5-5-2018

The Effects of Maternal Diet and Energy Demands on Offspring Development, Growth, and Health

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B.S., University of Connecticut, 2016

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

at the

University of Connecticut

2018

APPROVAL PAGE

Master of Science Thesis

The Effects of Maternal Diet and Energy Demands on Offspring Development, Growth, and Health

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DEDICATION

This thesis is dedicated to my fiancé Colin Gauvin, my sister Anna, and my family, both immediate, extended, and future in-laws.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my major advisor, Dr. Kristen Govoni, for her support, guidance, and knowledge throughout my entire undergraduate and graduate career, including our time spent together in WiMSE. I would also like to thank my associate advisors, Dr. Steven Zinn and Dr. Sarah Reed, for their time and guidance throughout this process; I would not be in graduate school if it were not for Dr. Zinn. Furthermore, I want to give a special thanks to associate advisor, Dr. Maria Hoffman, for her never-ending knowledge, patience, and camaraderie. You will forever be Govoni Lab mom.

To my fellow graduate students (present and previous), Brandon Smith, Arielle Halpern, Dominique Martin, Dr. Amanda Jones, Dr. Sambhu Pillai, and Stephanie Brown, and the rest of the Animal Science graduate students, thank you for your support, friendship, and listening to practice seminars time and time again.

To undergraduate students, Veronica Pleasant, Randi Szabo, Alexandra Cabra, Lauren Engels, and Nikki Harley, thank you for your assistance and enthusiasm over the years.

To my family, both immediate and extended, thank you for your continuous support and encouragement throughout this entire process.

And finally, thank you to my fiancé, Colin Gauvin, who believed in me, challenged me to do my best, and supported me every step of the way.

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ABSTRACT

Maternal programming can have numerous detrimental effects on offspring development, growth, and health. To improve livestock production efficiency, it is necessary to determine the mechanisms that can result in reduced growth, health, and product quality. We hypothesized that 1) restricted- and over-feeding during gestation would inhibit offspring muscle development, and 2) high maternal milk production would affect factors involved in offspring growth and immunity. To investigate the effects of poor maternal nutrition on offspring muscle development and growth, pregnant Western White-faced ewes (n = 82) were randomly assigned to one of three diets at day 30 of gestation through parturition; a control- (100% NRC requirements; CON), restricted- (60% NRC; RES), or over-fed (140%; OVER) diet. At day 45, 90, and 135 of gestation and within 24 hours of birth, ewes and fetuses were euthanized for collection of offspring longissimus dorsi, semitendinosus, and triceps brachii (n = 10 to 15 fetuses per treatment per time point). Muscle sections were immunostained with Pax7 antibody and data were analyzed using PROC MIXED in SAS. An interaction of maternal diet by time point was observed in the semitendinosus (ST) and triceps brachii (TB), where Pax7(+) cells were decreased in RES offspring at day 45, 90, and 135 in the ST ($P \leq 0.02$) and day 90 in the TB ($P \leq 0.002$). Within OVER offspring, Pax7(+) cells were decreased at day 90 in the ST ($P \leq 0.02$) and day 45 and 90 in the TB ($P \leq 0.04$). No effect of maternal diet by time point was observed in the LD ($P = 0.57$). An interaction of maternal diet by litter size was also observed, where singletons and twins had reduced Pax7(+) cells in the ST and TB ($P \leq 0.02$) but triplets had an increased percentage of Pax7(+) cells in the LD ($P \leq 0.04$). In conclusion, maternal restricted- and over-feeding negatively affects prenatal muscle development over time but these effects are muscle specific. To investigate the effects of maternal milk production during gestation on calf growth

and immunity, morphometric measurements and blood samples were obtained from dairy calves born to high (average lactation of 14,865 kg of milk; n = 17) or low (average lactation of 10,069 kg of milk; n = 18) milk producing cows within 24 hours of birth. Offspring will be referred to as HIGH or LOW, respectively. Blood samples were analyzed to determine concentrations of insulin, glucose, haptoglobin, interferon gamma, and other biochemical factors relating to growth and immunity. An effect of maternal lactation by gender was observed for skull width ($P = 0.09$), nose-occipital length ($P = 0.03$), glucose ($P = 0.05$), direct bilirubin ($P = 0.07$), calcium ($P = 0.08$), and magnesium ($P = 0.07$). Additionally, an effect of lactation was observed for total protein and globulin ($P \leq 0.01$). No effect of lactation by gender, lactation, or gender was observed for insulin ($P = 0.53$), serum IgG ($P = 0.23$), colostrum IgG ($P = 32$), or haptoglobin ($P = 0.24$). In conclusion, maternal lactation status can affect several factors relating to offspring growth and health. Future studies are required to further assess the effects of maternal programming on offspring development, growth, and health to improve current livestock production.

INTRODUCTION

Livestock production is an integral part of the global economy and comprises 40% of the worldwide agriculture industry (FAO, 2017). Within the United States, meat and dairy products are key sources of protein for human consumption, contributing to 70% of total protein intake (Daniel, 2011; National Dairy Council, 2015). Reinforced by major technological advances over the past several decades, livestock production is one of the fastest growing sectors in agricultural production and sustains nearly 1.3 billion people (FAO, 2017). However, it has been reported that the human population will increase by 2.3 billion by the year 2050 (United Nations, 2015). Despite the surge in production efficiency, this raises concerns within the area of food security as this population increase is expected to result in a global food shortage (United Nations, 2015). Consequently, livestock producers must alter current production methods to ensure an ample and sustainable food supply through refining production efficiency and improving animal health.

There are many factors, including climate, land quality, and disease, that can negatively influence livestock growth and health, and subsequently their productivity. One such factor that can have a major impact on animal production is maternal programming, which can be defined as changes to the inter- or intra-uterine environment that alter fetal growth and development (Wu et al., 2006). This occurrence has been shown to negatively affect a variety of offspring tissues, organs, and cell lineages both pre- and post-natally (Limesand et al., 2006; Wu et al., 2006; Nathanielsz et al., 2007; Zhu et al., 2008; Long et al., 2014; Reed et al., 2014; Hoffman et al., 2016; Pillai et al., 2016a). Despite accumulating phenotypic and molecular evidence supporting the role of maternal programming in livestock production, additional research is needed to fully understand the negative consequences of programming to better improve livestock growth and health in the future.

REVIEW OF LITERATURE

I. MATERNAL PROGRAMMING

Maternal programming is defined as changes to the maternal environment during critical periods of fetal development that have the ability to alter the trajectory of offspring growth and development (Nathanielsz et al., 2007). While in utero, the major milestones of fetal development, including organogenesis, are met (Du et al., 2017). These critical periods of development set the course for long-term growth in mammalian livestock, and ultimately influence production efficiency and product quality (Du et al., 2017). Accordingly, negative insults to the maternal environment during gestation can result in alterations to key production traits, including growth, body composition, and health (Hoffman et al., 2017). In addition to negative effects initiated by maternal programming having the ability to persist into post-natal life, there is evidence that these effects can exist across multiple generations (Nathanielsz et al., 2007; Shasa et al., 2015). Specifically, maternal programming can result from genomic imprinting, where the maternal environment influences fetal gene expression, particularly through epigenetic modifications (Wu et al., 2006). These alterations provide the basic molecular mechanisms for the impact of maternal programming on poor postnatal development and growth, and subsequent susceptibility to disease (Wu et al., 2006).

Hales and Barker (1992) hypothesized that maternal programming is the true origin of adult diseases, specifically the development of metabolic disorders. To support this theory, they proposed the thrifty phenotype hypothesis which states that fetal adaptations to a negative uterine environment during the periods of critical development ensures survival in the environment once they are born (Godfrey and Barker, 2001; Hales and Barker, 2001). However, while these adaptations can be advantageous in the short-term, they have the potential to critically affect

long-term survivability and overall health (Hales and Barker, 2001). A poor prenatal maternal environment predicts the environment in which the fetus will be born and thus, the offspring development and growth is programmed to adapt to survival under suboptimal postnatal conditions (Hales and Barker, 2001).

There are several factors that can cause maternal programming in production settings, which include but are not limited to, stress, disease exposure, poor maternal nutritional intake, and maternal energy demands during gestation (Wu et al., 2006). For example, grazing animals in tropical or subtropical climates can experience heat stress and as a result, reduced feed intake (Reynolds et al., 2005; Wallace et al., 2005). During gestation, decreased nutrient flow to the fetus can negatively impact growth rates, organ development, and affect metabolic function through intrauterine growth restriction (IUGR; Limesand et al., 2005; Boddicker et al., 2014). Additionally, maternal stress during gestation can result in preterm delivery, which is associated with a greater risk for neonatal mortality and morbidity in livestock animals (Vonnahme et al., 2013). The effects of maternal disease exposure during gestation on offspring development have also been well documented. Prenatal viral infections, especially at the earlier stages of gestation, can result in congenital defects or pregnancy loss (Merek Veterinary Manual, 2018). For example, pregnant ewes with Cache Valley virus may produce lambs with microcephaly, scoliosis, or muscular hypoplasia (Merek Veterinary Manual, 2018). While each of these factors can have detrimental effects on offspring growth and survivability, the role of maternal nutrient intake and energy demands during gestation have been of recent research interest due to their major impact on efficient livestock production and the frequency with which these factors occur in production settings (Du et al., 2017).

II. POOR MATERNAL NUTRITION DURING GESTATION

There is considerable evidence demonstrating that maternal nutrition affects offspring growth and development while in utero. Poor maternal nutrition can be caused by a suboptimal dietary intake during gestation, where total nutrient intake is restricted or consumed in excess (Morrison et al., 2016). This can be further defined as either restricted or excessive macro- or micro-nutrient intake during fetal development and growth (Hoffman et al., 2017). Numerous studies have determined that an inadequate intake of nutrients by the dam during the critical periods of development and growth can have profound effects on offspring tissue and body composition. For example, the restricted or increased consumption of macronutrients such as protein or lipids can result in reduced offspring growth and reduced lean body mass (Jansson et al., 2006; Desai et al., 2014). Furthermore, increased intake of these macronutrients has been found to decrease offspring muscle mass and increase adiposity, which can lead to reduced meat product quality in livestock (Long et al., 2015). While many studies have concentrated on the effects of changes to total or macronutrient intake on offspring development, evidence that proper micronutrient intake during pregnancy is vital for optimal fetal growth has also been provided (Vonnahme et al., 2013; Grieger and Clifton, 2015; Morrison et al., 2016). For example, supplementing restricted-fed ewes with selenium during gestation has been found to decrease elevated cortisol concentrations in lambs, which is critical for proper organ development (Vonnahme et al., 2013). Correlations between the impact of zinc, calcium, folate, and vitamin D supplementation and deficiencies during gestation and altered fetal development and growth have also been established (Grieger and Clifton, 2015). For example, supplementation of calcium in conjunction with vitamin D increases fetal femur and humerus growth in pregnant women (Young et al., 2012). Additionally, zinc deficiencies influence

embryonic and fetal development through teratogenic mechanisms, whereby offspring born to zinc-deficient mothers are born with skeletal defects, growth retardation, and brain abnormalities (Hurley and Swenerton, 1966). Folate deficiency has also long been understood to increase the risk for neural tube defects in offspring (Greenberg et al., 2011). These findings illustrate the importance of maternal nutrition during gestation on offspring development and growth, including both macro- and micro-nutrient intake. Maternal nutrition is of particular interest because the development of the offspring is closely associated with the maternal plane of nutrition, as all of the nutrients which the fetus receives are from maternal origins (Du et al., 2017). However, the severity of the impact of poor maternal nutrition upon offspring growth and development is dependent upon several factors, including the duration, type of nutrients being altered, and the timing of maternal nutritional insult (Wu et al., 2006; Du et al., 2010; Reed et al., 2014; Hoffman et al., 2014, 2017)

While there are numerous instances in which livestock can experience inadequate nutrition during gestation, two common instances of poor maternal nutrition in production operations are under- and over-feeding (Wu et al., 2006; Du et al., 2017). Animals raised on forage, particularly sheep and cattle, can be subjected to poor forage quantity and quality depending on the season and geographical locations. For example, it has been reported that grazing ewes without additional supplementation consume 50% less nutrients than the National Research Council (NRC) recommendations (Thomas and Kott, 1995). Furthermore, Fontaneli et al. (2005) reported that the sheer size of extensive production systems makes it unlikely for supplementation to be provided to grazing ruminants. Due to the seasonality of sheep and cattle reproduction, this opportunity for inadequate forage intake coincides with pregnancy and could result in poor maternal nutrition and detrimental offspring programming (Du et al., 2017). In

comparison, over-nutrition occurs more frequently in closely managed production settings, where practices such as flushing are utilized and animals are more likely to receive ad libitum feed (Hoffman et al., 2017). Grazing animals can further experience over-nutrition if fed on rich pastures (Hoffman et al., 2017). Therefore, there are various instances in which production species may be exposed to states of poor maternal nutrition and subsequently impact offspring development and growth.

From the period of oocyte maturation until parturition, offspring are susceptible to the effects of maternal nutrition (Wu et al., 2006). During embryonic development, placental structures are established and organogenesis is initiated. Therefore, inadequate nutrients consumed by the dam during this period can drastically affect the ability of these structures to develop properly (Wu et al., 2006). As embryonic development transitions into fetal development, organs begin to rapidly grow, with maximum growth occurring during the last six weeks of gestation (Wu et al., 2006). A maternal nutritional insult during fetal developmental can affect optimal late term prenatal growth, which will accordingly limit desired production growth, tissue accretion, and metabolism postnatally (Wu et al., 2004, 2006). The following sections will describe the specific effects of under- and over-nutrition on offspring growth and development.

