EFFECT OF MATERNAL METABOLIZABLE PROTEIN SUPPLEMENTATION DURING LATE GESTATION ON FETAL VISCERAL ORGAN WEIGHT WITH APPLICATION OF PROTEOMICS TO INVESTIGATE SKELETAL MUSCLE DEVELOPMENT IN SHEEP

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MASTER OF SCIENCE

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ABSTRACT

The experiment investigated supplementation of maternal metabolizable protein in an isocaloric diet during late gestation on fetal organ growth and skeletal muscle development in sheep. Although fetal body weight was unaffected by treatment, visceral organ weights were sensitive to MP treatment as alterations in small intestinal mass and perirenal adipose tissue content suggest impacts on postnatal growth. Two-dimensional gel electrophoresis and mass spectrometry compared the sarcoplasmic and myofibrillar protein fractions of fetal skeletal longissimus muscle, identifying spots from the sarcoplasmic and myofibrillar protein fractions (30 and 12, respectively). Increased expression of fructose-bisphosphate aldolase in fetal muscle from protein-supplemented and protein-restricted ewes when compared to those from control ewes suggests a positive influence on rate of glycolysis and muscle turnover in these offspring. Further research on maternal supplementation with metabolizable protein in isocaloric diets is needed to improve fetal growth efficiency, thereby optimizing offspring performance.

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DEDICATION

To Wanda,

Tell me and I forget,
Teach me and I may remember,
Involve me and I learn.

Thank you for inspiring me
in lab and life.

Science is truly best with chocolate…and a friend.

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LIST OF ABBREVIATIONS

˚C .....degrees Celsius

µL.....microliter

µm.....micrometer

2D.....two dimensional

ADF..... acid detergent fiber

ADG.....average daily gain

ADP..... adenosine diphosphate

ATP .....adenosine triphosphate

ALDOA..... Aldolase A

ANPC..... Animal Nutrition and Physiology Center

AOAC ..... Association of Official Analytical Chemists

BCS .....body condition score

BF.....back fat

BUN .....blood urea nitrogen

BW .....body weight

Ca .....calcium

CCR..... curved crown rump

CHCA .....a-cyanohydroxy cinammic acid

CID.....collision induced dissociation

CIDR .....controlled internal drug release

Co.....cobalt

CP..... crude protein

Cu.....copper

Cy ..... cyanine

Cy2 ..... cyanine 2 yellow dye

Cy3 ..... cyanine 3 red dye

Cy5 ..... cyanine 5 blue dye

CyDye ..... cyanine dye

d ..... day

Da ..... Dalton

DDG ..... dried distiller grain

DIGE ..... difference in gel electrophoresis

DM ..... dry matter

DNA ..... deoxyribonucleic acid

DP ..... digestible protein

DTT ..... DL-dithiothreitol

EBW ..... eviscerated body weight

EDTA ..... ethylenediamine tetraacetic acid

FW ..... fetal weight

g ..... gravity

g ..... gram

GIT ..... gastrointestinal tract

HPLC ..... high pressure liquid chromatography

h ..... hour

I ..... iodine

IM ..... intramuscular

Int ..... integrase

IPG ..... immobilized pH gradient

IU ..... international unit

kg ..... kilogram

LC ..... liquid chromatography

LEA ..... loin eye area

LISS ..... low ionic strength sarcoplasmic buffer

LM ..... longissimus

MALDI ..... matrix-assisted laser desorption/ionization

ME ..... metabolizable energy

Mg ..... magnesium

mg ..... milligram

mgf ..... Mascot generic format

MHz ..... mega hertz

min ..... minute

mL ..... milliliter

MLC1 ..... myosin light chain 1

mM ..... millimolar

mm ..... millimeter

Mn ..... manganese

mo ..... month

MP ..... metabolizable protein

MRF ..... myogenic regulatory factors

MRF-4 ..... myogenic transcription factor 4

mRNA ..... messenger ribonucleic acid

MS ..... mass spectrometry

MSB ..... myofibrillar solubilizing buffer

Myf-5 ..... myogenic factor 5

Myo-D ..... myogenic determination

N ..... nitrogen

NaCl ..... sodium chloride

NADPH ..... nicotinamide adenine dinucleotide phosphate-oxidase

NaOH ..... sodium hydroxide

NASS ..... National Agriculture Statistics Service

NDF ..... neutral detergent fiber

NH^3 ..... ammonium

NRC ..... National Research Council

NPN ..... nonprotein nitrogen

P ..... phosphorus

Pax ..... paired box gene

PGM1 ..... Phosphoglucomutase 1

pH ..... potential hydrogen

pI ..... isoelectric point

PM ..... psoas major

ppm ..... parts per million

RE ..... retinol equivalent

RNA ..... ribonucleic acid

rpHPLC ..... reversed phase high performance liquid chromatography

RUP ..... ruminally undegradable protein

SAS ..... Statistical Analytical Software

SDS-PAGE ..... sodium dodecyl sulfate polyacrylamide gel

Se ..... selenium

SE ..... standard error

SM ..... semimembranosus

T2D ..... technology transfer database

TL ..... fasciae latae

TNE ..... Tris, sodium chloride, EDTA buffer

TnT ..... troponin-T

TOF ..... time-of-flight

UIP ..... undegradable intake protein

UniProt ..... Universal Protein resource

UV ..... ultraviolet

V ..... volt

Vit ..... vitamin

vs. ..... versus

vol ..... volume

Wnt ..... wingless type

wt ..... weight

Zn ..... zinc

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

Introduction

Sheep and lamb inventory in the United States on January 1, 2012, totaled 5.35 million, down 2 % from 2011 [National Agriculture Statistics Service (NASS), 2012]. The quality of the livestock operations producing these animals has been collectively determined by each producer's reproductive, nutritional, and rangeland management practices. Current sheep research has focused primarily on increasing lamb production by increasing the number of lambs born per ewe. The 2011 lamb crop of 3.51 million head had a lambing rate of 109 lambs per 100 ewes one year or older, and this percentage continues to increase (NASS, 2012).

Past studies conducted in early-life nutrition in sheep began in the 1970s and were focused on the early neonatal period and the latter part of pregnancy. These periods were selected due to the fact that the nutrient demands on the fetus are highest at these times (Robinson et al., 1983). Offspring born to ewes undernourished during late pregnancy produce fewer lambs during their adult life because those offspring have a lower incidence of twin lambs (Gunn et al., 1995; Rhind et al., 1998). These observations indicate that one or more physiological mechanisms are affected by the nutritional state of the offspring during late fetal development. Information concerning the underlying mechanisms that encourage muscle growth and nutrient utilization in large animal species are largely lacking. We are especially concerned with factors that limit fetal growth in sheep because in the field, neonatal mortality rates are high in lambs having birth weights below breed averages (Purser et al., 1959; 1964; Alexander, 1974). Previous work by Barcroft (1946) found fetus variation in length and weight is proportionately greater at birth than at any other stage of pregnancy, indicating that much of this variability can be explained by different levels of maternal nutrition during late gestation (Wallace, 1948;

Thomson, 1959; Russell et al., 1968; Alexander, 1974). A better understanding in this regard provides a means to maximize livestock productivity while improving animal health and wellness.

The most important factor effecting success of most commercial sheep operations is reproductive efficiency (Lupton, 1998), defined as the BW of the lamb weaned or marketed per ewe exposed. Sheep are raised for food and fiber, and while reproductive efficiency is very important, it is also one of the most difficult factors to improve. With profit margins continuing to decrease, an increased effort has been placed on maximizing lamb viability (NASS, 2012).

Current agricultural production goals have focused on the need for energy conservation and improved efficiency of economic resources. Currently, the sheep industry is focused on increasing the number of lambs per ewe while improving the growth efficiency of those offspring. New advancements in animal science research offer the possibility for significant improvements in efficiency of meat and fiber production from sheep.

Maternal Nutritional Status

Maternal nutrition during pregnancy has a critical role in the regulation of placental-fetal development and thereby affects the lifelong health and productivity of offspring (Belkacemi et al., 2010). The dam must alter the partitioning of available nutrients to allow for maintenance of her own body tissues and the increasing demand for micronutrients by the gravid uterus. The major challenge pregnant ewes must overcome is providing adequate energy and protein to support embryonic and fetal growth, maintenance of the animal's physiology needs, mammary gland growth, colostrum and milk yield (Amanlou et al., 2010). Suboptimal maternal nutrition can result in low birth weight, with considerable effect, on perinatal morbidity and mortality of the newborn.

Pregnancy nutrition and its impact on offspring growth and performance have become increasingly prominent over the past decade. Maternal nutrition status is an important factor associated with nutrient partitioning and eventually the growth and development of major fetal organ systems (Wallace, 1948; Wallace et al., 1999; Godfrey and Barker, 2000; Wu et al., 2006; Caton et al., 2009, Caton and Hess, 2010). Mature body size of an ovine breeding female can range from less than 40 kg to more than 130 kg, having a major influence on total nutrients required and on adaptations to various conditions (NRC, 2007). There is a substantial increase in nutrient requirements for the late pregnant ewe compared with maintenance (Table 1.1).

One of the most essential nutrient requirements for both a pregnant ewe and her growing fetus is that of protein. Protein supplementation during late gestation is a common management practice for maintaining dam body condition score (BCS) and has been shown to enhance reproductive success of heifer calves (Martin et al., 2007) and increase weaning weight of steer calves (Stalker et al., 2006).

It appears that restricting maternal protein in late gestation has a greater impact on ewe weight than restricting energy. Hoaglund et al. (1992) used 30 Targhee ewes in an experiment investigating protein and energy supplementation. In the study, ewes were offered either no supplement, soybean meal (to meet ruminal protein needs), or blood meal + soybean meal (to provide additional protein above needs throughout gestation). The ME treatments provided either 80% or 100% of ME requirements during gestation. The group was also offered ad libitum access to long-stem barley straw. Ewes fed either of the protein supplementation treatments gained more weight and lost less body condition when compared to that of the unsupplemented ewes. There was no effect of ME intake on ewe BW or BCS, suggesting that protein may be more limiting than energy.

Table 1.1. Recommended nutrient intakes and dietary nutrient content for a mature 70-kg ewe at various physiological states[1]

Physiological State	Energy concentration in diet	Dry matter[2]	ME[3]	MP[4]
	Kcal/kg	kg/d	Mcal/d	g/d
Maintenance	1.91	1.68	2.25	60
Gestation, Early (Single lamb; BW = 3.9 to 7.5 kg)	1.91	2.09	2.80	81
Gestation, Late (Single lamb; BW = 3.9 to 7.5 kg)	1.91	2.58	3.45	105

[1]Adapted from NRC, Nutrient Requirements of Sheep, 2007.
[2]The daily dry matter intake, expressed as kg, of a diet having the indicated energy concentration (previous column) that is required to meet energy requirements.
[3]Energy requirements expressed as metabolizable energy (ME) as kcal/d.
[4]Protein requirement expressed as metabolizable protein (MP).

Fetal programming, also known as developmental programming or the Barker hypothesis, is based on epidemiological data that show that low birth weight due to maternal malnutrition has long-term effects on adult health (Barker et al., 2002). The hypothesis of fetal programming implies that changes in the uterine environment as a result of nutritional stress at certain stages of conceptus growth and development might permanently change tissue structure and function (Drake and Walker, 2004).

In ruminants, metabolizable protein (MP) is the combination of ruminal undegradable intake protein (UIP; protein that escapes rumen microbial breakdown) and bacterial crude protein (CP) that enters the small intestine and can be broken down and absorbed by the animal [Figure 1.1] (Loe et al., 2004). The ewe's requirement for MP is met from the digestible fractions of the microbial CP synthesized in the rumen in proportion to ME intake and the dietary CP that

escapes degradation in the rumen (Annison et al., 1959). The requirement for MP to support fetal growth in pregnancy is very low in the early stages and only becomes significant in cattle in the final 2 mo of gestation or the final month in sheep (Fuller, 2004). This protein requirement takes into account the main activities of the body and what is needed to maintain essential functions including lactation, pregnancy, and weight change. The amount of MP required for lactation is substantial during late pregnancy and has a significant effect on the amount of protein a ruminant needs to support pregnancy. Maximal fetal development is important to increase the growth potential of animals (Du et al., 2010). Limitations on protein intake inflict a restriction on the microbial population in the rumen, which has specific nutrient requirements for optimal growth (Robinson, 1982). Maternal nutrition during pregnancy is important in determining optimal fetal development, pregnancy outcome, and ultimately lifelong wellbeing of offspring.

Fetal Development and Nutrition

Fetal nutrient availability is dependent upon maternal food intake, availability of nutrients in the maternal circulation, and the ability of the placenta to efficiently transport substrates to the fetal circulation (Battaglia and Meschia, 1988). The placenta forms the interface between maternal-fetal circulations and, as such, is critical for fetal nutrition and oxygenation. In turn, the placental supply of nutrients to the fetus depends on its size, morphology, blood supply, and ability of transport (Belkacemi et al., 2010). An adjustment in the maternal nutrient supply may result in altered placental structure and function, consequently hindering fetal nutrition. Wallace et al. (1999) investigated the consequences of nutritionally-mediated placental growth restriction on ovine fetal organ growth, conformation, and body composition during late gestation. In this study, dams were offered a high or moderate level of complete diet to encourage rapid or

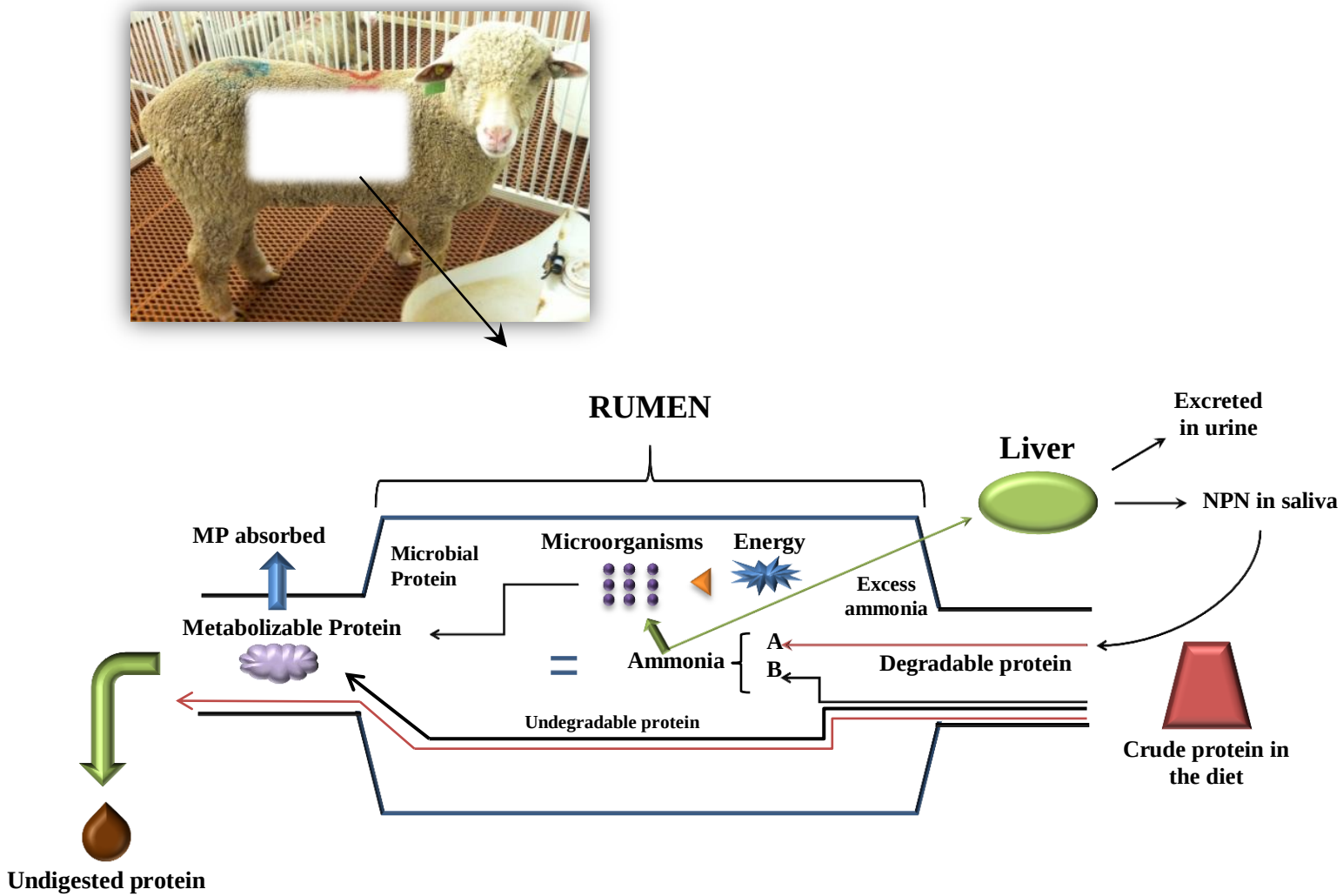

Figure 1.1. Metabolizable protein digestion in the rumen.

moderate maternal growth rates. After 100 d of gestation, placentome weight was 50% lower in high compared with moderate groups and was associated with a 37% reduction in fetal weight ($P < 0.01$). All variables of fetal conformation and absolute fetal organ weights, with the exception of the adrenal glands, were lower ($P < 0.05$) in fetuses from high compared with moderate groups. Wallace et al. (1999) reported that over-nourishing sheep is associated with not only a restriction in placental growth, but also the slowing of fetal growth during the final third of pregnancy.

Maternal undernutrition can also influence placental-fetal development. McCrabb et al. (1990) investigated the relationship between placental and fetal weight after placental growth was slowed by maternal undernutrition. In this study, single-lamb-bearing ewes were well-fed or severely undernourished between the d 30 and d 96 of gestation. They reported an increase ($P < 0.01$) in placental weight measured on d 96 (21%) and d 140 (30%) of pregnancy in undernourished ewes. Fetal growth, however, was not affected by maternal undernutrition in the experiment. In agreement with this study, Faichney and White (1987) found that dietary restriction stimulated placental growth in sheep to the extent that fetal growth could be maintained when the restriction occurred during the third trimester but not when applied throughout the second and third trimesters.

