PROTEIN METABOLISM IN RESPONSE TO METABOLIC ACIDOSIS IN RUMINANTS

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ABSTRACT

PROTEIN METABOLISM IN RESPONSE TO METABOLIC ACIDOSIS IN RUMINANTS

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Use of readily fermentable diets in ruminant production systems is common practice at periods of high energy demand, such as at the onset of lactation. However, increased production and absorption of lactate and propionate can occur, resulting in a declining blood pH. This lower blood pH is a symptom of metabolic acidosis. In monogastrics, the physiologic buffering to counteract metabolic acidosis occurs via a whole body response, including increased skeletal muscle proteolysis via the ubiquitin-mediated proteolytic pathway (UPP) after sarcomeric cleavage by caspase-3. Attenuation of proteolysis using exogenous supplementation of glutamine has been shown in monogastrics experiencing metabolic acidosis. However, whether the UPP is upregulated at the onset of metabolic acidosis in ruminants is unclear. In addition, it is unclear whether additional physiologic demands, such as the onset of lactation, could exacerbate the UPP response to metabolic acidosis. The objectives of the current research are to 1) examine regulation of the UPP at the onset of lactation in dairy cows, 2) examine protein degradation and amino acid response to varying severities of metabolic acidosis in ruminants, and 3) determine if glutamine supplementation provides a method for attenuation of proteolysis in ruminants during metabolic acidosis. The results presented in this thesis demonstrate that the onset of lactation does significantly upregulate mRNA expression of components of the UPP. However, induction of mild, moderate or a more

severe acidosis did not upregulate the UPP in skeletal muscle. Induction of a severe metabolic acidosis instead downregulated mRNA expression of caspase-3 in slow-twitch skeletal muscle, in a muscle type specific manor, potentially providing an explanation as to the lack of regulation of the UPP during acidosis. Increased plasma glutamine in acidotic sheep also suggests that glutamine is not limiting in ruminants during acidosis, and no observed change in proteolytic components in response to glutamine supplementation in acidotic sheep further suggests that other regulatory amino acids could be more beneficial to ruminants with metabolic acidosis.

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List of Acronyms

4EBP	4E binding protein
AA	Amino acid
ADP	Adenosine diphosphate
AG	Anion gap
ATP	Adenosine triphosphate
BCAA	Branch-chain amino acid
BE	Base excess
BEb	Base excess of blood
BEecf	Base excess of extracellular fluid
BW	Body weight
Ca^{2+}	Calcium
cDNA	Complementary DNA
Cl^-	Chloride
CO_2	Carbon dioxide
CPS1	Carbamoyl phosphate synthetase 1
Ct	Threshold cycle
DCAD	Dietary cation: anion difference
DDG	Dried distiller's grain
DEPC	Diethylpyrocarbonate
DM	Dry matter
DMI	Dry matter intake
EDL	Extensor digitorum longus
EGTA	Ethylene glycol tetraacetic acid
eIF	Eukaryotic initiation factor
FSR	Fractional synthesis rate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GH	Growth hormone
GSK3	Glycogen synthase kinase 3
H_2O	Water
H^+	Hydrogen ion
Hb	Hemoglobin
HCl	Hydrochloric acid
HCO_3^-	Bicarbonate
iCa	Intracellular calcium
IGF-1	Insulin-like growth factor 1
K^+	Potassium
Mg^{2+}	Magnesium
MHC	Major histocompatability complex
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
N	Nitrogen
Na^+	Sodium
NFC	Non-fibre carbohydrate

NH_4^+	Ammonium
NH_4Cl	Ammonium chloride
O_2Sat	Oxygen saturation
P70S6K	P70S6 kinase
pCO_2	Partial pressure of carbon dioxide
PI3K	Phosphoinositol-3-kinase
pO_2	Partial pressure of oxygen
RT-PCR	Real-time polymerase chain reaction
S^{2-}	Sulfide
S6K	S6 kinase
SID	Strong ion difference
TCA	Tricarboxylic acid
TCO_2	Total carbon dioxide
tHb	Total hemoglobin
TMR	Total mixed ration
UPP	Ubiquitin-mediated proteolytic pathway
VFA	Volatile fatty acids

CHAPTER 1

General Introduction

The use of a high grain diet is a common method to provide a readily fermentable, energy dense nutrient source to ruminants. This is particularly useful during periods in which additional physiologic demands are present, such as in periods of growth or the onset of lactation. However, increases in the concentrate portion of the ruminant diet can also be accompanied by shifts in the microbial populations that favour growth of lactate-producing microorganisms, such as *Streptococcus bovis* and lactobacilli (Herrera et al., 2009). Increased production and absorption of lactate results in a decrease in blood pH, a condition known as metabolic (or systemic) acidosis (Nocek, 1997). To buffer the increasing excess of H^+ and accommodate the increasing need for inter-organ nitrogen transfers in monogastrics, a whole body response ensues (Taylor and Curthoys, 2004). Onset of skeletal muscle proteolysis in monogastrics is rapid, providing additional amino acids, in particular glutamine, in the bloodstream for transport to the kidneys (Hughey et al., 1980; Reaich et al., 1992; Šafránek et al., 2003). Though other amino acids, such as glycine (Pitts et al., 1965), also provide aid in terms of buffering, glutamine appears to be the most crucial amino acid for monogastric buffering. Additional endogenous provision of glutamine is also supported by increased intestinal absorption of glutamine (Epler et al., 2003; Pan et al., 2004), as well as increased hepatic glutamine synthetase activity (Taylor and Curthoys, 2004). Renal uptake of glutamine increases (Moret et al., 2007), where glutamine is deaminated by glutaminase, releasing ammonium (NH_4^+) for urinary excretion, and glutamate (Garibotto et al., 2009). Glutamate can be further deaminated by

glutamate dehydrogenase to form α-ketoglutarate and excrete a second NH_4^+ molecule (Nissim et al., 1985; Garibotto et al., 2009). Ultimately, glutamine will produce 2 NH_4^+ and 2 HCO_3^-, and an increasing requirement for the buffering capacity of this amino acid will lead to an increased need for availability of glutamine.

Activation of skeletal muscle proteolysis by metabolic acidosis is accomplished via upregulation of the ubiquitin-mediated proteolytic pathway (UPP; Mitch et al., 1994). The UPP is the only ATP-dependent proteolytic pathway and is involved in many disease states in monogastrics, including cachexia, diabetes, renal failure and sepsis (Lecker et al., 2006; Rajan and Mitch, 2008). Ubiquitin molecules are attached in a chain-like fashion to the target protein via conjugating enzymes. Binding of ubiquitin to the conjugating enzyme E1 consumes one molecule of ATP, creating a high-energy thiol ester intermediate (Ciechanover, 2006). Ubiquitin is consequently transferred to conjugating enzyme E2, then E3. The conjugating enzyme E3 finally attaches the ubiquitin molecule to the target protein (Du et al., 2005). Once a sequence of at least four ubiquitin molecules has attached to the target protein, the ubiquitin chain is recognized by the 26S proteasome. The 26S proteasome is composed of 2 components, the ATP-dependent 19S cap, and the 20S core, which is further characterized by two conjoined catalytic β-subunits flanked on either side by an α-subunit (Hershko et al., 2000). The target protein is typically unfolded, the ubiquitin molecules cleaved in an ATP-requiring reaction, and the target protein is catabolized to smaller oligopeptide fragments (Hershko and Ciechanover, 1998; Jagoe and Goldberg, 2001). These fragments can be further broken down into individual amino acids, or can be carried by MHC 1 for antigen presentation on the cell surface (Saric and Goldberg, 2005). However, the UPP cannot

degrade large proteins, and hence precursor cleavage of the sarcomere is accomplished via caspase-3 (Du et al., 2005), and to some extent by the calcium-dependent calpains and lysosomal cathepsins (Lecker et al., 2004).

Attenuation of the proteolytic impact of metabolic acidosis has been successful with exogenous provision of glutamine. Provision of glutamine either by infusion (Wilmore, 2001) or nasogastric tube (Coeffier et al., 2003) decreases protein loss in humans with metabolic acidosis. However, whether this theory holds true for ruminants is less certain.

Ruminants have the amazing capacity to recycle nitrogen (Lobley et al., 1995), and the compensatory response to metabolic acidosis in ruminants is poorly characterized. Whether ruminants undergo ubiquitin-mediated proteolysis to provide additional amino acids for buffering is unknown. In addition, it is not clear if ruminants rely on glutamine for inter-organ nitrogen transport under circumstances of acidosis as heavily as do monogastrics. Livestock production systems generally contain animals that also have secondary requirements, such as growth or lactation, and hence these additional physiologic requirements could also impact UPP activity. Characterization of the proteolytic and amino acid response to independent physiologic demands in ruminants, such as lactation or metabolic acidosis, is important in order for development of mitigation strategies.

CHAPTER 2

Literature Review: Metabolic acidosis, protein turnover and glutamine usage

2.1 Introduction

The onset of metabolic acidosis triggers a whole body response to buffer declining blood pH. Orchestrated responses by the small intestine, liver, kidney and muscle attempt to buffer excess H^+ and provide additional sources of inter-organ nitrogen transport (Taylor and Curthoys, 2004). Glutamate is extensively utilized by the intestinal mucosa, and provides substrate for producing amino acid derivatives (Reeds et al., 2000; Wang et al., 2009). Conversely, glutamine formation is only accomplished by ATP-dependent ligation of NH_4^+ to glutamate via glutamine synthetase, which is found predominantly in liver, muscle, lung and adipose but is also found in herbivore kidneys (Taylor and Curthoys, 2004). Glutamine is in turn catabolized by hydrolytic cleavage in the kidneys via one of three isoforms of mitochondrial glutaminase, consequently releasing NH_4^+, glutamate and HCO_3^- (Garibotto et al., 2009). Glutamate dehydrogenase further deaminates glutamate, forming α-ketoglutarate (Nissim et al., 1985), allowing a total release of 2 NH_4^+ and 2 HCO_3^- per molecule of glutamine. The NH_4^+ is excreted in urine, while the HCO_3^- is recycled for further buffering. Provision of this glutamine comes from two key sources, namely via intestinal absorption and muscle proteolysis. The ubiquitin-mediated proteolytic pathway is the primary pathway involved in proteolysis during disease states (Lecker et al., 2006), and is accompanied by some suppression of protein synthesis to fulfill the physiologic requirement for increased glutamine (Šafránek et al., 2003). Exogenous provision of glutamine has provided some

relief to monogastric proteolysis during disease states (Wilmore, 2001; Wang et al., 2009); however, whether the same holds true for ruminants is unknown. Metabolic acidosis can occur in ruminants fed a readily fermentable diet, such as a high grain diet. This energy dense diet is often fed to increase the energy content of the diet in circumstances where there is increased biological productivity, such as the finishing period in meat production or the onset of lactation in dairy production. An increase in microbial production of volatile fatty acids and lactate induces a shift in microbial population caused by declining rumen pH (Nocek, 1997), spurring an increase in lactate-producing microbes and ultimately an increase in production and absorption of lactate (Huntington et al., 1981b). The response of protein metabolism, with specific emphasis on proteolysis, is poorly characterized in ruminants. Here we will discuss the physiologic response to metabolic acidosis in monogastrics and identify some potential areas of focus for ruminants.

2.2 Blood gas and acid:base status: Confirmation of metabolic acidosis

As a consequence of many monogastric disease states, a systemic, or metabolic, acidosis will result (Newsholme et al., 2003; Gunnerson, 2005; Morris and Low, 2008b). Understanding and measurement of 'acidosis' has proven to be complex and tedious. Classifications, or types, of acidosis exist, and dispute amongst researchers as to the benefits and pitfalls of each calculation for various types of metabolic acidosis is evident in the literature (Kellum, 2000; Kamel and Halperin, 2006; Morris and Low, 2008a; Fidkowski and Helstrom, 2009). For analytical purposes, metabolic acidosis must first be differentiated from respiratory acidosis. If a change in the partial pressure of carbon

dioxide (pCO_2) is observed, this is indicative of decreased carbonic acid dissociation to yield CO_2 and H_2O (Kellum, 2000), and is considered respiratory acidosis. If, however, no change of pCO_2 is observed, further analysis into disturbances of acid-base and gas balances can be made. Other indicators can then aid in further identification of a metabolic acidosis, such as decreases in the total CO_2 (TCO_2) concentration (Constable, 2000), and plasma bicarbonate (HCO_3^-) concentration. Plasma HCO_3^- concentration declines as metabolic acidosis progresses, and is an excellent indicator of acidosis as long as no other secondary illnesses are causing an increase in HCO_3^- (Kamel and Halperin, 2006); however, Constable (2000) also notes that the plasma HCO_3^- concentration, which is calculated using the Henderson-Hasselbalch equation, is not fully discrete from respiratory shifts in pCO_2.

Ultimately, three calculation options have emerged for determining potential acid-base disturbances, namely the anion gap (AG), Stewart's Strong Ion Difference (SID), and modified base excess (BE). All of the above calculations are acceptable; however, preference is given to individual calculations depending on the acidosis, or the mixture of acidosis types, occurring. The most basic of the three calculations is BE of blood (BEb) and extracellular fluid (BEecf), incorporating hemoglobin (Hb) and HCO_3^- (Constable, 2000) as shown below:

$$BEb = (1 - 0.023 \times [Hb]) \times [HCO_3^-] - 24.4 + (7.7 + 2.33[Hb]) \times (pH - 7.40)$$

$$BEecf = 0.93 \times \{[\text{actual } HCO_3^-] - 24.4 + 14.83 \times (pH - 7.40)\}$$

A standard bicarbonate measurement can be used specifically for humans to eliminate dependency on pCO_2 values (Constable, 2000). This calculation is based on the quantity of acid or base that must be added to the sample in order to restore it to a pH of 7.40, and assumes that blood is 37°C at pCO_2 of 40 mmHg (Kellum, 2000). Subcategories of BE can be made based on either what physiologic indice the BE is estimated in, ie. in blood or extracellular fluid (Constable, 2000), or by the cause of the BE, such as plasma protein or chloride concentration (Fidkowski and Helstrom, 2009). Though BE provides some standardization due to acidosis from a metabolic component, BE cannot identify the source of the acid: base disturbance (Kellum, 2000).

In response to this need for identification of the metabolic component, the AG calculation was developed as follows (Morris and Low, 2008b):

$$AG = ([Na^+] + [K^+]) - ([Cl^-] + [HCO_3]^-)$$

Corrections can also be included in this calculation for plasma albumin, phosphate, and lactate concentrations (Gunnerson, 2005). Even the simplest form of this equation is effective for determination of lactic acidosis (Morris and Low, 2008a; Morris and Low, 2008b), as the anion: cation difference accounts for unmeasured anions. However, in circumstances where metabolic acidosis is inversely related to either both cations or both anions included in the AG equation, AG becomes inaccurate in determination of acid: base inequilibriums. For example, incidences of hyperchloremia caused by hydrochloric acid (HCl), AG have been shown to be inadequate since the increase in Cl^- is counterbalanced by a decrease in HCO_3^- resulting from H^+ buffering (Morris and Low,

2008b; Fidkowski and Helstrom, 2009). The SID calculation more accurately predicts this form of acidosis, as shown below (from Morris and Low, 2008a):

$$SID = ([Na^+] + [K^+] + [Ca^{2+}] + [Mg^{2+}]) - ([Cl^-] + [lactate])$$

This calculation is also termed the apparent SID, as there are some ions that are not included in the calculation (Kellum, 2000); however, this measure still provides a useful measurement and is understood as being the physiochemical approach to acid: base measurement (Fidkowski and Helstrom, 2009).

2.3 Glutamine metabolism in normal and acidosis states in monogastrics

Perhaps due to glutamine's pivotal role in nitrogen transfer, glutamine is the most abundant free amino acid in the body. Glutamine is transported from tissues that are disposing nitrogen, such as muscle and lung, to tissues that utilize or excrete of excess nitrogen (Newsholme et al., 2003). Renal glutaminase activity is necessary for excretion of NH_4^+ in the urine. In addition, the intestine, liver, lymphocytes and kidney all have rapid rates of glutamine utilization, where glutamine is used as a precursor for the synthesis of proteins, amino sugars and nucleotides (Newsholme et al., 2003; Taylor and Curthoys, 2004).

In response to low blood pH in monogastrics, body proteins are catabolized and shifts in amino acid utilization occur in order to aid in buffering excess H^+ and inter-organ nitrogen transport. The amino acid glutamine is the primary nitrogen acceptor/donor (Taylor and Curthoys, 2004) and hence plays a crucial role in buffering

excess H^+ and transporting this excess H^+ for renal excretion as ammonium (NH_4^+). Acute metabolic acidosis is characterized by a 184% increase in arterial glutamine concentrations (Hughey et al., 1980). However, during chronic metabolic acidosis, arterial glutamine concentrations decrease to 70 - 90% of normal glutamine concentrations (Hughey et al., 1980; Schröck and Goldstein, 1981; Squires et al., 1976). Multiple organs are responsible for these fluctuations. This review will focus on the key organs participating in this physiologic shift, namely the kidneys, liver, muscle and small intestine.

The splanchnic bed, more specifically the small intestine, is the primary source of glutamine via dietary absorption and possesses a large glutamine requirement for normal function and maintenance. The primary products of glutamine oxidation are CO_2, with glutamate and NH_4^+ produced (Wu et al., 1995). In the intestine, approximately 31% of glutamine is converted to glutamate (Windmueller and Spaeth, 1974) via glutaminase. Glutamate is then further metabolized by glutamate dehydrogenase to produce α-ketoglutarate, also termed 2-oxoglutarate, which enters the tricarboxylic acid (TCA) cycle and produces pyruvate (Reeds et al., 2000). After first pass through the intestinal mucosa, approximately 20% of absorbed glutamine remains unchanged (Windmueller and Spaeth, 1974), with other derivatives such as alanine, arginine, aspartate, citrulline, ornithine, and proline being formed from the carbon skeleton (Wu et al., 1995; Rhoads and Wu, 2009). During chronic metabolic acidosis, intestinal absorption of glutamine increases, particularly via jejunal glutamine transporters (Epler et al., 2003; Pan et al., 2004), yet no data to show the consequent impact on glutamine delivery as a result of

dietary and/or intestinal flux of glutamine in ruminants (Peterson et al., 1971; Heitmann and Bergman, 1980).

The liver possesses a unique glutamine catabolic pathway, where glutamine deamination occurs in the mitochondria of periportal cells, and the resulting NH_4^+ is then bound with HCO_3^- to produce carbamoyl phosphate (**Figure 2.1**). Carbamoyl phosphate is utilized for urea synthesis in the urea cycle (Watford, 2000). The nitrogen on glutamate from glutamine deamination can also be transaminated to aspartate for use in the urea cycle (Taylor and Curthoys, 2004). Glutamine synthesis occurs in the third perivenous cell layer away from the hepatic venule where glutamine synthetase is localized, while urea synthesis occurs in cells closest to the portal venule (Meijer et al., 1990; Taylor and Curthoys, 2004). During metabolic acidosis, the liver decreases ureagenesis in an attempt to conserve glutamine, compensated by upregulation of urea transporters (Klein et al., 2002). There is also an increase in hepatic glutamine synthetase activity to increase glutamine formation and release into the bloodstream for renal uptake.

Renal glutamine utilization occurs through the initial uptake of glutamine through the apical brush border membrane of the proximal convoluted tubule. Deamination of glutamine occurs via action of phosphate-dependent glutaminase in the mitochondria, yielding NH_4^+ and HCO_3^- for excretion and/or recirculation (Garibotto et al., 2009). In addition, as depicted in **Figure 2.2**, a second ammonium ion is cleaved from glutamate via glutamate dehydrogenase to produce α-ketoglutarate, NH_4^+ and HCO_3^- (Nissim et al., 1985; Garibotto et al., 2009), α-ketoglutarate then enters the TCA cycle for malate production and oxaloacetate and/or pyruvate production for eventual conversion to glucose (Nissim et al., 1987). Metabolic acidosis induces an increase in renal glutamine

uptake, particularly via Na+ dependent glutamine transporters (Windus et al., 1984; Solbu et al., 2005; Moret et al., 2007). This results in increased ammoniagenesis and NH_4^+ urinary release via increased glutaminase and glutamate dehydrogenase (Curthoys et al., 2007), laterally creating increased HCO_3^- export and glucose production (Garibotto et al., 2004).