Offspring Body Weight and Growth

Alterations to prenatal growth are known to impact offspring birth weight (BW) and can affect the average daily gain (ADG) of production animals in postnatal life, despite variable results from individual studies (Ford et al., 2007; Hoffman et al., 2014, 2016; Van Emon et al., 2015). Maternal nutrient-restriction is often associated with reduced BW while maternal over-nutrition is associated with increased BW (Hoffman et al., 2014, 2016). Despite contrasting

results, both reduced and increased BW can increase the probabilities of postnatal mortality, demonstrating that offspring BW is a predictor of overall health and survivability (Dwyer et al., 2016; Hoffman et al., 2017). Interestingly, poor maternal nutrition does not always affect birth weight (Ford et al., 2007; Hoffman et al., 2014; Van Emon et al., 2016; Hoffman et al., 2016). However, this variability between studies could be due to differences in experimental design and the use of dissimilar animal and nutritional models (Hoffman et al., 2017).

These same studies have also found inconsistency in the effects of poor maternal nutrition on ADG, with maternal nutrient restriction resulting in decreased or no change in offspring ADG, and maternal over-nutrition resulting in either decreased or increased ADG (Van Emon et al., 2015; Hoffman et al., 2016). However, similar to the differences seen in offspring BW, these variable results could be due to differences in the overall study design (Hoffman et al., 2017). Additionally, because eutherian animals depend greatly on the rates and efficiency of metabolic transformations of nutrients for optimal growth performance, poor maternal nutrition can reduce offspring feed efficiency and negatively alter meat quality later in life (Wu et al., 2006). Therefore, for production animals to grow efficiently and at a desired rate of gain, it is essential for there to be an adequate maternal nutritional intake during gestation.

Offspring Stem Cell Function, Hormones, and Gene Expression

In addition to maternal nutrition during gestation affecting overall body growth and composition of offspring, stem cell function, hormonal regulation, and gene expression can also be affected (Wu et al., 2006; Ford and Long, 2011; Reed et al., 2014; Pillai et al., 2016a). For example, Pillai et al. (2016a) recently demonstrated that maternal diet during gestation reduces the proliferation and metabolism of mesenchymal stem cells (MCSs) postnatally. Mesenchymal stem cells contribute to the development of bone, adipose, and skeletal muscle tissue, and a

reduction in the ability of these cells to proliferate could lead to a reduced stem cell pool, and subsequently affect the differentiation and maintenance of these critical tissues (Pillai et al., 2016a). These findings are in agreement with previous reports, where rats fed a low-protein diet during gestation resulted in offspring with MSC proliferation and differentiation into osteoblasts (Oreffo et al., 2003). Additionally, poor maternal nutrition can alter hormones and growth factors involved in metabolic regulation and fetal growth (Hoffman et al., 2016). For example, several studies have demonstrated the effects of poor maternal nutrition on key factors involved in metabolism including increased circulating insulin concentrations, increased insulin:glucose ratio, and reduced leptin concentrations (Long et al., 2011; Wu et al., 2012; Hoffman et al., 2016). Maternal nutrition has also been found to affect the somatotropic axis, which has a critical role in overall growth (LeRoith, 2001; Hoffman et al., 2014). For example, maternal restricted- and over-feeding during gestation reduces serum insulin-like growth factor-1 (IGF-1) and associated binding proteins, which are directly related to early postnatal growth (Hoffman et al., 2014). Additionally, poor maternal nutrition can affect factors relating to the regulation of the hypothalamic-pituitary-adrenal axis, where nutrient restriction results in reduced neonatal survival due to a premature fetal cortisol surge (Wu et al., 2012). Wu et al. (2012) further demonstrated that maternal over-nutrition causes hyperglycemia, hyperinsulinemia, and insulin resistance in offspring. These findings provide evidence that inappropriate maternal nutrition during gestation may lead to altered offspring metabolic status and poor neonatal growth, which could potentially increase the risk for metabolic diseases later in life (Hoffman et al., 2017).

Variations in gene expression relating to these processes provide a potential mechanism contributing to altered development and growth in offspring (Hoffman et al, 2017). Poor maternal nutrition through over-feeding has been found to increase the myostatin gene, which

acts by inhibiting muscle growth (Reed et al., 2014). Genes relating to skeletal muscle nutrient uptake, fatty acid oxidation, protein accretion, triglyceride accumulation, and adipogenesis have also been found to be affected by over nutrition (Hoffman et al., 2016). Alterations in the expression of these genes could result in increased intramuscular adipose, and impaired tissue growth. Furthermore, over-feeding during gestation can alter micro- and small-RNAs in offspring, which are forms of epigenetic modifications that regulate transcript availability (Hoffman et al., 2016). Moisá et al. (2016) reports that restricted-feeing during gestation results in an increased abundance of microRNAs supporting adipogenesis in skeletal muscle, which could have negative consequences on product quality. Thus, altered gene expression offers a mechanism for the phenotypic deviations observed after inadequate nutrition is consumed during gestation.

Offspring Bone, Adipose, and Muscle

Poor maternal nutrition can affect development and growth of bone, adipose, and skeletal muscle, which are key tissues in livestock production and health (Mehta et al., 2002; Kleemann et al., 2015; Sasha et al., 2015; Pillai et al., 2016a; Hoffman et al., 2017). Bone is critical for protecting soft tissues and organs, maintaining mineral balance, and provides a structural scaffolding for the body (Hoffman et al., 2017). This tissue also has an important role in hematopoiesis, thus making proper bone development essential for optimal offspring growth and health (Floencio-Silva et al., 2015). However, due to bone receiving a greater nutritional priority over adipose and muscle, the effects of poor maternal nutrition on this tissue are not as apparent during prenatal development (Ford et al., 2007). Nevertheless, it has been shown that maternal nutrient restriction can result in reduced bone weight, length, and density, while maternal over-

nutrition can impair skeletal development (Tygesen et al., 2007; Lanham et al., 2010; Kleeman et al., 2015).

In contrast to bone, adipose tissue and skeletal muscle are the most susceptible tissues to inadequate maternal nutrition (Du et al., 2017). Adipose tissue is desirable in production animals in finite amounts, specifically because intramuscular adipose, or marbling, affects overall meat quality. Additionally, adipose is an important tissue to consider due to its role in energy metabolism. It has been demonstrated that livestock animals subjected to both maternal restricted- and over-nutrition can produce offspring with increased adiposity (Ford et al., 2007, 2009; Reed et al., 2014; Long et al., 2015; Sasha et al., 2015; Hoffman et al., 2017). This includes increased adipose deposition in or surrounding critical organs and increased subcutaneous adipose and intramuscular fat (Long et al., 2015; Hoffman et al., 2017). These changes in adiposity have concerning implications for animal health and production. Due to its metabolic role, increased adipose can affect animal growth rates, metabolic status, and can reduce meat quality and, therefore, reduced production efficiency. However, studies have also reported that poor maternal nutrition results in decreased adiposity (Reed et al., 2014). Recently, we showed that maternal nutrient-restriction during gestation results in increased intramuscular fat at birth, but is reduced by three months of age (Reed et al., 2014). As with increased adiposity, this can also be undesirable because specific amounts of intramuscular lipid accumulation are necessary for adequate marbling during the fattening stage, which improves the tenderness of meat (Du et al., 2017). Furthermore, similar studies suggest that when animals reach adulthood, adiposity tends to increase due to altered metabolic status and compensatory growth (Hoffman et al., 2017).

While the proper development and growth of bone and adipose tissues are important in livestock production, the effects of poor maternal nutrition on skeletal muscle are of particular interest due to the metabolic and economic importance of this tissue. Skeletal muscle is crucial for the processes of glucose and fatty acid oxidation (Zhu et al., 2008) and is very sensitive to insulin-mediated glucose uptake (Brown, 2014). When the nutritional status of a fetus is altered, nutrients are partitioned away from skeletal muscle in favor of more critical organs such as the brain and heart (Hales and Barker, 1992). However, postnatal skeletal muscle serves as an important amino acid reservoir for key organs during periods of stress or reduced nutrient intake, thereby making proper prenatal muscle development essential (Wolfe, 2006). Alterations in muscle mass, connective tissue content, and the deposition of intramuscular adipose are all potential consequences of poor maternal nutrient intake and can alter offspring meat and carcass quantity and quality, thereby resulting in overall reduced production efficiency (Daniel et al., 2007; Reed et al., 2014). The following sections will discuss the importance of muscle, the process of muscle development, or myogenesis, and its regulation.

III. MUSCLE DEVELOPMENT AND GROWTH

Muscle Function and Structure

Skeletal muscle comprises the most abundant tissue in the body of vertebrates, contributing to approximately 50% of total body weight, and has a critical role in locomotion and heat generation (Biressi et al., 2007). This tissue is heterogeneous in nature, being composed of distinct contractile units, or fibers, which depend on different metabolic substrates for action (Schiaffino, 2011). The myofiber is the cellular unit of adult skeletal muscle, and comprised of

hundreds of post-mitotic nuclei (Zammit et al, 2006). Muscle fibers can be classified as either type I or type II based on their speed of contraction and are further distinguished from each other based on their need for different energy substrates (Biressi et al., 2007). Specifically, type I fibers, or slow-twitch fibers, are mitochondria dense and rely on oxidative metabolism for contraction (Brown, 2014). These fibers are slower to reach peak tension but are relatively fatigue-resistant and thus necessary for endurance activities. Type II fibers, or fast-twitch fibers, can be divided into two subcategories; type IIa, which can function on both oxidative and glycolytic metabolism, and type IIb, which rely solely upon glycolytic metabolism (Brown, 2014). These fibers reach peak tension quicker than type I, but type IIb are easily fatigued due to the decreased concentration of mitochondria and are therefore dependent upon glycolytic metabolism for ATP synthesis (Brown, 2014). However, fiber types are not permanently fixed throughout postnatal life. Muscle fibers show significant plasticity and have the ability to adapt to altered metabolic states, thereby altering fiber type composition, which can have an effect upon meat palatability and quality (Maltin, 2008; Daniel et al., 2007; Schiaffino et al., 2013). The following sections will discuss the phases of myogenesis and its regulation.

Prenatal Myogenesis

Muscle development is a complex process that occurs in several distinct phases and requires expression of several paired box (Pax) and myogenic regulatory factors (MRFs) that act in concert with each other to regulate each step. The MRF family includes Myogenic Differentiation 1 (MyoD), Myogenic Factor 5 (Myf5), myogenin, and MRF4, all of which are basic helix-loop-helix transcription factors. During embryonic development, the embryo undergoes somitogenesis, which involves the formation and differentiation of somites from the mesoderm (Aoyama and Asamoto, 1988). This process is initiated by nearby signals, including

Wnts and Sonic Hedgehog proteins, which arise from the neural tube, notochord, and ectoderm (Cossu et al., 2000). Somites arise specifically from the paraxial mesoderm and progress in a cranio-caudal pattern through segmentation of the mesoderm on either side of the neural tube to form the dermomyotome and sclerotome (Christ and Ordahl, 1995). Under the influence of signals from surrounding tissues, including the expression of the Pax genes, somites of the dermomyotome become compartmentalized and regionally specified to give rise to diverse myogenic structures later in development (Christ and Ordahl, 1995). Myogenic progenitor cells originate from the newly differentiated dermomyotome and will eventually differentiate into mononucleated muscle cells, or myocytes (Biressi et al., 2007).

Proliferating myogenic progenitor cells co-express transcription factors Pax3/7 (Biressi et al., 2007). Paired Box 7, in particular, is an early marker of myogenic progenitor cells and is involved in maintaining these cells before differentiation into the myogenic lineage (Zammit et al., 2006). This transcription factor also has a role in upregulating the expression of transcription factors MyoD and Myf5, both of which are required for myoblast proliferation and differentiation (Seale et al., 2000; Zammit et al., 2006; Yablonka-Reuveni et al., 2008). Myogenic Differentiation 1 and Myf5 are further activated by the signaling of Wnt and Shh proteins (Cossu et al., 1999). Before differentiation, the downregulation of Pax7 is required for further progression through myogenesis (Zammit et al., 2006; Yablonka-Reuveni et al., 2008). Myoblasts exit the cell cycle and fuse together to form multinucleated primary myofibers during early gestation, a process mediated by transcription factors myogenin and MRF4 (Yablonka-Reuveni et al., 1995). This formation of primary myofibers from embryonic myoblasts is termed primary myogenesis, and occurs at approximately 32 to 38 days of gestation in sheep (Maltin,

2008). Due to the tight regulation of the myogenic regulatory factors, any disruption in the pattern or timing of their expression can adversely affect muscle development and growth.

As prenatal development transitions from embryonic to fetal development, a second phase of myogenesis occurs. In sheep, secondary myogenesis begins at day 62 of gestation (Maltin, 2008). During this period of muscle development, fetal myoblasts undergo differentiation as described previously and fuse together to form numerous, relatively smaller secondary fibers (Biressi et al., 2007). These smaller fibers form at the site of innervation of the existing primary fibers, which allows them to be encapsulated by the same basal lamina surrounding the primary fiber (Duxson et al., 1995). After formation, secondary fibers form around the primary fibers, which act as a pre-existing scaffolding for secondary fiber formation (Rhodes and Konieczny, 1989; Biressi et al., 2007). Primary and secondary fibers can be further distinguished from each other by their expression of myosin heavy chain (MyHC) isoforms, where primary fibers contain both embryonic (fast) and I/β (slow) MyHC but secondary fibers do not express I/ β MyHC.