The placenta reaches maximum cell proliferation between d 50 and 60 of gestation followed by culmination of DNA and mass accumulation between d 75 and 80 of gestation (Ehrhardt and Bell, 1995). The fetus however, accumulates almost 80% of its eventual mass at birth during the final third of pregnancy (Wallace et al., 1997). Maternal nutrition is an important determinant of optimal fetal development. However, previous studies call attention to placental function as it facilitates the maternal-fetal transfer of nutrients which is critical for development

of a healthy fetus. From this information we can see that a combination of factors originating from maternal, placental, and fetal nutritional sources act together to regulate the growth of the fetus.

Muscle Growth in Sheep Fetus

To understand the effects on sheep due to varying levels of maternal MP supplementation or restriction during late gestation, it is first necessary to understand the muscle physiology of the sheep fetus. Muscle fibers are the structural units of skeletal muscle. The formation of new muscle fibers is termed myogenesis, a differentiation process where multipotent stem cells are converted into committed muscle cells (Yan et al., 2012). In livestock, all muscle fibers are formed during the prenatal stage. Therefore, understanding the prenatal development of skeletal muscle is important because events occurring at this stage have dramatic impact on postnatal development and growth (Dauncey and Harrison, 1996).

There are 3 distinct phases of myogenesis during gestation, eventually concluding early in the third trimester (Maier et al., 1992; Wilson et al., 1992). Development of primary skeletal muscle fibers is initiated during the embryonic stage (Cossu and Borello, 1999). Primary fiber formation takes place in the first trimester (d 15 to d 50) and serves as a template for the formation of secondary muscle fibers. Mesenchymal stem cells commit to a myogenic lineage and form the primary and secondary muscle fibers. Prior to this formation, these cells are controlled by a group of regulatory proteins, including Wingless and Int (Wnt), paired box gene (Pax) 3 and Pax 7 (Maroto et al., 1997; Hyatt et al., 2008). Wnt signaling is an important factor in activating myogenesis, while Pax 3 and Pax 7 induce the expression of myogenic regulatory factors (MRF). MRF include myogenin, MRF-4, Myo-D and Myf-5, and their expression leads to muscle differentiation. Following formation of primary fibers, the first wave of secondary

fiber formation occurs from the end of the first trimester to the middle of the second trimester (d 48 to d 80). Secondary myotubes form in preparation for the final wave of secondary (tertiary) fiber formation. At this time (d 75 to d 105) a second wave of fiber formation allows myotubes to fill in the spaces not already occupied by existing fibers, completing myogenesis early in the third trimester (Yates et al.,2012). The secondary waves of myogenesis produce the majority of muscle fibers. Consequently, the fetal stage during which secondary myogenesis is taking place is critical for skeletal muscle development (Greenwood et al., 2000; Du et al., 2010). Moreover, since the number of muscle fibers formed during the fetal stage is dependent upon the proliferation of myogenic cells, which are highly sensitive to nutrients, maternal nutrition dramatically affects skeletal muscle development during this time (Zhu et al., 2004).

After myogenesis (approximately d 105) is accomplished, muscle growth persists through muscle fiber hypertrophy (Greenwood et al., 1999). The size of the muscle fiber depends on myoblast and corresponding myonuclei incorporation at this time, resulting in increased DNA content (Allen et al., 1979; Trenkle et al., 1978; Davis and Fiorotto, 2009). Nutrition during this stage is especially important because sheep are produced primarily for meat. A decrease in the number of muscle fibers due to maternal nutrition may permanently reduce muscle mass and negatively impact animal performance. The number of fibers in an adult muscle depends on the number of primary myotubes first formed, and muscle fiber numbers are sensitive to prenatal environmental conditions (Wilson, 1992).

Proteins within the muscle are divided into several classes based on their cell location and solubility. Bate-Smith (1934) revealed differences between intracellular and extracellular proteins. Making up 55% of total protein, the myofibrillar protein fraction contains, contractile (responsible for muscle contraction), regulatory (control of muscle contraction), and cytoskeletal

(support and maintenance of myofibril) proteins that are insoluble in water. There are about 9 major proteins in this group that are found within a myofibril of an intact muscle (Table 1.2). During times of nutritional stress, these proteins are an important source of gluconeogenic carbon and essential amino acids (Young, 1970; Waterlow et al., 1978).

Table 1.2. Major myofibrillar proteins in skeletal muscle[1]

Protein	Location	% Myofibrillar Protein
Myosin	Thick filaments	43
Actin	Thin filaments	22
Tropomyosin	Thin filaments	5
Troponin (TnI + TnT = TnC)	Thin filaments	5
Titin	Full Sarcomere	8
Nebulin	Thin filaments	3
Myosin binding protein C	Thick filaments	2
Alpha-actinin	Z-lines	2
M-protein	M-lines	2
Desmin	Z-lines	< 1

[1]Adapted from Romans, J. R., W. J. Costello, C. W. Carlson, M. L. Greaser, and K. W. Jones. 2001. The meat we eat. Interstate Publishers, Inc., Danville, IL. p. 900.

Table 1.3. Major sarcoplasmic proteins in skeletal muscle[1]

Enzyme	Content (mg/g)
Phosphorylase	2.0
Amylo-1,6-glucosidase	0.1
Phosphoglucomutase	0.6
Phosphoglucose isomerase	0.8
Phosphofructokinase	0.35
Aldolase	6.5
Triose phosphate isomerase	2.0
Glyceraldehyde phosphate dehydrogenase	11.0
Phosphoglycerate kinase	1.4
Phosphoglycerate mutase	0.8
Enolase	2.4
Pyruvate kinase	3.2
Lactic dehydrogenase	3.2
Creatine kinase	5.0

[1]Adapted from Scopes, R. K. 1973. J. Biochem. 134:197.

Sarcoplasmic proteins (Table 1.3) account for 30-35% of total muscle proteins and are found inside the cell membrane. These proteins are soluble in water and include myoglobin, oxidative, glycolytic and lysosomal enzymes (Smyth et al., 1999). Myoglobin is an oxygen-binding protein found within this group that is responsible for the typical red color in meat. Both myofibrillar are sarcoplasmic proteins have specific roles in development of fetal skeletal muscle tissue important in the life of the animal.

Intrauterine Nutrient Supply and Muscle Development of Offspring

Maternal nutrition affects fetal development, specifically fetal skeletal muscle development (Zhu et al., 2004). The trajectory of skeletal muscle development and growth is slowed in fetuses that are nutrient-restricted. Ultrasonic measurements of undernourished fetuses show that muscle masses are reduced (Padoan et al. 2004).

The timing of nutrient restriction is a very important determinant of skeletal muscle growth of the fetus. Nutritional insults during early to mid-gestation interfere with myotube formation and reduce fiber density in skeletal muscle (Yates et al., 2012). Zhu and others (2004) found that maternal nutrient restriction between the middle of first to the middle of the second trimester in sheep lowers the number of secondary fibers per fasciculi in the fetal longissimus dorsi (LM) muscle. In another study, Quigley (2005) determined that secondary fiber density is also lower in the fetal semitendinosus muscle from ewes recovering from malnourishment at peri-conception. These data confirm the decrease in fetal myofibers due to maternal nutrient restriction during the first trimester.

Several studies have investigated the effects of maternal under-nutrition in late gestation on the development of skeletal muscle in sheep. Estêvão et al. (2011) observed a substantial effect on the development of muscle mass in lambs from dams fed a restricted diet (50% of

nutrient requirements) when compared with those fed ad libitum during the last six wk of gestation. Results from this study indicate that weight at birth and muscle weight are lower in lambs from dams fed 50% of nutrient requirements during the last 6 wk of gestation when compared to dams fed ad libitum. Everitt and others (1968) found that myofiber number is reduced when the dam goes through severe nutritional insult during late gestation. A study conducted by Greenwood and others (1999) reported that maternal nutrient restriction during late gestation reduces fetal sheep muscle fiber size but not muscle fiber number. Additional research is needed in order to study the influence of a more specific nutrient form, such as protein supplementation or restriction, on the dam during these critical periods of fetal skeletal muscle development.

Ovine fetal skeletal muscle DNA, ribonucleic acid (RNA), and protein concentrations have also been altered by maternal nutrient restriction during late gestation. Quigley et al. (2005) found an increase in total muscle fiber number and protein: DNA ratio in fetuses whose mothers received a high feed intake over the peri-conception period compared with those whose mothers received a low intake, although no change in muscle weight was observed. Others (Greenwood et al., 2000) found that DNA, RNA, and protein are significantly reduced in low birth weight lambs when compared with that of high weight lambs in a study investigating effects of birth weight on growth and development of skeletal muscle in neonatal lambs.

Despite differences in mass and cellularity, no differences are observed in the frequency of Type I or II muscle fibers due to late gestational nutrient restriction (Tygesen and Harrison, 2005). This is likely because the critical stage for fetal skeletal muscle development, specifically fiber formation, is early to mid-gestation in sheep. Alterations in proliferative rate necessary to change muscle fiber numbers occurred earlier than the period of nutrient restriction, and the

skeletal muscle had most likely reached steady-state by late gestation. Muscle cell proliferation has been observed before d 85, and differentiation commences around d 85 (Fahey et al., 2005), supporting this hypothesis. Restricted nutrient supply to the fetus also affects postnatal metabolic homeostasis in skeletal muscle by reducing glucose oxidation rates, impairing insulin action, and lowering the proportion of oxidative fibers (Yates et al., 2012). In animal models of placental insufficiency, skeletal muscle fibers have less myonuclei at birth, resulting in fewer myoblasts and reduced muscle fiber size.

Numerous studies have demonstrated that nutrient manipulation during gestation has adverse effects on offspring. Lambs grow slower within the first 2 weeks post-partum, preventing their ability to properly utilize energy for fat and protein deposition, while having a lower intramuscular concentration of DNA, resulting in reduced postnatal skeletal muscle growth (Greenwood et al., 1998, 2000). Additionally, a reduced myofiber number in lambs from undernourished dams restricts the potential for postnatal compensatory growth of skeletal muscle (Wu et al., 2006).

Together, these data reveal the overwhelming adverse effects of maternal nutrient restriction on fetal skeletal muscle growth. From the start of conception, fetal myoblasts can be compromised by alterations in maternal nutrition, lowering myonuclei content and altering fiber phenotypes, thus preventing normal metabolic regulation. Although common measurements have not shown changes in fetal skeletal muscle composition when maternal nutrients are modified during late gestation, it does not mean that other aspects of muscle growth are not being affected. Proteomics may be applied to take a closer look at the protein profile of muscle, identifying post-

translational modifications, protein-protein interactions, and small changes in proteins that may provide further insight into the possible effects of maternal nutrition on fetal skeletal muscle development during late gestation.

Proteomics

Skeletal muscle development of the fetus is influenced by a number of different factors such as maternal genetics and environmental factors. During the lifetime of an animal the genes remain constant, while the expression of genes to mRNA and proteins is regulated by a large number of factors such as environment and developmental conditions which may subsequently influence meat quality (Hollung and Veiseth-Kent, 2012). However, understanding the molecular mechanisms by which these quality traits are determined requires further investigation. The proteome is the complete protein complement expressed by the genome (Wilkins et al., 1996). While the genome contains the informationon which genes and alleles are present in the genome, the proteome contains information on which genes are actually being expressed and translated into proteins. In contrast to the genome, the proteome is constantly changing, influencing protein synthesis and degradation. Thus, understanding the variation and different components of the proteome with regard to different experimental treatments may lead to knowledge that may be used in optimizing growth and development of muscle foods. Proteomics are the means by which the proteome is analyzed. With these tools, studies may now be conducted without any prior hypothesis on the mechanisms involved.

The field of proteomics has grown steadily in the last decade due to advances in mass spectrometry (MS), genomics, and bioinformatics (Lippolis and Reinhardt, 2008). Essentially, there are 2 analytical strategies used in proteomics. One is based on two-dimensional difference in-gel electrophoresis (2D DIGE) for separation of proteins, followed by identification of

separated proteins by MS. The second is based on liquid chromatography (LC) in one or more dimensions coupled with MS. Proteomics experiments can target a certain known protein of interest, or an indirect or shotgun approach can be taken. In the shotgun approach large complex proteomes are analyzed for changes in protein presence, expression, or modification. The large amount of data resulting from this experimental approach can yield unexpected results and may depend on the sensitivity of the mass spectrometer and the number of proteins the experiment can identify. In contrast to a Western blot, shotgun proteomics requires no specific antibody for detection of a protein and can aid in identifying many different proteins simultaneously.

Mass Spectrometry

Mass spectrometry measurements are carried out in the gas phase on ionized analytes. The mass spectrometer consists of the ion source, mass analyzer (measures the mass-to-charge ratio (*m/z*)), and the detector that registers the number of ions at each *m/z* value (Mann et al.,2001). Tandem MS or LC/MS may also be performed in order to separate peptides on reverse-phase high performance liquid chromatography (HPLC). Then the most abundant proteins are fragmented by collision-induced-dissociation (CID). This process identifies the "daughter ions" by *m/*z ratio, separating into *b* and *y* ions (Ho et al., 2003). The charge remains on the amino terminal or on the carboxy terminal fragment with the *b* and *y* ions, respectively. Matrix-assisted laser desorption/ionization (MALDI) is used in order to identify proteins from a gel spot or band collected. The sample is suspended in a matrix of crystals on a plate where ultraviolet (UV) lasers excite the analyte ion.

By means of MS we can delve further into the global analysis of protein composition, post-translational modifications, and the true nature of expression intensities (Aebersold and Mann, 2003; Guerrera and Kleiner, 2005; Yates et al., 2009). Mass spectrometry-based

proteomics identifies distinct protein species by analyzing trypsin-generated peptide mixtures of proteins of interest. Just in the last few years, this technique has successfully catalogued several hundred of the most abundant and soluble muscle-associated protein species and identified several thousand distinct protein isoforms present in skeletal muscle tissues (Capitanio et al., 2005; Hojlund et al., 2008; Raddatz et al., 2008; Parker et al., 2009).

Technical advances have improved the sensitivity and accuracy of mass spectrometers necessary for proteomic work (Lippolis and Reinhardt, 2008). Despite these improvements, a number of issues cause difficulty during protein identification. For example, the number of proteins that comprise the human proteome is approximately 30,000 proteins, not counting alternative splicing and post-translational modifications (Cho, 2007). Also, the range of protein expression interferes with detection of low abundance proteins in typical biological samples (Lippolis and Reinhardt, 2008). One of the most common examples of this obstacle is that of plasma. Nearly half of the protein in plasma is albumin, and the top 10 proteins in plasma make up nearly 90% of the total protein (Cho, 2007). As a result of these challenges, many protein separation methods before MS have been developed in order to create less complex mixtures for a more complete identification of proteins. Fractionation can be achieved by subcellular fractionation, enrichment strategies, chromatography, or gel electrophoresis (Stasyk and Huber, 2004). The goal of all protein or peptide separation methods is to better enable detection of all the proteins in a proteome. Mass spectrometry-based proteomics are useful when confirming a particular protein or protein modification. The method can also be used as a screening tool for protein-protein and protein-DNA interactions. However, MS proteomics should not be used as a stand-alone method for biological/physiological studies. Mass spectrometry can be biased toward the more predominant peptides, which may result in an incorrect identification.

Fluorescence Two-Dimensional Difference In-Gel Electrophoresis

The fluorescent 2D DIGE method involves direct labeling of the lysine groups on proteins with cyanine (Cy) dyes before isoelectric focusing. This advancement in 2D DIGE technology generates the ability to label 2 to 3 samples with different dyes and electrophorese all the samples on the same 2D gel, thus reducing spot variability and the number of gels in an experiment, while yielding simple and accurate spot matching (Tannu and Hemby, 2006). The cyanine dye (CyDye) DIGE fluor minimal dye had an *N*-hydroxysuccinimidyl ester reactive group. CyDye is designed to form a covalent bond with the epsilon amino group of lysine in proteins via an amide linkage. The single positive charge of the CyDye replaces the single positive charge of the lysine at neutral and acidic pH, keeping the isoelectric point (pI*)* of the protein relatively unchanged. The labeling reaction is dye limiting and the ratio of CyDye to sample (100 to 300 pmol: 50 µg) ensures that the dyes label approximately 1 to 2% of lysine residues. Therefore, each labeled protein carries only one dye label and is visualized as a single protein spot.

The most common experimental design for a 2D DIGE experiment is one in which a pooled internal standard is labeled with cyanide cyanine 2 yellow dye (Cy2) dye and the treatment and/or control groups are labeled with either cyanine 3 red dye (Cy3) or cyanine 5 blue dye (Cy5) dyes. The internal standard acts to decrease biological and experimental between-gel variation and improves statistical analysis. Utilizing this design, the individual protein data from control and/or treatment samples are normalized against the Cy2-labeled sample.

Utilizing the above experimental design for 2D DIGE, Anderson et al. (2012) reported the release of myosin light chain 1 (MLC1) from the myofibrillar fraction of postmortem aged bovine LM muscle as a potential indicator of proteolysis and tenderness in beef. Labeling d 1

postmortem with Cy3, d 14 postmortem with Cy5, authors were able to measure intensities of both the sarcoplasmic and Myofibrillar protein fractions. In this study, 12 spots the differed in relative abundance ($P < 0.10$) between the two treatment groups (high star probe vs. low star probe value steaks) were identified.