Skeletal muscle represents a dense amino acid reservoir (**Figure 2.3**). Approximately 85-90% of nitrogen released from human muscle is in the form of glutamine under normal circumstances (Jungas et al., 1992). One of the most rapid compensatory mechanisms at the onset of metabolic acidosis is muscle proteolysis, which provides glutamine to supplement diminishing glutamine pools (Schröck and Goldstein, 1981; Reaich et al., 1992) via several proteolytic pathways (Lecker et al., 1999). In addition, upregulation of muscle glutamine synthetase occurs in many disease states associated with acidosis (Lecker et al., 2006).

2.4 Proteolysis in response to metabolic acidosis

Shifts in protein turnover naturally provide additional amino acids required for buffering, such as glutamine, without total reliance on shifting intestinal absorption or exogenous supplementation. Whether it be suppression of protein synthesis or increased protein degradation, both could lead to an overall increase in free amino acid availability (Šafránek et al., 2003). Though acute acidosis increases IGF-1 concentrations in kidney (Fawcett et al., 2000) and liver (Bereket et al., 1996) and can induce hypertrophy in these tissues, protein degradation becomes predominant, particularly in skeletal muscle (Lecker et al., 1999). Proteolysis is accomplished via several proteolytic pathways, including

lysosomal cathepsins, calcium dependent calpains, pro-apoptotic caspases, metalloproteases, and the ATP-dependent UPP (Lecker et al., 1999; Saric and Goldberg, 2005; Lecker et al., 2006). Though cathepsins B and L are decreased in renal tissue at the onset of metabolic acidosis to contribute to hypertrophy (Fawcett et al., 2000), these same isoforms are the predominant cathepsins upregulated in skeletal muscle during disease states, particularly cathepsin L (Bechet et al., 2005). In terms of specific activities, cathepsin L can degrade most myofibrillar proteins, while cathepsin B has more specific targets and degrades myosin heavy chain, troponin, and tropomyosin (Bechet et al., 2005). Calcium-dependent calpains target the Z-disc of the sarcomere for degradation (Bartoli and Richard, 2005; Costelli et al., 2005). In addition, calpains are responsible for cleavage of several key proteins involved in muscle atrophy (Bartoli and Richard, 2005). Caspase-3 is also a participant in sarcomeric degradation, and is involved in dissociation of actomyosin (Du et al., 2005). Increases in muscle protein catabolic rates have been partially attributed to degradation of actomyosin and myofibrils to actin and myosin, respectively, by increased caspase-3 activity. The UPP then degrades actin and myosin into oligopeptides for further breakdown to individual amino acids by cytoplasmic peptidases (Du et al., 2005). Though degradation of the sarcomere by these proteases is required for further degradation by the UPP, the UPP is being explored as the most significant regulator of proteolysis in disease states.

2.5 The ubiquitin-mediated proteolytic pathway

The ubiquitin-mediated proteolytic pathway has been extensively studied as a major pathway involved in protein degradation and has been implicated in many diseases,

including metabolic acidosis (Mitch et al., 1994; Lecker et al., 2006; Rajan and Mitch, 2008). In this pathway, a 76 amino acid ubiquitin molecule is first bound to the conjugating enzyme E1 via ATP utilization and is consequently activated by E1 (Ciechanover, 2006; **Figure 2.4**). The high-energy thiol ester is an intermediate, and ubiquitin is transferred to the conjugating enzyme E2 to create a second high-energy thiol ester intermediate. A member of the E3 family then covalently transposes ubiquitin to lysine on the target protein. Once a chain of at least 4 ubiquitin molecules is formed on the substrate protein, it is recognized by the downstream region of the 26S proteasome complex. The 26S proteasome is comprised of a 20S core, formed of 2 outer α-subunits and 2 inner β-subunits, and 19S complexes flanking either end of the tubular 20S core (Ciechanover, 2006; Du et al., 2005; Hershko and Ciechanover, 1998; Hershko et al., 2000). Numerous α- and β- subunits of the 20S and 19S complexes exist, characterized by different localities within the cell (Brooks et al., 2000) and specific tissue expression (Farout et al., 2003). The substrate protein is normally unfolded by the 19S complex and is then degraded by catalytic sites in the β-subunits of the 20S complex (Hershko and Ciechanover, 1998). The ubiquitin molecules are removed in an ATP-requiring deconjugation reaction via isopeptidases that are physically associated with the 26S proteasome (Jagoe and Goldberg, 2001). The released oligopeptides can be further degraded to individual amino acids by cytoplasmic peptidases (Du et al., 2005). Because ubiquitin-mediated proteolysis itself is an ATP-requiring process, diminished exposure of incubated muscle samples to ATP reduces the proteolytic rate (Jagoe and Goldberg, 2001).

2.6 Protein synthesis and response to metabolic acidosis

Protein synthesis is the counterbalance to degradation, and shares many common signaling agents. For example, insulin-like growth factor- 1 (IGF-1) is a hormone produced and secreted by the liver under growth hormone (GH) stimulation, and is a potent stimulator of protein accretion. In humans, inhibition of IGF-1 as a result of metabolic acidosis is due to GH insensitivity and could be partially corrected by provision of GH, resulting in increased ammonium secretion (Wiederkehr and Krapf, 2001). Protein synthesis is mediated by activation of PI3K/Akt (Bodine et al., 2001; Rommel et al., 2001). Phosphoinositol-3 kinase (PI3K) activity is stimulated by IGF-1, and PI3K in turn stimulates Akt (Hoffman and Nader, 2004). Interaction with protein degradation is evident at this stage. Akt suppresses activity of the proteolytic forkhead transcription factor FOXO under anabolic circumstances (Léger et al., 2006); however, when Akt is downregulated, FOXO stimulates transcription of Atrogin-1 and MuRF-1, which are ubiquitin E3 ligases that signal muscle atrophy (Léger et al., 2006; Koyama et al., 2008). Akt phosphorylation is inhibited by glucocorticoids, which not only impair Akt activity but also impair FOXO phosphorylation, ultimately upregulating Atrogin-1 and MuRF-1 (Zhao et al., 2009). However, upon activation of Akt via phosphorylation, Akt then stimulates muscle accretion via two pathways, the first is via inhibition of glycogen-synthase kinase 3 (GSK3) for permissive activation of eIF2B, which mediates binding of methylated tRNA to the ribosomal 40S subunit (Vary and Lynch, 2007). The second pathway stimulated by Akt is the mTOR pathway. Inhibition of the tuberous sclerosis complex (TSC) by Akt allows for mTOR stimulation of p70S6 kinase (p70S6K), and mTOR mediated inhibition of 4EBP to allow for eIF4E to bind (Glass,

2005). Cell size is increased by p70S6K (Ohanna et al., 2005), while eIF4E binds to eukaryotic mRNA at the 7 methyl GTP cap (Vary and Lynch, 2007). Ultimately, increased ribosomal translation of mRNA for protein synthesis can occur via mTOR stimulation.

Though stimulation of proteolysis is the most apparent characteristic during metabolic acidosis, there is also depression of protein synthesis specifically in skeletal muscle (Šafránek et al., 2003; Caso et al., 2004), possibly accomplished by a reduction in the efficiency of ribosomal translation, and this is thought to be only in muscles composed of slow-twitch fibres (Caso and Garlick, 2005; Kleger et al., 2001).

2.7 Muscle Type Differentiation

Differential regulation of protein metabolism within muscle is an evolving concept. Fast-twitch glycolytic fibres have been proposed to be the first fibre type degraded under circumstances of body weight loss (Lehnert et al., 2006) which could also prove true for conditions of metabolic acidosis. Slow-twitch muscle (red *gastrocnemius*) has been observed to have significantly increased concentrations of ADP and AMP compared to fast-twitch muscle (white *gastrocnemius*) under acidotic conditions when muscle was tensed via wave stimulus (Dudley and Terjung, 1985). *In vivo* measurements of fast-twitch muscle (*vastus lateralis*) collected during exercise trials in humans under normal and acidotic conditions, demonstrated that acidosis depressed lactate concentrations, pyruvate production and oxidation, glycogen utilization, pyruvate dehydrogenase activity and glycogen phosphorylase activity while significantly increasing concentrations of glucose-6-phosphate, glycogen, acetyl-CoA and CO_2

(Hollidge-Horvat et al., 1999). In conjunction, sepsis was shown to stimulate proteasome activity in fast-twitch muscle (*extensor digitorum longus*), but did not have any effect on proteasome activity in slow-twitch muscle (*soleus*) in rats (Hobbler et al., 1999). However, whether this premise holds true for ruminants is unknown.

2.8 Induction of metabolic acidosis in ruminants by increasing dietary inclusion of non-fiber carbohydrate

Increasing the non-fibre carbohydrate (NFC) portion in ruminant diets is a common practice during periods of high-energy demand, such as growth or early lactation. When the readily fermentable content of the diet is increased, rumen microbes can easily break down this NFC material and produce volatile fatty acids (VFA) as a byproduct (**Figure 2.5**). An excess of VFA, especially propionate, is produced as a result of increasing the highly fermentable portion of the diet (Kreikemeier et al., 1987). Increased VFA production can create an acidic environment in the rumen, known as rumen acidosis, which also allows for lactate-producing rumen microbes, such as *Streptococcus bovis*, to temporarily flourish (Nocek, 1997; Herrera et al., 2009). Morphological changes in rumen tissue result from this lower rumen pH, not only increasing the incidence of keratosis and perakeratosis (Nocek, 1997), but also causing an increase in papillae height (Odongo et al., 2006), potentially resulting from VFA regulation of IGF-1 (Shen et al, 2004) or IGF binding proteins (Steele et al., 2009).

A continued increase in lactate production by *S. bovis* and accumulation further decreases the rumen pH. The rumen microbial environment continues to transform, as the *S. bovis* population is replaced by lactobacilli, which continue to produce lactate and

further decrease rumen and blood pH. In terms of lactate absorption and turnover, the D-form of lactate is metabolized less readily than L- lactate (Nocek, 1997). Relative to animals fed a high-forage diet, ruminants fed a high-NFC diet have increased ruminal L- and D- lactate concentrations (Huntington et al., 1981a), a more rapid D-lactate net portal absorption (Huntington et al., 1981b), increased L-lactate turnover (Huntington et al., 1981b) and an acute decrease in plasma L-lactate (Huntington et al., 1981a). However, the type of grain offered as the NFC portion can affect severity of microbial shifts (Huntington, 1997) and consequential shifts in lactate (Castillo et al., 2009), creating variability in the response to dietary NFC challenge. However, in conjunction with shifts in lactate production, absorption and turnover, other factors such as glucose concentration (Huntington et al., 1981b) and animal performance in terms of gain and fat deposition (Kreikemeier et al., 1987), are altered when feeding a NFC-rich diet. Other factors associated with an NFC challenge, such as the carbohydrate and energy density interactions, ultimately make it difficult to determine whether any resulting changes in protein turnover result from the acidosis itself, or other dietary factors. In addition, induction of this model can be difficult, as the rumen environment must first be altered before significant systemic effects can be seen. Nutritional induction of a hyperchloremic metabolic acidosis has been widely accepted in monogastric and ruminant research as an acceptable model to investigate the physiologic repercussions of acidosis (Heitmann and Bergman, 1978; Mutsvangwa et al., 2004; Las et al., 2007).

2.9 Use of dietary cation:anion difference for nutritional induction of hyperchloremia metabolic acidosis

Fluctuations in cation:anion balance occur during metabolic acidosis, as potassium (K^+), chloride (Cl^-) and calcium (Ca^{2+}) concentrations all increase in the blood (Las et al., 2007). Manipulation of dietary cation:anion difference [DCAD; ($Na^+ + K^+ - Cl^-$)/100 g of DM] can also attenuate metabolic acidosis by providing additional Na^+ and K^+ for absorption. Increased absorption leads to an increase in Cl^- absorption for exchange of Cl^- with HCO_3^- (Block, 1994). This is reflected in studies that have increased DCAD:S [($Na^+ + K^+ - Cl^- - S^{2-}$)/100g of DM] in early lactation diets of Holstein cows and found significantly increased plasma HCO_3^- concentrations (Chan et al., 2005). This could be expected, as the Henderson-Hasselbach acid/base equation clearly demonstrates a direct relationship;

$$\text{Blood pH} = 6.1 + \log_{10}(HCO_3^-/(0.03 \times pCO_2)$$

A meta-analysis of the influence of DCAD revealed that dietary Na^+ and K^+ did not alter blood concentrations of these ions; however, a quadratic relationship between DCAD and blood pH, HCO_3^-, DMI, milk yield, milk fat yield and milk fat percentage is evident (Hu and Murphy, 2004). Use of additives to alter the DCAD in dairy cow diets fed at the onset of lactation demonstrated that even a DCAD of -3 mEq/100 g DM could significantly lower milk fat, protein and lactose percentage of milk compared to milk from cows fed positive DCAD diets (Hu et al., 2007). However, no research to date has identified the specific mechanisms of milk yield and component fluctuations as a result of changes in DCAD or metabolic acidosis.

2.10 Influence of lactation on protein turnover

Though the intention of the current research is to investigate the effects of metabolic acidosis on protein turnover in ruminants, caution must be exercised when choosing the appropriate model. Loss in body protein at the onset of lactation due to tissue protein degradation is common (Bauman and Currie, 1980; Sainz et al., 1986; Wilson et al., 1988; Meijer et al., 1995), and much research has focused on ways to attenuate this loss of protein, in particular using dietary manipulation (Naismith et al., 1982; Komaragiri and Erdman, 1997; Dann et al., 2006). Examination of the proteolytic mechanisms responsible has demonstrated that the ubiquitin-mediated proteolytic pathway is upregulated in skeletal muscle of sows (Clowes et al., 2005). Suggestion of involvement of the ubiquitin-mediated proteolytic pathway at the onset of lactation in cattle was also made by Chibisa et al. (2008), who observed a 22% increase in ubiquitin and a 55% increase in E2 skeletal muscle mRNA expression in cows at 15 d post-partum compared to -14 d pre-partum. Microarray results have also suggested regulation of the proteasome at the onset of lactation (Xiao et al., 2004; Loor et al., 2005; Lemay et al., 2007); however, no validation of these results using PCR has been made, and some microarray results show down-regulation of the proteasome (Xiao et al., 2004; Lemay et al., 2007), suggesting caution in comparing studies that collect muscle samples at different time points relative to the onset of lactation. In addition, as mentioned previously, glucocorticoids inactivate Akt and stimulate FOXO-mediated activation of E3 ligases (Zhao et al., 2009), ultimately providing another source of catabolic stimulation at the onset of lactation when receptivity to glucocorticoids and catecholamines increases (Beerda et al., 2004). Induction of metabolic acidosis with HCl infusion caused a

significant increase in expression of proteasome components in lactating dairy cows (Mutsvangwa et al., 2004); however, whether the effects of lactation and metabolic acidosis interacted in an additive or synergistic manner to exacerbate shifts in proteolysis is unknown. It is the intention of the following studies to contribute to identification of any influence of the onset of lactation on ubiquitin-mediated proteolysis, and employ an animal model that will appropriately examine solely the influence of metabolic acidosis.

2.11 Current Understanding of amino acid usage and protein turnover in ruminants during metabolic acidosis

As stated above, glutamine is an important amino acid for inter-organ nitrogen transport during metabolic acidosis in monogastrics. However, whether this holds true for ruminants is less understood. First, it must be noted that there is significant variation in urea and glutamine production between species even under acidotic conditions, as sheep maintain urea and glutamine production around 595 and 175 umol N/kg wet liver per min from extracellular pH 7.2-7.6, respectively, while rats have drastic shifts in urea and glutamine production from 363 to 949 and from 49 to -45 umol N/kg wet liver per min from extracellular pH 7.0 to 7.7, respectively (see Lobley et al., 2001 for summary).

Lobley et al. (2001) demonstrated that glutamine production was essentially unresponsive to declining extracellular pH in sheep, but an increase in plasma flux of glutamine was observed after lipopolysaccharide challenge, demonstrating that at least some disease challenges can influence glutamine concentrations in ruminants. Earlier research in sheep using ruminal addition of NH_4Cl provided evidence that a net increase in renal removal and net decrease in hepatic removal of glutamine resulted in decreased

plasma concentrations of glutamine during periods of acidosis in ruminants (Heitmann and Bergman, 1978). However, unlike in monogastrics, this study, along with later research, observed no change in net movement of amino acids in the hindquarters under acidotic conditions (Heitmann and Bergman, 1978; Heitmann and Bergman, 1980). In the second study, ruminal addition of NH_4Cl was hypothesized to supply ruminal microbes with a nitrogen source for additional amino acid synthesis, resulting in a net increase in most amino acids in portal-drained visceral measurements and increased net hepatic removal of these amino acids, with no reliance on muscle proteolysis for provision of amino acids (Heitmann and Bergman, 1980). Infusion of NH_4Cl into the mesenteric vein of lambs, rather than addition of NH_4Cl to the rumen, demonstrated no change in portal drained visceral flux of any amino acid except glutamine (Lobley et al., 1995), supporting the previous study that suggested that the rumen microbial population was responsible for a shift in appearance of amino acids due to the addition of NH_4Cl to the rumen. This study also demonstrated an increased appearance of glutamate in the hepatic vein compared to both the portal vein and aortal concentrations, and suggested that glutamate formation was being accomplished in the perivenous hepatocytes, as well as glutamine cleavage in the periportal hepatocytes, and could impede urea formation (Lobley et al., 1995). Given the increased requirement for glutamine in the above studies upon induction of metabolic acidosis, it seems counterintuitive that no change in hindquarter proteolysis would occur for increased provision of glutamine. Nonetheless, it is possible that ruminants are able to entirely compensate for declining blood pH with a concurrent increase in inter-organ nitrogen transport via increased hepatic glutamine synthetase

activity, decreased renal release of glutamine, and increased ureagenesis, as long as no additional physiologic challenges, such as lactation, are present.

2.12 Supplementation to attenuate effects of metabolic acidosis

Provision of glutamine to patients with metabolic acidosis provides an interesting prospect for glutamine use as a pharmaceutic or therapeutic agent. Untreated metabolic acidosis stimulates intestinal glutamine absorption (Epler et al., 2003), while increased appearance of glutamine in the intestinal lumen also increases jejunal mucosa glutamine concentrations (Adegoke et al., 1999; Adegoke et al., 2003). Infusion of glutamine into human patients after abdominal surgery reduces the rate of nitrogen loss, indicating a decrease in protein catabolism or a potential increase in protein synthesis (Wilmore, 2001). Provision of glutamine to human patients via a nasogastric feeding tube was also shown to downregulate mucosal expression of an ATP-dependent proteolytic system, more so than nasogastric feeding of glycine, alanine, serine, proline, aspartate, asparagine and histidine combined (Coeffier et al., 2003). However, supplementation of specific amino acid mixes could still prove to be a useful method of providing additional buffering capacity in conjunction with increasing protein accretion or decreasing protein degradation (Wang et al., 2009). For example, the branched chain amino acid leucine was observed to decrease protein wasting in rat skeletal muscle cell cultures (Bevington et al., 2001) and work additively with insulin to suppress protein degradation (Sadiq et al., 2007). The results indicate that there is potential for the development of an amino acid therapeutic supplement that could both increase buffering capability while suppressing protein degradation or enhancing protein synthesis (Vary, 2007).

Bicarbonate production as a result of carbonic acid cleavage is necessary to buffer excess H^+ in the blood during metabolic acidosis. To examine the ability of dairy cattle to recognize and self medicate acidosis, rumen acidosis was induced in dairy cattle using wheat:barley grain pellet substitution for 25% of daily total mixed ration (TMR). Cows were then either offered free-choice buckets of sodium bicarbonate (Keunen et al., 2003) or free-choice sodium bicarbonate enriched water (Cottee et al., 2004). Neither circumstance yielded a significant difference in sodium bicarbonate intake during rumen acidosis. Recent research has also reported no significant decrease in ubiquitin mRNA expression in patients with chronic renal failure after oral administration of sodium bicarbonate (Roberts et al., 2002). Treatment with monensin can inhibit effects of subacute ruminal acidosis (SARA) on rumen pH; however, treatment with monensin may alter post-ruminal fibre digestion, possibly a result of increased intestinal availability due to decreased ruminal digestion (Osborne et al., 2004).

Production of NH_3 via protein catabolism during metabolic acidosis is also a crucial compensatory mechanism, as ammonia is required for binding of free H^+ resulting from cleavage of carbonic acid. Hence, inhibition of protein catabolism without supplementation of a buffering source would lead to a further decrease in blood pH and ultimate morbidity. Inhibition of the 26S proteasome has been observed by lactacystin, an irreversible natural inhibitor of the 20S proteasome via binding to threonine on the active sites (Goldberg et al., 1995; Dick et al., 1996). Reversible synthetic tripeptide aldehyde 26S proteasome inhibitors, including LLN and MG132, have also been observed to significantly decrease muscle atrophy (Tawa et al., 1997). In addition, tea polyphenols have recently been observed to be a natural reversible alternative to inhibit 20S

proteasome binding for protein catabolism (Nam et al., 2001). Though this provides an interesting opportunity for suppression of protein catabolism, the inhibitory effects of tannins on protein absorption must also be considered if in search of plant polyphenol supplements (Makkar, 2003).

