubate at room temperature for five minutes.

6. Incubate tubes at 50 °C for five minutes. Remove tubes from heat and place on a magnetic strand for 1 minute or until the supernatant clears. Transfer supernatant into a labeled 1.5 mL microcentrifuge tube.

Purify RNA

*Use RNeasy Mini-Elute Clean Up Kit (Qiagen)

1. Adjust sample volume to 100 µL Rnase free water and add 350 µL RLT buffer. Mix well.

2. Add 550 µL 100% ethanol and mix well. Transfer half of the sample to RNA easy mini elute spin column and collection tube. Centrifuge for 15 seconds at 8000 x g. Discard flow through.

3. Repeat for remaining sample volume. Place spin column on new 2 mL collection tube. Add 500 µL RPE buffer with ethanol added. Centrifuge for 15 seconds at 8000 x g. Discard flow through.

4. Add 500 µL 80% ethanol to spin column and centrifuge for 2 min @ 8000 x g. Place column on 2 mL collection tube.

5. Open lid of spin column and centrifuge at full speed for 5 minutes. Discard collection tube.

6. Place column on new 1.5 mL collection tube. Add 12 µL RNase free water and spin for 1 minute at full speed. Add 5 µL collected volume back onto the column and re-spin. Store eluted volume at -80 °C until usage.

APPENDIX VIII: DNA Library Preparation

Part I. RNA Fragmentation:

1. For each sample take 8-10 µL of ribodepleted RNA and add 1 µL 10x Rnase reaction buffer and 1 µL Rnase III. Flick the tube or pipette up and down to mix and then centrifuge briefly.

2. Incubate reaction for 3 minutes at 37 °C. Immediately following incubation add 20 µL of nuclease free water and place reaction on ice.

Part II. Purify Fragmented RNA

1. Prepare magnetic beads by vortexing in order to resuspend the beads. Add 5 µL beads to a tube and add 90 µL binding solution concentrate to each well. Pipette up and down 10 times to mix.

2. Transfer fragmented RNA to tube with beads on magnetic stand. Add 150 µL of 100% ethanol to each tube and pipette up and down 10 times. Incubate for 5 min at room temperature off the magnetic stand.

3. Place tube on stand for five to six minutes or until the supernatant clears. Then wash beads with 150 µL wash solution and incubate for 30 seconds. Remove the supernatant. Allow beads to dry for 1-2 minutes.

4. Elute RNA by adding 12 µL pre-warmed nuclease free water to each sample off the stand. Pipette up and down ten times to mix.

5. Place tube on stand for one minute and allow the solution to clear. Remove supernatant and evaluate RNA quality and size of fragment distribution using the Agilent Bioanalyzer and RNA pico kit.

Part III. Library Preparations:

1. Prepare hybridization mixture:
   Ion adaptor mix V2: 2 µL
   Hybridization solution: 3 µL

2. Add 5 µL of hybridization mixture to 3µL of fragmented RNA sample. If RNA input is low use all of the fragmented RNA

3. Pipette up and down ten times to mix and place in thermocycler for the following:

   68 °C for 10 min
   30 °C for 5 min

4. On ice add RNA hybridization mixture to 2 µL of hybridization enzyme mix and 10 µL ligation buffer. Pipette up and down five times to mix.

5. Add ligation reaction to the thermal cycler at 30°C for 1 hour.

6. Perform reverse transcription by preparing master mix for samples as follows:

   Nuclease free water: 2 µL
   10x RT buffer: 4 µL
   dNTP mixture: 2 µL
   Ion RT primer v2: 8 µL

7. Add 16 µL master mix to ligation mixture and vortex well. Incubate at 70°C for ten minutes and then snap cool on ice.

8. Add 4 µL 10x superscript III, vortex and incubate at 42°C with heated lid for 30 minutes. Return to Part II and use this portion of the protocol to purify the cDNA.

Part IV: Amplify the cDNA

1. Prepare master mix:

   45 µL PCR super mix
   1 µL Ion 3' PCR mix

2. Transfer 6 µL of cDNA samples to new PCR tube and add 47 µL PCR master mix.

3. Add 1 µL Ion torrent barcode to each sample and pipette up and down five times to mix.

4. Preform PCR using the following conditions:

   Hold: 94°for 2 min

   2 cycles: 94°C for 30 sec
   50 °C for 30 sec
   68 °C for 30 sec

   16 cycles: 94 °C for 30 sec
   62°C for 30 sec
   69°C for 30 sec

Hold: 68°C for 5 min

5. Return to Part II and perform another purification step.

6. Evaluate the yield and size of cDNA produced using an Agilent Bioanalyzer and High Sensitivity DNA kit

*If less that 50% of the cDNA sample is between 50-160 bp in length then samples can be pooled and move forward with additional sequencing preparations. If not additional purification steps may be needed.

APPENDIX IX: EdgeR Data

Table 1. Top 20 differentially expressed genes for OVER vs CON lambs as determined by mapping to the *Bos taurus* reference.