Although the use of this technology in the field animal science is relatively new, it holds great potential as a method for furthering our understanding of complex biological systems that control the physiology and pathology of our livestock species. The use of 2D DIGE has been used in meat science to identify the protein profile of muscle, including postmortem changes that alter the structure of the myofibril to identify potential indicators of tenderness (Hollung et al., 2007). Hamelin et al. (2006) observed post-translational modifications and protein fragments in his proteomic analysis of ovine muscle hypertrophy. Key proteins associated with energetic metabolism, contractile apparatus, detoxification, and regulators were identified and investigated. In their study, the same protein was found in different spots at different pI and molecular weight locations within a muscle, between different muscles, or both. These different locations are thought to correspond to different isoforms that can result from the expression of different genes. Also, these isoforms may be triggered by post-translational modifications such as oxidation, glycosylation, phosphorylation, and proteolytic cleavage. Upon analyzing the semimembranosus (SM) vastus medialis (VM), LM, and fasciae latae from Romanov ewes and Belgian Texel rams, it was observed that isoforms differentially expressed between genotypes showed similar patterns of between-muscle expressions. This study demonstrated the capability proteomics holds to discover how the functional abilities of muscle may require different protein isoforms, leading to a greater understanding of the energetic metabolism of muscle. In another study concerning hypertrophy, Bouley et al. (2004) utilized 2D DIGE followed by MS in order

to identify the key proteins that determine bovine meat quality. This method resulted in the identification of 75 proteins, including several troponin-T (TnT) isoforms, helping us to better understand the physiology of our muscle foods.

There have been few published studies to date investigating offspring organ and muscle development as affected by maternal nutrition using the proteomic approach. Sarr et al. (2010) observed numerous differences in abundance of the adipose tissue proteome of neonatal piglets from high protein and low protein supplemented German Landrace sows when compared with the control. Several of these differences included enzymes that participate in successive steps of glycolysis, a metabolic pathway that contributes to lipid accumulation in adipose cells (Temple et al., 2007). The protein transaldolase-1 was found to be greater in low protein piglets than control piglets, providing a link between the glycolytic pathway and the pentose phosphate pathway to produce reduced NADPH for lipogenesis (Wamelink et al., 2008). With the use of proteomics, it was possible to determine glucose conversion into fatty acids as an early upregulated route in response to the prenatal low protein diet. Once integral muscle proteins are sequenced via proteomics, correlation of these findings with genomic, transcriptomic, and metabolic databanks in order to establish the overall relationship in striated voluntary muscle tissues will increase understanding of fetal skeletal muscle development (Yi et al., 2008). Investigation of the fetal skeletal muscle proteome as influenced by maternal protein supplementation may lead to a better understanding of the molecular mechanisms behind fetal growth efficiency.

Maternal nutrition during late gestation is crucial to the successful development and growth of the fetus. Further research is necessary to better understand the role of supplementing protein in an isocaloric diet to further realize the effects of maternal nutrition on fetal organ

growth and skeletal muscle development. A better understanding of the effects of maternal MP supplementation in an isocaloric diet on the effects of gestational nutrition on fetal growth efficiency will allow for the development of management strategies to optimize livestock performance.

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CHAPTER 2. INFLUENCE OF METABOLIZABLE PROTEIN SUPPLEMENTATION IN LATE PREGNANCY ON VISCERAL ORGAN MASS AND INDICES OF MUSCLE GROWTH IN SHEEP

Abstract

The objective of this study was to evaluate the effects of maternal metabolizable protein (MP) supply during late gestation on fetal growth and organ development in sheep. Multiparous singleton pregnant ewes (n = 18) were randomized to receive 1 of 3 diets that were isocaloric and formulated to supply 60% (**MP60**), 100% (**MP100**), or 140% (**MP140**) of MP requirements during late gestation (d 100 to 130). Metabolizable protein requirements were calculated as: MP (g/d) = (CP, g/d × (64 + (0.16 × undegraded intake protein of diet)))/100. Pregnant ewes and fetuses were euthanized and necropsied on d 130 ± 1 SD of gestation. Data were analyzed using PROC GLM of SAS. There was no effect ($P \geq 0.12$) of maternal MP supplementation during late gestation on fetal BW, empty BW, curved crown rump length, heart girth circumference, or adrenal, brain, heart, kidney, lung, pancreas, large intestine, spleen, and stomach weights when expressed as g or as g/g BW. Fetuses from MP140 ewes had increased ($P \leq 0.05$) small intestinal mass (8.8 ± 0.4 g/g BW) at d 130 compared with fetuses from MP60 (7.3 ± 0.5 g/g BW) and MP100 (7.0 ± 0.5 g/g BW) ewes. Fetuses from MP60 ewes had increased ($P \leq 0.05$) perirenal fat mass (21.4 ± 1.59 g/g BW) compared with fetuses from MP100 (15.5 ± 1.5 g/g BW) and MP140 (17.2 ± 1.5 g/g BW) ewes. Although all pregnant ewes were provided adequate energy, restricting levels of MP supply during the defined period of gestation increased fetal perirenal fat whereas high levels of MP supply increased fetal small intestinal mass, all while not altering fetal BW.

Introduction

Nutrition of the ewe during late gestation directly relates to the growth of the fetus (Binns et al., 2002). Fifty percent of fetal growth takes place during the final 4 wk of pregnancy in addition to major redistribution of the ewe's body reserves to support udder development and colostrum production (Robinson, 1983). Supplementation with protein sources is necessary to meet nutrient supply as failure to increase feed intake can lead to reduced lamb birth weight (Annett et al., 2005).

Although several studies report negative (Ocak et al., 2005) or no effects (McNeil et al., 1997; Dawson et al., 1999) of MP supplementation during late gestation on the performance of pregnant ewes and particularly negative effects on colostrum yield and post-natal lamb survival, there is limited information regarding the effect of MP supplementation during late gestation on fetal visceral organ development. In a study offering isoenergetic diets providing 41 Afshari ewes metabolizable protein at 100%, 114%, or 124% of requirements, Amanlou et al. (2011) observed no effect of maternal protein supplementation during the last 3 wk of gestation on lamb birth weight or BW at weaning. Schauer et al. (2010) offered protein supplements in the last third of gestation providing 0.11% of BW/d of CP until lambing based on intake and protein requirements (NRC, 1985). In agreement with the previous study, Schauer et al. (2010) reported no effect of protein supplementation during late gestation on lamb birth weight.

The maternal gastrointestinal tract responds to both stage of pregnancy and nutritional level during gestation (Scheaffer et al., 2004; Reed et al., 2007), indicating that the dam may compensate for inadequate nutrition, sparing her offspring. Protein supplementation during the last third of pregnancy is crucial to support fetal growth and the increasing demands of the ewe to support mammary tissue development. Metabolizable protein requirements of sheep increase

with advancing gestation, with the greatest demand in late pregnancy. The last third of pregnancy is also an ideal time to nutritionally manage dams as they are commonly brought in from pasture just prior to lambing. If dams are not provided adequate protein during late gestation, lambs will likely experience slower growth and average daily gain (ADG). This study tested the hypothesis that altering levels of maternal MP in isocaloric diets during late gestation impacts fetal growth and organ development.

Materials and Methods

This study was conducted at the North Dakota State University Animal Nutrition and Physiology Center (ANPC). All animal care and handling procedures were approved by the Institutional Animal Care and Use Committee.

Experimental Design

Rambouillet ewes were transported to the ANPC (Fargo, ND) in July of 2011 from Hettinger, North Dakota. Upon arrival ewes were synchronized for estrus using progesterone inserts (CIDR, Pfizer Animal Health, New York, NY) for 7 d. Initially, 52 ewes were divided evenly into 4 separate pens with ad libitum access to hay and water. Ewes were mated with rams of proven fertility equipped with mating harnesses and marking crayons to obtain mating dates. Mating was recorded every 12 h. Pregnancy was confirmed and embryos were enumerated at 32 ± 4 d post-breeding via trans rectal ultrasonography (Aloka SSD-3500; Aloka America, Wallingford, CT) with a 7.5 MHz, linear trans-rectal probe, as described by Schrick and Inskeep (1993). Ewes carrying multiple conceptuses were administered 20 mg intramuscularly (IM) of Lutalyse (Pfizer Animal Health, New York, NY) and reintroduced to the rams 2 wk later. On d 90 of gestation dams carrying singleton pregnancies (n = 18) were housed in individual pens (0.91 x 1.2 m) at ANPC for the remainder of the experiment. All dams were acclimated to a

common diet (Table 2.1) and ad libitum access to water. Diets were analyzed for DM, ash, and CP following AOAC (1990), and NDF and ADF using an Ankom Fiber Analyzer (Ankom Technology, Fairport, NY).

Table 2.1. Ingredient composition and analyzed nutrient composition of diets fed to ewes from d 100 to d 130 of gestation

	Treatment[1]		
Item	MP60	MP100	MP140
Ingredient, % of dietary DM			
Supplement			
Corn	30.00	19.00	-
DDG[2]	4.00	24.00	43.00
Soyhulls	9.00	-	-
Trace mineral[3]	0.49	0.49	0.49
Fescue straw	56.51	56.51	56.51
Analyzed dietary composition, % DM			
DM	89.24	89.92	90.78
CP	6.57	10.25	14.60
NDF	58.60	59.36	65.42
ADF	34.28	32.02	34.05
Ash	6.42	6.54	7.11

[1]Maternal diets (DM basis) were balanced for mature ewes baring singletons during the last 4 weeks of gestation according to NRC (2007). Treatments: MP60 = 60% of MP requirements, MP100 = 100% of MP requirements, MP140 = 140% of MP requirements.
[2]Dried distillers grains with solubles.
[3]Trace mineral content: 16.0 to 17.0% Ca; 8.0% P; 21.0 to 23.0% salt; 2.75% Mg; 3 ppm Co; 5 ppm Cu; 100 ppm I; 1,400 ppm Mn; 20 ppm Se; 3,000 ppm Zn; 113,500 IU/kg vitamin A; 11,350 IU/kg vitamin D; and 227 IU/kg vitamin E.

Metabolizable protein treatments began on d 100 of gestation. Dams were supplemented with 140% of the MP requirement (MP140), 100% of the MP requirement (MP100), or 60% of the MP requirement (MP60) based on NRC (2007) requirements. Diets were isocaloric and balanced to meet the energy requirements of 70-kg mature ewes bearing singletons (NRC, 2007). Metabolizable protein requirements were based on the CP requirements of a ewe consuming a 40% RUP ration (NRC, 2007). Metabolizable protein supplied was calculated as: MP (g/d) =

(CP, g/d × (64 + (0.16 × undegradable intake protein (UIP) of ration))))/100. To account for variability in nutrient concentration of dried distiller grain (DDG) and other diet constituents, feedstuffs were analyzed for nutrient density just prior to the start of the treatment period. Ewes were blocked by BW into 1 of 3 treatments, housed individually, and weighed every 7 d to adjust the rations of MP supplement and corresponding hay for changes in BW throughout the experiment. All ewes were individually offered their respective MP supplement, and there were no refusals. The hay portion of the diet was given to the ewes shortly after the MP supplement had been consumed. Orts were collected once every 7 d, and there were rarely refusals throughout the treatment period.

Maternal BW was determined on a weekly basis. Maternal body condition was scored (1 to 5 scale, with 1 = emanciated and 5 = obese) by 2 separate evaluators on d 90, 110, and 130 of gestation (± 2.0 d; SE). Ultrasound measurements for ewe back fat thickness and LM area at the 12^{th} rib were determined using an Aloka 500-SSV (Aloka America, Wallingford, CT) with a linear transducer probe on d 90, 110, and 130 of gestation (± 2.0 d; SE). Maternal jugular vein blood samples were collected 1 h prior to feeding on d 100, 110, 120, and 130 of gestation (± 0.8 d; SE). Serum was separated by centrifugation and stored at -20˚C until determination of blood urea N (BUN) concentration for d 100 and d 130.

Tissue Collection and Analyses

On d 130 of gestation dams were not fed. All dams were weighed just prior to being stunned by captive bolt and exsanguinated. Final ewe BW was reported as the average BW measured on d 129 and d 130 of gestation just prior to exsanguination. The blood was quantitatively collected and weighed. The gravid uterus was immediately collected, and the fetus was removed and weighed. Fetal membranes were removed, and placentomes were dissected

from the uterine wall and counted. Placental caruncular and cotyledonary tissues were separated and weighed. The umbilical artery was located, and blood samples were collected into tubes, allowed to clot on wet ice, and later centrifuged at 2,000 x *g* for 20 min at 4˚C. Serum was stored at -20°C until further analysis. Both maternal and fetal viscera were removed, and the liver, mammary, and pancreas were dissected from the viscera and weighed. Fetal curved crown rump (CCR) length and abdominal girth were recorded. Fetal ponderal index was calculated using the following equation: ponderal index = fetal weight (kg) / CCR length (m^3). Fetal organ weights were recorded. Attached adipose tissue and digestive contents were removed from the gastrointestinal tract, and weights of liver, kidneys, stomach, small intestine, and lower gastrointestinal tract were recorded. In addition to weight, fetal hearts were further processed; the average left and right ventricle thicknesses were assessed with digital calipers at the base, mid, and top portions of the ventricle. Lastly, prominent portions of right and left sides of the LM, semimembranosus (SM), and psoas major (PM) were dissected from each lamb to provide adequate sample for tissue analysis. The samples were immediately frozen in liquid nitrogen and stored at -80˚C until further analysis.

Tissue Cellularity Estimates

Frozen muscle tissue samples (5 g) were homogenized using a Polytron with PT-10s probe (Brinkmann, Westbury, NY) in 1 × TNE (10 m*M* Tris, 0.2 m*M* NaCl, 1 m*M* ethylenediamine tetraacetic acid (EDTA), pH 7.4), and RNA and DNA were extracted by the method of Schmidt-Thannhause (1945) as described in Munro and Fleck (1966). DNA was determined using diphenylamine reagent (Burton, 1956). RNA was determined using orcinol reagent prepared as described by Lawson et al., (1988). Protein concentration in the homogenate

was determined using BioRad Quick Start Bradford Protein Reagent (BioRad, Hercules, CA; Bradford et al., 1976) after solubilizing in 0.1 *M* NaOH.

Statistical Analysis

Fetal visceral organ data were analyzed using the PROC GLM procedure of SAS (SAS Inst. Inc., Cary, NC) with MP treatment (MP60 vs. MP100 vs. MP140) and fetal weight in the model. Ewe organ weights, uterine weight, and placental measurements were analyzed using PROC GLM with MP treatment in the model. Measurements taken over time were analyzed using PROC MIXED procedures of SAS with MP treatment, time during MP treatment, and time by treatment as the source of variation in the model. Means were separated using the LSMEANS option of SAS and were considered significant when $P \leq 0.05$.

Results and Discussion

Maternal Measurements at d 130 of Gestation

Ewe BW was not affected ($P = 0.84$) by MP supplementation or restriction during late gestation (Figure 2.1; Table 2.2). There was an effect of MP treatment observed in maternal BW change (Figure 2.2), with ewes receiving the MP100 treatment gaining more weight throughout the treatment period than ewes receiving the MP60 treatment, with MP100 being intermediate. Ocak et al. (2005) reported that feeding of pregnant ewes 140% of the CP requirements during the last 6 wk of pregnancy increased ewe live weight at lambing, which is in contrast with our observations in the present study. The CP diet offered was not formulated to be isocaloric. Ewes were offered additional calories when compared to the control, which provided 100% of CP requirements in the study conducted by Ocak et al. (2005). Supplementation of CP in the maternal diet may have increased maternal body protein, while the supplementation of MP in isocaloric diets in the present study did not significantly affect the dam's body condition.

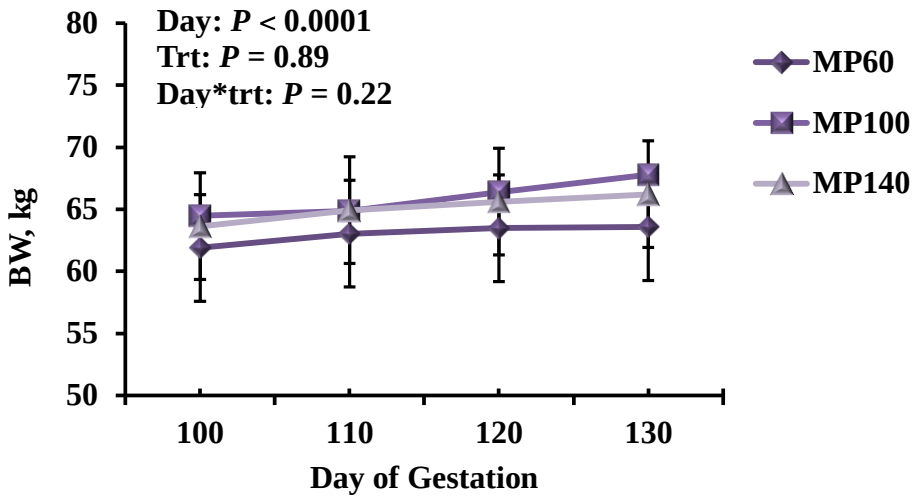

Figure 2.1. Average BW of ewes fed diets formulated to supply 60% (MP60), 100% (MP100), or 140% (MP140) of MP requirements (NRC, 2007) during late (d 100 to d 130) gestation.

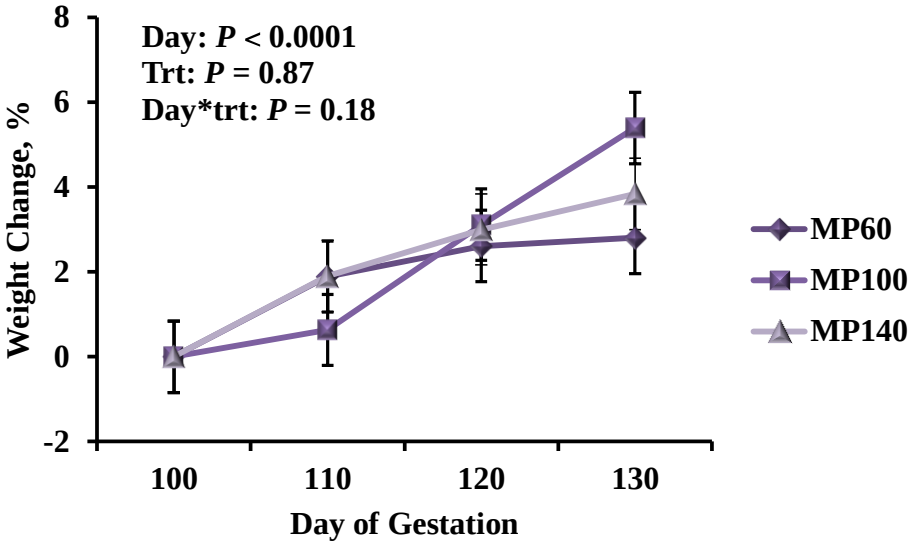

Figure 2.2. Average BW change of ewes fed diets formulated to supply 60% (MP60), 100% (MP100), or 140% (MP140) of MP requirements (NRC, 2007) during late (d 100 to d 130) gestation.