2.13 Conclusions: Dietary implications on protein turnover due to metabolic acidosis

High-energy feeds offered in early lactation commonly contain high amounts of readily fermentable carbohydrates, which are a primary cause of rumen acidosis in dairy cattle. Increased absorption of VFA and lactate lead to metabolic acidosis. Increased glutamine absorption has been observed under condition of chronic metabolic acidosis in monogastrics. Because of glutamine's involvement in HCO_3^- production and the suggestion of increased glutamine absorption under circumstances of chronic metabolic acidosis, the therapeutic efficacy of glutamine could be examined by feeding increased glutamine prior to experimental induction of metabolic acidosis. Dietary supplementation of this amino acid in conjunction with HCO_3^- supplementation could be a feasible prophylactic measure to diminish the severity of metabolic acidosis and ultimately reduce the rate of protein turnover. However, current understanding of shifts in protein turnover as a result of metabolic acidosis in ruminants is poorly understood. Characterization of the animal and induction models must first occur. It is hypothesized that metabolic acidosis will upregulate muscle mRNA and protein expression of the ubiquitin-mediated proteolytic pathway in ruminants. The objectives of the current studies are to: 1) Determine the expression of the ubiquitin-mediated proteolytic pathway in dairy cows at the onset of lactation, 2) Examine the influence of varying severities of metabolic

acidosis on protein degradation and amino acid usage in ruminants, and 3) Determine if supplementation with an exogenous source of glutamine could be beneficial during circumstances of metabolic acidosis in ruminants.

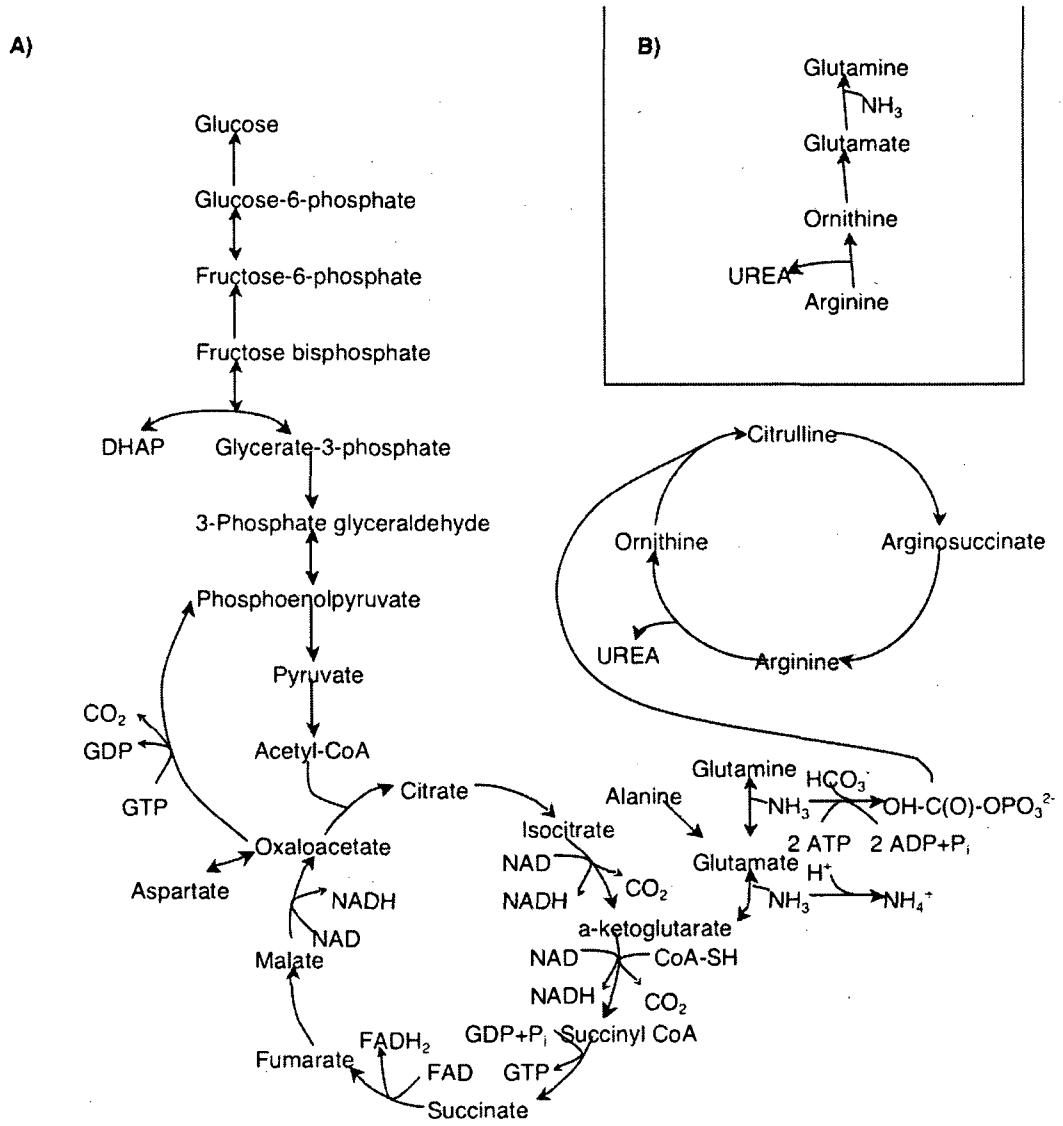


Figure 2.1. Glutamine metabolism in the liver. A) Glutamine metabolism in periportal cells, and B) Glutamine metabolism in perivenous cells. Adapted from Taylor and Curthoys, 2004.

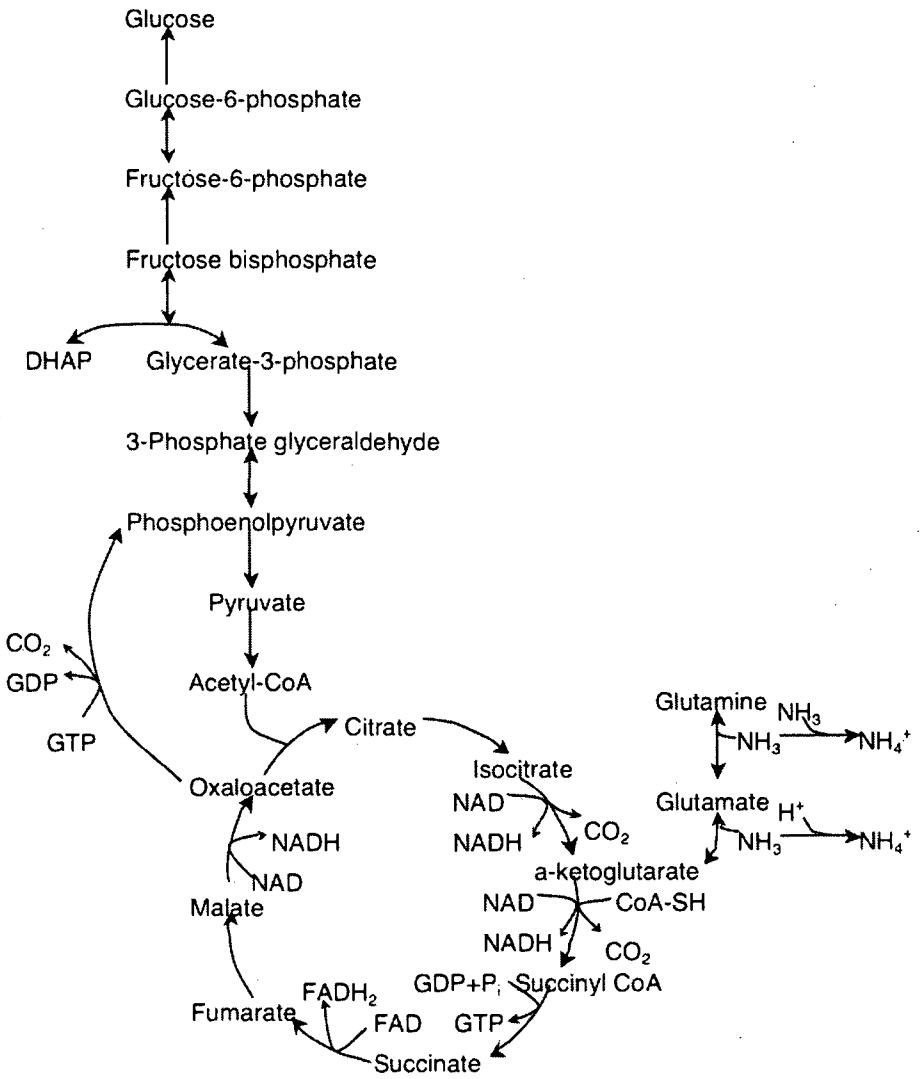


Figure 2.2. Glutamine metabolism in the kidney. Adapted from Taylor and Curthoys, 2004.

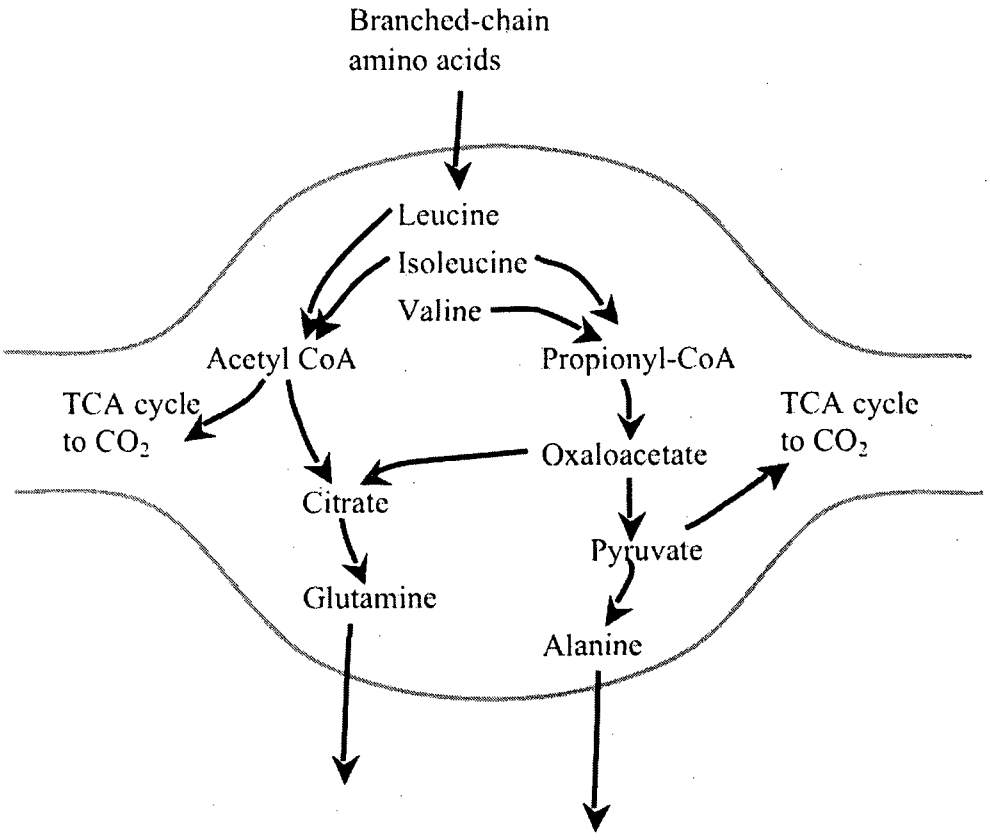


Figure 2.3. General glutamine metabolism in skeletal muscle. From Jungas et al., 1992.

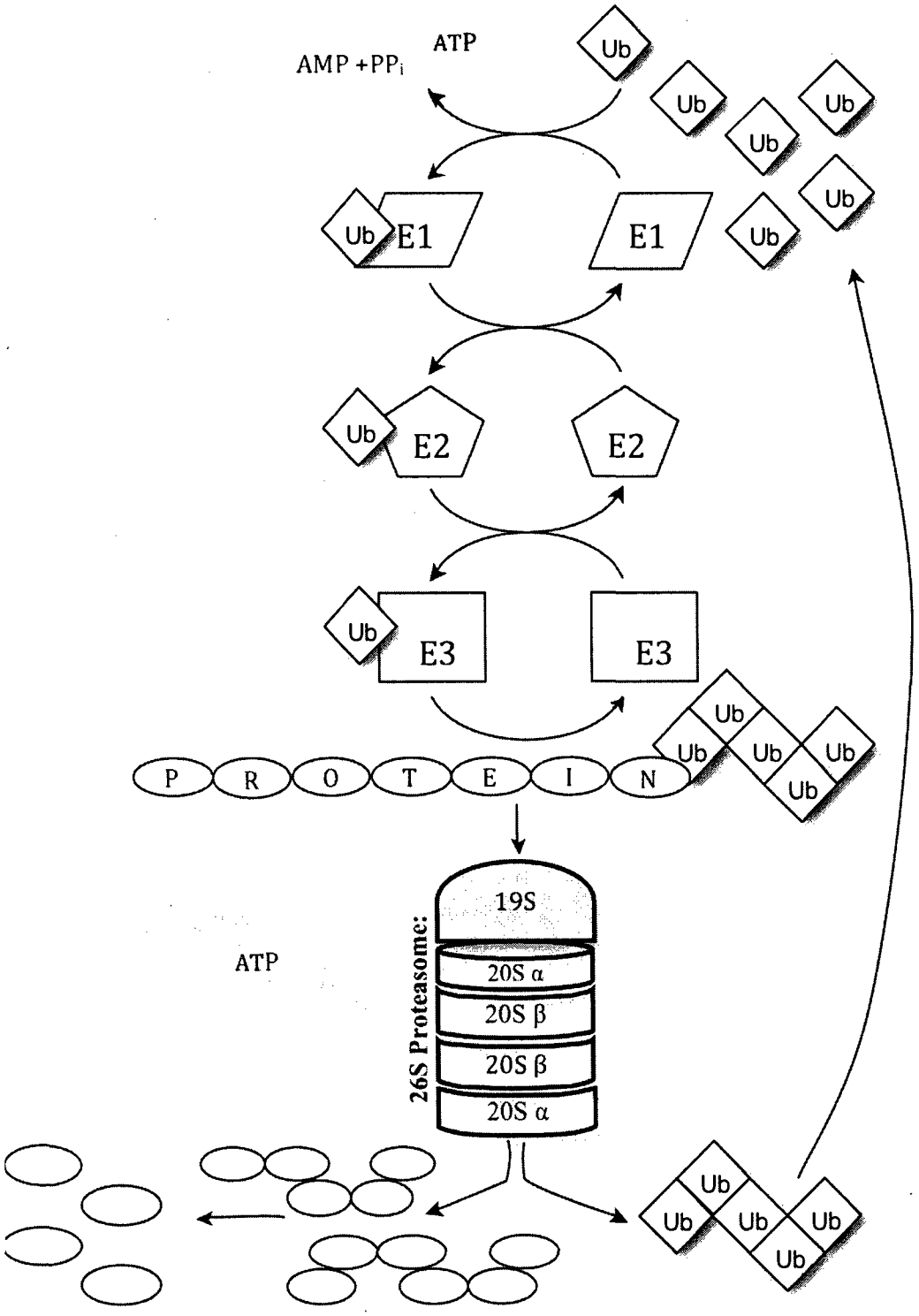


Figure 2.4. The ubiquitin-mediated proteolytic pathway. Adapted from Ciechanover, 2006. Ub, Ubiquitin.

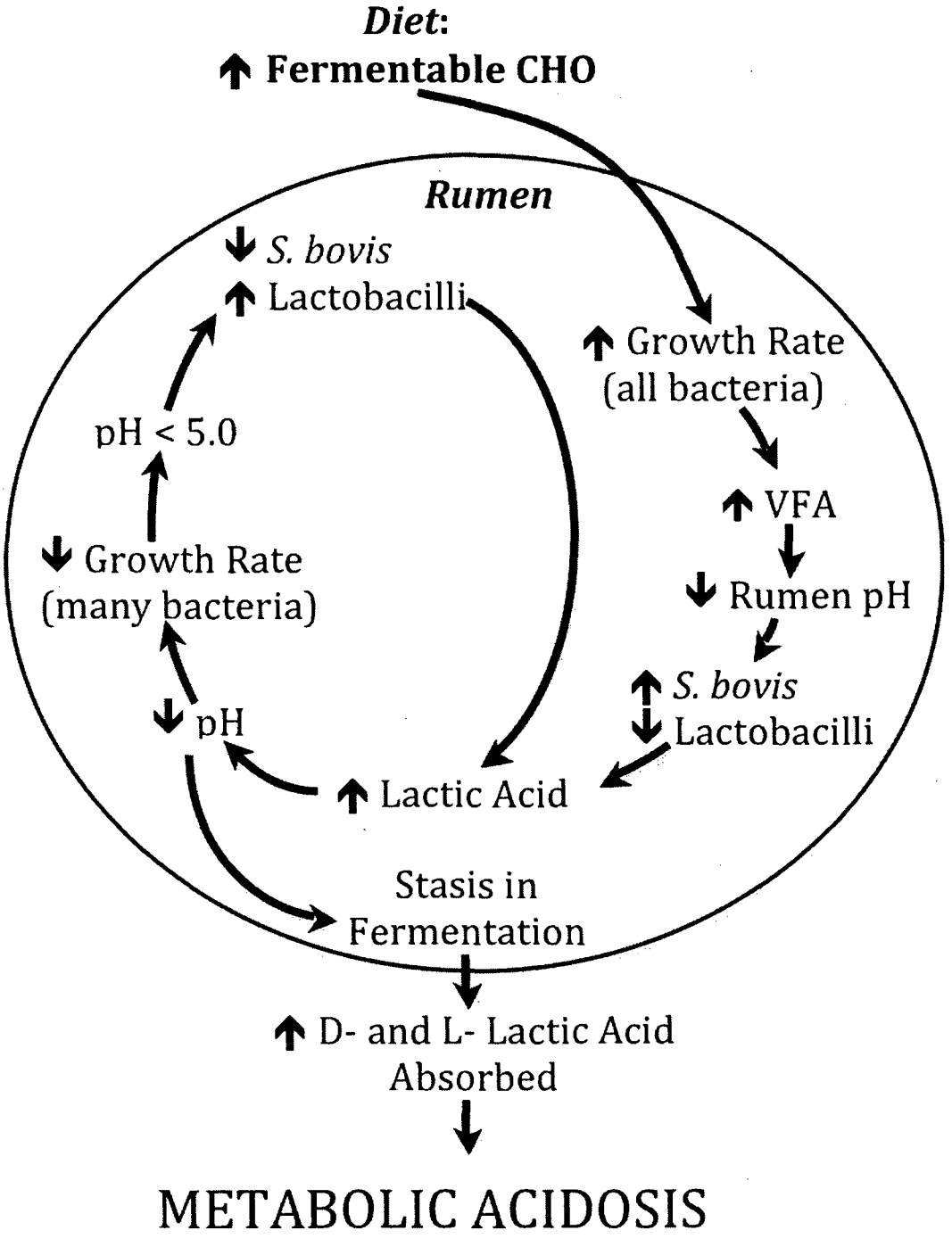


Figure 2.5. Induction of metabolic acidosis in ruminants using a diet rich in non-fiber carbohydrate. From Nocek, 1997.

CHAPTER 3

Lactation induces upregulation of the ubiquitin-mediated proteolytic pathway in skeletal muscle of dairy cows but does not alter hepatic expression[1]

3. 1 ABSTRACT

The current study investigates regulation of mRNA expression of components of ubiquitin-mediated proteolysis in transition dairy cows. *Longissimus dorsi* muscle (Experiment 1) and liver (Experiment 2) biopsies were collected from Holstein dairy cows at 27 and 16 days pre-partum, respectively, and 3 and 10 days post-partum, respectively. Regulation of C8, E2, and ubiquitin mRNA expression was determined. Upregulation of skeletal muscle C8 (P = 0.09) and ubiquitin (P = 0.004) mRNA expression occurred post-partum compared to pre-partum. No regulation of hepatic mRNA expression was observed. In conclusion, ubiquitin-mediated proteolysis may contribute to skeletal muscle protein degradation during the periparturient period, and could provide a potential mechanism for attenuation of body protein loss at the onset of lactation.