A third phase of myogenesis, or tertiary myogenesis, can occur following the formation of primary and secondary fibers (Mascarello et al., 1992). This phase involves the formation of tertiary fibers, which differ from the other classes of fibers based on their myosin composition (Mascarello et al., 1992). While primary and secondary fibers contain greater quantities of slow myosin heavy chain (MHC), tertiary fibers contain fast MHC, and often embryonic MHC (Mascarello et al., 1992). Tertiary fibers fuse to secondary fibers by embryonic day 62, and add another layer of complexity to muscle development (Wilson et al., 1992).

Satellite Cell Establishment

During prenatal muscle development, a separate pool of stem cells are established during somitogenesis and embryonic myogenesis (Mauro, 1961; Zammit et al., 2006; Yablonka-Reuveni et al., 2008). Satellite cells are muscle stem cells which lie in a mitotically quiescent state between the basal lamina and sarcolemma of the muscle fiber (Zammit et al., 2006; Yin et al., 2013). Similar to myogenic progenitor cells, there is evidence that satellite cells also originate from the somite, specifically the dermomyotome (Gros et al., 2005; Schienda et al., 2006; Zammit et al., 2008). During the breakdown of the dermomyotome, myogenic progenitor cells continue to proliferate to establish the progenitor pool (Yin et al., 2013). A subset of these proliferating progenitor cells expressing Pax3/7 persist into late fetal development and ultimately become enveloped beneath the basal lamina of development myofibers, where they adopt a satellite position (Gros et al., 2005; Relaix et al., 2006; Yin et al., 2013). While in a state of quiescence, these cells continue to express transcription factors Pax3/7 (Schiaffino et al., 2013). Paired Box 7 has a key role in maintaining this population of stem cells postnatally, and upon activation, MyoD expression is rapidly upregulated for these cells to differentiate into myocytes (Yablonka-Reuveni et al., 2008). Additionally, it has been established that this transcription factor is an ideal marker of satellite cells (Zammit et al., 2008). Satellite cells expressing Pax3 are associated with the regeneration of specific skeletal muscles whereas Pax7 is ubiquitously expressed by all satellite cells (Yin et al., 2013). While satellite cells continue to express the Pax proteins, they do not express muscle-specific proteins, including transcription factors belonging to the MRF family (Zammit et al., 2008). Despite the lack of myogenic-specific protein expression, it is generally accepted that satellite cells are a committed myogenic stem cell population (Schienda et al., 2006). This population of stem cells is largest in number towards the

end of prenatal development and during early postnatal development (Mauro, 1961). As an individual ages, the number of satellite cells associated with myofibers are reduced to less than 5% of the population (Pallafacchina et al., 2013).

Postnatal Myogenesis

Myogenesis is unique in that a majority of development occurs prenatally. Specifically, net muscle fiber number does not increase after parturition, meaning that growth is highly dependent upon hypertrophy of existing fibers through protein accretion and the incorporation of satellite cells (Glore and Layman, 1983; Greenwood et al., 2000; Nissen et al., 2003; Zhu et al., 2006; Rehfeldt et al., 2011). Myofiber hypertrophy occurs when the rate of protein accretion exceeds the rate of protein degradation (Schiaffino et al., 2013). A majority of protein accretion occurs in early postnatal life, when muscles are rapidly growing (Davis and Fiorotto, 2009). As an individual ages, protein accretion rates decrease until they are balanced with degradation rates (Davis and Fiorotto, 2009). Skeletal muscle growth is positively regulated by the insulin-like growth factor 1-phosphoinositide-3-kinase-Akt/protein kinase B-mammalian target of rapamycin (IGF1-PI3K-Akt/PKB-mTOR) pathway, resulting in increased protein synthesis and negatively regulated by the myostatin-Smad3 pathway (Schiaffino et al., 2013).

Hypertrophy also occurs through the incorporation of satellite cells, which supports postnatal myogenesis (Zammit et al., 2006). These cells are activated in response to injury or during muscle cell turnover, making them crucial for maintenance of myofibers (Seale et al., 2000; Zammit et al., 2006). Satellite cells are activated by several factors, including growth factors, mitogens, and intrinsic factors such as sphingosine-1-phosphate production (Bischoff, 1986; Kästner et al., 2000; Clemmons, 2009). Satellite cells re-enter the cell cycle within 24 h of activation, differentiate into myocytes, and ultimately fuse to existing fibers under the regulation

of MRFs after migrating inward towards the myofiber requiring repair (Bischoff, 1986; Tatsumi et al., 1998; Yablonka-Reuveni, 2008). Approximately 80% of satellite cells are activated readily, which represents the responsive population (Yin et al., 2013). The remaining 20% of satellite cells, or the reserve population, enter the cell cycle at a slower rate and only become proliferative when there is the need for extensive muscle regeneration or repair (Yin et al., 2013).

While a majority of satellite cells are extremely responsive to activation and quickly differentiate into myoblasts, a subset of proliferating satellite cells will cease MyoD expression while continuing to express Pax7, withdraw from the cell cycle, and return to a state of quiescence to populate the stem cell pool (Zammit et al., 2006; Le Grand and Rudnicki, 2007). Yin et al. (2013) suggests that the reserve population, which are less responsive to activation signals, also aid in maintaining this population of quiescent satellite cells. While in quiescence, Pax7 continues to stimulate minor amounts of transcription in these cells (Zammit et al., 2006). This process is defined as self-renewal, and is critical for maintaining the repair and maintenance of skeletal muscle.

IV. IMPACT OF POOR MATERNAL NUTRITION ON MUSCLE GROWTH AND DEVELOPMENT

There are several studies demonstrating the impact of maternal nutrition on offspring muscle development and growth. Our lab and others have found that poor maternal nutrition, either through under- or over-feeding during gestation, can affect myogenesis as well as muscle composition and growth (Quigley et al., 2005; Zhu et al., 2006; Ford et al., 2007; Reed et al., 2014). Specifically, we demonstrated that offspring born to restricted-fed ewes during gestation

displayed an increase in the muscle fiber cross-sectional (CSA) at birth, but by three months of age the CSA was reduced compared with control offspring (Reed et al., 2014). Additionally, intramuscular fat was reduced in offspring born to restricted-fed mothers at three months (Reed et al., 2014). These findings provide evidence that inadequate maternal nutrition may result in undesirable muscle composition in offspring, and consequently reduced meat product quality. Additionally, this particular study demonstrated the importance of evaluating the effects of poor maternal nutrition at multiple time points. For example, changes observed in muscle at birth appear to be economically beneficial (e.g., increased muscle) but by three months of age, muscle mass is significantly reduced. Using a similar model but re-acclimating ewes to a standard diet mid-gestation, Zhu et al. (2006) reported that 50% nutrient restriction resulted in decreased myofiber number and increased type IIb isoforms in lambs at eight months of age, leading to decreased product quantity and quality. Similarly, Ford et al. (2007) demonstrated that lambs from restricted-fed ewes slaughtered at nine months post-parturition weighed significantly more compared with lambs from control-fed ewes and had reduced muscle and increased adipose tissue. These animals also displayed dysregulated glucose uptake, characteristic of humans with onset diabetes mellitus (Ford et al., 2007). Thus, maternal nutrient restriction during gestation can have a negative impact on offspring muscle development and growth, and these changes observed in neonatal offspring may persist into adulthood, thereby affecting meat production quality and efficiency.

Maternal over-feeding, or maternal obesity, during gestation can have similar consequences on offspring muscle development and growth. In the previously described study, Reed et al. (2014) demonstrated that offspring born to over-fed mothers also displayed increased myofiber CSA at birth, and a reduction in CSA at 3 months of age, indicating a reduced capacity

for postnatal growth. However, contrary to offspring born to restricted-fed mothers, intramuscular fat was increased in offspring born to over-fed mothers at both birth and three months (Reed et al., 2014). These changes in muscle composition through increased lipid accumulation suggest changes to meat quality, and consequently palatability (Hoffman et al., 2017). Others have also established that maternal obesity can result in increased collagen and the development of fibrosis, both of which can reduce meat quality and tenderness (Yan et al., 2011; Huang et al., 2012). Thus, both maternal restricted- and over-feeding during gestation can affect the development and desired composition of offspring muscle, thereby affecting meat production as a whole.

Limited studies have been conducted to investigate the negative effects of poor maternal nutrition on offspring muscle development and growth at prenatal time points in sheep. Quigley et al. (2005) demonstrated that nutrient restriction during the peri-conception period in sheep reduced total myofiber number by 20% in offspring muscle, and specifically saw a reduction in secondary myofiber formation. Costello et al. (2008) conducted a similar study in which they subjected ewes to a 50% nutrient restriction peri-implantation (days 1 to 31 of gestation) or during late gestation (days 104 to 127 of gestation). They observed reduced fetal myofiber density in both groups as well as increased concentrations of factors relating to glucose uptake. However, this could be indicative of temporary fetal adaptations to maternal diet which could influence the risk for metabolic disease later on (Hales and Barker, 2001).

It is necessary to understand the implications of poor maternal nutrient intake during gestation on offspring development and growth to improve livestock production. Specifically, the effects on muscle development are of particular interest due to its economic importance. While the effects of under- and over-nutrition postnatally have been broadly researched, the

mechanisms by which muscle is affected prenatally are still largely unknown. More specifically, it is important to determine the effects of maternal nutrient inadequacy at the critical periods of myogenesis to understand how myofiber number and composition is being affected postnatally. Considering that net myofiber number is set at birth, more research is needed to understand the direct implications of inadequate maternal nutrient intake on prenatal myogenesis and muscle growth. Previously, we demonstrated that poor maternal nutrition does not alter expression of transcription factors, including Pax7 and members of the MRF family, involved in offspring muscle development at postnatal time points (Reed et al., 2014). However, changes in satellite number may be more evident at prenatal time points, where incorporation of satellite cells into the myofiber is at its greatest (Mauro, 1961; Pallafacchina, 2013; Reed et al., 2014). Due to the tight regulatory role of myogenesis by the Pax transcription factors and members of the MRF family, alterations to their expression may explain the phenotypic changes observed in offspring muscle postnatally. As stated previously, Pax7 in particular is a key marker of progenitor cells and satellite cells and has a critical role in the upregulation of factors involved in myoblast proliferation and differentiation (Zammit et al., 2006). Therefore, this factor can be used to determine whether the number of myogenic progenitor cells and satellite cells are altered at prenatal time points, which could explain the reduction in CSA and fiber number postnatally.

V. MATERNAL NUTRIENT PARTITIONING

Maternal Energy Demands during Gestation

As previously stated, maternal diet and energy demands during gestation are of particular interest within the livestock industry as these factors can lead to unwarranted fetal programming

and ultimately a reduction in desired production characteristics in offspring. Effectual postnatal growth depends highly upon the precursor of efficiency, which is prenatal development, and fetal nutrient transfer becomes the limiting factor for determining the growth trajectory of the offspring (Redmer et al., 2004). While the effects of poor nutrient intake by the dam on offspring development and growth have been intensely evaluated, the effects of maternal energy demands on offspring are less documented in livestock species.

Homeorhesis can be defined as a coordinated redirection of resources necessary to support a new physiological steady state (Bauman and Currie, 1980). The period of gestation represents a classic homeorhetic state, where the energy derived from nutrient intake and absorption must be partitioned between the mother and developing fetus (Bauman and Currie, 1980). Partitioning of nutrients is vital for the proper development and growth of the conceptus as well as neonatal survival since all of the nutrients which the fetus receives are of maternal origins (Bauman and Currie, 1980; Wallace, 2000; Du et al., 2017).

Maternal nutrients are partitioned to various tissues and organs according to their metabolic rate during pregnancy (Redmer et al., 2004). Second only to the brain, heart, and central nervous system, the placenta and fetus receive the greatest nutrient allocation during pregnancy (Wu et al, 2006). The placental nutrient transfer capacity as well as placental size regulates the growth trajectory of the fetus and will ultimately affect birth weight and subsequent postnatal growth and development (Redmer et al., 2004). In most mammalian livestock species, the earliest and most critical stages of development, including organogenesis and placental development, occur during the very first period of gestation (Reynolds et al., 1995). Contrastingly, approximately 90% of fetal growth occurs during the final stages of pregnancy

(Redmer et al., 2004). Thus, appropriate maternal nutrient partitioning is essential from conception until parturition for optimal development and growth of production animals.

Maternal Energy Demands in Conjunction with Gestation and Lactation

In livestock species, particularly in dairy cattle, lactogenesis becomes another factor to consider that is largely dependent upon efficient nutrient partitioning. Lactogenesis, or the onset of milk secretion, generates a shift from uterine nutrient transfer to the mammary gland, which will ultimately serve as neonatal nourishment upon parturition (Bauman and Currie, 1980). For example, alterations to the rates of lipogenesis and lipolysis in maternal adipose tissue are required for milk synthesis by the mammary gland (Bauman and Currie, 1980). The nutritional requirements for lactation in dairy cows are greatly increased compared with other species due to the increased energy demand of milk production (Knight et al., 2001). Thus, lactation is a metabolically demanding process that leads to a mobilization of body energy reserves, thereby resulting in the partitioning of nutrients (González-Recio et al., 2012). However, the energy requirements of lactation must be met while the cow maintains a concurrent pregnancy, specifically in multiparous cows. Dairy cows are bred on average to calve by 24 months of age and calve approximately every 385 days thereafter to optimize production (Kamal et al., 2016). Intensive production practices dictate that an efficient cow will have a maximized peak milk yield in addition to maintaining a short calving interval (Esslemont and Kossaibati, 2000). Accordingly, much of gestation coincides with lactation in multiparous cows and subjects these dams to energetically physiological extremes. Outside of intensively-managed dairy cow production settings, lactation combined with a concurrent pregnancy would only transpire under favorable nutritional circumstances (Knight, 2001). Under poor nutritional conditions, rebreeding during the lactation period typically does not occur until later to avoid physiological extremes on

the dam (Knight, 2001). Thus, nutrient allocation between lactation and gestation in domestic dairy cattle could subject developing offspring to a nutritionally adverse environment while in utero.