Parallel to BW, ewe BCS was not affected by treatment throughout gestation. Ewes had healthy body condition at the start of the experiment and therefore less likely to use dietary protein to replenish their own body reserves. The current study also used multiparous ewes,

which may have had a lower requirement for maintenance compared with that of a growing, primiparous ewe. There was no impact of MP treatment on ewe LEA or BF ultrasound measurements taken on d 100 and d 130 of gestation. However, there was an influence of MP on BF, with ewes receiving the MP140 treatment having less BF on d 130 of gestation when compared to the start of treatment. Ewe liver, mammary, and pancreas weights were not affected ($P \geq 0.17$) by MP treatments during late pregnancy (Table 2.2). Previous studies by others (Scheaffer et al., 2004; Reed et al., 2007; Carlson et al., 2009; Caton et al., 2009) found visceral organ weights are unresponsive to changes in nutrient intake during gestation. From these data we can conclude that even when calories are reduced, there is no change in fetal organ masses.

Metabolizable protein supplementation or restriction during late gestation did not affect ewe gravid or empty uterine weight ($P \geq 0.94$) or placentome weight and associated caruncle and cotyledon weights (Table 2.2). Placentome number in sheep is established by d 40 of gestation and remains constant thereafter (Schneider, 1996; Heasman et al., 1999). A previous study (Vonnahme et al., 2006) investigated the capability of ewes to protect their own fetus from a bout of maternal undernutrition by converting placentomes to more efficient forms. Different placental responses may function to maintain normal nutrient delivery to the developing fetus during periods of nutritional insult. In the present study, ewes were not treated until d 100 of gestation, well after placentome number is established. Therefore, as was expected from previous research, placentome number was unaffected ($P = 0.73$) by protein alteration, even when restricting ewes to 60 % of MP requirements during the last third of pregnancy.

Blood Metabolites

Blood urea nitrogen (BUN) concentration has been suggested to be a realistic predictor of nutrient utilization (Nolan et al., 1970; Egan and Kellaway, 1971) of sheep. In the present

Table 2.2. Ewe BW, organ weights, carcass and placental measurements on d 130 of gestation as influenced by MP supplementation during late (d 100 to d 130) gestation

	Treatment[1]				
Item	MP60 n = 6	MP100 n = 6	MP140 n = 6	SE	*P* - value
Initial body weight, kg	60.39	60.32	60.08		
Body weight, kg	63.56	67.77	66.21	4.29	0.84
Liver, g	558.17	631.93	688.57	46.09	0.17
Mammary, g	595.33	518.52	478.02	102.19	0.72
Pancreas, g	61.78	67.82	67.60	6.22	0.74
Back fat, cm	0.28	0.32	0.33	0.02	0.31
Loin eye area, cm^2	8.66	10.08	9.31	0.63	0.57
Gravid uterus weight, g	6411.60	6366.65	6436.03	302.22	0.98
Empty uterus weight, g	625.80	617.63	639.58	43.83	0.94
Total placentome weight, g	390.42	431.47	367.15	28.86	0.31
Placentome number	69.83	77.00	73.83	6.29	0.73
Caruncle weight, g	92.75	106.83	101.73	10.91	0.67
Cotyledon weight, g	279.02	317.17	248.47	20.48	0.09

[1]Treatments: MP60 = 60% of MP requirements, MP100 = 100% of MP requirements, MP140 = 140% of MP requirements (NRC, 2007).

study, BUN concentration reflected ewe MP treatment during late gestation (Figure 2.3). No differences among treatments were detected for BUN concentrations on d 100 ($P > 0.17$) of gestation prior to initiation of treatment. As expected, differences in BUN resulting from dietary MP levels were observed on d 130 of gestation; MP140 ewes had higher ($P < 0.001$) BUN concentration on d 130 of gestation when compared to the start of treatment. MP60 ewes had a lower ($P < 0.001$) BUN concentration on d 130 of gestation when compared to the start of treatment. Ewes receiving the MP100 treatment maintained a consistent BUN concentration throughout the treatment period.

The use of BUN levels in ruminants has has been used to assess the value of feed protein on the basis that BUN levels appear to reflect ammonia production in the rumen (Lewis, 1957).

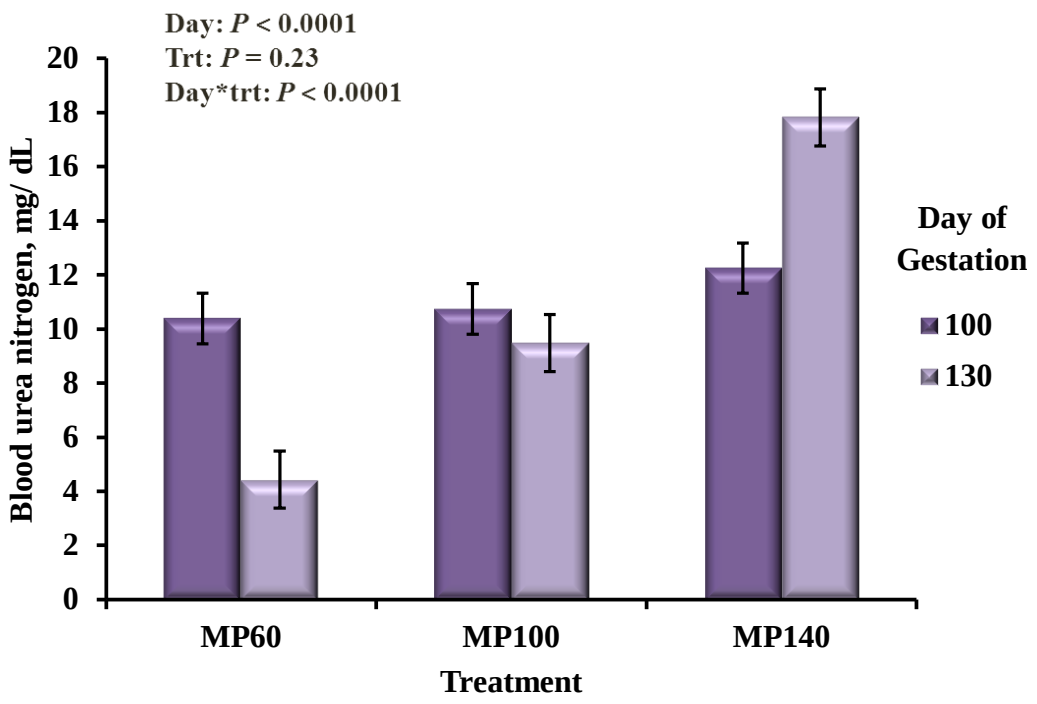

Figure 2.3. Blood urea nitrogen levels of ewes fed diets formulated to supply 60% (MP60), 100% (MP100), or 140% (MP140) of MP requirements (NRC, 2007) during late (d 100 to d 130) gestation.

Increased concentrations of BUN in ewes fed a higher supply of MP suggest greater nitrogen excretion. Sunny et al. (2007) suggests that although urea nitrogen in sheep is highly dependent on plasma urea concentration, it is also dependent on the efficiency of capture within the digestive tract. Salvage is greatly influenced by microbial fermentation events occurring within the gastrointestinal tract (GIT) and further investigation of the relationship between plasma urea concentration and urea nitrogen recycled to the GIT is needed.

Fetal Measurements at d 130 of Gestation

Fetal BW or eviscerated body weight (EBW) were not affected ($P \geq 0.67$) by maternal MP supplementation during late gestation (Table 2.3). The amino acid transfer from maternal circulation to fetal tissue is adjusted during its course, and McNeil et al. (1997) reported the low

ability of the placenta to transfer excess amino acids to the fetus. In contrast to this observation, Ocak et al. (2005) found that high protein diet pre-partum increased lamb birth weight. Carlson et al. (2009) reported that fetal weight (FW) is similar in lambs from ewes that were nutrient restricted or maintenance-fed throughout late gestation; however, others (Trahair et al., 1997; Vonnahme et al., 2003; Luther et al., 2007) have found that fetal weight is reduced when ewes are restricted in early to mid-gestation (d 78 to d 90), while Swanson et al. (2008) demonstrated that maternal nutrient restriction during the last two thirds of pregnancy in sheep reduces birth weights. In the case of nutrient excess, Wallace et al. (1996, 1999, 2001) found that over nourishing the singleton-bearing ewe throughout gestation results in rapid maternal growth at the expense of the nutrient requirements of the gravid uterus, resulting in low-birth weight lambs.

These findings substantiate the adverse effects forced upon the growing fetus caused by the alteration of maternal nutrient supply at critical windows of development. However, it is important to note that the above studies did not use diets formulated to be isocaloric. The lack of similar response in the current study is most likely attributable to the offering of MP in isocaloric diets. These data may also differ due to the level of MP supplementation or the timing, length, and (or) type of restriction. Fetal growth is the greatest over the final third of gestation and changes in the maternal diet during this period can have the greatest effect on birth weight in sheep. Nutrient restriction during late gestation can also lead to adverse effects on offspring's' postnatal metabolism and performance. Tygesen et al. (2007) found that a 50% reduction of maternal nutrient supply in the last six weeks of gestation reduces the birth weight and pre-weaning growth of the offspring due to lower milk intake, The differences that Tygesen et al. (2007) observed in the growth pattern of restricted lambs vs. control lambs suggests that prenatal undernutrition during late gestation may program postnatal metabolism.

Fetal CCR and abdominal girth were not different ($P \geq 0.50$) between groups. Maternal MP treatment had no effect on fetal brain, lung, adrenals, heart, and kidney, liver, spleen, and pancreas weights ($P \geq 0.40$; Table 2.3) when expressed alone or per unit of FW. Fetal heart weight, heart weight relative to FW, and left and right ventricular thicknesses were not different ($P \geq 0.43$) between treatment groups (Table 2.3). Fetal total perirenal fat mass (Table 2.3), when expressed per unit of FW, was greater ($P = 0.03$) in fetuses from MP60 dams compared with all other treatments.

Perirenal adipose tissue has marked proliferation of mitochondria between d 80 and 90 of gestation, with the tissue developing into brown fat and cell profiles characterized by many mitochondria with numerous distinct cristae (Gemmel and Alexander, 1978). Several studies (Alexander 1978; Budge et al., 2000; Symonds et al., 1998) found maternal nutrition to influence lipid reserves and brown adipose tissue deposition in newborn lambs. Interestingly, increased nutrition does not necessarily result in increased adipose tissue deposition. Budge et al. (2000) investigated the effects of maternal overfeeding during pregnancy (150% vs. 100% in controls) and reported fetuses from well-fed ewes have less total perirenal brown adipose tissue than control fed fetuses. In contrast, Alexander et al. (1978) found that pregnant ewes fed a restricted diet from 12 wk gestation onwards causes a decrease in brown adipose tissue deposition (g per kg body weight) of the offspring. Our results from the present study, along with the results above, suggest there is an optimal energy requirement of brown adipose tissue in the growing fetus, regardless of how much of the diet is composed of protein. Due to the fact that most fat in newborn lambs is internal, brown adipose tissue from well-fed fetuses has an increased thermogenic potential when compared with those mothers control-fed throughout pregnancy (Budge et al., 2000).

Table 2.3. Ovine fetal organ development on d 130 of gestation as influenced by level of maternal MP supplementation during late (d 100 to d 130) gestation

	Treatment[1]				
Item	MP60 (n = 6)	MP100 (n = 6)	MP140 (n = 6)	SE	*P*-value
Fetal weight, g	3461	3611	3424	156	0.67
Fetal eviscerated weight, g	2598	2723	2630	114	0.73
CCR[2] length, cm	50.8	51.3	50.4	1.4	0.91
Abdominal girth, cm	35.0	33.0	35.1	1.4	0.50
Brain, g	49.46	50.66	51.76	1.98	0.72
Lung, g	112.42	113.68	115.78	5.95	0.92
Lung/FW[3], g/kg	33	31	34	1.56	0.49
Adrenal, g	0.32	0.35	0.33	0.02	0.67
Adrenal/FW, g/kg	92	97	95	7	0.87
Heart, g	25.81	26.25	25.87	1.62	0.98
Heart/FW, g/kg	7.47	7.23	7.53	0.24	0.68
Left ventricle thickness, mm	5.97	5.57	6.14	0.31	0.43
Right ventricle thickness, mm	4.31	4.52	4.29	0.25	0.43
Kidney, g	20.41	19.58	20.08	1.01	0.85
Kidney/FW, g/kg	6.1	5.4	5.9	4.45	0.58
Perirenal fat, g	21.38	15.58	17.28	1.60	0.06
Perirenal fat/FW, g/kg	6.2[a]	4.4[b]	5.0[b]	0.44	0.03
Liver, g	95.15	93.00	92.61	6.36	0.95
Liver/FW, g/kg	27.6	25.7	26.9	1.27	0.56
Spleen, g	5.41	5.71	5.89	0.32	0.58
Spleen/FW, g/kg	1.57	1.58	1.72	0.08	0.40
Pancreas, g	3.08	3.13	3.19	0.26	0.95
Pancreas/FW, g/kg	0.88	0.86	0.93	0.05	0.74
Stomach complex[4], g	25.13	25.02	23.48	2.26	0.85
Stomach complex/FW, g/kg	7.29	6.91	6.96	0.61	0.89
Small intestine, g	25.39	25.54	29.88	2.08	0.22
Small intestine/FW, g/kg	7.37[b]	7.06[b]	8.79[a]	0.45	0.05
Large intestine, g	9.39	8.53	9.20	0.61	0.59
Large intestine/FW, g/kg	2.7	2.4	2.7	1.3	0.17

[1]Treatments: MP60 = 60% of MP requirements, MP100 = 100% of MP requirements, MP140 = 140% of MP requirements (NRC, 2007).
[2]CCR = curved crown rump.
[3]FW = fetal weight.
[4]Stomach complex = (reticulum + rumen + omasum + abomasum) – digesta.
[a,b]Values within rows with different superscripts are significantly different ($P \leq 0.05$).

Thus, a sufficient amount of adipose tissue at birth would better facilitate thermoregulation, and the lamb would be less susceptible to death from hypothermia.

Although fetal small intestinal mass did not differ ($P = 0.22$) between groups when expressed alone, fetuses from MP140 dams had increased ($P = 0.05$) small intestinal mass (Table 2.3) when expressed per unit of FW compared with all other treatments. Fetal large intestinal mass was unaffected ($P = 0.17$) by altering levels of maternal MP during late gestation. The effect of maternal protein supplementation during specific stages on gestation on the small intestine has not been extensively studied. The alterations in fetal small intestinal mass may be explained by changes in cellularity, cell proliferation, and vascularity in response to both maternal diet and fetal nutrient demand with advancing gestation. Increased small intestinal weights are important when considering postnatal growth of the lamb. Successful adaptation to the extrauterine environment is dependent on the ability of the neonate to obtain nutrients from ingested milk rather than from the placenta (Avila et al., 1989). Thriving gastrointestinal development during fetal life is dependent upon proper nutrition during gestation.

Visceral organ mass response to a differing amount of maternal MP during late gestation, such as in this study, and during early to mid-gestation are likely dependent upon the different developmental periods of the fetus and its organ systems (Fowden et al., 2006; Nathanielsz, 2006; Symonds et al., 2007). Normally, organogenesis occurs during early to mid-gestation, followed by rapid fetal growth in the last third of gestation (Fowden et al., 2006). However, each organ and tissue grows and develops at a unique rate. Various organ systems may respond differently to specific timing and severity of nutrient supplementation or restriction because of differing growth trajectories and maturation time points (Caton and Hess, 2010). In the present

study growth may have occurred due to increased efficiency of nutrient usage by the dam and/or the fetus following MP restriction, thus, fetal organ growth and development was minimally affected.

Tissue Cellularity

Regardless of muscle type, fetal skeletal muscle protein content, protein:DNA and RNA:DNA ratios were not affected ($P \geq 0.26$) by maternal MP treatment (Table 2.4). Previous studies have suggested that, in sheep, myogenesis is completed between d 80 and 125 of gestation (Ashmore et al., 1972; Swatland and Cassens, 1973; Maier et al., 1992). It appears that altering maternal MP during the treatment interval of our study (d 100 to d 130) did not alter fetal skeletal muscle hypertrophy. Fetal LM total DNA content tended to be greater in those from MP100 ewes compared with those from MP60 and MP140 ewes ($P = 0.09$). Fetal LM DNA content was not sensitive to maternal nutrition when ewes were offered either high or low levels of MP; however an adequate amount of maternal protein may have had a positive effect on fetal LM hyperplasia (Swanson et al., 1999; Scheaffer et al., 2004; Soto-Navarro et al., 2004). Fetal SM total RNA content from MP140 ewes tended ($P = 0.06$) to be greater compared with that of MP60 and MP100 treated ewes (Table 2.4). RNA content is dependent upon the muscle location and the fraction of slow and fast muscle fibers. It has been established that isometric contractile characteristics of fast and slow-twitch muscle is determined in fetal sheep between d 90 and 140 of gestation (Javen et al., 1996). Increased fetal muscle RNA from MP140-supplemented ewes could be explained by developmental changes in muscle gene expression due to the significant increase in maternal MP during such a critical period of fetal growth. In agreement with past research (Fahey et al., 2005; Daniel, et al., 2007), the three fetal muscles evaluated in the present study were not affected equally by maternal nutrition. During late gestation, nutrients received

by the fetus are distributed in order of physiological importance to the corresponding organ systems: brain, liver, heart, etc. Our results on indices of fetal muscle growth infer that fetal muscle development is sensitive to periods of fetal nutrient manipulation. Reducing or supplementing maternal protein during late pregnancy had little effect on fetal muscle cellularity, although differences in DNA and RNA content demand further investigation into fiber type and impacts on subsequent mature muscle.