3.2 INTRODUCTION

Onset of lactation elicits a significant demand on energy mobilization for generation of milk components. Although fat mobilization from adipose has received

[1] Reprinted with permission. Originally printed in Can. J. Anim. Sci. (2009) 89: 309-313.

much attention due to its large caloric potential (Naismith et al., 1982), investigation of the molecular basis of protein mobilization during the onset of lactation has not been studied extensively. Proteolysis to provide amino acids for milk synthesis during early lactation commonly occurs, as intake alone cannot meet the large amino acid demand (Plaizier et al., 2000). Estimates of body protein loss in dairy cattle range from approximately 9 kg estimated from 14 d pre-partum to 15 d post-partum (Chibisa et al., 2008) to 12 kg (Komaragiri et al., 1998) and 21 kg (Komaragiri and Erdman, 1997) estimated from 14 d pre-partum to 35 d post-partum. Though these losses could be attributed to either an increase in protein degradation or a decrease in protein synthesis, Baracos et al. (1991) reported no significant differences in hind-limb tissue fractional synthesis rates (FSR) between dry goats and lactating goats in their third week of lactation. Research using rats concluded that significant losses in protein mass within 2 to 10 days post-partum were more attributed to high levels of fractional degradation rates than FSR (Pine et al., 1994).

Research of the 26S proteasome has demonstrated that this ATP-dependent proteolytic system is involved in many physiological states, and is integrally involved in degradation of proteins into short oligopeptides for amino acid recycling and oligopeptide provision for antigen screening. Lactation-induced regulation of the ubiquitin-mediated proteolytic pathway (UPP) components has been observed in porcine skeletal muscle (Clowes et al. 2005) and murine mammary gland (Lemay et al., 2007). However, contradicting results have been reported for regulation of UPP mRNA expression in bovine tissues collected from transition cows. No significant differences in expression of ubiquitin or ubiquitin carrier E2 components were observed in dairy cows at 15 d post-

partum compared to 14 d pre-partum values in a recent experiment (Chibisa et al., 2008), though numeric increases were noted. In addition, changes in bovine hepatic mRNA expression during the periparturient period, detected using cDNA microarray technology, indicated downregulation of expression of the 26S proteasomal ATPase (Loor et al., 2005). Subsequent microarray analysis of bovine liver revealed upregulation of 26S subunits and ubiquitin-associated proteases during the periparturient period (Loor et al., 2006). Neither of these findings were validated by Real-time PCR (RT-PCR). Shifts in hepatic protein degradation elicited specifically at the onset of lactation have not been intensively studied. Shifts in protein degradation by the UPP could contribute to the drastic changes incurred in hepatic tissue at the onset of lactation, in conjunction with the previously documented increased hepatic hypertrophy (Reynolds et al., 2003). The UPP could be increased in order to aid in turnover of the increasing number of functional proteins, such as enzymes and proteins involved in milk component synthesis, fat metabolism and gluconeogenesis, and also to quickly degrade misfolded proteins. It is hypothesized that the UPP is involved in protein degradation in skeletal muscle of cows at the onset of lactation to provide amino acids for milk synthesis and energy requirements. In turn, it is hypothesized that mRNA expression of UPP components in the liver will increase in order to aid in the increasing need to regulate hepatic proteins and enzymes. The objective of the current study was to investigate whether the onset of lactation would significantly affect mRNA expression in liver and muscle of the 26S C8 subunit, the conjugating enzyme E2, and ubiquitin compared to expression levels observed during the late pre-partum period.

3.3 MATERIALS AND METHODS

Periparturient Holstein dairy cows were maintained at the Ponsonby Research Station, University of Guelph, Canada, or the Elora Research Station, University of Guelph, Canada, and handled in accordance with the Canadian Council on Animal Care regulations. The University of Guelph Animal Care Committee approved their use for these experiments.

3.3.1 Muscle Biopsy (Experiment 1)

Longissimus dorsi muscle biopsies were collected from four multiparous Holstein dairy cows (3.5 ± 0.6 lactations; 805.6 ± 46.9 kg BW) at approximately 27 d pre-partum (20 to 36 d) and 3 d post-partum (average milk production of 28.2 ± 4.5 kg), as a subset of animals taking part in a larger experiment. To obtain a muscle sample, the site of incision was determined by measuring 10 cm from the last rib and 8 cm from the backbone. A 20 cm area around the incision site was shaved, scrubbed and disinfected with rubbing alcohol before 1-2 mL of local anesthetic (Xylocaine®, 2% lidocaine without epinephrine, AstraZeneca Canada Inc., Mississauga, ON) was injected at the incision site. A 1 cm incision was made for insertion of the biopsy needle. Insertion was to approximately 5 cm in depth and 4 samples were collected, each by rotating the biopsy needle 90° to cut a small muscle sample. The incision site was closed using staples, which were removed at least 10 d later. Obvious fat and blood was quickly removed using sterile forceps and gauze before biopsy samples were snap frozen in liquid nitrogen and stored at -70°C until analysis. The incision post-partum was made at the same sight, only slightly to the side of the scar tissue from the original biopsy.

3.3.2 Liver Biopsy (Experiment 2)

Liver biopsies were obtained from fifteen primiparous Holstein dairy cows (634.1 ± 14.6 kg BW) at approximately 16 d pre-partum (14 to 21 d) and 10 d post-partum (average milk production of 25.7 kg ± 1.1 kg) as a subset of animals taking part in a larger study. Cows were dosed to effect with an analgesic (approximately 0.7 mL/cow; Rompun®, Bayer HealthCare, Toronto, ON) into the coccygeal vein. A section of hair coat was shaved on the right side of the animal, beginning approximately 15 cm from the backbone and extending within the entire area between the 8^{th} and 13^{th} rib. The area was then scrubbed, rinsed and disinfected with chlorhexidine solution (0.5% chlorhexidine gluconate) before being scrubbed and rinsed a second time. A 70% ethanol solution was then applied liberally within the shaved area and allowed to flow over the projected site. The site of incision was identified at the intersection of the line made from the right hip down to the elbow joint of the front right leg of the animal and between the 10^{th} and 11^{th} rib. A local anesthetic (approximately 10 mL/cow; Xylocaine®, containing 2% lidocaine hydrochloride injection USP, AstraZeneca Canada Inc.) was dosed to effect subcutaneously in a figure 7 pattern at the determined incision site. A 3-cm incision was made within the anesthetized area through the intercostal muscle layer, and a trocar and cannula (length of 30 cm; diameter of 1 cm) was inserted into the incision site, puncturing the peritoneum. The trocar was then retracted and approximately 2 g of liver sample was collected using the cannula and slowly removed from the body cavity. The incision site was closed using staples, which were removed at least 10 d later. Samples were snap frozen in liquid nitrogen and stored at -80°C until analysis.

3.3.3 Diet Composition

Diets were formulated according to the NRC (2001) guidelines to meet requirements for pre-partum and post-partum dairy cows, and are listed in Table 3.1. Cows were offered a TMR at 0700 and 1500 h daily with unlimited access to water.

3.3.4 Real-time PCR

Approximately 100 mg of muscle tissue and 200 mg of liver tissue was used for RNA isolation as previously described (Greenwood et al., 2008) using the TRIzol method (Invitrogen, Burlington, ON). Briefly, tissue was homogenized in 1 mL TRIzol/mg of tissue before the addition of 0.2 mL chloroform/mL TRIzol. Samples were centrifuged at 12,000 *x g* for 15 min, and 0.5 mL isopropanol/mL TRIzol was added before a 1-h precipitation at room temperature. Samples were centrifuged at 12,000 *x g* for 10 min to create an RNA pellet. Supernatant was then removed and the pellet was washed twice with 75% ethanol and resuspended in diethylpyrocarbonate (DEPC)-treated water. RNA was quantified using absorbance readings at 260 and 280 nm. Samples were then treated to remove genomic contamination (DNase, Invitrogen), and reverse transcribed to produce cDNA. Subsamples were diluted with DEPC water to create a final 1:15 dilution. Uniplex Taqman real-time PCR was performed on triplicate samples using the Prism 7000 Real-time PCR machine (Applied Biosystems, Foster City, CA) to determine mRNA expression of 26S subunit C8, E2, ubiquitin, and β-actin (housekeeping) using exon-spanning primer and probes (FAM-labeled) listed in Table 3.2. Statistical analysis of threshold cycle (Ct) values obtained from PCR using the β-actin primers and probe was first performed to ensure that there was no significant change in β-actin mRNA expression between pre-partum and post-partum values. Genes of interest were selected to encompass sequences for proteins involved in key steps in the UPP pathway, including

sequences for the protein that is required for target protein identification (ubiquitin), one of the conjugating enzymes responsible for the attachment of the ubiquitin molecule to the target protein for identification (E2), and a subunit of the proteasome responsible for degradation of the target protein (C8). The Comparative Ct method was used to compare pre- and post- partum values by normalizing target gene Ct values using the β-actin Ct values within the same sample for the individual pre- or post- partum sample (ΔCt). The normalized pre-partum values were then used as the calibrator value within each animal to generate comparative Ct (–ΔΔCt) values depicting the change in mRNA expression of the target gene compared to pre-partum. The comparative Ct values are subsequently expressed as the fold change observed ($2^{-\Delta\Delta Ct}$).

3.3.5 Statistical Analysis

Changes in mRNA expression ($2^{-\Delta\Delta Ct}$) were used for statistical analysis using a paired t-test (SAS Institute, Inc. 2004), where the pre-partum value of each animal was compared to their post-partum value for each gene of interest. Pre-partum and post-partum β-actin Ct values were also analyzed using this statistical model to ensure stability of the housekeeping gene. Results were declared significant at $P < 0.05$ and data were expressed as mean ± SEM.

3.4 RESULTS AND DISCUSSION

Relative mRNA expression of ubiquitin ($P = 0.004$) was significantly upregulated by 2.1 fold, and C8 expression tended ($P = 0.09$) to be upregulated, in skeletal muscle tissue collected post-partum compared to pre-partum. A 3.4 fold increase in skeletal

muscle E2 mRNA expression was observed post-partum compared to pre-partum, though not significant ($P = 0.16$), as depicted in Figure 3.1. Despite the small number of skeletal muscle samples available, the shifts in mRNA expression observed in the UPP components demonstrate regulation of this proteolytic pathway during the early post-partum period. A recent study found that mRNA expression of ubiquitin, E2 and the ATPase 26S subunit in skeletal muscle collected from Holstein dairy cows was not significantly different at 15 d post-partum relative to 14 d pre-partum, although E2 was lower at 38 d post-partum compared to 15 d post-partum (Chibisa et al., 2008). Of the UPP components examined in periparturient sow muscle at 6 d pre-partum and 9 to12 d post-partum, Clowes et al. (2005) observed the greatest upregulation of E2 mRNA expression, with lesser, though still significant, upregulation of mRNA for the 26S C9 subunit and ubiquitin. Differences in strength of mRNA upregulation could simply be due to the E2 isoform amplified, as many E2 have been characterized (Saric and Goldberg, 2005). Other sources of variation include diet, energy balance, and exact timing of upregulation of these components relative to parturition, because there could be a pre-partum or post-partum period at which mRNA expression is most strongly affected, which could account for slight variations between studies, such as those observed between the current study and previous research (Loor et al., 2006; Chibisa et al., 2008).

In contrast to skeletal muscle, no significant ($P > 0.10$) regulation of liver mRNA expression of C8, E2 or ubiquitin was observed 10 d post-partum compared to 16 d pre-partum (Figure 3.2). As mentioned previously, Loor et al. (2005, 2006) reported cDNA microarray data from liver biopsies collected during the periparturient period from cows fed ad libitum or restricted to 80% of intake. Though expression of 26S proteasomal

subunits and proteases associated with the UPP were observed to be increased at parturition (Loor et al., 2006), downregulation of the 26S proteasomal ATPase was also observed (Loor et al., 2005) and RT-PCR was not performed to validate either of these observations. We had hypothesized that protein degradation may increase in hepatic tissue at the onset of lactation, as there is a drastic increase in metabolic activity, both in terms of enzymatic activity and transport of resulting products, for example, glucose. However, our results demonstrate that any shift in protein metabolism is most likely not performed by shifting UPP activity.

Previous research has demonstrated that increased protein degradation, rather than a decrease in protein synthesis, is the primary contributor to body protein loss in lactating animals (Baracos et al., 1991; Pine et al., 1994). Though there are many proteolytic pathways that could contribute to protein degradation at the onset of lactation, such as the calcium-dependent calpains, which were upregulated at 15 d post-partum relative to pre-partum values in dairy cattle (Chibisa et al., 2008), the UPP has been observed to be the primary pathway involved in proteolysis induced by many physiological states in monogastrics, including fasting and disease states (Curthoys et al., 2007; Lecker et al., 2006). Therefore, identification of changes in mRNA expression of UPP components was the focus of the current experiment. Though transcriptional regulation could prevent changes in protein abundance despite significant regulation at the mRNA level, and enzymatic regulation of protein pathways will also influence body protein loss, there is evidence to suggest that ubiquitin mRNA expression is tightly correlated to 20S activity (R=0.93; Martin et al., 2002). This finding supports the idea that the observed

upregulation of UPP components in the current study indicates increased proteolytic activity of this pathway.

In the current study we were able to focus on components of the UPP using the highly specific and accurate method of Taqman RT-PCR, and have demonstrated that shifts in components of the UPP did not occur in hepatic tissue as a result of the onset of lactation. We conclude there is no persistent lactational switch in hepatic UPP expression. Whether this imparts a resulting shift in protein turnover is yet to be determined, as shifts in protein synthesis were not included in the scope of the current study. However, we have demonstrated upregulation of skeletal muscle UPP, which could contribute to protein mobilization during the onset of lactation.

Table 3.1. Diet composition of pre-partum and post-partum total mixed rations offered ad libitum to Holstein dairy cows in both exps. 1 and 2

Ingredient/TMR	Pre-partum	Post-partum
	%, DM	
Hay	7.1	5.3
Alfalfa Silage	12.1	22.7
Corn Silage	60.5	29.3
High Moisture Corn	5.5	24.4
Supplement[1]	14.8	18.3
Chemical composition		
DM, %	43.0	38.0
	%, DM	
CP (N x 6.25)	12.9	14.7
Soluble Protein	5.4	5.5
ADF	27.0	24.8
NDF	45.6	39.6
Lignin	8.3	10.1
Ether extract	2.3	3.1
Ash	6.0	6.7
NEL, Mcal kg^{-1}	1.45	1.49

[1] Supplement contained soybean meal, canola meal, wheat bran, soybean hulls, molasses and vitamin/mineral mix

Table 3.2. Exon-spanning primer and FAM-labeled probe sequences used for determination of mRNA expression of C8, E2, Ubiquitin and β-actin (housekeeping) using Taqman Real-time PCR

Gene	Primers	Probe	Accession No.
C8	F: GAAGAAGGTTCCAACAAACGACTTT R: ACGAGCGTCTGCCAACAAA	CCTGCTACTGCCATTCC	NM_001034235
E2	F: AGCCGCCAACTGTTAGGTTTT R: TGTTGGACTCCATCTATTCTGAAGGA	CCATCAGCATACACATTTG	NM_001037459
Ubiquitin	F: CTTCGCATTCATTCACAGGTCAAAA R: CCTCCAGGGTGATGGTCTTG	TCTTCGTGAAAACCC	NM_174133 AF038129
β-Actin	F: CGTGAGAAGATGACCCAGATCA R: TCACCGGAGTCCATCACGAT	CTGCCATGTACGTGGC	NM_173979

Figure 3.1. Relative mRNA expression of C8 ($P = 0.09$), E2 ($P = 0.16$) and Ubiquitin ($P = 0.004$) in skeletal muscle samples collected from Holstein dairy cows at approximately 27 days pre-partum and 3 days post-partum. All post-partum values are expressed as fold change relative to pre-partum values within each animal ± SEM.

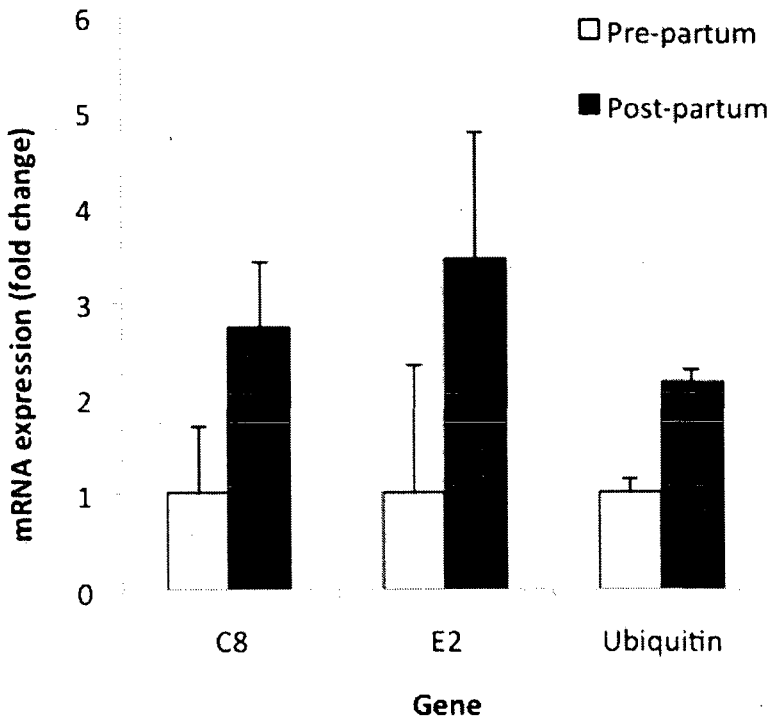
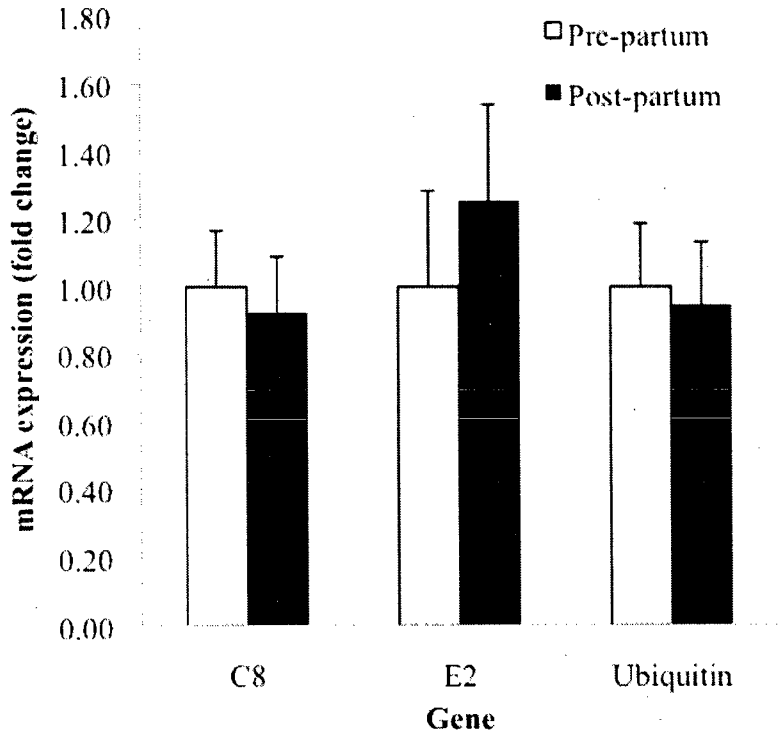


Figure 3.2. Relative mRNA expression of C8 ($P = 0.75$), E2 ($P = 0.54$), and Ubiquitin ($P = 0.84$) in liver samples collected from Holstein dairy cows at approximately 16 days pre-partum and 10 days post-partum. All post-partum values are expressed as fold change relative to pre-partum values within each animal and average values are depicted ± SEM.