In most production settings, dairy cows are typically provided with an ad libitum diet, with feed intake characterized as a dry matter intake (DMI; Merck Veterinary Manual, 2018). Factors affecting DMI include the stage of lactation, maternal body size, fermentative digestion, and the stages of gestation (Knight et al., 2001). Milk production is initiated at parturition, and peaks at approximately two months post-parturition, and then gradually declines at a rate of 2% per week thereafter (Knight, 2001; Wu et al., 2006). However, DMI peaks anywhere between two to five months post-calving, regardless of providing adequate diets during peak lactation (Wu et al., 2006). Bar-Peled et al. (1998) found that DMI is negatively impacted by increased hormonal factors associated with milk synthesis, including oxytocin. With the increase in milk output during this time coinciding with a lag in DMI, a negative nutrient balance can occur, especially with regards to energy and protein deficits, during the first two months of lactation (Bauman and Currie, 1980; Bar-Peled et al., 1998; Wu et al., 2006; Fenwick et al., 2008; González-Recio et al., 2012). Beever et al. (1998) reported that this period of energy imbalance has the ability to extend 20 weeks into the lactation cycle. This period of under-nutrition ultimately coincides with the earliest stages of pregnancy, particularly early embryonic development (Wu et al., 2006).

Additionally, DMI as a percentage of body weight is reduced during the non-lactating period preceding parturition, with voluntary feed intake decreasing by up to 35% during the last three weeks of gestation (Grummer et al., 1995). This increases the likelihood of a negative energy balance during these final stages of pregnancy, which coincides with the stage of rapid

fetal growth (Ferrell et al., 1991; Wu et al., 2006). Therefore, a state of undernutrition can often occur during late pregnancy in multiparous cows and the physiological extremes of pregnancy combined with the preceding lactation status can potentially impair offspring growth from implantation to parturition (Wu et al., 2006). Accordingly, for efficient dairy cow production operations to persist, it is necessary to determine whether the maternal lactation status during gestation has direct effects upon calf development, growth, and health.

Calf Mortality and Nutrient Partitioning

Dairy calf mortality rates are relatively high, with 5.8% of calves dying due to non-predatory causes with the main causes of mortality derived from digestive (30.6%) and respiratory issues (36.7%; USDA APHIS, 2011). This is problematic as female calves are necessary for replacements to the milking herd over time while male calves are integral to meat production. Consequently, optimal calf production relies on the ability to reduce mortality rates. Neonatal calf mortality may be due to numerous factors, but as the energy demand of lactation divides biological resources between milk production and fetal development, there could be the potential for altered nutrient availability for the developing offspring, particularly organogenesis, and thus, the predisposition of these animals to poor growth and health (Wu et al, 2006; Kamal et al., 2016).

During the lactation period, glucose and IGF-1 are negatively correlated with milk output during the production phase (Taylor et al., 2004; Ingvartsen and Friggens, 2005). Accordingly, the availability of these factors to the fetus may be negatively affected, thereby impacting proper fetal development and growth (Kamal et al., 2014). Additionally, intrauterine glucose and IGF-1 concentrations may be reduced even throughout later phases of lactation due to persistent milk output which modern dairy cows can achieve (Ingvartsen and Friggens, 2005). As previously

stated, these mid and final stages of lactation coincide with the latter stages of gestation, when a majority of fetal growth occurs and could thereby affect overall growth and calf health (Kamal et al., 2014).

There is limited research regarding the effects of maternal milk production during gestation on calf growth and health. Previously, Kamal et al. (2014) showed that increased maternal milk production during gestation in dairy cattle reduced calf growth. Specifically, calves born to high milk producing cows had reduced birth weights and increased circulating insulin concentrations, suggesting alterations to offspring growth and metabolism at early postnatal time points (Kamal et al., 2014, 2015). As previously mentioned, reduced birth weights have been associated with the development of various metabolic diseases later in life, including glucose intolerance and metabolic syndrome (Hales and Barker, 2001). Moreover, insulin acts as a key growth factor in fetal development, and decreased fetal concentrations of this hormone prenatally or in early postnatal life can lead to reduced growth (Hales and Barker, 2001). However, Kamal et al. (2015) reported that increased maternal milk production increased calf insulin concentrations, and this could be indicative of reduced insulin sensitivity. Insulin concentrations and insulin sensitivity are closely related where increased concentrations of this hormone is usually indicative of reduced insulin sensitivity in key tissues including skeletal muscle (Kamal et al., 2015). In an attempt to understand the role of nutrient partitioning on calf development and growth, Kamal et al. (2016) also evaluated the effects of maternal milk production on gross placental morphology in the same set of animals. Interestingly, no effects of maternal milk production were observed on placental growth, and they concluded that this tissue may be sustained at the expense of other tissues in an effort to reduce the negative effects on offspring development and growth. However, the limitations on this study included evaluating

only the fetal portion of the placenta and excluding the maternal portion, and within the fetal placental membranes, only the gross phenotypic morphology was ascertained.

In view of the leading causes of calf mortality and increased instances of disease in this neonatal population, determining whether maternal milk production during gestation affects passive transfer in dairy cattle should also be addressed. Passive transfer is the process by which immunoglobulins, including Immunoglobulin G (IgG), are transferred to the neonate for the purpose of immune system activation via intestinal absorption (Butler et al., 1983; Fetcher et al., 1983). Calves, among other ruminant animals, are born agammaglobulinemic and rely upon this transfer of immunoglobulins within the first 24 hours of life (Quigley, 2004). Once intestinal absorption of colostrum takes place, IgG is resecreted by the crypt cells of the intestine back into the lumen, where these factors aid in reducing the incidence of gastrointestinal diseases (Quigley, 2004). Failure of passive transfer occurs when inadequate concentrations of immunoglobulins are absorbed in the intestinal epithelia. It has been reported that one in five heifer calves experience failure of passive transfer (USDA AHPIS, 2011). Therefore, determining the factors predisposing these animals to intestinal diseases is essential to limit calf mortality and improve production efficiency. Recently, Meyer and Caton (2016) demonstrated that IUGR, initiated by placental insufficiency, reduces offspring intestinal mass, decreases enterocyte proliferation, and alters crypt depth and villus density in offspring at days 20 and 180 post-parturition. Based on these findings, it is possible that maternal milk production during gestation could have similar programming effects on the small intestine of offspring and negatively impact passive transfer of immunoglobulins. However, to our knowledge, the effects of maternal milk production on passive transfer have yet to be explored.

VI. RATIONAL AND HYPOTHESIS

In considering the evidence of maternal programming within the livestock industry and the adverse effects which programming can have on offspring development and growth, it is necessary to identify the mechanisms behind these alterations to improve overall production efficiency. Both maternal diet and energy demands during gestation have been found to negatively affect fetal and neonatal growth, and predisposition to optimal health. However, the mechanisms underlying these changes are not well understood. Therefore, we hypothesized that maternal programming, through maternal diet and energy demands during gestation, would negatively alter offspring development, growth, and health. Our first objective was to determine the effects of maternal restricted- and over-feeding during gestation on offspring muscle development, specifically the effect on the myogenic progenitor cell population. Our second objective was to determine the effects of maternal milk production during gestation on offspring growth and immunity.

CHAPTER I: POOR MATERNAL NUTRITION DURING GESTATION ALTERS PRENATAL MUSCLE DEVELOPMENT IN OFFSPRING

INTRODUCTION

There is considerable evidence demonstrating that maternal nutrition plays a major role in offspring development and growth. Poor maternal nutrition, which can be defined as either restricted or excessive macro- or micro-nutrient intake during gestation, has been found to negatively affect a variety of tissues, organs, and cell lineages both pre- and post-natally (Wu et al., 2006; Zhu et al., 2008; Nesterenko and Aly, 2009; Long et al., 2014; Reed et al., 2014; Hoffman et al., 2016a; Pillai et al., 2016a). Despite accumulating phenotypic evidence of the effects of poor maternal nutrition on offspring development, the mechanisms behind these changes are not completely understood. One potential mechanism stimulating these changes is fetal programming, which is defined as changes to the maternal environment during critical periods of fetal development that have the ability to alter the trajectory of offspring development and growth (Nathanielsz et al., 2007). Individuals exposed to negative alterations, including inadequate maternal nutrition, while in utero may be predisposed to metabolic, endocrine, and cardiovascular diseases later in life as a long-term consequence of maternal programming (Wu et al., 2006). Furthermore, there is also evidence that these effects can persist across multiple generations (Nathanielsz et al., 2007; Shasa et al., 2015).

While poor maternal nutrition has been found to affect multiple tissues and developmental processes, the impact on skeletal muscle development is of interest due to its role in glucose and fatty acid oxidation, glycogen storage, and establishment of adult muscle mass (Zhu et al., 2008). When the nutritional status of a fetus is altered, nutrients are partitioned away from skeletal muscle in favor of more critical organs such as the brain and heart (Hales and

Barker, 1992). However, postnatal skeletal muscle serves as an important amino acid reservoir for key organs during periods of stress or reduced nutrient intake, thereby making proper prenatal muscle development critical (Wolfe, 2006). Additionally, the role of poor maternal nutrition in skeletal muscle development is of great importance within the livestock industry, as muscle serves as an important protein source for human consumption. Alterations in muscle mass, connective tissue content, and the deposition of intramuscular adipose are all potential consequences of poor maternal nutrient intake and as a result, can alter meat and carcass quantity and quality, thereby reducing production efficiency (Daniel et al., 2007; Reed et al., 2014). Importantly, the net muscle fiber number does not increase postnatally. Consequently, alterations to prenatal myogenesis could potentially have long-term effects on overall muscle quantity and composition (Zhu et al., 2006; Rehfeldt et al., 2011; Reed et al., 2014). Maternal nutrient restricted- and over-nutrition during gestation can alter muscle mass and muscle fiber cross-sectional area (CSA), reduce muscle fiber number, and increase adiposity (Huang et al., 2010; Yan et al., 2013; Reed et al., 2014; Hoffman et al., 2016a). Specifically, we found that offspring born to restricted- and over-fed mothers displayed an increase in the muscle fiber CSA at birth, but by three months of age the CSA was reduced compared with control offspring (Reed et al., 2014). Additionally, intramuscular fat was increased in offspring born to over-fed mothers at both birth and three months of age and reduced in offspring born to restricted-fed mothers at three months of age. These findings suggest that prenatal muscle development is impaired and the consequences of poor maternal nutrition persist into postnatal growth and consequently affect production quality and quantity.

The development of skeletal muscle is a complex process that requires several myogenic regulatory factors that act in concert with each other to regulate prenatal myogenesis, including

Paired Box 7 (Pax7) and members of the Myogenic Regulatory Factor (MRF) family. Pax7 is a marker of myogenic progenitor cells and is involved in maintaining the population of muscle stem cells, or satellite cells, postnatally (Zammit et al., 2006). This transcription factor also plays a role in upregulating the expression of MyoD and Myf5, both of which are involved in myoblast proliferation and differentiation (Seale et al., 2000; Zammit et al., 2006; Yablonka-Reuveni et al., 2008). Downregulation of Pax7 is required for further progression through myogenesis (Zammit et al., 2006; Yablonka-Reuveni et al., 2008). Myoblasts exit the cell cycle and fuse together to form primary myofibers during early gestation, a process mediated by myogenin and MRF4. Secondary myofibers then form around primary fibers to increase myofiber number (Rhodes and Konieczny, 1989; Lee et al., 2013). Due to the tight regulation of these MRFs, any disruption in the pattern or timing of their expression can adversely affect muscle development.

While the effects of poor maternal nutrition during gestation on postnatal growth are well established, the effects on prenatal development and growth are less understood. Further studies are required to understand and validate changes observed in offspring muscle postnatally, especially within the period of prenatal myogenesis. Therefore, we hypothesized that poor maternal nutrition during gestation would alter the development of offspring muscle prenatally through the alteration of the number of myogenic progenitor cells.

MATERIALS AND METHODS

Animals

All animal experiments were reviewed and approved by the University of Connecticut Institutional Animal Care and Use Committee (A13-059).

Eighty-two multiparous Western White-faced ewes were estrus synchronized using a progesterone controlled intravaginal drug release device (Easi-Breed CIDR Sheep Insert, vaginally, Zoetis Inc., Parsippany, NJ), after which a single intramuscular injection of Prostaglandin $F_2\alpha$ was administered (Lutalyse, Zoetis Inc.), as previously described (Pillai et al., 2017). Ewes were then housed with one of four breeding rams and breeding date was determined via rump markings on ewes. After confirmation of pregnancy using ultrasound at day 20 of gestation, animals were individually housed. Starting at day 30.2 ± 0.2 of gestation, ewes were assigned to one of three diets; control-fed (100%; n = 27), restricted-fed (60%; n = 28) or over-fed (140%; n = 27) based on the National Research Council (NRC) requirements for TDN for a ewe carrying twins (National Research Council, 1985; Figure 1). Diets were adjusted weekly based on individual body weight.