Table 2.4. Effects of maternal MP supplementation during late (d 100 to d 130) gestation on cellularity measurements of ovine fetal muscle

	Treatment[1]				
Item	MP60	MP100	MP140	SE[2]	*P* - value
Longissimus					
RNA, mg/g	2.08	2.32	2.07	0.10	0.13
DNA, mg/g	0.67	0.79	0.60	0.05	0.09
Protein, mg/g	87.34	80.89	83.94	6.60	0.39
Protein:DNA	132.31	105.79	139.32	12.41	0.40
RNA:DNA	3.16	2.97	3.49	0.18	0.38
Dry matter, %	16.86	17.42	17.13	0.41	0.28
Semimembranosus					
RNA, mg/g	1.82	2.12	2.17	0.08	0.06
DNA, mg/g	0.58	0.71	0.78	0.06	0.42
Protein, mg/g	50.72	55.15	54.47	2.05	0.34
Protein:DNA	98.79	77.62	70.53	9.87	0.48
RNA:DNA	3.56	2.99	2.82	0.40	0.83
Dry matter, %	17.19	17.14	17.26	0.28	0.80
Psoas Major					
RNA, mg/g	2.55	2.89	2.28	0.30	0.65
DNA, mg/g	0.65	0.81	0.60	0.09	0.57
Protein, mg/g	92.11	82.80	84.47	9.82	0.90
Protein:DNA	146.35	102.29	176.68	22.58	0.26
RNA:DNA	3.97	3.58	4.52	0.49	0.71
Dry matter, %	17.13	18.05	18.57	0.57	0.48

[1]Ewes fed 60% (MP60), 100 % (MP100), or 140% (MP140) of MP requirements during late gestation (NRC, 2007).
[2]Standard error of the means for MP60, n = 6; MP100, n = 6; and MP140; n = 6.

Conclusion

Maternal nutrition has many theoretical implications for offspring because of its influence on the nutrients available to the uteroplacenta and consequently the developing conceptus. The nutrient supply available to the fetus could greatly affect not only the development of the animal in utero, but also the developmental programming of the growing lamb when it reaches the external environment. Any possible means to improve nutrient acquisition by the dam could improve nutrient availability for offspring. Further research is needed to determine if the fetus received differing diets. Our data on fetal growth suggest that supplementing ewes with MP during late gestation could increase lamb nutrient efficiency, thus better preparing them for the external environment and productive efficiency as a mature animal.

In summary, fetal organ mass and skeletal muscle growth were sensitive to maternal MP during late gestation. Late-gestational MP also influenced ewe BW, body composition, and BUN level. Our results show that maternal supplementation with MP in isocaloric diets altered potential energy reserves of the fetus, but did not seem to have an obvious effect on BW change of lambs. The MP supplementation has some beneficial effects and needs further research on potential benefits in field production.

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CHAPTER 3. PROTEOMIC ANALYSIS OF FETAL OVINE SKELETAL MUSCLE AS INFLUENCED BY MATERNAL METABOLIZABLE PROTEIN SUPPLEMENTATION IN LATE PREGNANCY

Abstract

The objective of the study was to identify proteins in ovine longissimus dorsi (LM) muscle in offspring as influenced by maternal metabolizable protein (MP) in late pregnancy. Two-dimensional difference in-gel electrophoresis (2D DIGE) coupled with mass spectrometry (MS) was used to compare the sarcoplasmic and myofibrillar protein fractions from fetal skeletal LM samples from dams fed either 60% (MP60), 100% (MP100), or 140% (MP140) of MP requirements during late (d 100 to d 130) gestation. On d 130 of gestation ewes were euthanized and LM samples were collected from fetuses. Skeletal muscle samples were analyzed using an immobilized pH 3 to 10 gradient (IPG) in the first dimension, followed by running proteins in their second dimension by SDS-PAGE. Mass spectrometry analyses with matrix-assisted laser desorption/ionization –time of flight (MALDI-TOF/TOF) was performed on in-gel spots of interest to identify proteins. Peptide identifications with > 95% probability and protein identifications with > 99% probability with at least two identified unique peptides were accepted. Thirty spots from the sarcoplasmic fraction and 12 spots from the myofibrillar fraction were selected for further identification by MS. Of these, there were 7 spots in the sarcoplasmic fraction corresponding to 5 proteins, respectively (tubulin beta-5 chain, alpha-enolase, creatine kinase m-type, fructose-bisphosphate aldolase [aldolase A], and phosphoglucomutase) and zero spots in the myofibrillar fraction that differed in relative abundance ($P \leq 0.10$) among MP treatments. Further analysis by Western Blotting was performed for aldolase A in the sarcoplasmic fraction and troponin-T (TnT) in the myofibrillar fraction. Aldolase A in the LM of

fetuses from MP140 and MP60 ewes was more abundant ($P = 0.05$) compared with those from MP100 supplemented ewes. The use of these methods in order to study fetal ovine skeletal muscle development resulted in the identification of many high abundant proteins, including many plasma proteins. Further investigation into prefractionation or other methods of in-gel sample preparation must be considered in order to look more closely at low abundant proteins that may have been masked during this experiment.

Keywords: late gestation, mass spectrometry, maternal nutrition, metabolizable protein, proteomics, sheep

Introduction

There is substantial evidence that maternal nutritional status is one of the extrinsic factors programming nutrient partitioning and ultimately growth and development of the fetus (Wallace, 1948; Wallace et al., 1999; Godfrey and Barker, 2001; Strickland et al., 2004; Wu et al., 2006). Current literature suggests that balancing diets for metabolizable protein (MP) supply is a more accurate reflection of true protein needs of ruminants than using digestible protein (DP) or crude protein (CP). In brief, MP is a combination of dietary and microbial protein that is digested postruminally and from which amino acids are absorbed from the intestine (NRC, 2007). Previous data with protein supplementation during gestation has shown that maternal under-nutrition can significantly reduce the number of muscle fibers and nuclei in the offspring (Bedi et al., 1982; Wilson et al., 1988; Ward and Stickland, 1991). Altering the maternal diet before muscle fiber formation also changes the muscle fiber development of the fetus. Fahey and others (2005) found decreased maternal nutrition before fiber formation (d 30 through d 70) results in lambs with fewer myosin heavy-chain fast fibers and more myosin heavy-chain slow fibers in the LM, while maternal undernutrition during other periods of pregnancy had no effect on the

number of muscle fibers in newborn lambs. Two-dimensional DIGE allows simultaneous separation of thousands of proteins. Current research using this method to study sarcoplasmic and myofibrillar protein fractions in skeletal muscle is limited, but these fractions contain the majority of proteins involved in metabolism and signal transduction pathways which are of interest in understanding regulatory mechanisms (Hamelin et al., 2006).

The aim of the present study was to develop a method to study and identify proteins from fetal skeletal muscle that may be differentially expressed by different levels of maternal MP supplementation during late gestation using 2D DIGE and MS. This type of approach allowed us to establish an ovine skeletal muscle protein map using an IPG of 3-10, which is an important step in applying proteomic analysis to the field of animal science.

Materials and Methods

Experimental Design

Rambouillet ewes were transported to the Animal Nutrition and Physiology Center (ANPC) in Fargo, North Dakota, in July of 2011. Upon arrival ewes were synchronized for estrus using progesterone inserts (CIDR, Pfizer Animal Health, New York, NY) for 7 d. Initially, 52 ewes were divided evenly into 4 separate pens with ad libitum access to hay and water. Ewes were mated with rams of proven fertility equipped with mating harnesses and marking crayons to obtain mating dates. Mating was recorded every 12 h. Pregnancy was confirmed and embryos were enumerated at 32 ± 4 d post-breeding via trans rectal ultrasonography (Aloka SSD-3500; Aloka America, Wallingford, CT) with a 7.5 MHz, linear trans-rectal probe, as described by Schrick and Inskeep (1993). Ewes carrying multiple conceptuses were administered 20 mg of Lutalyse intramuscularly (Pfizer Animal Health, New York, NY) and reintroduced to the rams 2 wk later. . On d 90 of gestation dams carrying singleton pregnancies (n = 18) were housed in

individual pens (0.91 x 1.2 m) at ANPC for the remainder of the experiment. All dams were acclimated to a common diet (Refer to Chapter 2; Table 2.1) and ad libitum access to water. Diets were analyzed for DM, ash, and CP following AOAC (1990), and NDF and ADF using an Ankom Fiber Analyzer (Ankom Technology, Fairport, NY).

Metabolizable protein treatments began on d 100 of gestation. Dams were supplemented with 140% of the MP requirement (MP140), 100% of the MP requirement (MP100), and 60% of the MP requirement (MP60). Diets were isocaloric and balanced to meet the energy requirements of 70-kg mature ewes bearing singletons (NRC, 2007). Metabolizable protein requirements were based on the CP requirements of a ewe consuming a 40% RUP ration (NRC, 2007). Metabolizable protein supplied was calculated as: MP (g/d) = (CP, g/d × (64 + (0.16 × UIP of ration)))/100. To account for variability in nutrient concentration of DDG and other diet constituents, feedstuffs were analyzed for nutrient density just prior to the start of the treatment period. Ewes were blocked by BW into 1 of 3 treatments and housed individually (MP60, n = 6; MP100, n = 6; MP140, n = 6) and weighed every 7 d to adjust the rations of MP supplement and corresponding hay for changes in BW throughout the experiment. All ewes were individually offered respective MP supplement. The hay portion of the diet was given to the ewes shortly after all had consumed the MP supplement.

Collection of Muscle Sample

Eighteen Rambouillet ewes were euthanized by exsanguination after stunning with captive bolt at ANPC on d 130 of gestation. Longissimus dorsi muscles were removed from the fetus, frozen in liquid nitrogen, and stored at -80˚C until analysis.

Extraction of Sarcoplasmic Fraction

The highly soluble sarcoplasmic fraction was extracted from the snap frozen LM samples. Approximately 1.5 g of muscle was homogenized in low ionic strength sarcoplasmic extraction buffer [50 m*M* Tris, 1 m*M* ethylenediaminetetraacetic acid (EDTA); pH 8.5 (LISS); Anderson et al., 2012] with a Polytron (Brinkmann, Westbury, NY). Samples were then centrifuged at 21,100 × *g* for 30 min at 4˚C. The supernatant (sarcoplasmic protein fraction) was then decanted (using a transfer pipet) into 15-mL polypropylene conical tubes and the protein concentration was determined using the Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA; Bradford et al., 1976). The pellet (myofibrillar protein fraction) was saved on wet ice until ready to process. The protein concentration of the sarcoplasmic fraction was adjusted to 6 mg/mL using cold LISS, separated into 0.5 mL aliquots, and stored at – 80˚C until analysis.

Extraction of Myofibrillar Fraction

To each pellet (myofibrillar protein fraction) saved previously, 5.0 mL of cold LISS was added (washing step) and samples were then centrifuged at 21,000 × *g* for 30 min at 4˚C. The supernatant was then discarded using a transfer pipet and the LISS washing step was repeated. The supernatant was then discarded and 10 mL of warm (≤ 37˚C) myofibrillar solubilizing buffer [8.3 *M* urea, 2 *M* thiourea, 2% (wt/vol) 3-[(3-cholamidopropyl) dimethyl-ammonia] -1-propanesulfonate (CHAPS), pH 8.5 (MSB); Bjarnadottir et al., 2011] was added to the myofibrillar protein fraction. The tubes were then vortexed and incubated in a warm water bath (≤ 37˚C) for 30 min. Each pellet was homogenized with a Polytron (Brinkmann, Westbury, NY) and centrifuged at 21,000 × *g* for 30 min at 25˚C. The supernatant was then transferred (using a transfer pipet) to a 15-mL polypropylene conical tube and stored at room temperature until same-

day protein concentration determination with the Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA; Bradford et al., 1976). The protein concentration of the myofibrillar fraction was adjusted to 5 mg/mL using cold extraction buffer, separated into 0.5 mL aliquots, and stored at – 80 ˚C until analysis.

Two-Dimensional Difference In-Gel Electrophoresis (2D DIGE) and Data Analysis

The sarcoplasmic and myofibrillar fractions of fetal ovine LM from ewes fed MP treatments throughout late gestation were further analyzed by 2D DIGE in order to observe the difference in protein expression among treatments. First, an internal standard for each (sarcoplasmic and myofibrillar) fraction was created using a pooled sample representative of the samples used in the study and ran as a standard labeled with Cy2 for all gels (Rozanas & Loyland, 2008; Westermeier & Scheibe, 2008). Then, samples from both fractions were labeled with Cy dyes according to the trials designed for the experiment (Table 3.1) and the manufacturer's directions (GE Healthcare, Piscataway, NJ).

Combinations of 15 µg of protein from the MP60 (Trial 2) or MP140 (Trial 1), 15 micrograms from the MP100, and 15 micrograms from internal standard were loaded on each IPG strip (pH 3 to 10) for a total of 45 µg of protein per strip (Table 3.1; Figure 3.1). Samples from the three treatments (MP60, MP100, and MP140) were labeled alternately with Cy3 and Cy5 to avoid a labeling bias. Two trials were created among the 3 treatments (Table 3.1). Trial 1 compared MP140 (n = 6) to MP 100 (n = 6), and Trial 2 compared MP60 (n = 6) to the same MP100 (n = 6) lamb LM samples from Trial 1. Trials 1 and 2 were run for both the sarcoplasmic and myofibrillar fractions, and duplicate strips were run to minimize variation (24 sarcoplasmic + 24 myofibrillar = 48 total IPG strips in the experiment).

Separation of proteins in the first dimension by isoelectric point (pI) was accomplished using Immobiline DryStrips (11 cm, pH 3-10, GE Healthcare, Piscataway, NJ) containing 2.5 m*M* DL-dithiothreitol (DTT). Focusing of proteins on IPG strips was performed on an Ettan IPGphor 3 isoelectric focusing system (GE Healthcare, Piscataway, NJ). Initially, a low voltage (500 V) was applied, followed by a stepwise increase to 8000 V to reach a total of 18,500 V hours. After isoelectric focusing, strips were equilibrated for 15 min in 10 mL of equilibration buffer (50 m*M* tris pH 8.8, 6 *M* urea, 30% (vol/vol) glycerol, 2% (wt/vol) SDS, and a trace of bromophenol blue) containing 65 m*M* DTT. This was followed by an equilibration for 15 min in 10 mL of equilibration buffer containing 135 m*M* iodoacetamide (Rozanas and Loyland, 2008).

Table 3.1. Cy dye labeling[1] of 2-dimensional difference in gel electrophoresis comparison of MP60, MP100, and MP140 fetal ovine LM samples

Strip	Cy 2	Cy3	Cy5
		Trial 1	
1	Standard[2]	MP100[3]	MP140
2	Standard	MP140	MP100
3	Standard	MP100	MP140
4	Standard	MP140	MP100
5	Standard	MP100	MP140
6	Standard	MP140	MP100
		Trial 2	
1	Standard	MP100	MP60
2	Standard	MP60	MP100
3	Standard	MP100	MP60
4	Standard	MP60	MP100
5	Standard	MP100	MP60
6	Standard	MP60	MP100

[1]Trials 1 and 2 were run for both myofibrillar and sarcoplasmic protein fractions
[2]Standard = pooled sample created for each protein fraction representative of all samples used in the study.
[3]Maternal diets (DM basis) were balanced for mature ewes baring singletons during the last 4 weeks of gestation according to NRC (2007). Treatments: MP60 = 60% of MP requirements, MP100 = 100% of MP requirements, MP140 = 140% of MP requirements.

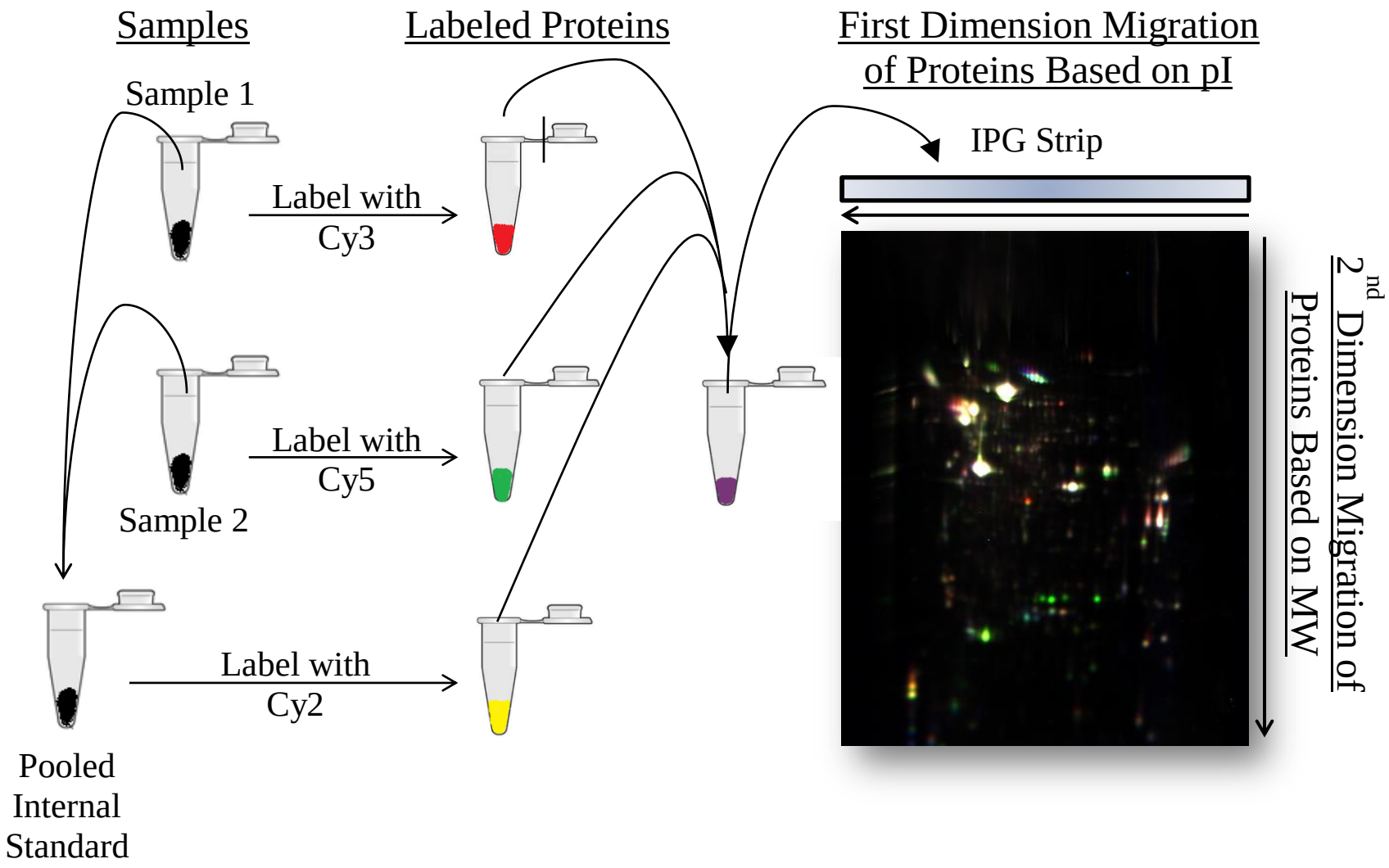

Figure 3.1. Two-Dimensional In-Gel Electrophoresis (2D DIGE). Sample preparation for 2D PAGE. Adapted from Posch, A. 2007. Methods in Molecular Biology.