CHAPTER 4

Plasma amino acid profile and expression of the ubiquitin-mediated proteolytic pathway in lambs with induced metabolic acidosis[1]

4.1 ABSTRACT

Metabolic acidosis is a condition often induced by ruminal acidosis. Identification of the specific proteolytic pathways affected by metabolic acidosis and characterization of amino acid concentration changes induced by metabolic acidosis in ruminants has yet to be confirmed. The objective of this study was to examine the effect of nutritionally induced metabolic acidosis on lamb plasma amino acid and tissue parameters, including mRNA and protein expression of components of the ubiquitin-mediated proteolytic pathway. Lambs (n = 10) were divided evenly into treatment groups receiving alfalfa pellets supplemented with either 1) a control canola meal supplement, or 2) HCl-treated canola meal supplement for a 10-d treatment period. On d 11 lambs were slaughtered and liver, muscle, and kidney samples were collected to determine mRNA expression of components of the ubiquitin-mediated proteolytic pathway and ubiquitin protein expression. Plasma concentrations of serine (P = 0.06), glycine (P = 0.002), and glutamine (P = 0.04) were greater in acidotic lambs compared to control animals, indicating that protein catabolism may be occurring. However, no alteration ($P > 0.1$) in messenger RNA expression of the proteasome subunit C8, ubiquitin-conjugating enzyme E2, or ubiquitin, or ubiquitin protein expression were observed. These results suggest that

[1] Reprinted with permission. Originally printed in J. Anim. Sci. (2008) 86: 2651-2656.

ubiquitin-mediated proteolysis is not the primary pathway of protein degradation in lambs afflicted with metabolic acidosis.

4.2 INTRODUCTION

Feeding high starch diets to ruminants is common practice in production systems when there is need to increase energy availability. However, increased fermentation rate in the rumen increases production of propionate and lactate, commonly resulting in ruminal acidosis (Nocek, 1997). Consequently, increased absorption of lactate across the ruminal wall can lead to metabolic acidosis. Physiological compensation for decreased blood pH includes increased bicarbonate buffering, inter-organ nitrogen transport, and ultimately H^+ excretion via NH_4Cl from the kidney. These actions are accomplished primarily through increased net glutamine output from the liver, increased nitrogen excretion from the kidney, and increased protein catabolism within muscle (Hollidge-Horvat et al., 1999; Garibotto et al., 2004; Taylor and Curthoys, 2004).

Metabolic acidosis has been widely studied in non-ruminants because it is a sign of cachexia, renal failure, and trauma injuries (Caso et al., 2004; Lecker et al., 2006; Curthoys et al., 2007). Research using non-ruminant animals has yielded strong evidence for the induction of the ubiquitin-mediated proteolytic pathway as the primary proteolytic mechanism stimulated under acidotic conditions to provide amino acids for nitrogen buffering (Du et al., 2005). The ATP-requiring ubiquitin pathway is unique for its usage of the 26S proteasome, which recognizes ubiquitin chains bound to target proteins by ubiquitin-conjugating enzymes (Hershko and Ciechanover, 1998; Ciechanover, 2006).

Despite detrimental effects of metabolic acidosis in livestock production systems, the effect of metabolic acidosis on intracellular protein degradation in ruminants has received little attention to date. Ruminants have unique compensatory mechanisms via nitrogen recycling in the rumen and shifts in nutrient absorption (Kingston-Smith and Theodorou, 2000), as well as repartitioning of nutrient utilization to restore acid-base balance (Lobley et al., 1995). Therefore, it is plausible that metabolic acidosis stimulates proteolysis differently in ruminants. In a previous companion study, our objective was to characterize blood acid-base parameters in lambs as a result of nutritionally induced metabolic acidosis using an anionic supplement (Las et al., 2007). In continuance of our objective, the current study examines the effect of this nutritionally induced metabolic acidosis on plasma amino acid and tissue parameters associated with protein catabolism to further characterize physiological response to decreased plasma pH and strong ion difference.

4.3 MATERIALS AND METHODS

4.3.1 Animals and Experimental Design

Ten fully fleeced yearling Rideau-Arcott wether lambs (54.3 ± 6.7 kg of BW) were allotted randomly to 2 treatments in a randomized complete block design as described previously by Las et al. (2007). All animal procedures were approved by the University of Guelph Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

4.3.2 Experimental Treatments

As described previously by Las et al. (2007), lambs were offered 2 equal portions of dehydrated alfalfa pellets (1.2 kg/d total containing 90% DM, 22% CP as % of DM, 1.2 Mcal of NEg/kg of DM) at 1000 and 1300 daily during the pre-experimental period. During the experimental period, control lambs were supplemented with a canola meal supplement whereas acidosis lambs were supplemented with a HCl-treated canola meal (NutriChlor, Nutritech Solutions, Abbotsford, Canada). Lambs were fed supplement from d 0 to d 10, receiving 0 g (d 0), 50 g (d 1), 100 g (d 2), 150 g (d 3), and 200 g/d (d 4 to 10) in 2 (0700 and 1100) equal portions mixed with 30 mL molasses while alfalfa pellets were offered at 1000 and 1500 during the experimental period. Laboratory analyses of the diets were given by Las et al. (2007). Treatments were discontinued on d 11 and lambs were slaughtered by captive bolt stunning and exsanguination.

4.3.3 Sample Collection

Lamb BW were recorded 2 d prior to commencement of the experiment. As described in Las et al. (2007), vinyl catheters were fitted into the left jugular vein of each lamb for repeated blood sampling during the experimental period. Baseline blood measurements were determined on d 0, followed by daily jugular blood sample collections (between 1100 and 1130) from d 1 to 10. Sterile 3-mL non-venting tubes containing lithium-heparin (Gaslyte, Vital Signs Inc, Englewood, CO) were used for blood collection. Within 3 min of blood collection on each day, blood acid-base parameters were measured, and have been reported in Las et al. (2007), along with plasma parameters measured after blood separation by centrifugation.

Approximately 3 g each of liver (center of right lobe), kidney (cross section including cortex and medulla), and muscle (sections of sternomandibularis) samples were

collected within 10 min of slaughter, and snap frozen in liquid nitrogen and stored at −70°C until analysis.

4.3.4 Plasma amino acid concentration

Reverse phase HPLC was performed to determine amino acid concentrations in plasma samples from each lamb. Samples were first pooled within animal from d 4 to 10 of the experimental period, and subsequently prepared using the method of Bidlingmeyer et al. (1984) with modifications for biological samples as described by House et al. (1994).

4.3.5 mRNA expression of C8, E2 and Ubiquitin in liver, kidney and muscle tissue

Relative real-time PCR was performed to examine fluctuations in messenger RNA (mRNA) expression of components of the ubiquitin-mediated proteolytic pathway. Total RNA was isolated from the kidney, liver, and muscle tissue using the TRIzol method (Invitrogen, Burlington, Canada). Total RNA was resuspended in diethylpyrocarbonate-treated water before quantification using absorbance readings at 260 nm/280 nm. Samples were treated for removal of genomic contamination (DNase, Invitrogen, Burlington, Canada), and analyzed for RNA integrity (2100 Bioanalyzer, Agilent Technologies, Brockville, Canada). All intact RNA was reverse-transcribed (5 μg RNA per sample), and complementary DNA was diluted to 1:50 for gene amplification by TaqMan real-time PCR (Prism 7000, Applied Biosystems, Foster City, CA). Exon-spanning primers and FAM-labeled probes (Table 4.1) were custom designed (Applied Biosystems) for the proteasome subunit C8, the ubiquitin-conjugating enzyme E2, ubiquitin, and β-Actin (housekeeping gene) genes using bovine sequences listed on GenBank (National Center Biotechnology Information, Bethesda, MD) that were

observed to have no similarities to other gene sequences through BLAST (National Center Biotechnology Information). Standard dilutions were amplified on each plate (1:10, 1:40, 1:160, 1:640, 1:2,560), and all samples were tested in triplicate. Standard dilutions were used to generate plate efficiency values ($E = 10^{-1/slope}$) and incorporated into determination of mRNA (Pfaffl, 2001). Control animals were pooled to generate calibrator values for all genes within each tissue type.

4.3.6 Ubiquitin protein expression

Expression of the ubiquitin protein was analyzed in liver, kidney, and muscle samples collected from each animal. One gram of wet tissue was combined with 250 m*M* sucrose, 10 m*M* HEPES-KOH, 1 m*M* EGTA, and protease inhibitor cocktail (Sigma-Aldrich, Oakville, Canada), and homogenized before being stored at −70°C until analysis. Protein concentration was determined (Bradford, 1976) using bovine serum albumin as the standard. Equal parts Laemmli buffer (Sigma-Aldrich, Oakville, Canada) and sample, containing 15 μg of protein, were combined and heated prior to being loaded into wells of pre-made 18% resolving Tris-HCl gels (BioRad, Mississauga, Canada) for protein separation by SDS-PAGE. Proteins were transferred from gels to polyvinylidene fluoride membranes (Millipore, Billerica, MA) before blocking for 1.5 h with blocking reagent (2% instant milk powder dissolved in 200 m*M* NaCl low stringency Tris buffer saline and Tween 20 solution). Membranes were then incubated with blocking reagent containing 1:1,000 of bovine ubiquitin monoclonal antibody of mouse origin (Fitzgerald Ind. Intl., Inc., Concord, MA) for 1.5 h at room temperature, washed, and subsequently incubated in 1:5,000 horseradish peroxidase linked secondary whole antibody of sheep origin (Amersham Biosciences, Piscataway, NJ) and blocking reagent at 4°C overnight.

Antibodies have been validated for use on bovine samples and considered appropriate for use with ovine samples due to high sequence conservation between bovine and ovine species. Membranes were washed before chemiluminescence determination using an Enhanced Chemiluminescence detection reagent kit (Amersham Biosciences, Piscataway, NJ). Membranes were stained with fast-green and an internal control was loaded on each gel. Density of bands and fast–green stained membranes of each lane were determined (Northern Eclipse, Empix Imaging, Mississauga, Canada). The band value was then normalized to the density of the entire fast-green stained lane as described by Howell et al. (2003).

4.3.7 Statistical Analysis

All results were analyzed using PROC MIXED of SAS (SAS Inst. Inc, Cary, NC) using the following model: $Y_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ij}$, where Y_i was the dependent variable, μ was the overall mean, α_i was the effect of dietary treatment, β_j was the effect of block (blocked by weight), $\alpha\beta_{ij}$ was the effect of the dietary treatment x block interaction, ε_{ij} was the random residual error. Block was insignificant ($P > 0.05$) for all measured variables and thus removed from the model. Results were declared significant at $P < 0.05$ unless otherwise stated.

4.4 RESULTS AND DISCUSSION

As described by Las et al. (2007), blood and urinary pH were lower while plasma bicarbonate and calcium concentrations were increased in acidotic lambs vs. control lambs. Though plasma evidence of metabolic acidosis was observed in acid-base

parameters (Las et al., 2007), only greater plasma concentrations of serine (P = 0.06), glycine (P = 0.002), and glutamine (P = 0.04) were observed in acidotic lambs (Table 4.2). Previous research has demonstrated that renal serine synthesis can occur via two different pathways, either via glycine catabolism or gluconeogenic precursors, and that glycine could be a major contributor to renal ammonia production (Lowry et al., 1987). In agreement with these observations, arterial concentrations of both glycine and serine were increased with the induction of metabolic acidosis in non-ruminants (Lowry et al., 1987; Garibotto et al., 2004), which was consistent with our current findings in lambs.

No change (P > 0.1) in mRNA expression of C8, E2, or ubiquitin was observed in kidney (Figure 4.1), liver (Figure 4.2), or muscle (Figure 4.3) tissue collected from acidotic lambs compared to control lambs using real-time PCR in agreement with previous Northern hybridizations using these same tissue samples (Greenwood et al., 2007). Also, no fluctuations in ubiquitin protein expression were observed in the kidney (P = 0.34; Figure 4.4), liver (P = 0.56; Figure 4.5), or muscle (P = 0.26; Figure 4.6) in acidotic lambs compared to control lambs.

Research has demonstrated the importance of the ubiquitin-mediated proteolytic pathway under conditions of severe metabolic acidosis in non-ruminants (Mitch et al., 1994; Hollidge-Horvat et al., 1999; Garibotto et al., 2004); however, the current study now provides evidence that the same proteolytic pathway is not the primary response pathway in mildly acidotic lambs. Exploration of the possibility that the ubiquitin-mediated proteolytic pathway is not the predominant pathway in metabolically acidotic lambs will lead to examination of other proteases, such as cathepsins and calpains. The Ca^{2+}-dependent calpains have been proposed to be precursors binding to the Z-disc, and

subsequently releasing actin and myosin fragments (Bartoli and Richard, 2005). Plasma calcium concentrations were increased in lambs offered the acidosis diet (Las et al., 2007), potentially providing the Ca^{2+} binding required for calpain activation. In addition, caspase-3 has also been described as a precursor for preliminary breakdown of muscle before proteasomes can successfully degrade the smaller filaments (Du et al., 2005). Furthermore, cathepsin L has been identified as a lysosomal protease that is upregulated in muscle as a result of fasting, tumor bearing, uremia, and diabetes mellitus (Lecker et al., 2004). Cathepsins B and D have also been observed to be upregulated during muscle wasting, which is symptomatic of several ailments including diabetes, trauma, fasting, cancer, and sepsis (Bechet et al., 2005). However, these cathepsin isoforms have also been observed to be downregulated in the kidney due to NH_4Cl loading (Fawcett et al., 2000), indicating potential differential regulation depending on tissue.

A second possibility is that ruminants can spare protein from degradation using other biological mechanisms to cope with increased nitrogen requirements without sacrificing their own tissue protein. It has been previously observed that during metabolic acidosis, ruminant metabolism has the unique capacity to favor increasing perivenous hepatocyte glutamine synthesis, in lieu of sacrificing HCO_3^- for carbamoyl phosphate synthesis for increased ureagenesis in periportal hepatocytes (Lobley et al., 1995; Milano et al., 2000). Renal glutamine deamination is also increased to dispose of excess ammonia as ammonium ions in urine (Heitmann and Bergman, 1980). As a result, these unique mechanisms could prevent significant protein degradation as a result of metabolic acidosis.

Model parameters must also be considered, as the current study utilized an anionic supplement to induce metabolic acidosis instead of inducing increased lactate and VFA absorption through offering a highly fermentable diet. However, the primary objective of the current research was to examine the blood parameters associated with use of a dietary cation-anion difference. Despite differences in how blood pH depression is achieved, the level of anionic supplement used in the current study elicits a drop in blood pH observed for other acidotic animal models (Lobley et al., 1995; Mutsvangwa et al., 2004; Moret et al., 2007). Also, the length of treatment was typical of experiments previously cited (Heitmann and Bergman, 1980; Lobley et al., 1995).

In conclusion, the results herein indicate that ubiquitin-mediated proteolysis was not the primary pathway of tissue protein degradation in lambs with induced metabolic acidosis. Further investigations will focus on different proteolytic pathways, such as the cathepsins and calpains, to determine if protein degradation is increased via these proteases under conditions of metabolic acidosis in ruminants.

Table 4.1. Primers and probes used for real-time PCR[1]

Gene	Primers[2]	Probe	Accession No.
C8	F: GAAGAAGGTTCCAACAAACGACTTT R: ACGAGCGTCTGCCAACAAA	CCTGCTACTGCCATTCC	NM_001034235
E2	F: AGCCGCCAACTGTTAGGTTTT R: TGTTGGACTCCATCTATTCTGAAGGA	CCATCAGCATACACATTTG	NM_001037459
Ubiquitin	F: CTTCGCATTCATTCACAGGTCAAAA R: CCTCCAGGGTGATGGTCTTG	TCTTCGTGAAAACCC	NM_174133 AF038129
β-Actin	F: CGTCTTCCCGTCCATCGT R: GGCCCATGCCCACCAT	CCGGCACCAGGGCGTA	NM_173979

[1]Messenger RNA sequences with exon junctions were used for primer and probe design (Applied Biosystems, Foster City, CA).

[2]F = forward; R = reverse.

Table 4.2. Plasma amino acid concentrations[1] in lambs offered control canola supplement (control) or HCl-treated canola supplement (acidotic)

Amino acid, μM	Control	Acidotic	SEM	*P*
Aspartate	4.4	4.9	0.5	0.6
Glutamate	78.1	66.8	5.5	0.2
Serine	54.4	69.4	4.8	0.06
Asparagine	44.0	48.1	2.0	0.2
Glycine	337.9	445.5	16.6	0.002
Glutamine	193.5	219.8	7.7	0.04
Taurine	49.4	43.1	7.3	0.6
Histidine	211.8	212.8	17.9	0.9
Citrulline	59.0	48.8	4.6	0.2
Threonine	102.9	89.0	7.7	0.2
Alanine	133.4	143.0	5.2	0.2
Arginine	15.6	16.5	2.2	0.8
Proline	100.9	104.6	5.2	0.6
Tyrosine	73.4	71.3	5.4	0.8
Valine	307.6	279.7	23.3	0.4
Methionine	19.1	17.5	2.0	0.6
Cystine	10.6	8.0	1.8	0.3
Isoleucine	101.7	87.8	5.8	0.1
Leucine	173.8	164.7	7.8	0.4
Phenylalanine	57.9	56.9	5.2	0.9
Tryptophan	54.5	47.2	2.5	0.1
Ornithine	139.2	146.3	15.1	0.8
Lysine	159.9	160.4	12.9	0.9

Figure 4.1. Relative mRNA expression of C8 ($P > 0.05$), E2 ($P > 0.05$) and ubiquitin ($P > 0.05$) in kidney tissue collected from n = 5 control lambs (control) and n = 5 acidotic lambs (acidotic). All genes were calibrated against β-actin and are relative to the pooled control group values. Depicted as least square means ± SEM.

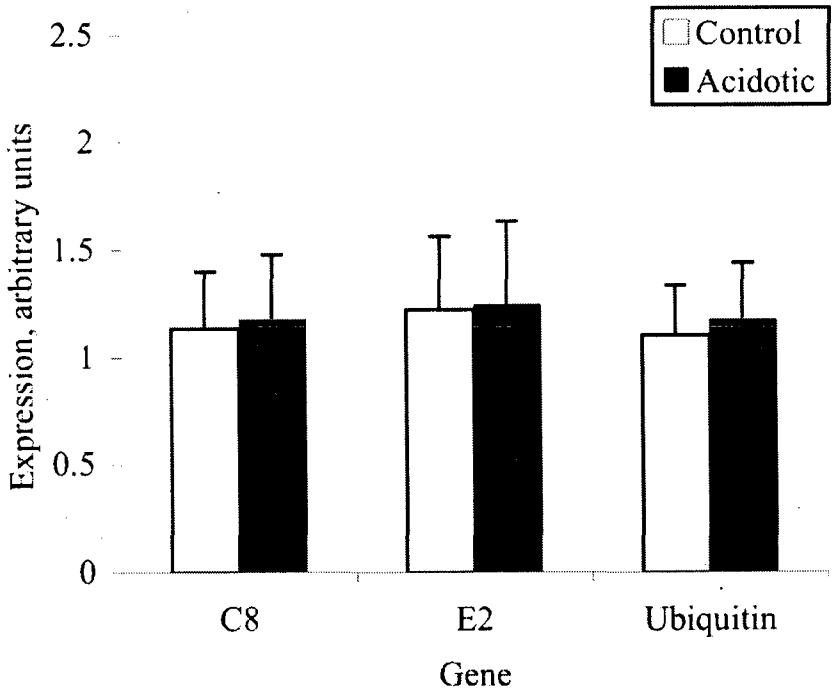


Figure 4.2. Relative mRNA expression of C8 ($P > 0.05$), E2 ($P > 0.05$), and ubiquitin ($P > 0.05$) in liver tissue collected from n = 5 control lambs (control) and n = 5 acidotic lambs (acidotic). All genes were calibrated against β-actin and are relative to the pooled control group values. Depicted as least square means ± SEM.

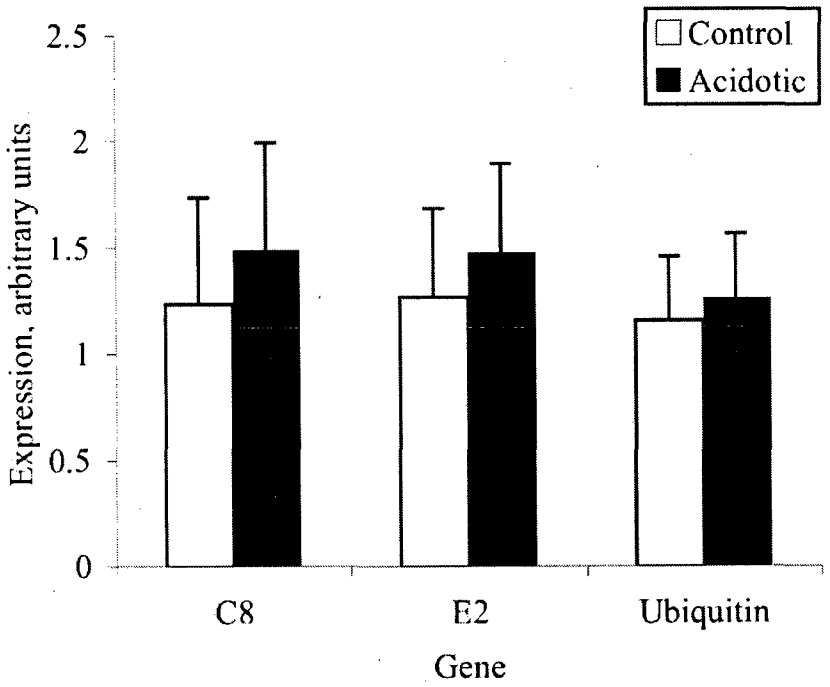


Figure 4.3. Relative mRNA expression of C8 ($P > 0.05$), E2 ($P > 0.05$) and ubiquitin ($P > 0.05$) in muscle tissue collected from n = 5 control lambs (control) and n = 5 acidotic lambs (acidotic). All genes are calibrated against β-actin and are relative to the pooled control group values. Depicted as least square means ± SEM.