Ewes (n = 5 to 7 per diet) remained on their respective diets until day 45, 90, or 135 of gestation. At each of these time points, a subset of ewes was euthanized with an i.v. injection of Beuthansia-D Special (390 mg/mL sodium pentobarbital and 50 mg/mL phenytoin; Merck Animal Health, Summit, NJ) based on body weight, followed by exsanguination. A hysterectomy was performed to remove the uterus and fetus for fetal sample collection. Another subset of ewes (birth; n = 5 to 7 per diet) was allowed to undergo natural parturition and lambs nursed for up to 24 hours, after which the lambs were euthanized as described above to collect samples. Offspring born to control-, restricted- and over-fed ewes will be referred to as CON, RES and OVER, respectively.

Sample collection and processing

Muscle samples for histological analysis were collected from the longissimus dorsi (LD), semitendinosus (STN), and triceps brachii (TB) at each time point from each fetus (n = 10 to 15 fetuses or lambs per dietary treatment per time point). Muscle samples were embedded in Tissue-Tek optimal cutting temperature medium (Fisher Scientific, Pittsburg, PA). Samples were stored at -80°C until further use.

Immunohistochemistry

Percentage of Pax7 positive [Pax7(+)] progenitor cells were visualized using immunohistological procedures (Town et al., 2004; Reed et al., 2014). Muscle samples were sectioned at 10 µm using a Microm HM 525 cryostat (Thermo Scientific, Waltham, MA) and fixed in 4% paraformaldehyde for 20 minutes followed by three 5 minute washes with PBS. Sections were blocked with 5% horse serum, 0.2% Triton-X100 in PBS for 20 minutes. To determine the percentage of Pax7(+) cells, sections were incubated with Pax7 concentrate antibody (1:1000; Developmental Studies Hybridoma Bank, Iowa City, IA) overnight in a 4°C humidified chamber. Sections were rinsed and incubated with secondary antibody (Goat anti-Mouse Alexa Fluor 488; 1:250; Invitrogen, Carlsbad, CA) to visualize Pax7(+) cells. Hoescht 33342 (1:2,000; Invitrogen) was used to visualize nuclei. Alexa Fluor 568 conjugated WGA (1:50; Invitrogen) was used to visualize the sarcolemma membrane.

All images for immunohistochemistry analyses were captured with an AxioCam camera (Zeiss, Inc., Jena, Germany) mounted to an AxioObserver microscope (Zeiss, Inc.) with 5 images taken per slide (n = 3 sections per slide per animal). Images were false colored and merged using ImageJ (National Institutes of Health, Bethesda, MD). The percentage of Pax7(+) cells was

determined by dividing the number of Pax7(+) nuclei by the total number of nuclei per 20X image.

Statistical Analysis

Percentage of Pax7(+) cell data were analyzed using PROC Mixed in SAS (Cary, NC; version 9.4). Data were analyzed as a split-plot design, where ewes were randomly assigned to a 3 × 4 factorial arrangement of treatments of diet and time point. In the sub plot, offspring were assigned to a 3 × 4 × 3 factorial arrangement of treatments, including maternal diet (3), time point (4), and offspring number (3), respectively, and listed as the fixed effects with all interactions. Statistical significance is discussed at $P \leq 0.05$.

RESULTS

Longissimus Dorsi

An effect of dietary treatment was not observed across time on the percentage of Pax7(+) cells within the LD of offspring ($P = 0.57$; Table 1). However, there was an interaction of diet by offspring number ($P = 0.04$; Table 2), where the percentage of Pax7(+) cells was increased by 68% in OVER triplets compared with CON triplets ($P \leq 0.03$). However, no differences were detected between RES and CON triplets ($P \geq 0.77$). Within singletons and twins, no differences were observed between CON, RES, and OVER ($P \geq 0.07$). Interestingly, within OVER litter sizes, Pax7(+) percentage was increased by 70% and 63% in triplets compared with singletons ($P \leq 0.04$) and twins ($P \leq 0.02$), respectively. No changes were observed for total nuclei over time across treatments ($P = 0.08$; Table 3). Additionally, no changes were observed for Pax7(+) nuclei over time across treatments ($P = 0.54$).

Semitendinosus

Contrary to the LD, there was an interaction of diet by time point within the STN of offspring ($P = 0.0002$; Table 1). The percentage of Pax7(+) cells was reduced by 48% in RES ($P \leq 0.02$) offspring when compared with CON at day 45 of gestation. At day 90, the percentage of Pax7(+) cells was reduced by 65% and 57% in RES ($P < 0.0001$) and OVER ($P < 0.0001$) offspring, respectively, compared with CON. Furthermore, at day 135 of gestation, the percentage of Pax7(+) cells were reduced by 31% in RES ($P \leq 0.026$) compared with OVER with CON being intermediate. There were no differences in percentage of Pax7(+) cells between RES, OVER, and CON at birth ($P \geq 0.13$). Additionally, there was a tendency for a diet by offspring number interaction ($P = 0.10$; Table 2). Within singletons, RES had a 43% reduction in the percentage of Pax7(+) cells compared with CON ($P \leq 0.02$). Within twins, the percentage of Pax7(+) cells were reduced by 36% in RES compared with CON ($P \leq 0.01$). Similarly, within triplet pregnancies, RES displayed a 37% reduction in the number of Pax7(+) cells compared with CON ($P \leq 0.04$). No differences were observed for total nuclei number over time across treatments ($P = 0.88$; Table 3). However, there was an effect of maternal diet on total Pax7(+) nuclei ($P = 0.0001$), where Pax7(+) nuclei were reduced in RES and OVER offspring at day 45 ($P \leq 0.03$) and day 90 ($P \leq 0.0001$) compared with CON.

Triceps Brachii

Similar to observations made in the STN, there was a significant interaction of dietary treatment by timepoint observed for the percentage of Pax7(+) cells in the TB ($P = 0.02$; Table 1) of offspring. The percentage of Pax7(+) cells was reduced by 36% in OVER ($P \leq 0.02$) compared with CON at day 45 of gestation. At day 90, the percentage of Pax7(+) cells was

reduced by 43% and 26% in the RES ($P \leq 0.001$) and OVER ($P \leq 0.05$), respectively, compared with CON. There were no differences in Pax7(+) cell percentage between diets at day 135 or birth ($P \geq 0.27$). An interaction of diet by offspring number was also observed in the TB ($P = 0.04$; Table 2). Within singletons, RES ($P \leq 0.01$) and OVER ($P \leq 0.0086$) had a 41% and 40% reduction in the percentage of Pax7(+) cells compared with CON, respectively. There were no differences observed between CON, RES, and OVER twins and triplets ($P \geq 0.17$). No differences were observed in total nuclei across treatments over time ($P = 0.18$: Table 3). However, there was an effect of maternal diet by time point on total Pax7(+) nuclei ($P = 0.0002$). Specifically, Pax7(+) nuclei were reduced in RES and OVER offspring at day 45 ($P \leq 0.005$) and day 90 ($P \leq 0.001$) compared with CON.

Figure 1.

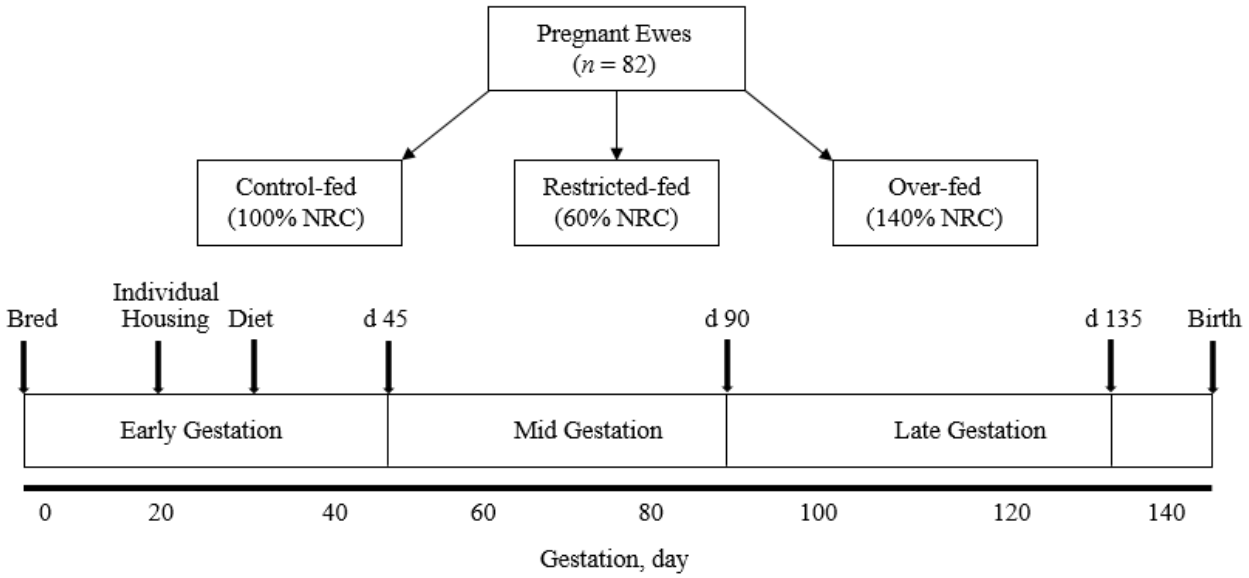

Figure 1. Experimental Design. To evaluate the effects of restricted- or over-feeding during gestation on offspring muscle development and growth, pregnant ewes (n = 82) were housed individually at day 20 of gestation (Pillai et al., 2017). At day 30 of gestation, ewes were randomly assigned into a 3 × 4 factorial arrangement. Treatment structure included three diets [control (100% NRC), restricted (60% NRC) or over (140% NRC)] and four time points (day 45, 90, and 135 of gestation and birth; n = 5 to 7 ewes per treatment). At day 45, 90, and 135 of gestation, subsets of ewes were euthanized and fetal muscle samples collected. A fourth subset of ewes was allowed to undergo natural parturition (birth), after which muscle samples were collected from the lambs within 24 hours of birth.

Table 1. Effect of maternal diet and day of gestation on percentage of Pax7(+) cells

	Treatment[1,2]				*P*-value
Muscle	CON	RES	OVER	SEM	Maternal diet by day of gestation
Longissimus dorsi					0.5713
d 45	12.76^{a}	10.44^{a}	12.36^{a}	0.95	
d 90	6.04^{b}	4.78^{b}	3.84^{b}	0.86	
d 135	9.88^{c}	7.29^{b}	7.24^{c}	0.56	
Birth	6.14^{b}	5.65^{b}	5.90^{bc}	0.56	
Semitendinosus					0.0002
d 45	4.33^{Aa}	2.25^{Ba}	2.98^{ABa}	0.42	
d 90	8.66^{Ab}	3.03^{Bab}	3.75^{Ba}	0.55	
d 135	5.34^{ABa}	4.34^{Bb}	6.30^{Ab}	0.49	
Birth	5.00^{Aa}	4.53^{Ab}	5.76^{Ab}	0.54	
Triceps brachii					0.0233
d 45	3.78^{Aa}	2.73^{Aba}	2.41^{Ba}	0.29	
d 90	4.59^{Aa}	2.60^{Ba}	3.39^{Ba}	0.28	
d 135	3.07^{Ab}	2.78^{Aa}	2.58^{Aa}	0.24	
Birth	3.56^{Aab}	4.22^{Ab}	3.59^{Aab}	0.23	

^{AB}Denotes LSMean differences for maternal diet by time point interaction ($P \leq 0.05$) within a time point

$^{a-c}$Denotes LSMean differences maternal diet by time point interaction ($P \leq 0.05$) within a treatment

[1]Offspring from ewes fed a control- (100% NRC), restricted- (60% NRC), or over-fed (140% NRC) diet are referred to as CON, RES, and OVER, respectively. Pregnant ewes began diets at day 30 of gestation, and subsets of these ewes were euthanized at day 45, 90, and 135 of gestation to obtain fetal muscle samples. Another subset of ewes was allowed to undergo natural parturition, after which lambs were necropsied within 24 hours of birth to obtain muscle samples (n = 10 to 15 fetuses or lambs from 5 to 7 ewes per diet per day of gestation). The percentage of Pax7(+) cells was determined in CON, RES, and OVER offspring at day 45, 90, and 135 of gestation and within 24 hours of birth.

[2]LSMeans ± SEM are reported.