Second dimension electrophoresis (separation by molecular weight using SDS-PAGE) was run on 12.5% acrylamide gels (acrylamide: *N,N'*-bis-methylene acrylamide = 100:1 [wt/wt], 0.1%SDS [wt/vol], 0.05% *N,N,N',N*-tetramethylethylenediamine (TEMED), 0.05% [wt/vol]ammonium persulfate, and 0.5 *M* Tris, pH 8.8) with a SE 600 vertical slab gel unit (Hoefer Scientific Instruments, Holliston, MA). Two strips were placed side-by-side on the top of each gel and run at a constant of 120 V until tracking dye had just run off completely. Gels were then imaged on an Ettan DIGE Imager (GE Healthcare, Picataway, NJ) with 3 images for each strip gel area (Cy2, Cy3, and Cy5, respectively). All images were uploaded into DeCyder 2D Software (v. 6.5; GE Healthcare, Piscataway, NJ) and analyzed to identify differences between MP treatments in relative abundance of individual spots.

After analysis of all spots was completed using DeCyder, a last set of gels i.e., pick gels were poured in order to manually pick gel spots for further protein identification and analysis by MS. Pick gels were 12.5% acrylamide gels (acrylamide: *N,N'*-bis-methylene acrylamide = 100:1 [wt/wt], 0.1 %SDS [wt/vol], 0.05% TEMED, 0.05% [wt/vol] ammonium persulfate, and 0.5 *M* Tris, pH 8.8) run on a Hoefer SE 600 Ruby unit (Hoefer Scientific Instruments, Hollison, MA). Four Immobiline IPG strips for pick gels were prepared as previously described, but with a load of 500 µg of pooled unlabeled protein from all 3 treatment samples (MP60, MP100, and MP140) combined. IPG strips were focused in the first dimension as previously described. For second dimension electrophoresis, two duplicate pick gels were run on each fraction (2 sarcoplasmic and 2 myofibrillar, respectively) at 100 V until tracking dye had just run off. After second dimension electrophoresis, pick gels (total of 4) were stained with colloidal coomassie blue solution (1.7% [wt/vol] ammonium sulfate, 30% [vol/vol] methanol, 3% [vol/vol] phosphoric acid, and 0.1% [wt/vol] Coomassie G-250 for 24 h, followed by destaining for up to 4 h. Proteins of interest and

those previously determined by differences detected in comparison studies using the DeCyder 2D software were manually excised from the gel, placed into individual snap cap tubes, and stored at -80˚C until digestion and identification by MS.

In-Gel Protein Digestion

Spots were destained and in-gel trypsin digested by standard laboratory protocol (Shevchenko et al., 1996). Gel spots were destained and proteins were reduced in-gel with 4 m*M* DTT in 50 m*M* ammonium bicarbonate for 15 min at 60˚C. Iodoacetamide was added to make 16 m*M* and alkylation was allowed to proceed for 30 min at room temperature in the dark. The reaction was quenched with an additional 3 m*M* DTT. The gel spot was then equilibrated with 55 m*M* ammonium bicarbonate, dehydrated with 100% acetonitrile, and rehydrated in 0.02 µg Trypsin Gold (Promega) in 40 m*M* ammonium bicarbonate. Digestion was allowed to proceed overnight at 37˚C. Approximately 50 µL of peptides were extracted. The samples were acidified with formic acid to make 0.1% final concentration.

Manual Spot Analysis

A subset of spots was initially analyzed by manual spotting. Peptides from these samples were captured by C18 solid-phase extraction using Zip-Tips by passing 13 µL through the tip. The remaining partially depleted sample was retained and stored at -80˚C. The recovered peptides were eluted and 0.5 µL was mixed with an equal volume of 10 mg/ml of a-cyanohydroxy cinammic acid (CHCA) in 75% [vol/vol] acetonitrile/0.1% formic acid and spotted onto a MALDI target. Samples were analyzed using an AB 4800 MALDI TOF/TOF. Top 20 MS precursors were selected and analyzed by MSMS from weakest to strongest. The remaining spots were analyzed by manual spotting as above except the half of the samples that were removed previously for LC-TEMPO analysis.

LC-TEMPO Analysis

This method is also described in EW004. Samples were analyzed by reverse phase high performance liquid chromatography (rpHPLC) and spotted onto a MALDI target plate using a TEMPO-LC integrated nanoflow HPLC/spotter. A total of 8.8 µL of sample was injected onto a Proteocol C18 0.3 c 10 mm trap (3 µm, 300A pore size). The samples were desalted with 2% acetonitrile/formic acid (vol/vol) for 10 min at a flow rate of 10 µL/min. Peptides were eluted in-line through a 0.1 x 100 mm Magic AQ C18 (5 µm) column using a 30 m gradient from 100% Buffer A to 60% Buffer A, /40% Buffer B (98% [vol/vol] acetonitrile, 0.1% [vol/vol] formic acid) at a flow rate of 1 µL/min. Eluate was mixed post-column with an equal volume of 10 mg/ml CHCA in 75% [vol/vol] acetonitrile and 0.1% [vol/vol] formic acid. The matrix/eluent mix was spotted at 18 sec/spot. The column was regenerated at 70% (vol/vol) acetonitrile/0.1% formic acid.

Protein Isolation and Identification by Mass Spectrometry

Spot sets were analyzed on the AB4800 MALDI TOF/TOF in reflector positive ion mode. For MS the *m/z* range was 800 to 4000. A total of 400 subspectra were accumulated per spectrum. For MSMS, the top 10 precursors were selected per spot with the weakest precursor first. A total of 600 subspectra were accumulated per MSMS spectrum.

Raw spectra were converted to T2D files using 4000 series Explorer 3.5.28193. The file extension T2D stands for technology transfer database; a binary format data file that is exported from an Oracle database. These files were converted to Mascot generic format (mgf) files using Peaks to Mascot. All spectra were searched with Mascot (v. 2.3.02, Matrix Science) against the Universal Protein resource (UniProt) database (v., May 5, 2012) restricted to the bovine complete proteome, human complete proteome, or the sheep proteome. The latter was

incomplete and contained many sequence fragments. The search parameters were trypsin with up to one missed cleavage, carbamidomethyl (N-term C), Gln>pyro-Glu (N-term Q), Oxidation (M). Mass values were monoisotopic, peptide mass tolerance was 100 ppm, and fragment ion tolerance was 0.5 Da. Mascot searches were combined and analyzed in Scaffold (v. 3.00.08) to validate peptide and protein identifications. Each spot was treated as a biological replicate. This permitted spot-to-spot comparison which helped to flag some identifications as carry-over contaminants from proceeding runs. Peptide identifications with > 95% probability and protein identifications with > 99% probability and at least two identified peptides were accepted.

SDS-PAGE and Western Blotting

Wang's protein extracts for gel electrophoresis were prepared using methods described by Huff-Lonergan et al. (1996) and Wang (1982), utilizing the modified 3x Wang's Sample Gel Buffer/Tracking Dye [3 m*M* EDTA; 3% (wt/vol) SDS; 30% (vol/vol) glycerol; 0.003% (wt/vol) pyronine Y; 30 m*M* Tris, pH 8.0]. The sarcoplasmic protein fraction was used in LISS and the myofibrillar fraction was used in MSB.

SDS-PAGE Electrophoresis

Samples in sample buffer/tracking dye (sarcoplasmic fraction in LISS and myofibrillar fraction in MSB) for aldolase A and TnT, respectively, were run on 12.5% acrylamide separating gels (acrylamide: *N*, *N*'-bis-methylene acrylamide = 37.5:1 [wt/wt], 0.1% [wt/vol] SDS, 0.05% (TEMED), 0.05% [wt/vol] ammonium persulfate, and 0.37 *M* Tris, pH 8.8) with 5% acrylamide stacking gels (acrylamide: *N*, *N*'-bis-methylene acylamide = 37.5:1 [wt/vol], 0.1% [wt/vol] SDS, 0.125% TEMED, 0.075% [wt/vol] ammonium persulfate, and 0.125 *M* Tris, pH 6.8) in a running buffer [25 m*M* Tris, 192 m*M* glycine, 1.7 m*M* EDTA, 0.1% [wt/vol] SDS] (Melody, et al., 2004). Electrophoresis was carried out on a BioRad Mini-PROTEAN Tetra Cell system (BioRad

Laboratories, PA). Gels for TnT were loaded with 7 µg of protein per lane and run at a constant voltage of 100 V for 3 h. Gels for aldolase A were loaded with 10 µg of protein per lane and run at a constant voltage of 100 V for 3.5 h.

Transfer Conditions

Proteins were transferred onto Millipore Immobilon-P polyvinylidene Diflouride transfer membrane (Millipore Corporation, Bedford, MA) using a TE22 Might Small Transphor electrophoresis unit (Hoefer Scientific Instruments; Holliston, MA) at a constant voltage of 90 V for 1.5 h. In a buffer [25m*M* Tris, 1.9 *M* glycine, 0.017 *M* EDTA, and 15% (vol/vol) methanol] maintained at 4˚C using a refrigerated circulating water bath.

Western Blotting

Post transfer, all membranes were blocked in PBS-Tween [80 m*M* disodium hydrogen orthophosphate, 100 m*M* sodium chloride, 0.1% (vol/vol) polyoxyethylene sorbitan monolaurate (Tween-20), and 5% (wt/vol) nonfat dry milk] for 1 h at room temperature (23˚C). After blocking, membranes were placed in their respective primary antibody diluted with PBS-Tween. Troponin-t blots were incubated overnight at 4˚C with the primary antibody (mouse monoclonal anti-rabbit troponin-T antibody, catalog No. T6277; Sigma Chemical Co., St. Louis MO) diluted 1:30,000. For aldolase A, the primary antibody (mouse monoclonal anti-human ALDOA antibody, catalog No. 226-M01; Abnova Antibodies, Taiwan) was diluted to 1:20,000 and incubated overnight at 4˚C. After primary antibody incubations were complete, membranes were allowed to warm to room temperature (23˚C) for 20 min and washed 3 times (10 m/wash) using PBS-Tween. Both TnT and aldolase A blots were incubated 1 h at room temperature with the secondary antibody (goat anti-mouse conjugated with horseradish peroxidase, Catalog No. A2554; Sigma Chemical Co.) diluted at 1:45,000. After completion of secondary antibody

incubation, all membranes were washed 3 times (10 m/wash) with PBS-Tween at room temperature (23˚C) to achieve a total wash rinse time of 30 min before chemiluminescence detection (Melody et al., 2004). Chemiluminescence was initiated using premixed reagents (ECL Prime kit; Amersham Pharmacia Biotech, Pascataway, NJ). Chemiluminescence was detected using a F2.8 28-70 mm zoom lens camera (Alpha Innotech Corp.). Densitometry measurements were completed using the AlphaEaseFC software (Alpha Innotech Corp.).

Western Blotting Statistics

Troponin-T and Aldolase A Western blot data were analyzed using GLM procedure in SAS (v.9.2; SAS Inst., Inc., Cary, NC) to determine differences between MP treatments. Least squares means were separated using the PDIFF option. The ratio of the intensity of the sample band to the intensity of the same band in control sample was used to analyze differences in aldolase A in the sarcoplasmic fraction of fetal LM between maternal MP treatments. The ratio of the intensity of the sample band to the intensity of the same band in the control sample was used to analyze differences in TnT isoforms in the myofibrillar fraction of fetal LM between maternal MP treatments

Results and Discussion

Two-Dimensional In-Gel Electrophoresis

Results from this study using 2D DIGE and Western blot analysis showed that differential expression of fetal muscle proteins occurred as a result of maternal MP supplementation in late gestation. In a first approach, an attempt to get an overview of protein expression in the LM of fetal ovine from ewes supplemented or restricted in MP throughout late gestation was made. For this purpose, two 2D DIGE pick gel maps of the sarcoplasmic and myofibrillar fractions (500 µg protein load) of LM (Figures 3.4 and 3.5, respectively) are

presented. These figures display the separation of proteins over the entire range of the pH 3 to 10 strip for each fraction, respectively. Using the DeCyder 2D software, background was subtracted from each image and spots were detected and matched across gels of the same trial, followed by spot volume normalization with the standard. Observations of the multiple repeat 2D DIGE gels showed that spot locations and intensities were highly similar between gels from the same sample. Approximately 300 spots were detected and compared in each gel for both sarcoplasmic and myofibrillar proteins. Thirty spots from the sarcoplasmic fraction and 12 spots from the myofibrillar fraction were selected for further identification by MS. The Scaffold proteome software used to analyze MS results also has the ability to categorize these proteins into their biological processes and cellular components (Figures 3.2 and 3.3). Of these, there were 6 spots in the sarcoplasmic fraction and 0 spots in the myofibrillar fraction that differed in relative abundance ($P \leq 0.10$) among MP treatments. The complete sheep proteome has yet to be sequenced, and the remaining spots were chosen because they were prominent on the pick gel and therefore of particular interest to the current study on fetal ovine skeletal muscle growth.

The separation of proteins on 2D gels and peptide mass fingerprint analyses allowed us to identify different classes of proteins. Proteins were divided into the following groups: energy metabolism, contractile apparatus, cell structure, cell defense, regulator, and other (Tables 3.2 and 3.3). The proteins identified, along with their associated molecular weight (MW) and pI are presented. Although there were no MW markers on Figures 3.4 and 3.5, the pH range is displayed. Also listed for each identified protein are the number of unique peptides and total sequence coverage, respectively, from MS analysis. As stated previously, a sample was required to have at least two unique peptides upon analysis to be considered. The identified proteins from the sarcoplasmic protein fraction are listed in Table 3.2. Eight were associated with energy

metabolism and matched to 6 different proteins: triosphosphate isomerase (2 spots), alpha-enolase, creatine kinase M-type, aldose reductase, glyceraldehyde-3-phosphate dehydrogenase (2 spots), and carbonic anhydrase. Tropomyosin beta chain was the only protein identified in the contractile apparatus category. Ten were associated with cell structure and matched to five different proteins: tubulin alpha-1B chain, tubulin alpha-4A chain, tubulin beta-5 chain, vimentin, actin (cytoplasmic), actin (alpha skeletal muscle), desmin, and aldolase A. Two heat shock proteins (HSP), HSP beta-1 and HSP 90-beta, were sorted into the cell defensive category. Four spots were categorized as regulatory and matched to 4 different proteins: alpha-1 antiproteinase, beta-enolase, poly(rC)-binding protein 1, and 14-3-3 protein gamma. Seven spots matched to 5 other proteins were also identified: endoplasmin, alpha-2-HS-glycoprotein, serotransferrin, serum albumin, and phosphoglucomutase-1.

Table 3.3 shows the proteins identifies in the myofibrillar fraction. Five were associated with energy metabolism and matched to 4 different proteins: creatine kinase M-type, AZP synthase subunit beta, fructose-bisphosphate aldolase, and glyceraldehyde-3-phosphate dehydrogenase. Four proteins related to contractile apparatus were matched to 5 spots: tropomyosin alpha-1 chain, tropomyosin beta chain, troponin-T, and myosin light chain. Two spots were linked to cell structure and matched to 2 proteins; actin and desmin. An additional protein identified was a probable C->U editing-enzyme APOBEC-2.

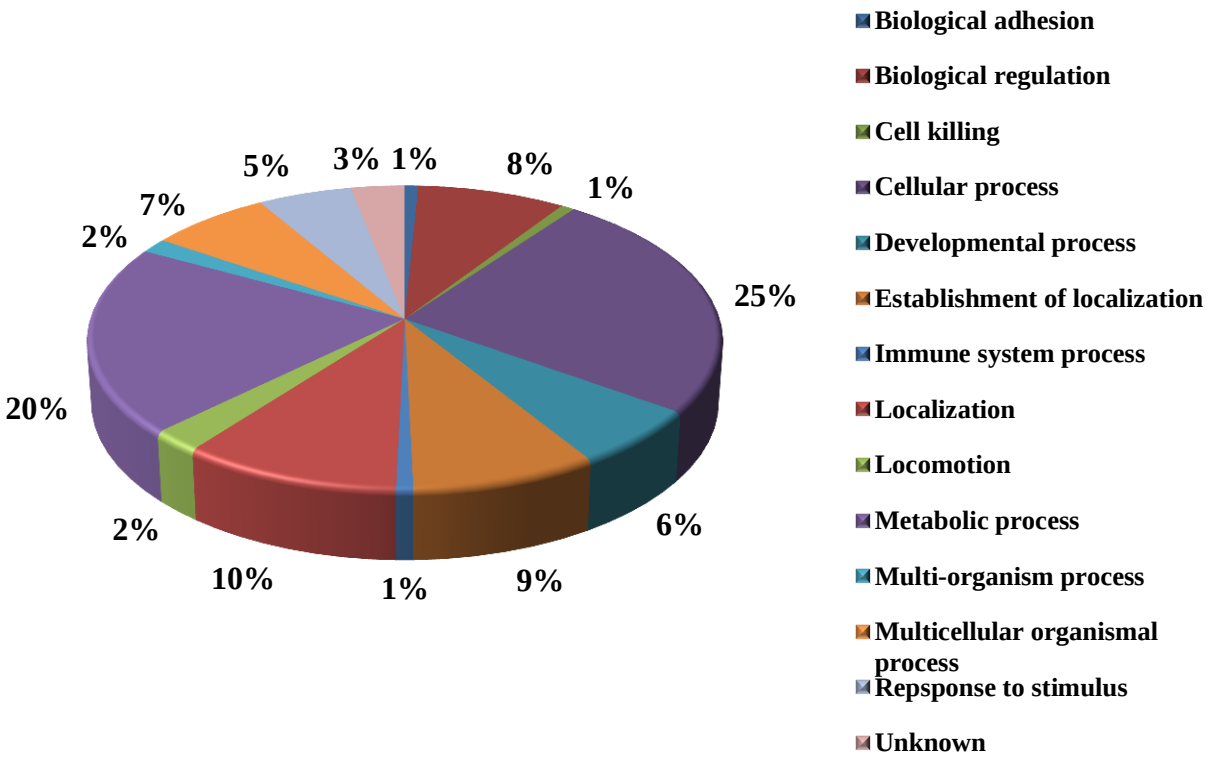

Figure 3.2. Biological processes of identified proteins as determined by Scaffold proteome software.