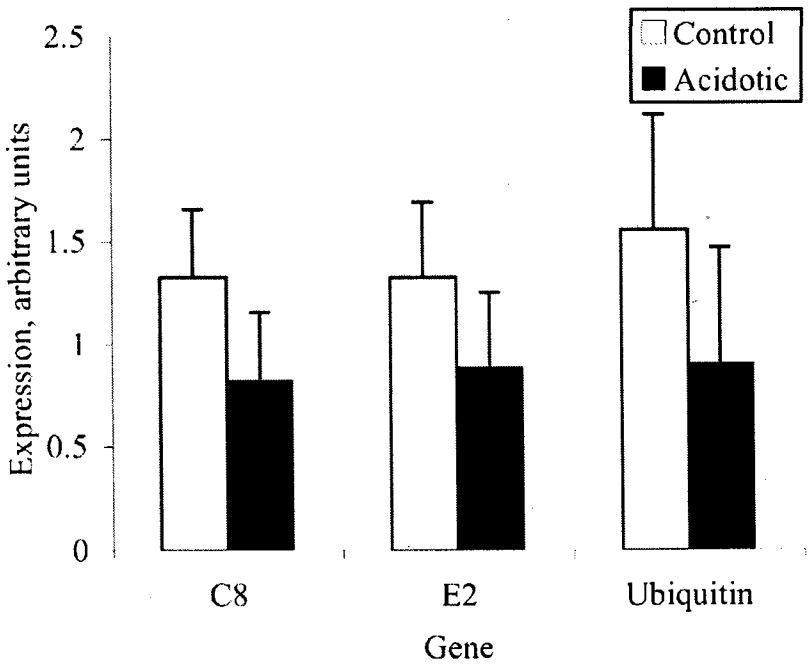
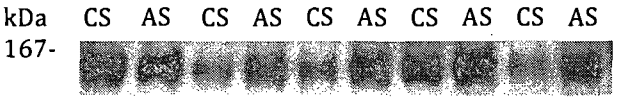


Figure 4.4. Immunoblot (A) and densitometric comparison (B) of ubiquitin protein expression ($P > 0.05$) in kidney tissue collected from n = 5 control lambs (control; CS) and n = 5 acidotic lambs (acidotic; AS). Densitometric levels were corrected by creating a ratio between ubiquitin and fast-green stained lane for each sample. Depicted as least square means ± SEM.

A



B

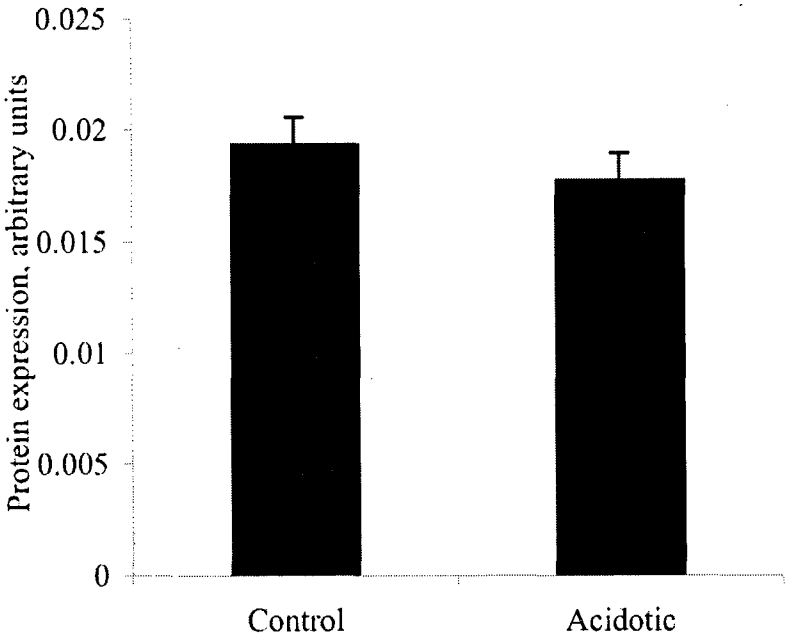
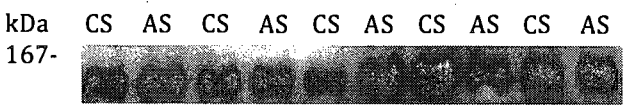


Figure 4.5. Immunoblot (A) and densitometric comparison (B) of ubiquitin protein expression ($P > 0.05$) in liver tissue collected from n = 5 control lambs (control; CS) and n = 5 acidotic lambs (acidotic; AS). Densitometric levels were corrected by creating a ratio between ubiquitin and fast-green stained lane for each sample. Depicted as least square means ± SEM.

A



B

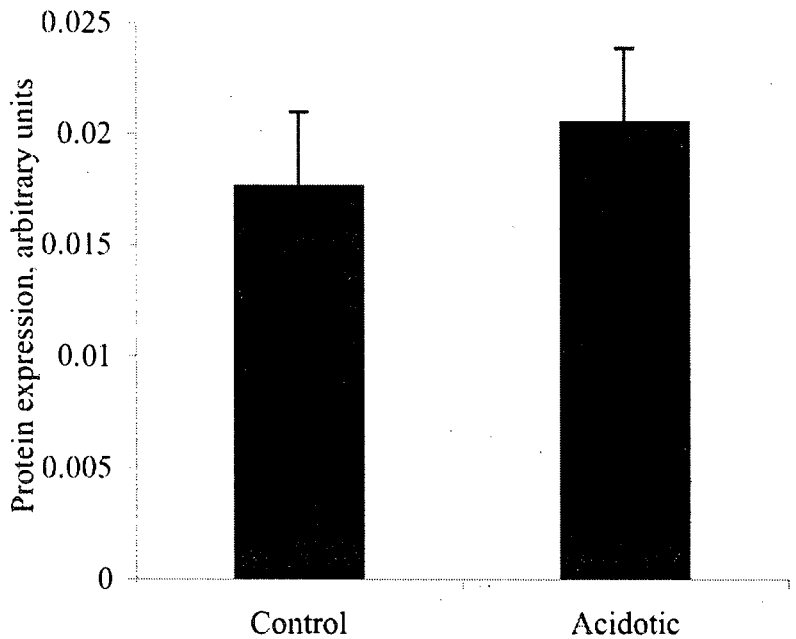
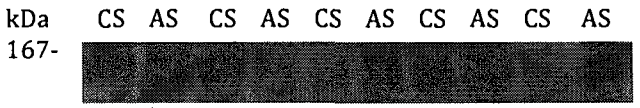
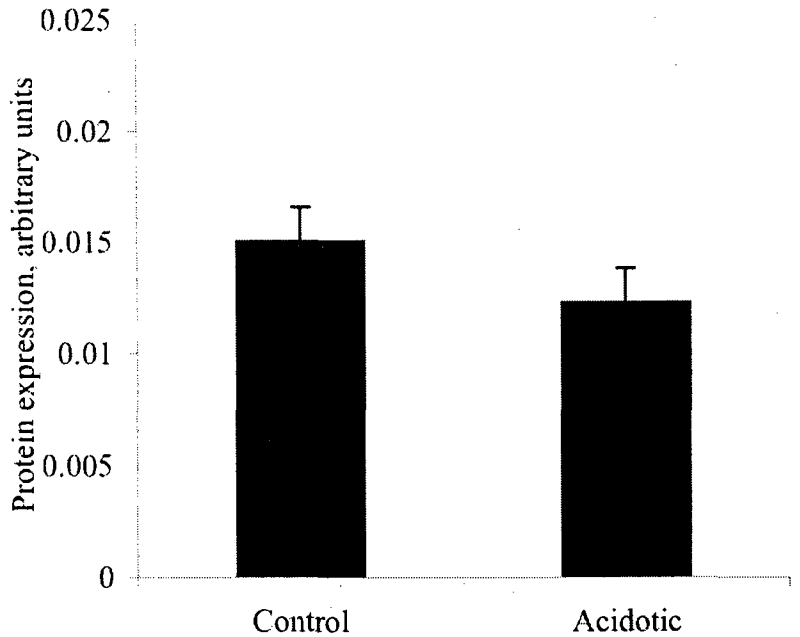


Figure 4.6. Immunoblot (A) and densitometric comparison (B) of ubiquitin protein expression ($P > 0.05$) in muscle tissue collected from n = 5 control lambs (control; CS) and n = 5 acidotic lambs (acidotic; AS). Densitometric levels were corrected by creating a ratio between ubiquitin and fast-green stained lane for each sample. Depicted as least square means ± SEM.

A



B



CHAPTER 5

Influence of glutamine infusion on ubiquitin, caspase-3, cathepsins L and B, and m-calpain expression in sheep with nutritionally induced metabolic acidosis[1]

5.1 ABSTRACT

Provision of amino acids (AA) has shown success in attenuating proteolytic activity in monogastrics suffering from metabolic acidosis. However, it is unknown whether AA supplementation can provide any beneficial effects to ruminants with nutritionally induced metabolic acidosis. The objective of the current study was to examine the effects of glutamine infusion on various protein degradation components across a number of tissues in sheep with induced metabolic acidosis. Sheep were assigned to a randomized complete block design with 2 x 2 factorial arrangement of treatments (n = 6 sheep/treatment) consisting of a control diet or acidosis diet, and receiving a saline or L-glutamine infusion. Sheep were fed diets for 10 d and slaughtered on d 11. Liver, kidney, and muscle samples were collected at slaughter and examined for relative mRNA expression of ubiquitin, C8, E2, cathepsin L, cathepsin B, caspase-3, and m-calpain, as well as protein expression of ubiquitin. Relative mRNA expression of C8 ($P = 0.02$), E2 ($P = 0.06$), and ubiquitin ($P = 0.07$) was lower in kidney in acidotic versus control sheep. Additionally, mRNA expression of m-calpain in kidney was greater ($P = 0.01$) as a result of glutamine infusion. There were no significant alterations ($P > 0.10$) in mRNA of any

[1] Reprinted with permission. Originally printed in J. Anim. Sci. (2009) 87: 2073-2079.

component as a result of acidosis in the liver or muscle. This study demonstrates the inability of metabolic acidosis to increase expression of the ubiquitin-mediated proteolytic pathway in skeletal muscle; however, downregulation of renal mRNA expression of these components is apparent during the induction of metabolic acidosis.

5.2 INTRODUCTION

Metabolic acidosis is a condition in which increased inter-organ nitrogen transport and H^+ buffering are required for animal survival. During rumen acidosis, production of lactate and propionate increase in the rumen and are consequently absorbed, decreasing blood pH (Nocek, 1997). However, the mechanistic responses to metabolic acidosis in ruminants are poorly understood, as the condition has thus far been predominantly studied in rodent or monogastric disease models (Curthoys et al., 2007; Lecker et al., 2006). The unique digestive capabilities of ruminants complements their distinctive metabolic properties, such as their ability to continuously recycle nitrogen (Owens and Zinn, 1988), as well as their dependency on absorption of gluconeogenic precursors (Fahey and Berger, 1988). These, amongst other, fundamental differences in ruminant metabolism may also alter the compensatory actions due to physiological insult. Recent sheep research suggests that the ubiquitin-mediated proteolytic pathway is not upregulated during acidosis (Greenwood et al., 2008), unlike nonruminants (Mitch et al., 1994). This could indicate that ruminants predominantly use a different proteolytic pathway under acidotic conditions, or have been able to compensate for an increased anionic load via other mechanisms. Provision of exogenous glutamine and leucine to

nonruminants with metabolic acidosis results in attenuation of protein degradation (Watford et al., 2000; Wilmore, 2001). It is important to establish a ruminant model and examine the influence of glutamine supplementation on proteolysis in ruminants. We have recently reported the impact of nutritionally induced metabolic acidosis and infusion of L-glutamine on acid-base balance, and plasma AA concentrations in sheep (Odongo et al., 2009). The objective of the current study was to examine shifts in tissue mRNA and protein expression of proteolytic components as a result of metabolic acidosis and L-glutamine treatment in the same animals.

5.3 MATERIALS AND METHODS

5.3.1 Animals, Experimental Treatments, and Experimental Design

Twenty-four yearling wether Canadian-Arcott sheep (63.6 ± 5.9 kg of BW) were obtained from the Ponsonby Research Station, University of Guelph, Canada, and were handled in accordance with the Canadian Council on Animal Care regulations. The University of Guelph Animal Care Committee approved their use for this experiment. The outline of the 2 x 2 factorial arrangement and treatments has been described by Odongo et al. (2009). Briefly, sheep (blocked by BW) were housed in individual pens with ad libitum access to water and fed dehydrated alfalfa pellets containing 900 g DM/kg, 220 g CP/kg DM, and 1.2 Mcal NEg/kg daily at 1000 and 1500 h. During the 10 d experimental period, sheep were offered an additional supplement of either a control canola meal supplement or an HCl-treated canola meal supplement (NutriChlor, Nutritech Solutions, Abbotsford, Canada) offered twice daily at 0700 and 1100 h. The

canola meal supplement was mixed with 60 mL of molasses and the supplement amount offered was increased through the experimental period as follows: d 0, 0 g/d; d 1, 100 g/d; d 2, 200 g/d; d 3, 250 g/d; d 4 to 10, 300 g/d. Diet composition and chemical analysis is described by Odongo et al. (2009). In addition, sheep were administered jugular infusions of either saline or L-glutamine (292 mg/L; Cambrex Corporation, East Rutherford, NJ) from d 4 to 10 at a rate of 0.3 g of L-glutamine per kg of BW or 1.8 mL/min of saline for 196 ± 3.1 min (mean ± SE) starting at 1300 h daily using a peristaltic pump as previously described (Plaizier et al., 2001). All treatments were discontinued on d 11, and sheep were killed by captive bolt and exsanguination. Muscle (sternomandibularis), liver (central right lobe), and kidney (cross section of cortex/medulla) samples were collected at slaughter, snap frozen in liquid nitrogen, and stored at -70°C until further analysis.

5.3.2 Real-time PCR

Total RNA was isolated using the TRIzol method (Invitrogen, Burlington, Canada), where frozen tissue was ground using chilled mortars and pestles and 200 to 600 mg of tissue was suspended in 1 mL TRIzol/mg of tissue and homogenized using an electric homogenizer before the addition of chloroform for phase separation. RNA was precipitated using isopropanol and the RNA pellet was washed twice with 75% ethanol before drying. Total RNA was resuspended in diethylpyrocarbonate-treated water for 260/280 nm absorbance readings. Intact RNA (5 μg) was treated to eliminate genomic contamination (DNase, Invitrogen, Burlington, Canada) and subsamples of treated RNA were examined for RNA integrity (Agilent 2100 Bioanalyzer, Agilent Technologies, Brockville, Canada). Intact RNA was reverse-transcribed and frozen at 1:50

concentrations diluted in diethylpyrocarbonate-treated water. Samples and standard dilutions (1:10, 1:40, 1:160, 1:640, 1:2560) were loaded onto plates in triplicate and examined for messenger RNA (mRNA) expression of the 26S proteasome C8 subunit, the E2 conjugating enzyme of the ubiquitin-mediated proteolytic pathway, ubiquitin, caspase-3, cathepsin B, cathepsin L, m-calpain, and β-actin (housekeeping gene) in 25 μL uniplex reactions with commercial master mix (Taqman Real-time PCR Master Mix with AMPerase UNG; Applied Biosystems, Foster City, CA) using real-time PCR (Prism 7000 Real-time PCR machine, Applied Biosystems, Foster City, CA). Exon-spanning primers and probes (Table 5.1) were designed based on mRNA sequences obtained by GenBank (National Center Biotechnology Information, Bethesda, MD) that were determined to have no sequence similarities to other ovine or bovine sequences through BLAST (National Center Biotechnology Information, Bethesda, MD). Plate efficiencies were determined using slope of standard dilutions ($E = 10^{-1/slope}$) and relative mRNA expression was determined using the Pfaffl method (Pfaffl, 2001). Individual real-time PCR results were calibrated to pooled control diet saline infused animal values within each tissue type.

5.3.3 Immunoblot Analysis

Tissue subsamples obtained from liver, kidney and muscle samples (1 g) were homogenized in extraction buffer (250 m*M* sucrose, 10 m*M* HEPES-KOH, 1 m*M* ethylene glycol tetraacetic acid) and protease inhibitor cocktail (Sigma-Aldrich, Oakville, Canada) before being frozen at -70°C. Protein concentration was determined (Bradford, 1976) using thawed sample homogenate. Equal parts Laemmli sample buffer (Sigma-Aldrich, Oakville, Canada) and 15 μg of protein were combined and heated at 100°C for

15 min before loading into wells of pre-made 18% Tris-HCl gels (BioRad, Mississauga, Canada). Proteins were separated by gel electrophoresis at 100V and electro-transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA) at 100V for 1 h. Membranes were incubated in blocking reagent [2% Carnation dried non-fat milk (Metro, Guelph, Canada) and TBS/Tween 20] for 1.5 h before being incubated in 1:1000 primary antibody of mouse origin against bovine ubiquitin (Fitzgerald Ind. Intl., Inc., Concord, MA) and blocking reagent at 4°C overnight. Membranes were washed with blocking reagent before undergoing incubation in 1:5000 horseradish peroxidase linked secondary antibody of sheep origin (Amersham Biosciences, Piscataway, NJ) and blocking reagent for 40 min. Antibodies were considered appropriate for ovine protein measurements due to high sequence conservation between ovine and bovine species, of which antibodies have been validated for use. Membranes were washed in TBS/Tween 20 solution before determination of protein abundance by chemiluminescence through addition of Enhanced Chemiluminescence detection reagents (Amersham Biosciences, Piscataway, NJ). Membranes were subsequently stained with fast-green, and ubiquitin band density was normalized to lane density after densitometric analysis using Northern Eclipse (Empix Imaging, Mississauga, Canada) as previously described (Howell et al., 2003; Greenwood et al., 2008). All samples were tested in duplicate and an internal control was loaded onto each membrane.

5.3.4 Statistical Analysis

All data were analyzed statistically using Proc MIXED of SAS (SAS Inst. Inc, Cary, NC). The statistical model employed was as follows: $Y_{ijk} = \mu + \tau_i + \alpha_j + \beta_k + \alpha\beta_{jk} + \varepsilon_{ijk}$, in which Y_{ijk} = the dependent variable, μ = the overall mean, τ_i = the effect of block

($_i$= 1, 2, 3), α_j = the effect of dietary treatment ($_j$ = 1, 2), β_k = the effect of infusion ($_k$ = 1, 2), $\alpha\beta_{jk}$ = the effect of dietary treatment by infusion interaction ($_{jk}$ = 1, 2, 3, 4), and ε_{ijk} = the random residual error. Treatment means were compared using Tukey's multiple comparison procedure. Results were declared significant at $P < 0.10$ unless stated otherwise, and data were expressed as mean ± SEM.

5.4 RESULTS AND DISCUSSION

As presented and discussed by Odongo et al. (2009), significantly lower urinary pH and blood pH, bicarbonate, urea, total CO_2, partial pressure of CO_2, and strong ion difference, in addition to other blood gas and acid-base parameters, demonstrated that metabolic acidosis was successfully achieved in sheep fed the acidosis diet. In terms of tissue parameters examined in the current study, induction of metabolic acidosis resulted in reductions in C8 ($P = 0.02$), E2 ($P = 0.06$), and ubiquitin ($P = 0.07$) mRNA expression in the kidney (Figure 5.1). In contrast, no significant regulation was observed as a result of acidosis in the liver ($P \geq 0.80$; Figure 5.2) or muscle ($P \geq 0.51$; Figure 5.3).