Table 2. Effect of maternal diet and offspring number on percentage of Pax7(+) cells

	Treatment[1,2]				*P*-value
Muscle	CON	RES	OVER	SEM	Maternal diet by day of gestation
Longissimus dorsi					0.0486
Singleton	10.12 ^{Aa}	7.55 ^{Aa}	6.48 ^{Aa}	1.66	
Twin	9.07 ^{Aa}	6.74 ^{Aa}	6.70 ^{Aa}	0.58	
Triplet	6.51 ^{Aa}	7.09 ^{ABa}	10.95 ^{Ba}	1.37	
Semitendinosus					0.1021
Singleton	6.86 ^{Aa}	3.15 ^{Ba}	5.03 ^{ABa}	0.73	
Twin	5.38 ^{Aa}	3.46 ^{Ba}	4.81 ^{ABa}	0.43	
Triplet	6.34 ^{Aa}	3.98 ^{Ba}	5.03 ^{ABa}	0.67	
Triceps brachii					0.0465
Singleton	4.84 ^{Aa}	2.84 ^{Ba}	2.89 ^{Ba}	0.21	
Twin	3.61 ^{Aab}	2.99 ^{Aa}	3.02 ^{Aa}	0.23	
Triplet	3.35 ^{Ab}	3.67 ^{Aa}	2.90 ^{Aa}	0.60	

^{AB}Denotes LSMean differences maternal diet by offspring number interaction ($P \leq 0.05$) within offspring number

^{ab}Denotes LSMean differences maternal diet by offspring number interaction ($P \leq 0.05$) within a treatment

[1]Offspring from ewes fed a control- (100% NRC), restricted- (60% NRC), or over-fed (140% NRC) diet are referred to as CON, RES, and OVER, respectively. Pregnant ewes began diets at day 30 of gestation, and subsets of these ewes were euthanized at day 45, 90, and 135 of gestation to obtain fetal muscle samples. Another subset of ewes was allowed to undergo natural parturition, after which lambs were necropsied within 24 hours of birth to obtain muscle samples (n = 10 to 15 fetuses or lambs from 5 to 7 ewes per diet per day of gestation). The percentage of Pax7(+) cells was determined in CON, RES, and OVER singleton, twin, and triplet offspring.

[2]LSMeans ± SEM are reported.

Table 3. Effect of maternal diet and day of gestation on total and Pax7(+) nuclei numbers

	Treatment[1,2]						*P*-value	
	CON		RES		OVER			
Muscle	Total nuclei	Pax7(+) nuclei	Total nuclei	Pax7(+) nuclei	Total nuclei	Pax7(+) nuclei	Total nuclei maternal diet by day of gestation	Pax7(+) nuclei maternal diet by day of gestation
Longissimus dorsi							0.0801	0.5487
d 45	395[a]	50[a]	403[a]	43[a]	428[a]	54[a]		
d 90	719[b]	37[b]	708[a]	31[a]	797[b]	29[b]		
d 135	563[c]	56[ac]	630[b]	47[b]	581[b]	42[ab]		
Birth	443[ad]	27[bd]	493[c]	27[c]	418[cb]	25[cb]		
Semitendinosus							0.8810	0.0001
d 45	546[Aa]	22[Aa]	570[Ba]	13[Ba]	531[Ca]	13[Ba]		
d 90	578[Aa]	50[Ab]	564[Aa]	17[Bb]	600[Ab]	23[Bb]		
d 135	466[Ab]	25[Ac]	504[Ab]	22[Ac]	482[Ac]	30[Ac]		
Birth	292[Ac]	14[Ad]	363[Ac]	16[Ad]	302[Ad]	17[Ad]		
Triceps brachii							0.1821	0.0002
d 45	466[Aa]	17[Aa]	363[Ba]	10[Ba]	349[Ca]	9[Ba]		
d 90	655[Ab]	28[Ab]	596[Ab]	16[Ba]	603[Ab]	21[Bb]		
d 135	350[Ac]	10[Ac]	388[Ac]	11[Aa]	322[Ac]	8[Ac]		
Birth	286[Ad]	10[Ac]	319[Ad]	8[Ab]	287[Ad]	13[Ad]		

[A-C]Denotes LSMean differences maternal diet by time point interaction ($P \leq 0.05$)

[a-d]Denotes LSMean differences maternal diet over time ($P \leq 0.05$) within a treatment

[1]Offspring from ewes fed a control- (100% NRC), restricted- (60% NRC), or over-fed (140% NRC) diet are referred to as CON, RES, and OVER, respectively. Pregnant ewes began diets at day 30 of gestation, and subsets of these ewes were euthanized at day 45, 90, and 135 of gestation to obtain fetal muscle samples. Another subset of ewes was allowed to undergo natural parturition, after which lambs were necropsied within 24 hours of birth to obtain muscle samples (n = 10 to 15 fetuses or lambs from 5 to 7 ewes per diet per day of gestation). The total number of nuclei and Pax7(+) nuclei were determined in CON, RES, and OVER offspring at day 45, 90, and 135 of gestation and within 24 hours of birth. [2]LSMeans are reported.

DISSCUSION

There have been multiple studies demonstrating the effects of poor maternal diet on offspring muscle development both pre- and post-natally (Quigley et al., 2005; Zhu et al., 2006; Ford et al., 2007; Reed et al., 2014). Previously, we have shown that poor maternal nutrition during gestation, through both restricted- and over-feeding, inhibits muscle development at postnatal time points (Reed et al., 2014). Consequently, this can have implications on meat quantity and overall production efficiency over time. However, because net myofiber number is set before parturition, any alterations to prenatal myogenesis can negatively affect muscle post-natally, thereby explaining the phenotypic changes observed in previous research (Rehfeldt et al., 2011; Reed et al., 2014).

Myogenesis is a tightly regulated process that occurs in distinct stages throughout prenatal development. In sheep, primary myogenesis, which comprises the first stage of muscle development, begins at approximately 32 to 38 days of gestation (Maltin, 2008). During this period, proliferating progenitor cells differentiate into myoblasts, and ultimately fuse together to form primary fibers (Zammit et al., 2006). This is followed closely by secondary myogenesis beginning at day 62 of gestation (Maltin, 2008). This period of muscle development consists of precursor and/or satellite cells forming secondary fibers, which fuse to the existing primary fibers (Maltin, 2008). Thus, the number of myogenic progenitor cells greatly influences the number of myofibers formed during development (Du et al., 2017). Furthermore, this population of cells affects the density of the satellite cell pool, which is vital for proper postnatal muscle growth (Du et al., 2017).

In an effort to understand the impact of restricted- and over-feeding during gestation on offspring muscle development prenatally, we investigated the effect of a poor maternal diet on

progenitor and satellite cells expressing Pax7 in fetal and early neonatal muscle. As previously stated, Pax7 acts as a key marker of both myogenic progenitor cells in early prenatal development and satellite cells during pre- and post-natal development, which allows for visualization of myogenic cellular populations (Seale et al., 2000; Zammit et al., 2006). This transcription factor is thought to be transcriptionally active in quiescent satellite cells, and throughout activation and proliferation (Zammit et al., 2006). Relaix et al. (2005) demonstrated that Pax7 is required for fetal muscle development to progress, and subsequently adult myofiber hypertrophy through the incorporation of satellite cells. Specifically, $Pax7^{-/-}$ results in the complete absence of skeletal muscles in the trunk and limbs (Relaix et al., 2005). Accordingly, Pax7 is critical for muscle development prenatally, and postnatal repair and maintenance.

In the present study, restricted- and over-feeding during gestation resulted in a reduction of Pax7(+) cells at multiple time points. These particular time points were chosen to represent the stages of myogenesis, including primary (day 45) and secondary (day 90) myogenesis, as well as the period of hypertrophy (day 135) which occurs towards the later stages of gestation (Maltin, 2008; Du et al., 2015). Although we did not observe an effect of maternal diet on Pax7(+) progenitor cells in the LD, we saw reductions in Pax7(+) progenitor cells in both the STN and TB. Within both of these muscles, there were smaller myogenic populations at days 45, 90, and 135 of gestation, which may indicate inhibited muscle fiber formation during both stages of myogenesis, and a reduction in satellite cell incorporation towards the end of gestation. However, no differences were observed at birth in either muscle. As stated previously, net fiber number is established by parturition, meaning that fiber formation by satellite cell fusion begins to decline towards the end of gestation (Gonzalez et al., 2013). Enumeration of Pax7(+) cells throughout gestation indicates a steady decline in cellular numbers as parturition approaches,

where Pax7(+) nuclei number reaches approximately 15% by late gestation (Gonzalez et al., 2013). This could explain the lack of changes observed in satellite cells expressing Pax7 at birth in each of the muscles evaluated. However, these data are inconsistent with a previous report by Gonzalez et al. (2013), who demonstrated that maternal nutrient restriction results in decreased Pax7(+) cells at the initial stages of gestation, where primary myogenesis is expected to occur, but the reduction was not continued throughout gestation and the progenitor cell population was presumed to not be irrevocably impaired. This inconsistency between findings could be due to differences in cellular enumeration methodology, where Gonzalez et al. (2013) counted Pax7(+) cells solely adjacent to dystrophin. In our current study, we enumerated total Pax7(+) cells, regardless of localization to dystrophin, to account for disrupted localization and this could explain the differences in percentages of Pax7(+) cells between studies. Additionally, cattle were utilized as the model in this study, whereas our present study utilized sheep and this could also lend to the differences observed between studies.

In addition to evaluating the effects of restricted- and over-feeding on offspring over time, we assessed the differences between litter sizes. In the present study, we observed differences between singleton, twin, and triplet offspring in the LD, STN, and TB of offspring. Similar to changes over time, alterations in the percentage of Pax7(+) cells within RES and OVER litter sizes could be indicative of delayed or attenuated primary and/or secondary myogenesis brought on by poor maternal nutrition. Sheep typically gestate twin pregnancies, but singletons and triplets are not uncommon. In addition to triplet offspring being subjected to poor maternal nutrition, this size litter can also be subjected to a state of IUGR through placental insufficiency (Wu et al., 2006). During pregnancy, the placenta is responsible for the exchange of nutrients from the dam to the growing fetus (Vonnahme et al., 2013), and larger litter sizes

may experience a competitive environment for nutrients (Wu et al., 2006). However, our model does not implicate IUGR in the observed changes, indicating that alterations in Pax7(+) cells in triplet offspring are likely due to maternal feed-restriction (Pillai et al., 2017). Within the LD, the only observed changes were within triplet offspring. Contrary to the previously described alterations, Pax7(+) cells were increased in these animals compared with CON. However, for myoblast differentiation to occur, Pax7 expression must be reduced (Zammit et al., 2006; Yablonka-Reuveni et al., 2008). This could indicate that primary and/or secondary myogenesis may be prolonged in OVER triplet offspring where increased Pax7(+) cells may be inhibiting differentiation.

To our knowledge, this is the only study which compares the effects of inadequate maternal nutrition on multiple skeletal muscles across gestation at multiple time points. Presently, we have demonstrated that many of the changes observed in the STN and TB are not observed in the LD of offspring across gestation. It is largely known that differences exist between skeletal muscles, including fiber type composition and cellular makeup (Briand et al., 1981). However, many similar studies only evaluate a single muscle type or gestational time point (Gonzalez et al., 2013; Yates et al., 2014), and our data indicate a necessity for assessing multiple muscles to obtain a better understanding of the overall effects of poor maternal nutrition on offspring muscle development and growth.

CONCLUSIONS AND IMPLICATIONS

In conclusion, poor maternal nutrition through restricted- and over-feeding may negatively affect offspring muscle development and growth, either through the inhibition or attenuation of primary and secondary myogenesis. Additionally, muscle growth through the

incorporation of satellite cells appears to also be reduced in these animals. However, these effects are muscle specific, and this study indicates the necessity of evaluating multiple muscles to determine the overall effects of maternal programming on muscle development and growth. These data are important when considering the improvement of livestock production efficiency and overall meat quantity and quality. Further research is still required to understand the long term effects of this initial insult on prenatal muscle development and to determine whether these effects are multigenerational.

CHAPTER II: THE EFFECTS OF HIGH MATERNAL MILK PRODUCTION ON DAIRY CALF GROWTH AND HEALTH

INTRODUCTION

Rearing dairy calves can be challenging as these animals are susceptible to disease due to the undeveloped status of their immune systems (USDA APHIS, 2011). For example, 5.8% of calves die due to non-predatory causes, with dairy operations experiencing a high rate of calf mortality due to digestive (30.6%) and respiratory issues (36.7%; USDA APHIS, 2011). One mechanism by which calves may be predisposed to altered growth and increased disease susceptibility is due to maternal programming, particularly nutritional constraints while in utero due to the metabolic demands of milk synthesis (Wu et al., 2006). Specifically, the gestation period of multiparous dairy cows coincides to a large extent with lactation, causing nutrient usage to be divided between these two energetically demanding events (Bauman and Currie, 1980; Kamal et al., 2016). This nutrient partitioning between the mammary gland and the uterus may subject the developing offspring to a state of nutrient restriction by diverting greater quantities of nutrients to dam maintenance and milk synthesis instead of towards calf development and growth (Bauman and Currie, 1980; Knight, 2001; Redmer et al., 2014). Therefore, dairy cows producing large quantities of milk during gestation may affect overall dairy production efficiency by negatively impacting the dairy calf population.

Research evaluating the effects of maternal milk output during gestation on calf growth and health is limited. Recently, Kamal et al. (2014, 2015) demonstrated that calves born to cows with increased milk production had reduced births weights and increased circulating insulin concentrations, suggestive of alterations to offspring growth and development at early postnatal time points. However, to the best of our knowledge, there are no studies on the effects of

maternal milk production on calf immunity, which is one of the leading causes of neonatal calf mortality (USDA APHIS, 2011). Therefore, we hypothesized that increased maternal milk production during gestation would affect key factors relating to calf growth and immunity. The objective of this study was to determine whether there is an effect of maternal milk production during gestation on calf growth and health.

MATERIALS AND METHODS

Animals

All animal experiments were reviewed and approved by the University of Connecticut Institutional Animal Care and Use Committee (A16-038).