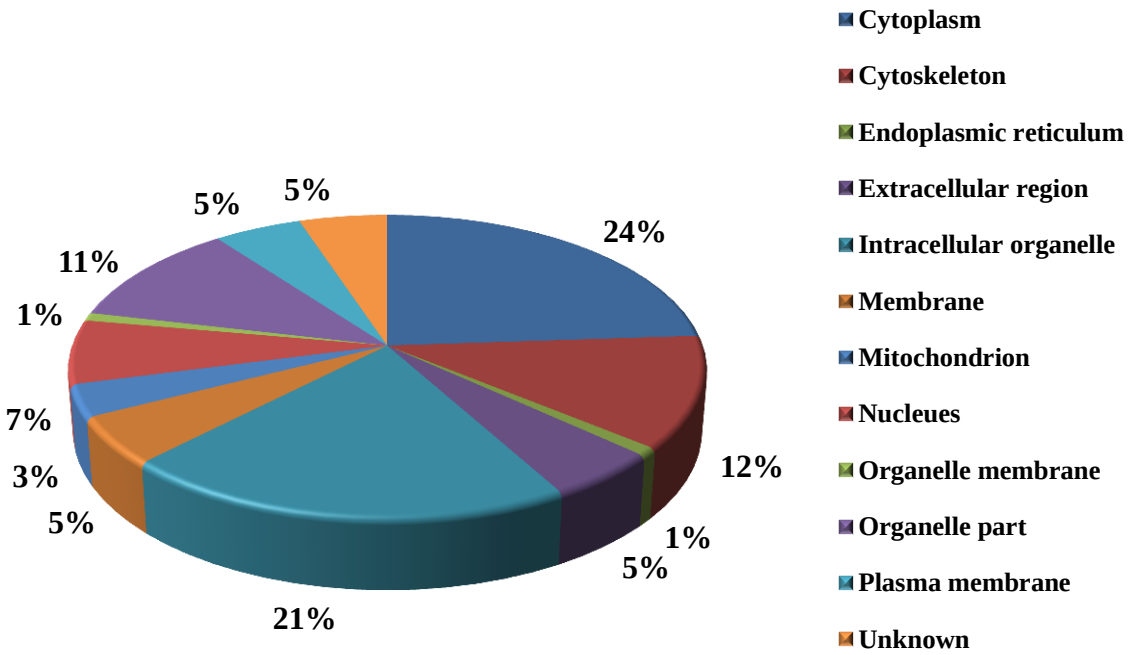

Figure 3.3. Cellular components of identified proteins as determined by Scaffold proteome software.

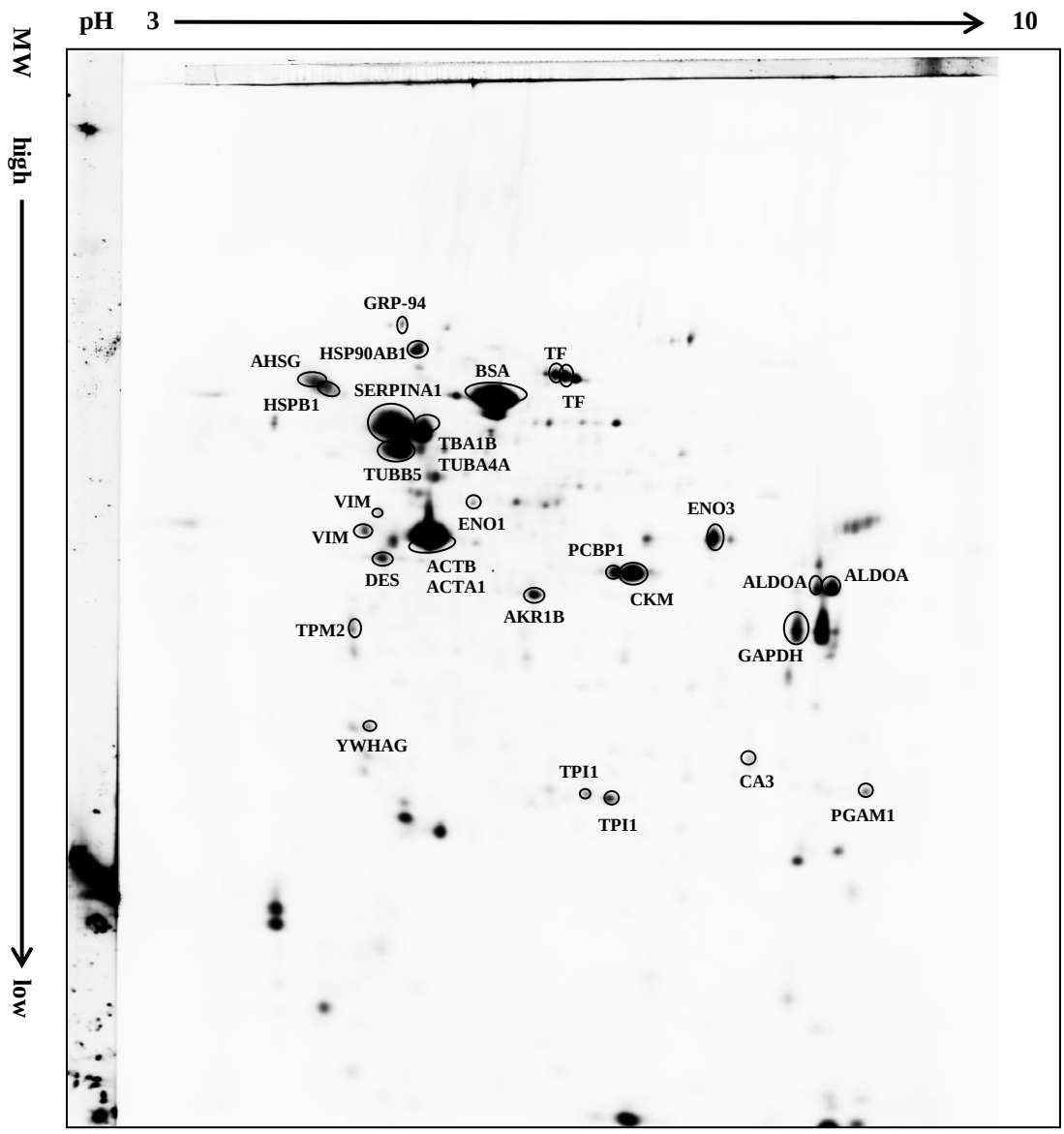

Figure 3.4. Representative two-dimensional difference in-gel electrophoresis map of fetal ovine LM sarcoplasmic proteins. Abbreviations: ACTA1 = Actin, alpha skeletal muscle; ACTB = Actin, cytoplasmic; AHSG = Alpha-2-HS-glycoprotein; AKR1B1 = Aldose reductase; ALDOA = Fructose-bisphosphate aldolase; BSA = Serum albumin; CA3 = Carbonic anhydrase 3; CKM = Creatine kinase M-type; DES = Desmin; ENO1 = Enolase 1; ENO3 = Enolase 3; GAPDH = Glyceraldehyde-3-phosphate dehydrogenase; GRP-94 = Endoplasmin; HSPB1 = Heat shock protein beta-1; HSP90AB1 = Heat shock protein HSP 90-beta; PCBP1 = Poly(rC)-binding protein 1; PGAM1 = Phosphoglucomutase-1; SERPINA1 = Alpha-1-antiproteinase; TBA1B = Tubulin alpha-1B chain; TF = Serotransferrin; TPI1 = Triosephosphate isomerase; TPM2 = Tropomyosin beta chain; TUBA4A = Tubulin alpha-4A chain; TUBB5 = Tubulin beta-5 chain; VIM = Vimentin; YWHAG = 14-3-3 protein gamma.

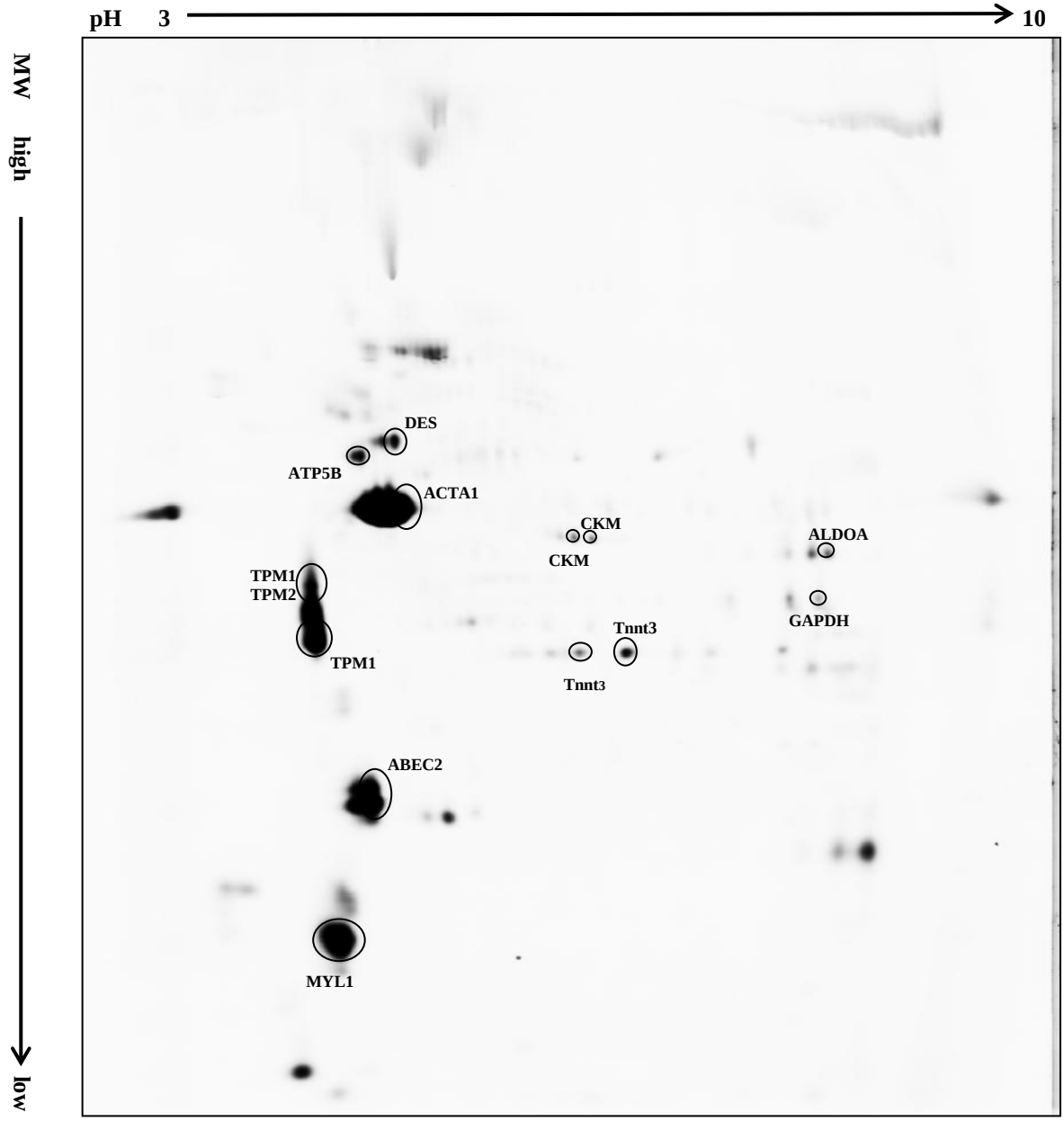

Figure 3.5. Representative two-dimensional difference in-gel electrophoresis map of fetal ovine LM myofibrillar proteins. Abbreviations: ABEC2 = Probable C->U-editing enzyme APOBEC-2; ACTA1 = Actin, alpha skeletal muscle; ALDOA = Fructose-bisphosphate aldolase; ATP5B = ATP synthase subunit beta, mitochondrial; CKM = Creatine kinase M-type; DES = Desmin; GAPDH = Glyceraldehyde-3-phosphate dehydrogenase; MYL1 = Myosin light chain 1/3, skeletal muscle isoform; Tnnt3 = Troponin T fast skeletal muscle type; TPM1 = Tropomyosin alpha-1 chain; TPM2 = Tropomyosin beta chain.

Table 3.2. Summary of protein identification from the sarcoplasmic fraction of fetal ovine LM

Spot ID	SWISS-PROT accession number[1]	Protein name[1]	MW, kDa theoretical	pI theoretical	Unique peptide	Sequence coverage, %
Energy metabolism						
6	TPIS_BOVIN	Triosphosphate isomerase	27	6.45	10	50
192	ENOA_BOVIN	Alpha-enolase	47	6.37	6	29
308	KCRM_BOVIN	Creatine kinase M-type	43	6.63	22	48
342	Q5E962_BOVIN	Aldose reductase	36	5.88	14	42
356	G3P_BOVIN	Glyceraldehyde-3-phosphate dehydrogenase	36	8.51	16	44
351	G3P_BOVIN	Glyceraldehyde-3-phosphate dehydrogenase	36	8.51	15	44
364	CAH3_BOVIN	Carbonic anhydrase 3	29	7.71	13	58
439	TPIS_BOVIN	Triosephosphate isomerase	27	6.45	18	75
Contractile apparatus						
311	TPM2_BOVIN	Tropomyosin beta chain	33	4.66	12	37
Cell structure						
164	TBA1B_BOVIN	Tubulin alpha-1B chain	50	4.94	19	39
164	TBA4A_BOVIN	Tubulin alpha-4A chain	50	4.93	4	39
181	TBB5_BOVIN	Tubulin beta-5 chain	50	4.78	16	40
204	VIME_BOVIN	Vimentin	54	5.05	13	32
216	VIME_BOVIN	Vimentin	54	5.05	13	26
280	ACTB_BOVIN	Actin, cytoplasmic	42	5.29	17	45
280	ACTS_BOVIN	Actin, alpha skeletal muscle	42	5.23	6	45
302	DESM_BOVIN	Desmin	54	5.21	28	49
315	A6QLL8_BOVIN	Fructose-bisphosphate aldolase	39	8.45	22	71
317	A6QLL8_BOVIN	Fructose-bisphosphate aldolase	39	8.45	15	43
Cell defensive						
3	E1BEL7_BOVIN	Heat shock protein beta-1	23	5.77	7	37
4	HS90B_BOVIN	Heat shock protein HSP 90-beta	83	4.96	16	20

[1]Protein names and accession numbers were taken from the UniProt database (http://www.uniprot.org).

Table 3.2. Summary of protein identification from the sarcoplasmic fraction of fetal ovine LM (continued)

Spot ID	SWISS-PROT accession number[1]	Protein name[1]	MW, kDa theoretical	pI theoretical	Unique peptide	Sequence coverage, %
Regulator						
2	A1AT_BOVIN	Alpha-1-antiproteinase	46	6.05	8	16
217	ENOB_BOVIN	Beta-enolase	47	7.60	23	44
309	PCBP1_BOVIN	Poly(rC)-binding protein 1	37	6.66	8	33
353	1433G_BOVIN	14-3-3 protein gamma	28	4.80	5	25
Other						
34	ENPL_BOVIN	Endoplasmin	92	4.76	29	33
87	FETUA_BOVIN	Alpha-2-HS-glycoprotein	38	5.26	5	9.7
105	G3X6N3_BOVIN	Serotransferrin	78	7.13	18	23
106	G3X6N3_BOVIN	Serotransferrin	78	7.13	18	23
132	ALBU_BOVIN	Serum albumin	69	5.82	25	30
376	PGAM1_BOVIN	Phosphoglucomutase-1	61	6.75	18	38
382	PGAM1_BOVIN	Phosphoglucomutase-1	61	6.75	13	31

[1]Protein names and accession numbers were taken from the UniProt database (http://www.uniprot.org).

Table 3.3. Summary of protein identification from the myofibrillar fraction of fetal ovine LM

Spot ID	SWISS-PROT accession number[1]	Protein name[1]	MW, kDa theoretical	pI theoretical	Unique peptide	Sequence coverage, %
Energy metabolism						
2	KCRM_BOVIN	Creatine kinase M-type	43	6.63	17	39
173	KCRM_BOVIN	Creatine kinase M-type	43	6.63	15	38
123	ATPB_BOVIN	ATP synthase subunit beta, mitochondrial	56	5.15	19	45
168	A6QLL8_BOVIN	Fructose-bisphosphate aldolase	39	8.45	12	37
186	G3P_BOVIN	Glyceraldehyde-3-phosphate dehydrogenase	36	8.51	2	9.3
Contractile apparatus						
185	TPM1_BOVIN	Tropomyosin alpha-1 chain	33	4.69	7	17
217	TPM1_BOVIN	Tropomyosin alpha-1 chain	33	4.69	17	48
185	TPM2_BOVIN	Tropomyosin beta chain	33	4.66	9	43
210	Q8MKI0_BOVIN	Troponin T fast skeletal muscle type	31	8.69	5	20
303	MYL1_BOVIN	Myosin light chain 1/3, skeletal muscle isoform	21	4.96	9	53
Cell structure						
3	ACTS_BOVIN	Actin, alpha skeletal muscle	42	5.23	18	54
125	DESM_BOVIN	Desmin	54	5.21	17	44
Other						
246	ABEC2_BOVIN	Probable C->U-editing enzyme APOBEC-2	26	4.84	5	17

[1]Protein names and accession numbers were taken from the UniProt database (http://www.uniprot.org).