The downregulation of renal mRNA expression of ubiquitin mediated proteolysis components in the current study indicates a potential downregulation of ubiquitin-mediated proteolysis and could be a consequence of feedback inhibition induced by increased ammonia flux. By examination of leucine kinetics and blood parameters in human subjects given combination oral acidifiers, Garibotto et al. (2004) observed that increased renal ammonia concentrations induced a decrease in cathepsin mediated protein degradation. In the current study, downregulation of the ubiquitin-mediated proteolysis

components suggests that ammonia may also elicit a downregulation of this proteolytic pathway.

However, examination of corresponding ubiquitin protein expression in kidney, liver, and muscle tissue demonstrated no significant change in ubiquitin protein expression in any of these tissues as a result of diet or infusion (Figures 5.4, 5.5, and 5.6). It must also be noted that immunoblot analysis yielded observation of larger molecular weight peptides (approximately 167 kDa) and relatively no chemiluminescence of the 8.5 kDa free ubiquitin. Observation of these larger bands is similar to previous reports, and it is widely documented that these bands depict polyubiquitinated proteins and are reflective of ubiquitin expression (Grossman et al., 2003; Kelly et al., 2007).

To further develop possible interaction with the ubiquitin-mediated proteolytic pathway, mRNA expression of caspase-3 was also determined. As the 26S proteasome cannot degrade microfilaments, it has previously been hypothesized that regulation of caspase-3 might occur in conjunction with ubiquitin-mediated proteolytic components, as caspase-3 can degrade actin into smaller fragments for the 26S proteasome (Du et al., 2005). However, in the current study, no significant differences were observed for caspase-3 mRNA expression in kidney ($P = 0.13$), liver ($P = 0.27$), or muscle ($P = 0.29$). Again, this suggests that ruminants have been able to avoid proteolysis despite a significant anionic load.

Though analysis of ubiquitin-mediated proteolytic components extends only to the mRNA and protein level in the current study, the lack of plasma AA response observed (Odongo et al., 2009) in conjunction with previous research demonstrating a significant correlation ($R = 0.93$) between ubiquitin and 20S activity in liver and muscle

tissue (Martin et al., 2002) provide additional support to our suggestion that acidosis was unable to stimulate the ubiquitin-mediated proteolytic pathway in the present study. In addition, the animal variation in skeletal muscle and hepatic mRNA response in the current study is indicative of no succinct response between animals and the inability of diet or infusion to regulate hepatic and muscle ubiquitin expression at the mRNA or protein level.

The acidotic state induced in the current study (Odongo et al., 2009) was a more severe treatment than our previous research (Las et al., 2007; Greenwood et al., 2008). A greater urinary pH depression, and greater plasma anion gap, lactate concentration, base excess, and strong ion difference was evident in acidotic animals within the current study compared to our previous research (Las et al., 2007; Odongo et al., 2009). However, even with a more severe metabolic acidosis, upregulation of ubiquitin-mediated proteolysis components was not observed. This suggests that sheep were able to tolerate and compensate for this anionic load via shifts in glutamine facilitated nitrogen transport to the kidney, as described by Heitmann and Bergman (1980). Analysis of plasma AA suggest that animals did not have increased proteolysis as a result of nutritionally induced metabolic acidosis, and only plasma leucine, lysine, and taurine concentrations were significantly altered as a result of diet or infusion (Odongo et al., 2009). In addition, there was no significant change in plasma glutamine concentration due to diet or glutamine infusion (Odongo et al., 2009). This lack of infusion effect indicates that either supplemental glutamine is being excreted, or that this additional exogenous source of glutamine is being used by tissues, perhaps for renal ammoniagenesis, as discussed by Odongo et al. (2009), and would correspond with the earlier suggestion that renal

ubiquitin-mediated proteolysis components were being downregulated by increased ammonia flux.

Though no significant differences were observed in mRNA expression of cathepsins B or L in kidney, liver, or muscle as a result of treatments (Figures 5.7, 5.8, and 5.9), hepatic m-calpain mRNA expression was significantly greater as a result of glutamine infusion (P = 0.01; Figure 5.8). Previous research (Heitmann and Bergman, 1980; Lobley et al., 1995) has demonstrated that ruminants are capable of avoiding increases in hepatic periportal urea synthesis, instead increasing hepatic perivenous glutamine synthesis. This allows for efficient transport of nitrogen to the kidney, whereby an increase in glutaminase activity allows for glutamate formation and ammonia excretion. Also, Endo et al. (1999) observed that calpain is specifically involved in degradation of various enzymes involved in glutamate and glutamine metabolism, including mitochondrial aspartate aminotransferase, as well as degradation of oxidatively modified hepatic glutamine synthetase (Rivett, 1985). Hence, it is plausible that increased glutamine availability in the liver as a result of L-glutamine infusion could have upregulated calpain mRNA expression for degradation of glutamine synthetase. Conversely, it is also possible that increased calpain activity promoted conservation of glutamate for glutamine formation instead of being deaminated to α-ketoglutarate. However, other research has demonstrated that a significant increase in calpain and calpastatin mRNA expression was not accompanied by increased activity under fasting conditions, as there was simply an increase in ribosomal loading of this mRNA (Ilian and Forsberg, 1992). In addition, it is uncertain whether AA, specifically glutamine, possess any capacity for transcriptional regulation of calpains, or whether the influence lies on

changes in inhibition of calpains by calpastatin. Though some transcriptional regulation of calpain by other regulators has been observed under various treatments (Sandmann et al., 2001), it is also believed that the calpain inhibitor calpastatin may be the most crucial regulator of this protease (Goll et al., 2003).

Oral glutamine is absorbed from the intestinal lumen in monogastrics, as identified by increased arterial plasma glutamine concentrations. Additional dietary glutamine, in turn, effectively suppresses endogenous glutamine turnover in rats (Watford et al., 2000) and humans (Wilmore, 2001). However, the current study did not demonstrate any attenuation of proteolytic indices as a result of glutamine infusion. The lack of an infusion effect of L-glutamine suggests differences between the monogastric and ruminant responses during acidosis. In the current study, it was hypothesized that infused glutamine was being utilized for nitrogen transport to the kidneys instead of increasing excess nitrogen via increased ureagenesis (Odongo et al., 2009).

The current results demonstrate a significant increase in calpain mRNA expression as a result of glutamine infusion, indicating potential involvement of m-calpain in glutamine metabolism. Though, at present, the specific interaction causing this shift in mRNA expression is unclear, there is potential direct involvement of glutamine to subside degradation of calpain targets, or an indirect result from calpastatin interactions. Additionally, the inability of nutritionally induced moderate metabolic acidosis to elicit a change in mRNA expression of proteases previously observed in the monogastric proteolytic response to metabolic acidosis demonstrates significant differences in the proteolytic response of ruminants compared to monogastrics under similar levels of blood acid-base insult. The observation that expression of the ubiquitin-mediated proteolytic

pathway does not appear to be significantly affected during acidosis in skeletal muscle and liver of ruminants suggests that ruminants have sufficient buffering capacity to dispose of an excess anionic load, rather than increasing proteolysis to augment inter-organ nitrogen transport. However, significantly lower mRNA expression of components of the ubiquitin-mediated proteolytic pathway in kidney during metabolic acidosis suggests some regulation of this proteolytic pathway in a tissue specific manor for involvement other than skeletal muscle degradation, perhaps due to increased glutamine diversion to the kidney and a consequent increase in renal ammoniagenesis.

Table 5.1. Primers and probes used for real-time PCR

Gene	Primers	Probe	Accession No.
C8	F: GAAGAAGGTTCCAACAAACGACTTT R: ACGAGCGTCTGCCAACAAA	CCTGCTACTGCCATTCC	NM_001034235
E2	F: AGCCGCCAACTGTTAGGTTTT R: TGTTGGACTCCATCTATTCTGAAGGA	CCATCAGCATACACATTTG	NM_001037459
Ubiquitin	F: CTTCGCATTCATTCACAGGTCAAAA R: CCTCCAGGGTGATGGTCTTG	TCTTCGTGAAAACCC	NM_174133 AF038129
Caspase-3	F: AGAAGTCTGACTGGAAAACCCAAAC R: GTCTCAATACCACAGTCCAGTTCTG	CTCGGCAGGCCTGAATA	NM_001077840 BC123503
Cathepsin B	F: CCGACCATCAAAGAGATCAGAGAT R: CCGGTCAGAAATGGCTTCCA	CCCCGAACGCCCAGCAG	NM_174031
Cathepsin L	F: GCCTGGACTCAGAGGAATCTTATC R: GGCAGAGAACACTCAGGCTTATAGTTA	AACGGACACAAATAGC	BC151425 NM_174032
m-calpain	F: CAGAGCTTCCAGGAGAACTATGC R: TGCCGAGTGCACGAAGAG	ACTTCCAGTTCTGGCAGT	BC134526
B-Actin	F: CGTCTTCCCGTCCATCGT R: GGCCCATGCCCACCAT	CCGGCACCAGGGCGTA	NM_173979

Figure 5.1. Relative mRNA expression of C8 (P diet = 0.02), E2 (P diet = 0.06), and ubiquitin (P diet = 0.07) in kidney tissue collected from lambs offered either control diet or acidosis diet (supplemented with HCl-treated canola meal), and with saline or L-glutamine infusion. All genes were calibrated separately against β-actin and are relative to the pooled control diet-saline infused treatment group. Relative mRNA expression of different gene types cannot be compared within tissue type, as each gene was calibrated separately; genes are represented within tissue only for diagrammatical purposes. Depicted as least square means ± SEM.

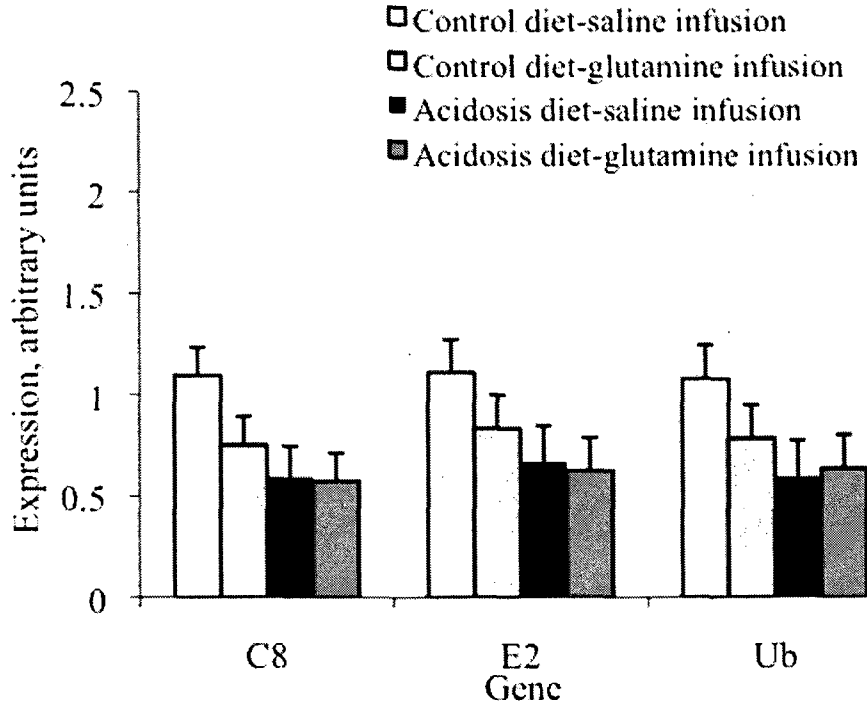


Figure 5.2. Relative mRNA expression of C8 ($P > 0.1$), E2 ($P > 0.1$), and ubiquitin ($P > 0.1$) in liver tissue collected from lambs offered either control diet or acidosis diet (supplemented with HCl-treated canola meal), and with saline or L-glutamine infusion. All genes were calibrated separately against β-actin and are relative to the pooled control diet-saline infused treatment group. Relative mRNA expression of different gene types cannot be compared within tissue type, as each gene was calibrated separately; genes are represented within tissue only for diagrammatical purposes. Depicted as least square means ± SEM.

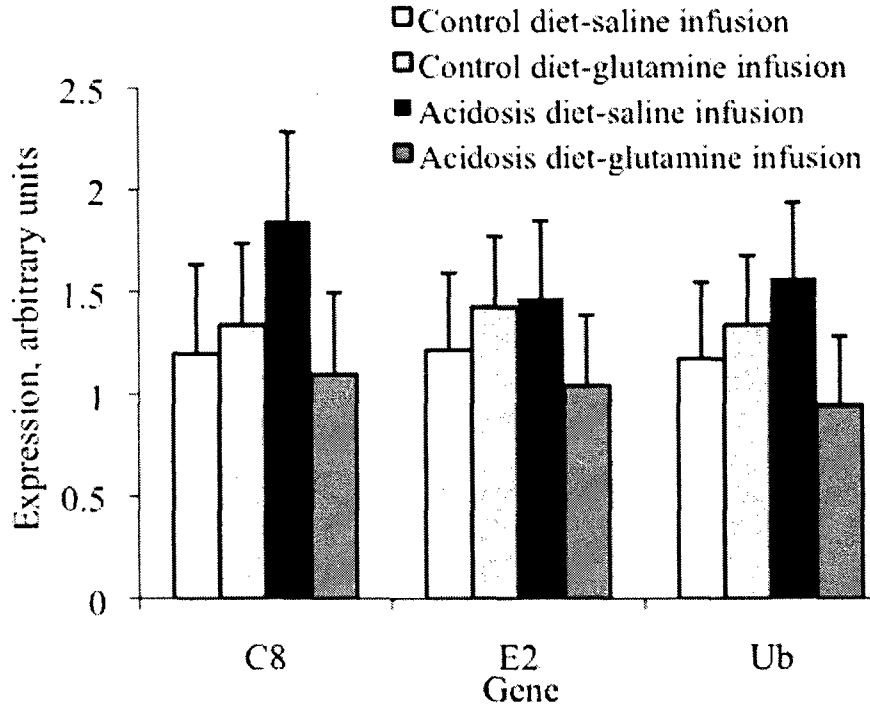


Figure 5.3. Relative mRNA expression of C8 ($P > 0.1$), E2 ($P > 0.1$), and ubiquitin ($P > 0.1$) in muscle tissue collected from lambs offered either control diet or acidosis diet (supplemented with HCl-treated canola meal), and with saline or L-glutamine infusion. All genes were calibrated separately against β-actin and are relative to the pooled control diet-saline infused treatment group. Relative mRNA expression of different gene types cannot be compared within tissue type, as each gene was calibrated separately; genes are represented within tissue only for diagrammatical purposes. Depicted as least square means ± SEM.

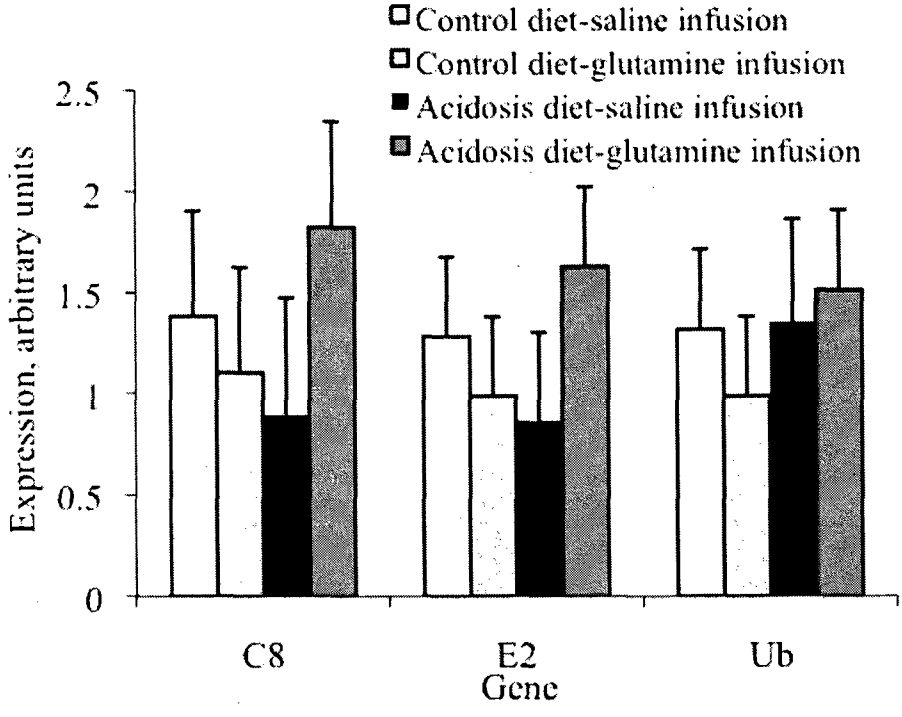


Figure 5.4. Immunoblot (A) and densitometric comparison (B) of ubiquitin protein expression ($P > 0.1$) in kidney tissue collected from lambs receiving either a control diet (CS) or acidosis diet (AS), and with saline (-S) or glutamine (-G) infusion. Ubiquitin densitometric calculations were normalized to fast-green stained lane density within each animal for each sample. Depicted as least square means ± SEM.

A

B

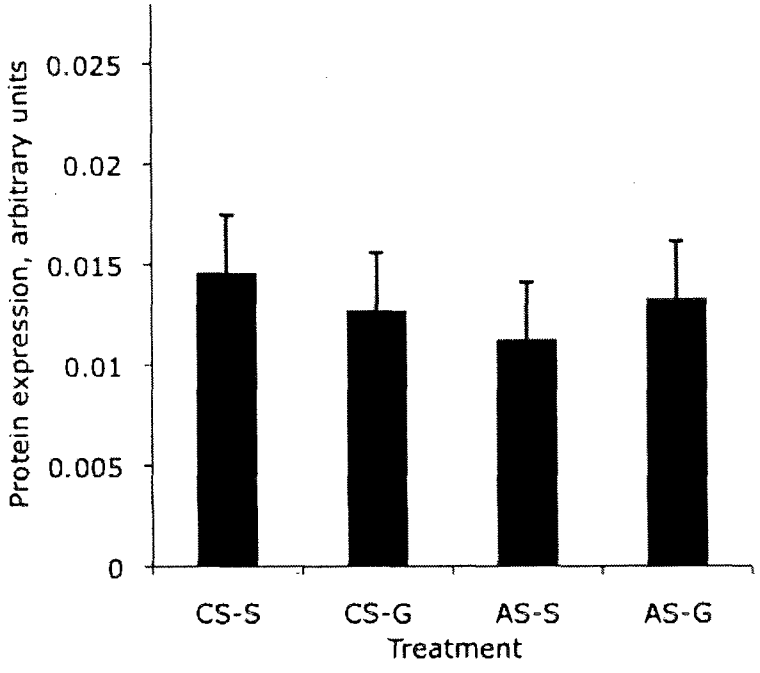
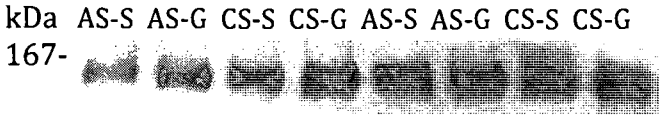


Figure 5.5. Immunoblot (A) and densitometric comparison (B) of ubiquitin protein expression ($P > 0.1$) in liver tissue collected from lambs receiving either a control diet (CS) or acidosis diet (AS), and with saline (-S) or glutamine (-G) infusion. Ubiquitin densitometric calculations were normalized to fast-green stained lane density within each animal for each sample. Depicted as least square means ± SEM.

A



B

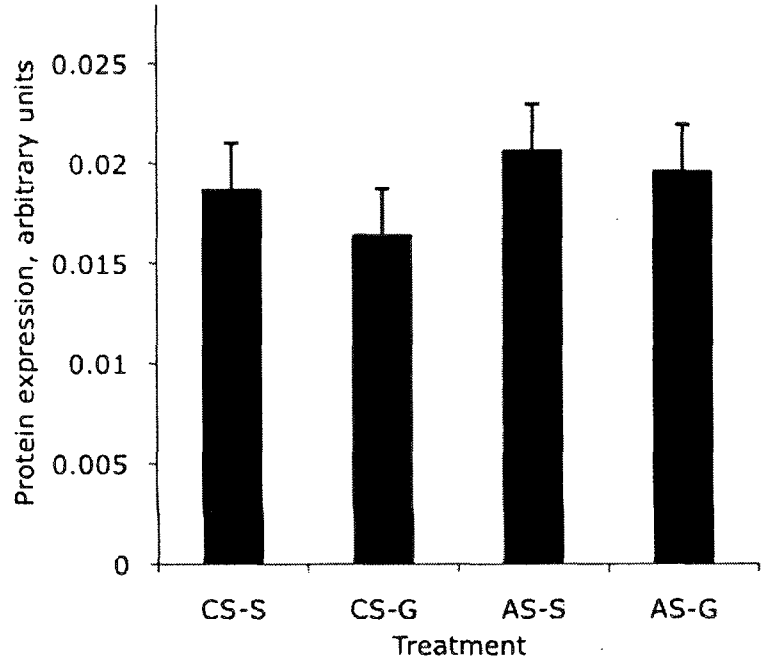
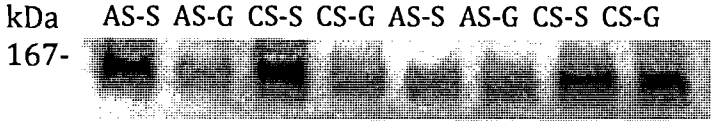


Figure 5.6. Immunoblot (A) and densitometric comparison (B) of ubiquitin protein expression ($P > 0.1$) in muscle tissue collected from lambs receiving either a control diet (CS) or acidosis diet (AS), and with saline (-S) or glutamine (-G) infusion. Ubiquitin densitometric calculations were normalized to fast-green stained lane density within each animal for each sample. Depicted as least square means ± SEM.