GENE	FOLD CHANGE	P VALUE
SOCS3	-5.64	1.95E-05
SNORD113	-3.69	4.71E-05
TPT1	3.48	6.81E-05
AKAP12	-1.46	0.0003
ANXA7	1.56	0.0004
ARRDC2	3.44	0.0004
GFPT2	-1.77	0.0005
SLC38A1	-1.75	0.0008
DDIT4	2.48	0.0016
THBD	-1.99	0.0020
SEC22C	1.98	0.0024
SLC7A1	-1.75	0.0024
MIDN	-1.89	0.0027
ANKRD1	-3.16	0.0035
PLEKHO2	-1.47	0.0035
RGS16	-2.02	0.0039
BET1	1.36	0.0046
ARID5B	2.92	0.0048
U1	-2.14	0.0048

Suppressor of cytokine signaling 3 (SOCS3), Small nucleolar RNA SNORD113/SNORD114 family, Translationally-controlled tumor protein, A kinase (PRKA) anchor protein 12,Annexin A7 (ANXA7), Arrestin domain containing 2 (ARRDC2), Glutamine-fructose-6-phosphate transaminase 2 (GFPT2), Solute Carrier Family 38, Member 11 (SLC38A1) DNA-damage-inducible transcript 4 (DDIT4), Thrombomodulin (THBD), SEC22 vesicle trafficking protein homolog C (SEC22C), Solute carrier family 7 (cationic amino acid transporter, y+ system), member 1 (SLC7A1), Midnolin Ankyrin repeat domain 1 (cardiac muscle) (ANKRD1),Pleckstrin homology domain containing, family O member 2 (PLEKHO2),Regulator of G-protein signaling 16 (RGS16), Blocked early in transport 1 homolog (BET1), AT rich interactive domain 5B (MRF1-like), U1 spliceosomal RNA

Table 2. Top 20 differentially expressed genes for RES vs CON lambs as determined by mapping to the *Bos taurus* reference.

GENE	FOLD CHANGE	P VALUE
TPT1	3.65	3.24E-05
SNORD62	2.99	0.0003
MYL3	-1.44	0.003
SNORD113	-2.66	0.004
ENTHD2	-1.46	0.006
DUSP10	-0.95	0.007
PPM1J	-1.19	0.008
FABP4	1.49	0.009
ACTN1	1.02	0.010
CPXM	-1.27	0.011
CRTAC1	-1.09	0.012
SOCS3	-2.78	0.012
ANXA7	1.08	0.014
RBM41	1.05	0.015
BMPR1B	1.46	0.015
BET1	1.10	0.021
U1	-1.69	0.021
METTL7A	1.32	0.022
FOS	-1.62	0.022
CSRP3	-1.14	0.023

Translationally-controlled tumor protein, Small nucleolar RNA SNORD62, Myosin, light chain 3, alkali; ventricular, skeletal, slow (MYL3), Small nucleolar RNA SNORD113/SNORD114 family, ENTH domain containing 2, Dual specificity phosphatase 10 (DUSP10), Protein phosphatase, Mg2+/Mn2+ dependent, 1J, Fatty acid binding protein 4, adipocyte (FABP4), Actinin, alpha 1 (ACTN1), Carboxypeptidase X (M14 family), member 1 (CPXM1),Cartilage acidic protein 1,Suppressor of cytokine signaling 3 (SOCS3), Annexin A7 (ANXA7),RNA binding motif protein 41,Bone morphogenetic protein receptor, type IB (BMPR1B),Blocked early in transport 1 homolog (BET1),U1 spliceosomal RNA, Methyltransferase like 7A (METTL7A),FBJ murine osteosarcoma viral oncogene homolog (FOS),Cysteine and glycine-rich protein 3 (cardiac LIM protein) (CSRP3)

Table 3. Top 20 differentially expressed genes for OVER vs RES lambs as determined by mapping to the *Bos Taurus* reference.

GENE	FOLD CHANGE	P VALUE
ARID5B	4.57	4.50E-05
ARRDC2	3.84	0.0001
ACTN1	-1.59	0.0001
CNN1	-2.08	0.0002
TRIM63	3.49	0.0003
YPEL3	2.77	0.0003
SESN1	2.95	0.0004
KLHL38	2.78	0.0004
EPHA2	-1.80	0.0005
AKAP12	-1.37	0.0006
U1	-3.31	0.0007
FBXO32	3.69	0.0011
MICALL1	-1.90	0.0012
PFKFB3	3.22	0.0012
CTGF	-1.29	0.0013
GADD45G	2.54	0.0016
SNORA11	-2.24	0.0024
SNORD38	1.97	0.0025
CPT1A	-1.33	0.0025

AT rich interactive domain 5B (MRF1-like),Arrestin domain containing 2 (ARRDC2), Actinin, alpha 1 (ACTN1), Calponin 1, basic, smooth muscle (CNN1), Tripartite motif containing 63 (TRIM63), Yippee-like 3 (YPEL3), Kelch-like family member 38,EPH receptor A2, A kinase (PRKA) anchor protein 12, U1 spliceosomal RNA, F-box protein 32 (FBXO32), MICAL-like 1 (MICALL1), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), Connective tissue growth factor (CTGF), Growth arrest and DNA-damage-inducible, gamma (GADD45G), Small nucleolar RNA SNORA11, Small nucleolar RNA SNORD38

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