Functional Analysis of Differentially Expressed Proteins

The proteins differentially expressed ($P \leq 0.05$) between treatments are associated with energy metabolism and cell structure. Tubulin beta-5 chain was more abundant ($P < 0.05$) in the LM of fetuses from ewes receiving the MP100 treatment when compared with those from MP60. In muscle cells, tubulin is a highly abundant structural protein essential for myotube formation. Although their function in skeletal muscle after differentiation is unclear, studies have been done to investigate the adaptation properties of tubulin in muscle (Sakurai et al., 2005). The results from the present study indicate decreased tubulin in LM in fetuses of dams receiving less than adequate protein, possibly hindering the muscle's cytoskeletal ability to respond mechanically to the environment.

The metabolic enzyme alpha-enolase was more abundant ($P < 0.05$) in the LM of fetuses from MP60-treated ewes when compared with that from MP100 ewes. Apart from its role in glycolysis, recent assays have identified alpha-enolase as a novel protein capable of binding RNA (Hernández-Pérez et al., 2011). Identified in the rat myoblasts, alpha-enolase is present in the nucleus, suggesting the enzyme involvement in regulation, transport, or modification processes related to RNA (Hernández-Pérez et al., 2011). There is evidence from several studies demonstrating the role of alpha-enolase in glycolysis and transcriptional regulation (Merkulova et al., 1997; Keller et al., 2007; Jeffrey, 2009). The metabolic state of muscle cells and the localization of proteins to different cellular components during development of the LM in fetuses from MP60 ewes may have caused an increase in alpha-enolase expression when compared with fetuses from MP100 ewes. The protein profile of muscle from fetuses of dams receiving less than adequate amounts of protein may have been adversely affected, resulting in differential appearance of the glycolytic enzyme.

Creatine kinase was more abundant ($P < 0.05$) in the LM of fetuses from ewes receiving the MP100 treatment when compared to the MP140 treatment. Creatine kinase is most commonly known for its ability to catalyze the reversible transfer of high energy phosphate from ATP to creatine. In muscle cells, this extra energy buffer plays a pivotal role in maintaining ATP homeostasis (Hettling, 2011). An enzyme with two different subunits, creatine kinase has been shown immunochemically to be expressed in a developmentally and tissue-specifically regulated manner (Robert et al., 1991). There are many proposed roles of creatine kinase in muscle including, regulation of phosphorylation, intracellular energy transport, transfer of phosphoryl groups via the phosphocreatine shuttle, and the ability to buffer ADP concentration in cytosol near sites of ATP hydrolysis. Adequate amounts of dietary protein or essential amino acids can increase muscle protein synthesis in healthy animals. In the present study, excess levels of maternal MP did not appear to enhance the glycolytic potential of fetal LM. A muscle creatine kinase deficiency could result in delay of postnatal muscle growth, impaired muscle regeneration and decreased muscle hypertrophy (Izumiya et al., 2008).

We identified aldolase A in 2 spots at different pI on our 2D gel map. Analysis of these 2 spots demonstrated the most alkaline spot was more abundant ($P < 0.01$) in the LM of fetuses from MP140 ewes, while the remaining spot was more abundant ($P < 0.05$) in the LM of fetuses from MP100 ewes. These data could be the result of a post-translational modification such as phosphorylation. Unfortunately, due to the fact that the sheep proteome in not sequenced, it is unclear what modification, if any, occurred. A study investigating nutritional status on muscle development in rabbits found that increased activity of aldolase A slowed down muscle development as a result of feed rationing (Zotte et al., 2004). Phosphoglucomutase 1 (PGM1) was more abundant ($P < 0.05$) in LM from fetuses of ewes receiving the MP100 treatment when

compared with that from fetuses of ewes receiving the MP60 treatment. PGM1 catalyzes the interconversion of glucose-1-phosphate and glucose-6-phosphate via the glucose-1,6-diphosphate intermediate, representing a key step in the glycolytic pathway (Levin et al., 1999). The growth potential of fetal skeletal muscle is dependent upon the increased expression of central enzymes involved in glycolysis (Bernard et al., 2009). Glycogen metabolism could be adversely affected by a decreased expression of PGM1 in fetuses of ewes receiving the MP60 treatment. A decrease in this important enzyme may lead to reduced glucose availability to the fetus, resulting in slowed muscle turnover.

Western Blotting

The same protein can be identified in different spots and at different pI and MW locations within a muscle. Different locality of the same protein on gels maps could be consistent with different isoforms. Isoform expression can result from the expression of different genes or may also be initiated by post-translational modifications such as oxidation, glycosylation, phosphorylation, or proteolytic cleavage. Mass spectrometry analysis resulted in 2 spots at different locations and intensities identified as aldolase A. In a one-dimensional Western blot for aldolase A 2 immunoreactive bands were detected (Figure 3.5). The ratio of the intensity of the sample band to the intensity of the same band in control sample was used to analyze differences in aldolase A in the sarcoplasmic fraction of fetal LM between maternal MP treatments.

Although we observed no effect of MP treatment on Band 1, Band 2 was more abundant ($P = 0.05$) in LM of fetuses from MP140 and MP60 ewes when compared to those from MP100 supplemented ewes. This is in agreement with our results from 2D DIGE (Table 3.4). We suspect that immunoreactive Band 1 might be aldolase B since we cannot contribute it to non-specific binding.

Table 3.4. Identified proteins from proteomic analysis differentially expressed among MP treatments in sarcoplasmic fraction of fetal ovine LM

Spot ID[1]	Name	Comparison	More abundant in	*P* - value	UniProt ID	Theoretical MW (kDa)
181	Tubulin beta-5 chain	MP60 vs. MP100	MP100	0.016	TBB5_BOVIN	50
192	Alpha-enolase	MP60 vs. MP100	MP60	0.013	ENOA_BOVIN	47
308	Creatine kinase M-type	MP140 vs. MP100	MP100	0.016	KCRM_BOVIN	43
315	Fructose-bisphosphate aldolase	MP140 vs. MP100	MP140	0.002	A6QLL8_BOVIN	39
317	Fructose-bisphosphate aldolase	MP140 vs. MP100	MP100	0.014	A6QLL8_BOVIN	39
376	Phosphoglucomutase 1	MP60 vs. MP100	MP100	0.018	PGAM1_BOVIN	61

[1]Spot name corresponds to spots identified in Fig. 3.2.

[2]Protein names and accession numbers were taken from the UniProt database (http://www.uniprot.org).

[3]Maternal diets (DM basis) were balanced for mature ewes baring singletons during the last 4 weeks of gestation according to NRC (2007). Treatments: MP60 = 60% of MP requirements, MP100 = 100% of MP requirements, MP140 = 140% of MP requirements.

Aldolase A, found in the developing embryo, is an enzyme that catalyzes a reversible aldol reaction during glycolysis. The increase in the glycolytic enzyme content seen in the LM of fetuses from MP140 and MP60 ewes may increase the volume of substrates through glycolysis. While providing adequate protein to the dam during gestation did not alter fetal aldolase A expression, fetal LM was sensitive to both high and low levels of maternal-supplemented MP, suggesting possible effects of maternal nutrition on fetal muscle glucose metabolism.

In order to further examine whether maternal nutrition in utero affects myogenesis, TnT, a myofibrillar protein identified that is important to skeletal muscle development, was investigated. Troponin T binds to tropomyosin and helps position it on actin, regulating contraction of striated muscle. It has been shown to generate protein diversity by alternative RNA splicing in all striated muscles. Fast and slow skeletal TnT isoforms can be expressed from several different mRNA in a developmentally regulated and tissue-specific manner (Gomes et al., 2002). The role of these different TnT isoforms in the regulation of muscle contraction is not well understood. In a one-dimensional Western blot for TnT (myofibrillar protein fraction), 6 bands were detected (Fig. 3.6). No effect of treatment was observed in Bands 1 to 5 of TnT analyzed from the myofibrillar fraction of ovine fetal LM. There was a tendency ($P = 0.08$) for Band 1 to be more abundant in the LM of fetuses from ewes receiving the MP140 treatment when compared with that of fetuses from MP60 ewes, with MP100 being intermediate.

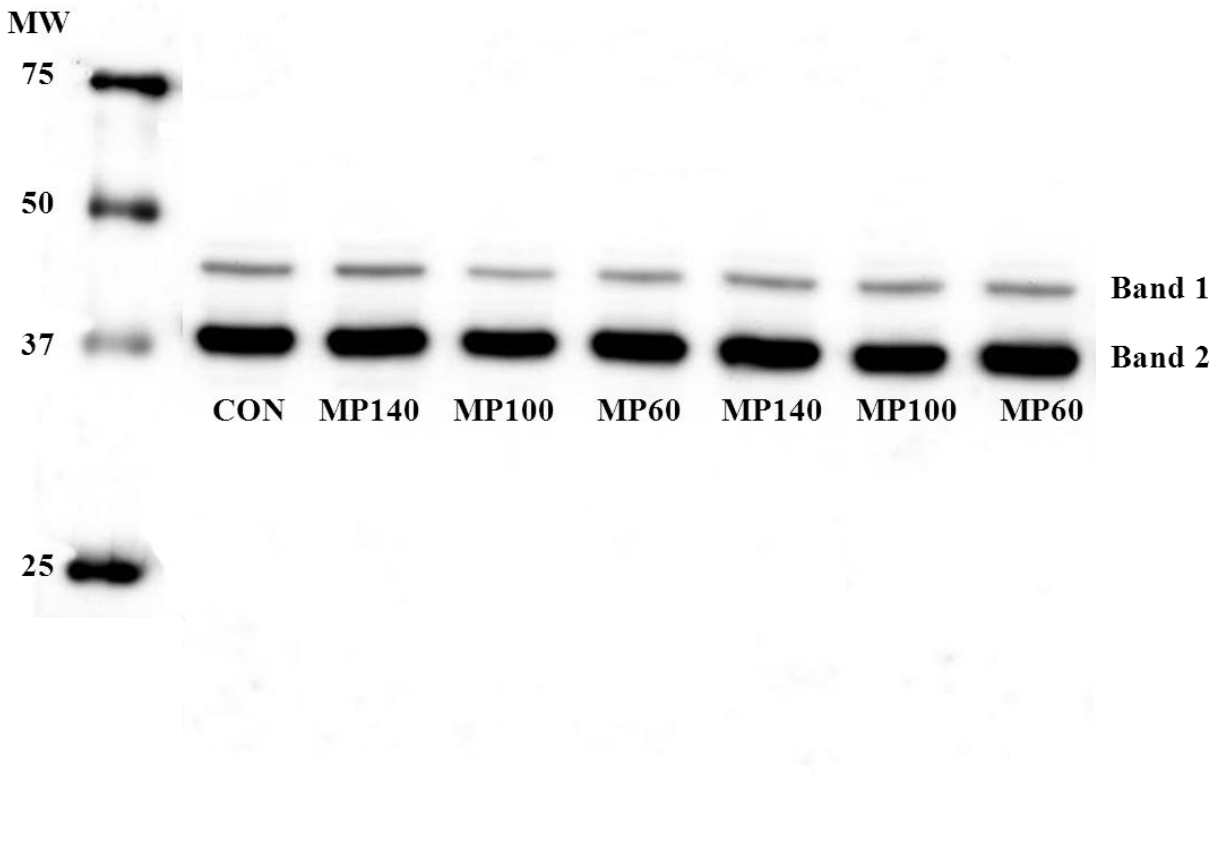

Figure 3.6. Representative aldolase A Western blot run on 12.5% acrylamide separating gel loaded with 10 µg of sarcoplasmic protein. Two major immunoreactive bands were detected. Maternal diets (DM basis) were balanced for mature ewes baring singletons during the last 4 weeks of gestation according to NRC (2007). Treatments: MP60 = 60% of MP requirements, MP100 = 100% of MP requirements, MP140 = 140% of MP requirements.

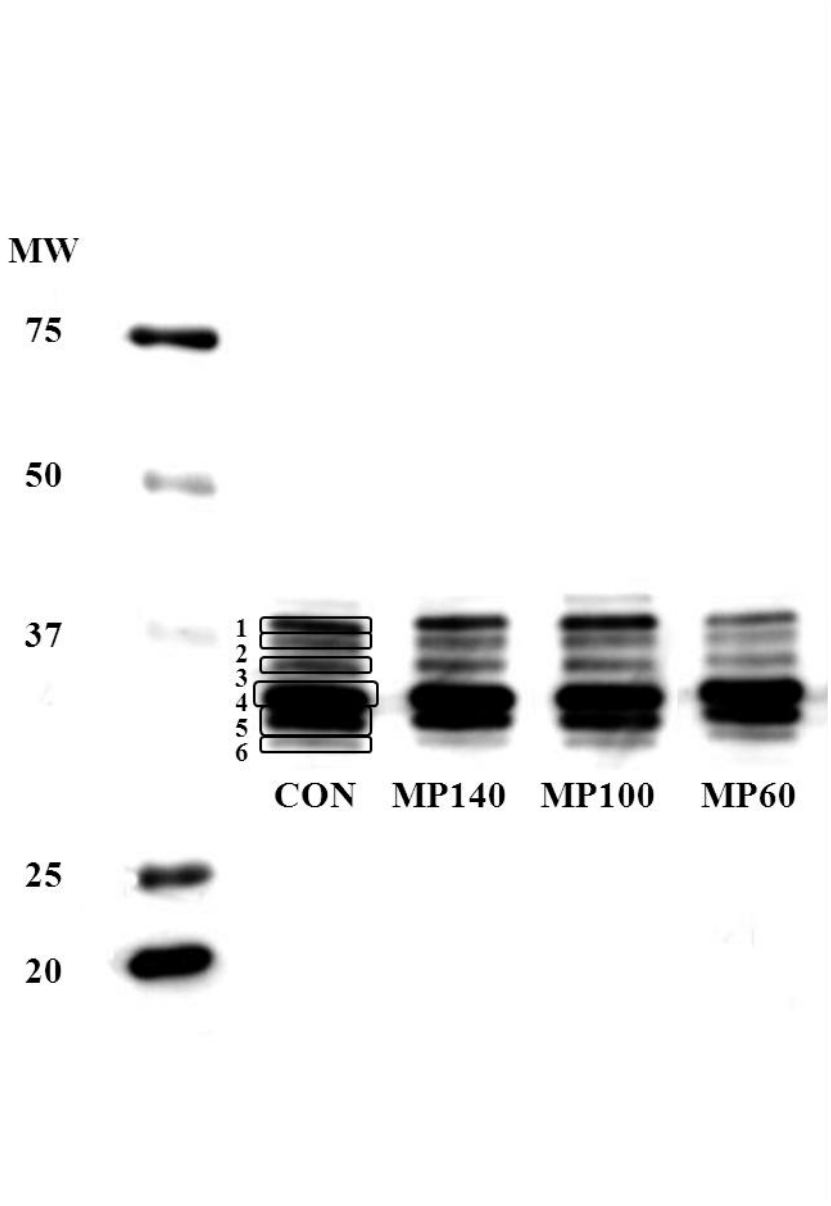

Figure 3.7. Representative Troponin-T Western blot run on 12.5% acrylamide separating gel loaded with 7 µg of myofibrillar protein. Five major immunoreactive bands were detected. Maternal diets (DM basis) were balanced for mature ewes baring singletons during the last 4 weeks of gestation according to NRC (2007). Treatments: MP60 = 60% of MP requirements, MP100 = 100% of MP requirements, MP140 = 140% of MP requirements.

Conclusion

The sheep is a valuable model for the study of developmental programming mechanisms that are critical to a better understanding of the nutrition and prenatal muscle development relationship. The lack of genetic and genomic resources on the sheep has limited the power of proteomic analysis, impending progress in emerging areas of sheep pregnancy biology and advanced muscle research. Ovine fetal LM was sensitive to maternal MP supplementation during late gestation. Using 2D DIGE to compare the proteome of muscles that differ between treatment, numerous proteins were identified that may be useful in understanding the metabolism of developing fetal sheep muscle. Tubulin beta 5-chain, alpha-enolase, and phosphoglucomutase differed in relative abundance in the sarcoplasmic fraction of fetal LM from MP60 and MP100-supplemented ewes. Creatine kinase and fructose-bisphosphate aldolase differed in relative abundance in the sarcoplasmic fraction of fetal LM from MP100 and MP140-supplemented ewes. The data presented in this study may influence current research on the series of genes expressed in the fetal ovine LM, which will strengthen annotation and assembly of the sheep genome. Notable, however, was our identification of many high abundant proteins. Using 2D-DIGE on fetal muscle has proven to be a useful method, but alterations in preparation of the gel sample must be considered in order to look more closely at low abundant proteins that may have been masked during this experiment.

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CHAPTER 4. OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

Developmental programming research in ruminant livestock species has focused on altering overall caloric intake. Currently, little is known about how specific nutrient (e.g. protein, energy, amino acids, fatty acids, minerals, vitamins) supplementation during gestation influences fetal organ growth and skeletal muscle development. Providing supplemental protein during late gestation is crucial to maintenance of the dam and growth of the fetus. Altering a subset of maternal nutrients, while still providing an isocaloric diet has proved to have some beneficial effects with regard to fetal organ growth and skeletal muscle development.

Fetal visceral organ growth was sensitive to maternal MP supplementation, enhancing small intestinal mass and perirenal fat content. Feeding isocaloric protein is important to fetal development, but demands further research into effects on postnatal growth. Additional investigation is also needed regarding maternal supplementation of isocaloric protein and its possible alterations to fetal skeletal muscle fiber type. Further understanding of metabolizable protein's role in fetal development will allow for the design of supplementation schemes and altered management strategies to improve fetal growth efficiency, thereby improving the offspring's health and performance.