Multiparous Holstein cows (n = 35; 1 set of twins) from the University of Connecticut dairy herd were used in this study. Cows were inseminated at approximately 45 days in milk (DIM) after exhibiting natural estrus. Those who did not exhibit heat by 50 DIM were placed on an Ovsynch protocol and inseminated ten days following synchronization or earlier if estrus was observed. Cows were fed a standard diet that met maintenance, growth, and lactation requirements (NRC, 2001). Milking was performed three times per day in the Kellogg Dairy Center (KDC; Storrs, CT) milking parlor and individual milkings were recorded using a standard computerized recording system.

Cows were separated into two groups (n = 17 to 18 per group) based on total milk output during the present lactation, which was obtained from the herd database at the KDC. Cows that had a total milk weight of $\geq 11{,}688 \pm 532$ kg (average of 14,865 kg) during the current lactation were classified as high milk producers and cows that had a total milk weight of $\leq 11{,}688 \pm 477$ kg (average of 10,069 kg) during the current lactation were classified as low producers. Calves

(n = 5 to 13 per group per gender) born to high or low milk producing cows will be referred to as HIGH or LOW, respectively.

Sample Collection and Preparation

Within 24 hours of parturition, individual calves were allowed to consume colostrum from the dam and were kept in 8x8 indoor pens after separation. Calves were weighed in kilograms on a large animal digital scale and body measurements including crown rump length, nose occipital length, skull width, whither height, and girth were taken with a flexible measuring tape. Additionally, 20 mL of whole blood was collected via a jugular venipuncture for serological analysis from each calf. Blood was aliquoted into non-anticoagulant tubes (Fisher Scientific) for serum collection and tubes containing heparin or ethylenediaminetetraacetic acid (EDTA; Fisher Scientific) for plasma collection. To obtain serum samples, blood was allowed to coagulate overnight at 4°C. The following morning, tubes were centrifuged at 1800 x *g* for 30 min at 4°C, after which serum was collected and frozen at -20°C until analysis. Plasma tubes were immediately placed on ice following blood collection, centrifuged, and collected as described previously, and frozen at -20°C until analysis.

Additionally, 20 mL of first colostrum was collected from each cow at calving by the KDC staff and kept refrigerated in 50 mL falcon tubes. At the time of sampling, colostrum samples were obtained and frozen at -20°C until further analysis.

Serological Analyses

Individual enzyme-linked immunosorbent assay (ELISA) kits were utilized to determine circulating concentrations of insulin (ALPCO; Salem, NH), haptoglobin (Abcam; Cambridge,

UK), interferon gamma (IFNγ; ThermoFisher; Waltham, MA), and immunoglobulin G (IgG; Bethyl Laboratories; Montgomery, TX) in serum, plasma or colostrum according to the respective manufacturer protocol. Serum insulin (undiluted; 6.9% CV), plasma haptoglobin (1:1,000; 7.6% CV), plasma IFNγ (undiluted), serum IgG (1:1,000,000; 7.7% CV), and colostrum IgG (1:750,000; 6.2% CV) concentrations were obtained using a standard curve for each receptive assay and absorbance values were read using a plate reader (BioTek Synergy 2; Winooski, VT). Additionally, serum was aliquoted and sent to the University of Missouri Veterinary Diagnostic Laboratory (Columbia, MO) where a blood biochemistry analysis (Food Animal Maxi Profile) was performed.

Statistical Analysis

All data were analyzed using PROC GLM in SAS (SAS Inst. Inc, Cary, NC, USA) with analysis of covariance using the current milk weight as a covariate. Fixed effects included maternal lactation status and gender and the interaction of maternal lactation status by gender. Statistical significance was considered at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

RESULTS

Morphometric measurements

An effect of maternal milk production by gender was not observed for each of the morphometric measurements, including body weight (BW), obtained from calves ($P \geq 0.14$, Table 1). However, there was a tendency for milk production by gender for skull width ($P = 0.09$, Table 1). Specifically, HIGH females had a 20% and 14% increase in skull width (14.5 cm ± 0.8; $P \leq 0.01$) compared with LOW females (12 ± 0.3 cm) and LOW males (12.7 ± 0.4 cm),

respectively. A significant effect of maternal milk production was observed for nose-occipital length ($P = 0.03$, Table 1), where LOW calves (20.2 ± 0.4 cm) had a 13% decrease in nose-occipital length compared with HIGH calves (23.2 ± 0.7 cm). A tendency for gender was also observed ($P = 0.06$) on nose-occipital length, where females (20.2 ± 0.5 cm) had decreased nose-occipital length compared with males (22.1 ± 0.3 cm).

Insulin and Glucose

There was a significant effect of maternal milk production by gender for glucose in calves ($P = 0.05$, Table 2). Specifically, LOW males (120.8 ± 17.5 mg/dL; $P \leq 0.008$), HIGH males (116.6 ± 7.9 mg/dL; $P \leq 0.05$), and HIGH females (118.1 ± 5.6 mg/dL; $P \leq 0.08$) had a 38%, 36%, and 37% increase in glucose concentrations compared with LOW females (74.7 ± 9.2 mg/dL), respectively. An effect of maternal milk production by gender was not observed for insulin ($P = 0.523$, Table 3).

Passive Transfer and Inflammatory Factors

An effect of maternal milk production by gender was not observed for factors relating to passive transfer and immunity, including haptoglobin, IgG, and gamma-glutamyl transferase ($P \geq 0.16$, Tables 2 and 3). However, an effect of maternal milk production was observed for several factors, including total protein and globulin ($P \leq 0.01$, Table 2). Specifically, total protein was reduced by 11% in LOW calves (5.5 ± 0.3 g/dL) compared with HIGH calves (6.2 ± 0.2 g/dL; $P \leq 0.01$). Similarly, LOW calves (2.6 ± 0.3 g/dL) had a 39% decrease in globulin concentrations compared with HIGH calves (4.2 ± 0.2 g/dL; $P \leq 0.02$). Interferon gamma concentrations were below the detectable limit of the assay (Table 3).

Additional Factors

A tendency for maternal milk production by gender was observed for direct bilirubin (P = 0.07), where LOW males (0.35 ± 0.04 mg/dL; $P \leq 0.04$) had a 22% reduction in direct bilirubin concentrations compared with LOW females (0.45 ± 0.03 mg/dL). Additionally, there were tendencies for the main effect of maternal milk production on calcium and magnesium ($P \leq$ 0.07). Specifically, HIGH calves had a tendency for increased calcium concentrations (11.91 ± 0.2 mg/dL) compared with LOW calves (11.12 ± 0.3 mg/dL; $P \leq 0.07$). Magnesium was also increased in HIGH calves (2.7 ± 0.08 mg/dL; $P \leq 0.04$) compared with LOW calves (2.3 ± 0.1 mg/dL). A main effect of gender was also observed on anion gap (P = 0.05), where males (25.6 ± 1 mEq/L; $P \leq 0.09$) had decreased anion gap compared with females (28.07 ± 0.5 mEq/L). An effect of maternal milk production by gender, lactation, or gender was not observed for remaining factors including urea nitrogen, creatinine, sodium, potassium, chloride, bicarbonate, albumin, phosphorus, total bilirubin, AST, and GGT ($P \geq 0.16$).

Table 1. Effect of dam milk production on calf size variables

	Treatment[1,2]				*P*-value		
	HIGH		LOW				
Variable	Male	Female	Male	Female	Lactation x Gender	Lactation	Gender
BW, kg	48.4 ± 3.4	44.7 ± 2.5	44.3 ±1.5	39.6 ± 1.6	0.8348	0.2855	0.1020
Crown-rump length, cm	93.5 ± 1.8	89.9 ± 1.6	91.8 ± 1.7	88.9 ± 2.1	0.8849	0.7245	0.1747
Nose-occipital length, cm	23.3 ± 0.5	23.1 ± 0.9	22.1 ± 0.3	20.2 ± 0.5	0.1447	**0.0329**	**0.0661**
Skull width, cm	13.4 ± 0.3[ab]	14.5 ± 0.8[a]	12.7 ± 0.4[b]	12 ± 0.3[b]	**0.0976**	**0.0514**	0.6931
Girth, cm	82 ± 1.4	80.8 ± 1.5	82.4 ± 0.9	78.8 ± 1.1	0.4028	0.7181	0.1046
Height, cm	81.3 ± 1.5	82.3 ± 1.3	81.8 ± 1.4	79.3 ± 1.0	0.8591	0.8827	0.1866

[ab]Denotes mean differences for lactation by gender interaction ($P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$)

[1]Offspring from cows with an average lactation of 14,865 kg are referred to as HIGH (n = 17) and offspring from cows with an average lactation of 10,069 kg are referred to as LOW (n = 18). Measurements were taken within 24 hours of birth and analyzed in SAS using PROC GLM with total milk weight during lactation period as a covariate.

[2]LSMeans ± SE are reported.

Table 2. Effect of dam milk production on calf serum variables

	Treatment				*P*-value		
	HIGH		LOW				
Variable	Male	Female	Male	Female	Lactation x Gender	Lactation	Gender
Glucose, mg/dL	116.6 ± 7.9[a]	118.1 ± 5.6[a]	120.8 ± 17.5[a]	74.7 ± 9.2[b]	**0.0552**	0.3155	**0.0731**
Urea nitrogen, mg/dL	9.7 ± 0.77	8.07 ± 1	9.6 ± 0.58	9.5 ± 1	0.4383	0.6533	0.3849
Creatinine, mg/dL	1.4 ± 0.16	1.4 ± 0.2	1.2 ± 0.1	1.6 ± 0.2	0.3310	0.9045	0.3653
Sodium, mEq/L	140.6 ± 1.2	139.7 ± 0.9	141.2 ± 2.8	140.4 ± 0.8	0.9685	0.8075	0.6307
Potassium, mEq/L	5.9 ± 0.2[b]	6.2 ± 0.2[a]	6.7 ± 0.2[a]	6.3 ± 0.2[a]	0.0966	0.3189	0.8965
Chloride, mEq/L	95.2 ± 0.9	94 ± 1.5	98.6 ± 1.7	96.7 ± 1.1	0.8072	0.1565	0.2479
Bicarbonate, mEq/L	24.8 ± 0.5	23.8 ± 0.8	23.5 ± 0.8	21.9 ± 0.9	0.7536	0.2550	0.1411
Anion gap, mEq/L	26.5 ± 1.1	28.4 ± 0.9	25.6 ± 1	28.07 ± 0.5	0.7613	0.6979	**0.0508**
Albumin, g/dL	2.4 ± 0.1	2.4 ± 0.07	2.5 ± 0.03	2.4 ± 0.03	0.6371	0.8697	0.9795
Total protein, g/dL	6.1 ± 0.3	6.3 ± 0.2	6.05 ± 0.3	5.5 ± 0.3	0.1637	**0.0098**	0.6988
Globulin, g/dL	4.1 ± 0.3	4.4 ± 0.2	3.1 ± 0.3	2.6 ± 0.3	0.2552	**0.0148**	0.7619
Calcium, mg/dL	11.91 ± 0.2	11.7 ± 0.2	11.11 ± 0.3	11.13 ± 0.1	0.6295	**0.0796**	0.6832
Phosphorus, mg/dL	7.1 ± 0.3	7.1 ± 0.3	7.3 ± 0.4	7.3 ± 0.3	0.4232	0.6153	0.6110
Magnesium, mg/dL	2.7 ± 0.08	2.7 ± 0.1	2.3 ± 0.1	2.4 ± 0.1	0.4459	**0.0746**	0.9228
Total bilirubin, mg/dL	0.93 ± 0.06	0.9 ± 0.09	0.9 ± 0.1	1.14 ± 0.1	0.3775	0.2921	0.5558
Direct bilirubin, mg/dL	0.38 ± 0.02[ab]	0.35 ± 0.02[ab]	0.35 ± 0.04[a]	0.45 ± 0.03[b]	**0.0744**	0.5580	0.3254
Aspartate aminotransferase, U/L	74.25 ± 5.8	82.8 ± 3.05	77.1 ± 6.4	75.8 ± 6.8	0.4837	0.3641	0.6232
Gamm-glutamyl transferase, U/L	3127 ± 270	4086 ± 451	2115 ± 558	2263 ± 740	0.5337	0.1811	0.4003
Creatine kinase, U/L	653 ± 170	653 ± 164	464 ± 80	678 ± 128	0.5445	0.7713	0.5445

[abc]Denotes mean differences and/or tendencies for lactation by gender interaction, and the main effects of lactation or gender ($P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$)

[1]Offspring from cows with an average lactation of 14,865 kg are referred to as HIGH (n = 17) and offspring from cows with an average lactation of 10,069 kg are referred to as LOW (n = 18). Serum was collected within 24 hours of birth and analyzed by the University of Missouri

Veterinary Diagnostic Laboratory (Food Animal Maxi Profile). Data were analyzed in SAS using PROC GLM with total milk weight during lactation period as a covariate.

[2]LSMeans ± SE are reported.

Table 3. Effect of dam milk production on growth and immunity factors

	Treatment[1,2]				*P*-value		
	HIGH		LOW				
Variable	Male	Female	Male	Female	Lactation x Gender	Lactation	Gender
Insulin, ng/mL	0.44 ± 0.14	0.83 ± 0.17	0.44 ± 0.15	0.62 ± 0.08	0.5325	0.6364	0.7401
Haptoglobin, mg/mL	0.98 ± 0.04	0.06 ± 0.008	1.1 ± 0.07	0.12 ± 0.01	0.2369	0.1380	**0.0001**
IFNγ, ng/mL	nd[3]	nd	nd	nd			
IgG, mg/mL (serum)	51.2 ± 4.5	94.5 ± 6.7	67 ± 5.2	73.5 ± 6.2	0.23	0.92	0.11
IgG, mg/mL (colostrum)	397.5 ± 6.7	213.8 ± 5.4	384 ± 8	309.7 ± 5.6	0.3233	0.6342	**0.0264**

[1]Offspring from cows with an average lactation of 14,865 kg are referred to as HIGH (n = 17) and offspring from cows with an average lactation of 10,069 kg are referred to as LOW (n = 18). Serum and plasma were collected within 24 hours of birth and analyzed utilizing enzyme-linked immuno absorbent assay (ELISA) kits. Insulin, haptoglobin, interferon gamma (IFNγ), serum IgG, and colostrum IgG concentrations were obtained using a standard curve for each receptive assay and absorbance values were read using a plate reader. Interferon gamma concentrations were below the detectable limit of the assay. Data were analyzed in SAS using PROC GLM with total milk weight during lactation period as a covariate.

[2]LSMeans ± SE are reported.

[3]nd = not detectable.

DISCUSSION

Homeorhesis can be defined as coordinated changes in the metabolism of various tissues to prioritize a new physiological state (Bauman and Currie, 1980). In dairy cattle, lactogenesis generates a shift from uterine nutrient transfer to the mammary gland, which will ultimately serve as neonatal nourishment upon parturition (Bauman and Currie, 1980). The nutritional requirements for lactation in dairy cows is greatly increased compared with other species due to the high energy demand of milk production, resulting in nutrient partitioning to support milk synthesis (Knight et al., 2001). However, the energy requirements of lactation must be met while a multiparous cow maintains a concurrent pregnancy. Dairy cows calve on average by 24 months of age and calve approximately every 385 days thereafter to optimize production (Kamal et al., 2016). Intensive production practices dictate that an efficient cow will have a maximized peak milk yield in addition to maintaining a short calving interval (Esslemont and Kossaibati, 2000). Accordingly, much of gestation coincides with lactation in multiparous cows and subjects these dams to energetically physiological extremes. Thus, nutrient allocation between lactation and gestation in domestic dairy cattle could expose developing offspring to a nutritionally adverse environment while in utero. However, evidence of the effects of maternal programming on calf growth and health is limited. Determining whether nutrient partitioning is affecting calf development is critical, considering current calf mortality rates.

In the present study, total milk output for the whole of lactation was utilized to separate high and low milk producing cows. This methodology provides a complete picture of the maternal environment that could be influenced by lactation before gestation as well as during gestation. There is evidence that the peri-conception period before breeding can influence offspring development (Quigley et al., 2005; Sen et al., 2016). For example, Quigley et al.

demonstrated that nutrient-restriction before conception results in decreased myofiber number in offspring muscle by day 75 of gestation. Considering the negative energy balance of dairy cows during the lactation period before rebreeding, the period of milk synthesis pre-breeding could affect the initial stages of gestation and offspring development (Wu et al., 2006).

In an effort to determine whether maternal milk production has an effect upon dairy calf development and health in this study, we evaluated several factors, including circulating factors relating to growth and immunity. As stated previously, Kamal et al. (2014, 2015) demonstrated that dairy calves born to high milk producing cows had decreased birth weights and increased circulating insulin. Lighter birth weight has been connected with the development of various diseases in later postnatal life and increased circulating insulin is related to decreased insulin sensitivity, suggesting alterations to calf growth and metabolism (Hales and Barker, 2001). In the present study, we did not observe an effect of maternal milk production on calf BW at birth but there was a tendency for decreased skull size (length and width) in LOW females, which may indicate nutrient partitioning due to the energy requirements of lactation. When the nutritional status of a fetus is altered, nutrients are partitioned away from less crucial tissues, such as muscle and adipose, in favor of more critical organs such as the brain and heart (Hales and Barker, 1992). However, no changes were observed in the remaining body measurements. Calves were evaluated at one postnatal time point, and it is well known that changes in offspring due to maternal programming occur over time postnatally as the animal continues to develop and grow (Ford et al., 2007; Reed et al., 2014; Hoffman et al., 2017).

To determine whether maternal energy demands affect offspring glucose metabolism, we evaluated the concentrations of insulin and glucose in calves. Insulin is a primary regulator of circulating glucose concentrations, and thus, a critical factor in overall metabolism. Additionally,

within prenatal development, fetal insulin acts as a key growth factor in offspring (Hales and Barker, 2001). Contrary to the findings of Kamal et al. (2014), no significant difference in insulin was observed in the present study. However, blood samples were obtained in the present study within 24 hours of birth, whereas samples were collected at three days of age following a meal in the aforementioned study and this could explain the discrepancy between our results. Interestingly, glucose was increased in HIGH females and males as well as LOW males compared with LOW females, which could be indicative of altered glucose metabolism in these three groups. Considering the regulatory role of insulin on glucose, increased glucose concentrations may signify an alteration to insulin sensitivity and glucose clearance in offspring tissue. These findings are similar to previous reports where maternal nutrient restriction resulted in altered glucose metabolism (Ford et al., 2007). For example, Ford et al. (2007) demonstrated that maternal nutrient-restriction during gestation resulted in a greater area under the curve (AUC) for glucose at day 63 and 250 of postnatal age in offspring. In conjunction with increased glucose, offspring also exhibited a greater AUC for insulin at day 63, but by day 250, the AUC for insulin was reduced (Ford et al., 2007). We did not observe a change in insulin in combination with increased glucose at birth in calves, but the findings from Ford et al. indicate that over time, glucose tolerance may steadily decrease and consequently a decline in insulin production. To determine the mechanisms of high maternal milk production on calf glucose homeostasis and insulin sensitivity, it would be beneficial to perform an intravenous glucose tolerance test and an insulin challenge at several time points during postnatal life.

Considering the relatively high dairy calf mortality rates due to non-predator causes (5.8%; USDA AHPIS, 2011), with the leading causes of death being respiratory and digestive diseases, we evaluated the effects of increased maternal milk production on factors relating to

immunity, and more specifically passive transfer. Dairy calves are born agammaglobulinemic and thus require the transfer of immunoglobulins from dam to calf through the consumption of colostrum for the establishment of the immune system (Quigley, 1998). In the present study, changes in total protein and globulin concentrations were observed, where HIGH females displayed increased total protein and globulin compared with LOW females. While we anticipated decreased concentrations of total proteins and globulins in calves born to high producing cows, there is evidence that age of the calves at sampling can have a large impact on serum protein concentrations (Tóthová et al., 2015). For example, evaluating concentrations of IgG is best in calves at 32 hours of age or greater, indicating that interpreting serum protein concentrations is best over time (Weaver et al., 2000). Additionally, age-related reference values are necessary when determining the optimal protein concentrations but such data in dairy calves is rarely obtainable (Tóthová et al., 2015). No differences were observed in other factors relating to immunity, including haptoglobin, which is the principal hemoglobin-binding protein in the blood and typically increased during inflammation. Nevertheless, the analysis of additional factors relating to immunity and stress may aid in determining whether increased maternal milk production is predisposing calves to disease through decreased immune systems.

Tendencies for increased calcium and magnesium were also observed in HIGH compared with LOW calves. Calcium is vital for cellular functions including signaling, neurotransmission, muscle contraction, and activation of immune responses (Saris and Carafoli, 2005; Parekh, 2006), while magnesium is involved in signaling and enzyme activation (Rose, 1968). However, there are limited data on increased calcium and magnesium concentrations in early neonatal calves. Subsequently, a larger population size is required to draw conclusions from these results.

CONCLUSIONS AND IMPLICATIONS

Although the mechanisms behind current calf mortality rates remain unclear, the findings of this study provide an intriguing basis for the predisposition of poor growth and health in dairy calves. Additional research, utilizing a larger sample population and multiple time points, is necessary to fully implicate maternal milk status in altered calf development and immunity, but determining the mechanisms behind this loss in production would greatly benefit the dairy industry as a whole.

GENERAL DISCUSSION

Livestock production is an essential component of the food supply because it provides an excellent protein source for human consumption. To meet current and future demands, livestock producers must improve current production methods to ensure an ample and sustainable food supply through refining production efficiency and improving meat and dairy animal health. While there are several factors that can negatively impact the livestock sector, it has been widely demonstrated that maternal programming can have detrimental effects upon efficient animal production and consequently animal products (Wu et al., 2006; Nathanielsz et al., 2007; Hoffman et al., 2017). Maternal programming occurs when there are changes to the inter- or intra-uterine environment that have the potential to alter the trajectory of offspring development and growth (Nathanielsz et al., 2007).

Overall, the objective of this research was to identify the mechanisms involved in facilitating the effects of maternal programming throughout prenatal development and early neonatal life. There are many factors that can result in maternal programming, including maternal nutrition and energy demands. Considering muscle is the primary tissue of importance in meat production, it is important to understand the impacts maternal programming has on offspring muscle development and growth. In the first chapter, we demonstrated that maternal programming through poor maternal nutrition, which is a common occurrence within current production settings (Wu et al., 2006; Du et al., 2017), may inhibit prenatal muscle development and growth in fetal lambs. Specifically, we found that restricted- and over-feeding reduces myogenic cellular populations expressing Pax7 at several key gestational time points which coincide with the stages of myogenesis. However, despite hypothesizing that poor maternal nutrition would reduce the CSA of myofibers in these same animals, other analyses in our

laboratory demonstrate that in the present study, maternal restricted- and over-feeding does not affect myofiber CSA in each muscle at each time point (Pillai et al., 2016b). Additionally, primary and secondary fibers were increased in RES and OVER offspring in the LD and STN, signifying that smaller myogenic populations may not be affecting myofiber formation pre-natally in these muscles. However, changes we detected in CSA of offspring born to restricted- and over-fed ewes in our previous study indicate that negative alterations to offspring myofibers may not be apparent until later post-natal time points (Reed et al., 2014). Consequently, evaluating the effects of poor maternal nutrition on both pre-natal and later post-natal time points within the same study may shed more light on the inconsistencies we observe in the present studies. An RNA-seq analysis was also performed on the LD of offspring, and an effect of maternal diet was not observed on Pax7 expression (Wynn et al., *unpublished*). Considering the changes we observed in myogenic populations were in the STN and TB, it would be beneficial to perform an RNA-seq analysis on these muscles to determine whether the expression of Pax7 is altered in the STN and TB although not in the LD.

Collectively, these data demonstrate the importance of evaluating the effects of maternal programming on muscle over time, particularly when we consider the phasic development of muscle characteristic of myogenesis. Additionally, these data provide evidence that evaluating multiple muscle types is important to obtain an overall understanding of how inadequate maternal nutrition affects offspring muscle development and growth. Since meat production includes the selection of many muscle types, it is important to understand the impact of maternal programming on the quantity and quality of skeletal muscle as a whole. Many studies only evaluate one muscle type, and therefore do not provide a complete understanding of the overall effects of poor maternal nutrition.

The research in this thesis also aimed to determine whether maternal programming is a factor in predisposing dairy calves to poor growth and health. With the current practice of rebreeding domestic dairy cows during peak lactation, this metabolically demanding process may lead to a mobilization of body energy reserves, thereby resulting in the partitioning of nutrients away from the developing calf (González-Recio et al., 2012). Based on the findings of Kamal et al. (2014, 2015), we were interested in whether high milk production during gestation is a factor linked to the relatively high mortality rates of dairy calves (USDA APHIS, 2011). Each calf death represents lost genetic potential for the dairy industry, and discovering the mechanisms behind these instances of mortality are critical for optimizing dairy production. Our present study evaluated several factors in dairy calves relating to growth and health in an effort to determine the effects of high maternal milk production on calf development and immunity. While many of the investigated factors remained unchanged between calves born to high and low milk producing calves, additional research utilizing a larger sample population and greater control is required to fully implicate maternal milk production in poor calf growth and health. Similar to our previous study on the effects of maternal nutrition on offspring muscle development, these data also represent the necessity of evaluating the effects of maternal milk production over time.

CONCLUSIONS AND IMPLICATIONS

In conclusion, identifying factors relating to maternal programming is critical for optimal livestock production. Maternal diet during gestation, either through restricted- or over-feeding, can greatly impact muscle development and growth. Through improved extension efforts, producers could proactively use this information to ensure they are properly managing their animals and optimize their production quantity and quality. The role of maternal energy demands

on offspring development and growth still remains largely unknown and requires additional research, but may potentially be a mechanism behind altered calf growth and health. Overall, these findings can be used to further advance meat and dairy production, thus helping to sustain an efficient and ample food supply for future generations.

APPENDIX I: Pax7 Staining Protocol

1. Section muscle 5-10µm think (3 sections/slide) using cryostat
2. Heat fix slides for 1 minute on heat block (at 1)
3. Circle sections/edge of slide with Vaseline or pen
4. Fix tissue for 20 minutes in 4% paraformaldehyde in PBS (located in fridge; light sensitive)
5. Wash 3x with PBS (2 minutes each)
6. Block for 20 minutes in 5% horse serum, 0.2% Triton-X100 in PBS (located in fridge); remove block
7. Place in primary antibody/fresh blocking solution overnight at 4°C in a humidified chamber with 200µL/slide
    a. Pax7 concentrate antibody (Hybridoma core) – 1:1000
8. Wash 3x with PBS following morning (5 minutes each)
9. Place in secondary antibody (goat anti-mouse AlexaFluor 488 – 1:250) and Hoescht 33342 dye (1:2000) in fresh blocking solution for one hour with 200µL/slide
10. Wash 3x PBS (2 minutes each)
11. Coverslip with 1:9 PBS:glycerol and image @ 20X

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