A



B

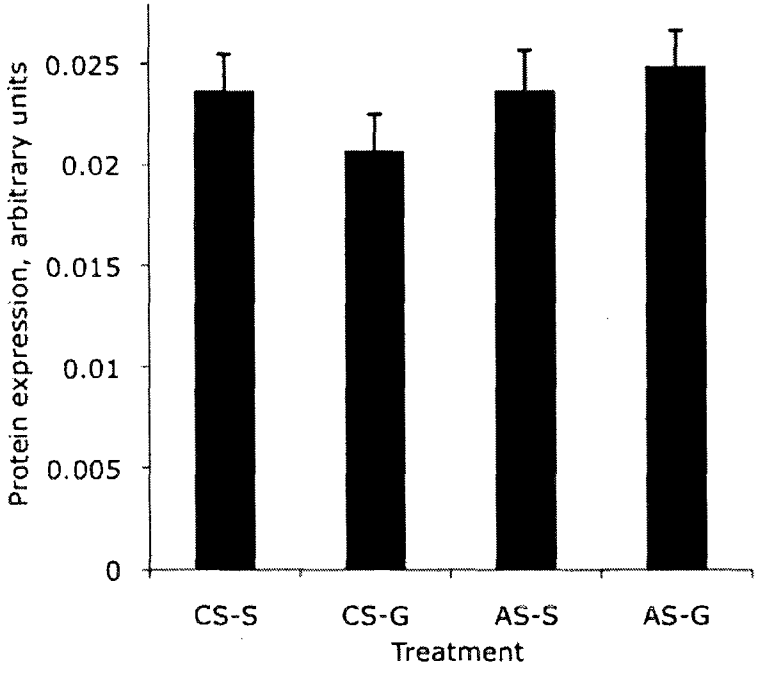


Figure 5.7. Relative mRNA expression of caspase-3 ($P > 0.1$), m-calpain ($P > 0.1$), cathepsin L ($P > 0.1$), and cathepsin B ($P > 0.1$) in kidney tissue collected from lambs offered either control diet or acidosis diet (supplemented with HCl-treated canola meal), and with saline or L-glutamine infusion. All genes were calibrated separately against β-actin and are relative to the pooled control diet-saline infused treatment group. Relative mRNA expression of different gene types cannot be compared within tissue type, as each gene was calibrated separately; genes are represented within tissue only for diagrammatical purposes. Depicted as least square means ± SEM.

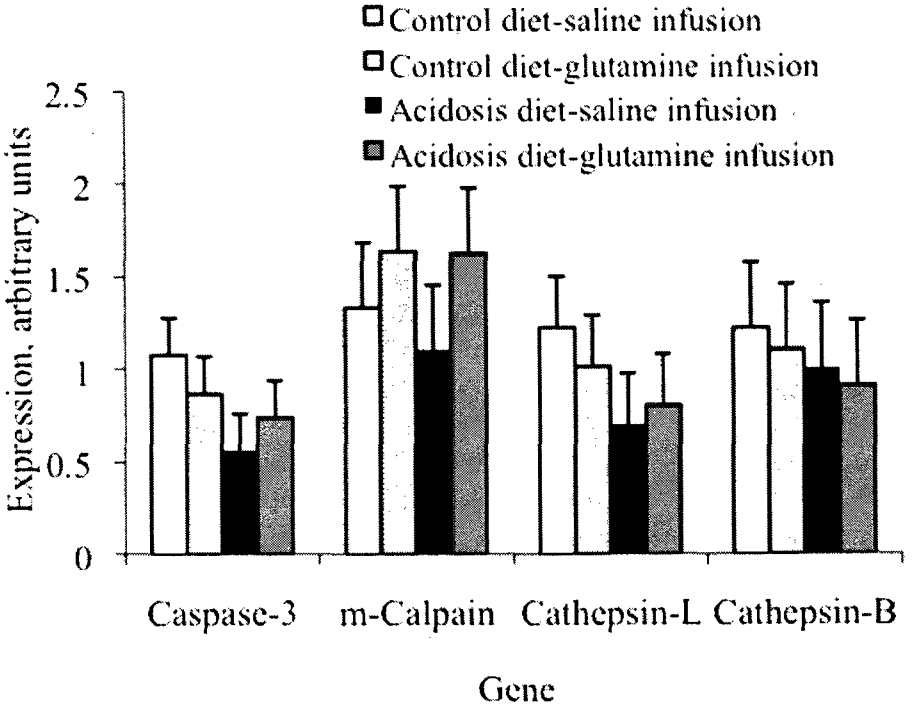


Figure 5.8. Relative mRNA expression of caspase-3 ($P > 0.1$), m-calpain (P infusion = 0.01), cathepsin L ($P > 0.1$), and cathepsin B ($P > 0.1$) in liver tissue collected from lambs offered either control diet or acidosis diet (supplemented with HCl-treated canola meal), and with saline or L-glutamine infusion. All genes were calibrated separately against β-actin and are relative to the pooled control diet-saline infused treatment group. Relative mRNA expression of different gene types cannot be compared within tissue type, as each gene was calibrated separately; genes are represented within tissue only for diagrammatical purposes. Depicted as least square means ± SEM.

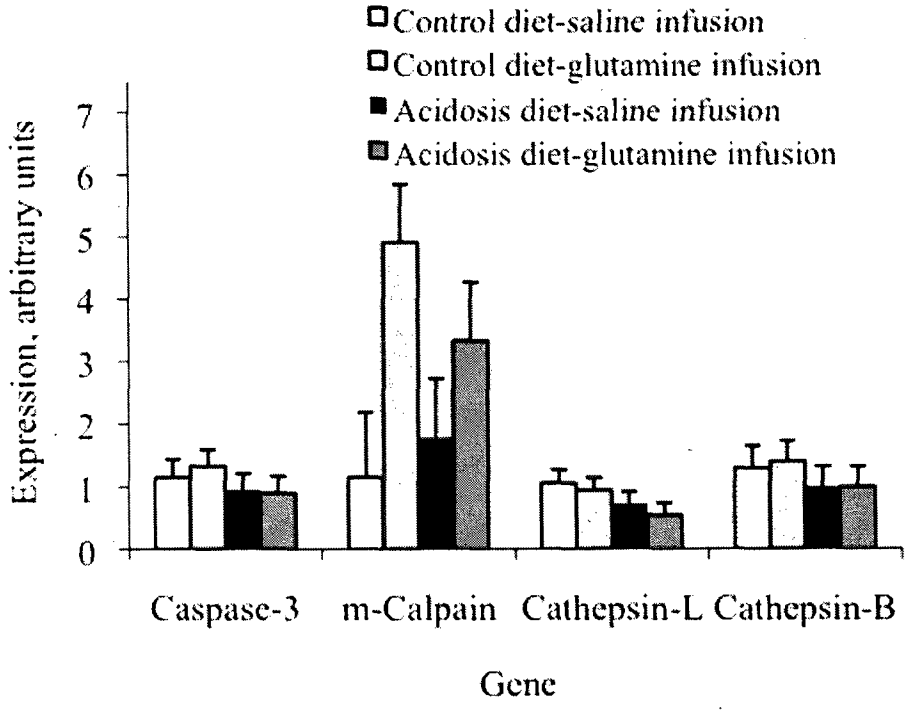
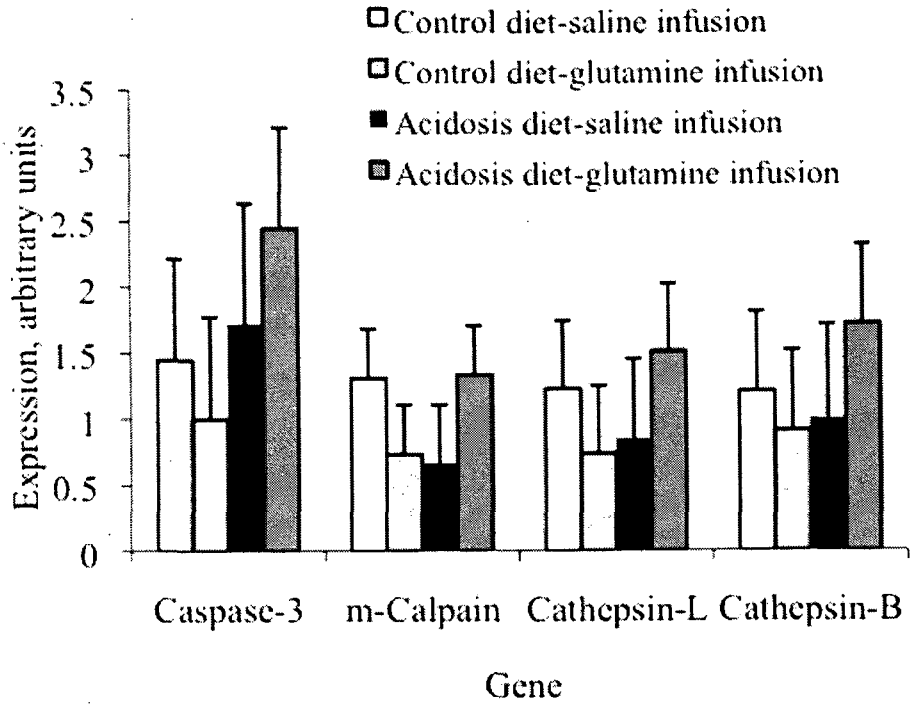


Figure 5.9. Relative mRNA expression of caspase-3 ($P > 0.1$), m-calpain ($P > 0.1$), cathepsin L ($P > 0.1$), and cathepsin B ($P > 0.1$) in muscle tissue collected from lambs offered either control diet or acidosis diet (supplemented with HCl-treated canola meal), and with saline or L-glutamine infusion. All genes were calibrated separately against β-actin and are relative to the pooled control diet-saline infused treatment group. Relative mRNA expression of different gene types cannot be compared within tissue type, as each gene was calibrated separately; genes are represented within tissue only for diagrammatical purposes. Depicted as least square means ± SEM.



CHAPTER 6

Plasma amino acids and mRNA expression of components related to protein turnover in lambs with nutritionally induced metabolic acidosis

6.1 ABSTRACT

Metabolic acidosis commonly occurs in ruminants fed a readily fermentable diet. In monogastrics, the ubiquitin-mediated proteolytic pathway (UPP) is upregulated in many disease states, including metabolic acidosis, to provide amino acids. In the current study, aspects of amino acid and protein turnover were examined in lambs with nutritionally induced metabolic acidosis to examine whether the same response occurs in ruminants as in monogastrics. Lambs (n = 30) were offered a base diet of dehydrated alfalfa pellets and grain mixture, and lambs were randomly assigned to one of three treatment groups, receiving a topdress of either 1) dried distiller's grain (DDG) receiving a maximum anion load of +5 mEq/100 g DM (Control treatment), 2) DDG + ammonium chloride (NH_4Cl) treated DDG to a maximum anionic load of -320 mEq/100 g DM (Moderate treatment), or 3) DDG + NH_4Cl receiving a maximum anionic load of -420 mEq/100 g DM (High treatment). All lambs were fed the control diet for a 9 d baseline period, then increasing amounts of DDG treated NH_4Cl by 25% every 3 d. Lambs were slaughtered on d 22 and kidney, liver, extensor digitorum longus muscle, and soleus muscle samples were collected for examination of mRNA expression. Blood gas, blood acid-base and urine pH measurements were made on d 9 and d 21, and plasma amino acid concentrations were

determined at four timepoints on d 21. Blood pH, bicarbonate, base excess of blood, base excess of extracellular fluid, total CO_2 and urinary pH were all significantly lower as a result of linear treatment effect ($P < 0.0001$). Plasma concentrations of glutamine ($P = 0.05$), glycine ($P = 0.02$), histidine ($P = 0.0002$), arginine ($P < 0.0001$) and asparagine ($P = 0.06$) increased linearly due to acidosis treatment. Renal glutamate dehydrogenase mRNA expression ($P = 0.02$) and hepatic glutamine synthetase mRNA expression ($P = 0.02$) were linearly increased due to acidosis. Soleus muscle caspase-3 mRNA expression ($P = 0.01$) was linearly decreased by acidosis. In addition, no regulation of caspase-3 was observed in extensor digitorum longus (EDL) muscle, and no treatment effect was observed in mRNA expression of UPP components in either soleus or EDL muscle. These results herein suggest that UPP mediated proteolysis to provide additional glutamine does not occur in lambs with metabolic acidosis. In addition, these results also suggest slow-twitch muscle regulation of caspase-3, potentiating a role for muscle specific roles in proteolysis.

6.2 INTRODUCTION

Cleavage of proteins by the 26S proteasome has received increasing attention, as it is the only ATP-dependent proteolytic pathway and appears to play a pivotal role in proteolysis under disease states in monogastrics (Lecker et al., 2006). Induction of the ubiquitin-mediated proteolytic pathway (UPP) as a result of metabolic acidosis is a compensatory adaptation observed in monogastrics to supplement diminishing glutamine pools for excess H^+ buffering and inter-organ nitrogen (N) transport. However, whether

increased proteolysis to provide additional glutamine/glutamate buffering occurs in ruminants is refutable, as previous investigation of milder acidosis have elicited no response in mRNA or protein expression of UPP components in growing sheep (Greenwood et al., 2009).

Ruminants have demonstrated the ability to repartition excess ammonia N. Conditions of mild or moderate acidosis require little change in hepatic glutaminase or glutamine synthetase activity, with adequate glutamine shuttling of excess N and H^+ to the kidneys (Xue et al., 2009). However, it remains unknown if there are limitations to this ability, or if additional glutamine synthesis is required during a more severe metabolic acidosis. In addition, other pathways involved in metabolic regulation, such as protein synthesis, could also create an amino acid imbalance and explain the lack of proteolytic shift in skeletal muscle from growing ruminants. A decline in the rate of protein synthesis could decrease the use of amino acids for protein formation and instead increase availability of amino acids for buffering activity, such as in monogastrics (Caso et al., 2004). Involvement of factors typically involved in apoptosis could also aid in restoration of homeostasis, for example cleavage of actomyosin must first be accomplished via caspase-3 (Du et al., 2004). Though we have previously examined mixed muscle fiber caspase-3 regulation from a milder form of metabolic acidosis (Greenwood et al., 2009), other research has demonstrated differential regulation of proteolysis in red versus white muscle fiber types (Hobbler et al., 1999).

The objectives of the current study were to examine plasma amino acid profiles of metabolically acidotic sheep, and determine whether metabolic acidosis regulates

components of glutamine/glutamate recycling, ubiquitin-mediated proteolysis or protein synthesis at the mRNA level.

6.3 MATERIALS AND METHODS

All animal procedures were done with the approval of the University of Guelph Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care. Thirty Rideau-Arcott whethers (42.8 ± 4.1 kg BW) were maintained at the University of Guelph Ponsonby General Animal Facility in individual pens. Each lamb was fed individually and had *ad libitum* access to water. Lambs were weighed at the beginning of the baseline period. Lambs were blocked by BW and then randomly assigned to treatments within block. Lambs within block were fed dietary DM at 2.5% of the average BW within block.

6.3.1 Diet

Lambs were maintained on a diet consisting of 57.7% (DM basis) dehydrated alfalfa pellets and 27.7% (DM basis) grain pellets (40% wheat, 40% barley, 20% corn), and topdressed with dried corn distillers grain (DDG; 14.6% DM basis) for 9 d as a baseline period prior to commencement of experimental treatments (total dietary anionic load of + 5 mEq/kg). For the duration of the study, one half of the daily alfalfa allotment was offered to lambs at 0900 h and 1700 h. Lambs were offered one third of their daily DDG and one third of their grain allotment (mixed with 10 mL molasses used as carrier for DDG) at 0700 h, 1100 h, and 1300 h. Beginning on d 10, ammonium chloride (NH_4Cl) treated DDG was substituted for part of the DDG portion of the diet of 20 lambs, the

remaining 10 on the original diet to serve as a control group. Amount of substitution provided in the treatment diets was increased by 25 % on d 10, 13, 16 and 19 of treatment, to provide a maximum anionic load of either 1) -32.0 mEq/100 g DM (moderate treatment; n=10 lambs), or 2) -41.7 mEq/100 g DM (high treatment; n=10 lambs). Chemical compositions and ingredients of diets are listed in Table 6.1. Lambs were maintained on the maximum anionic load within treatment for 3 d (until d 21), and were slaughtered on d 22 within 4 h of receiving their first allotment of DDG, NH_4Cl treated DDG, and grain mixture. Kidney (cross sections of cortex and medulla), liver (sections of centre lobe), extensor digitorum longus (EDL) muscle and soleus muscle tissue samples were collected within 10 min of slaughter, snap frozen in liquid nitrogen, and subsequently stored at -70°C until further analysis.

6.3.2 Blood and Urine Sampling

Blood samples were collected beginning at 1400 h on the last day of the baseline period (d 9), and at 0530 h, 1000 h, 1400 h and 1900 h on the last day prior to slaughter (d 21). Samples were collected by jugular venipuncture into 6 mL Sodium Heparin coated Vacutainers® (Becton Dickinson, Franklin Lakes, NJ) at all time points, and a second blood sample was collected in 6 mL EDTA coated Vacutainers® (Becton Dickinson) at 1400 h. A subsample of each heparinized blood sample collected at 1400 h was used for blood gas and acid-base determination. All other blood samples were placed on ice until centrifuged at 3,000 *x g* for 10 min for plasma collection. Plasma was separated and frozen at -70°C until analysis.

Urine samples were collected as previously described (Odongo et al., 2006) for urine pH determination immediately after blood samples were collected at the 1400 h timepoint on both the last day of baseline period (d 9) and the last day before slaughter (d 21).

6.3.3 Blood Gas and Acid-Base Concentration

Blood pH, partial pressure of CO_2 (pCO_2), partial pressure of O_2 (pO_2), hematocrit, sodium (Na^+), potassium (K^+), intracellular calcium (iCa), bicarbonate (HCO_3^-), total CO_2 (TCO_2), base excess of blood (BEb) and extracellular fluid (BEecf), O_2 Saturation (O_2Sat) and total hemoglobin (tHb) were determined immediately using an IRMA TruPoint blood analyzer (Trupoint Blood Gas Analysis System, ITC, Edison, NJ). Plasma chloride analysis was performed by the University of Guelph Animal Health Laboratory (University of Guelph, Guelph, Canada) using a Hitachi 911 Analyzer.

6.3.4 Plasma Amino Acid Concentration

Plasma amino acid concentrations were determined in samples collected beginning at 0530 h, 1000 h, 1400 h and 1900 h on the last day prior to slaughter (d 21). Plasma amino acid concentrations were measured at four points throughout the day in order to identify if any amino acid concentrations were transiently altered due to anionic load. Sample concentrations were determined in duplicate using Ultra-Performance Liquid Chromatography (AccQ•Tag_{ultra} UPLC, Waters Inc., Mississauga, Canada) as previously described (Boogers et al., 2008). Briefly, heparinized plasma samples (100 μL of plasma) were mixed with 20 μL of solution containing the internal standard (257 μM; L-2-aminoadipic acid, Sigma, Oakville, Canada) and tricarboxylic acid (to decolor plasma) and mixed thoroughly prior to centrifugation at 12,000 *x g* for 5 min. In a total recovery vial, 30 μL of the supernatant was added to 80 μL of borate buffer and 20 μL of reaction

liquid (Reagent kit, Waters Inc., Mississauga, Canada). The solutions were then vortexed and incubated at 50°C for 10 min. The solution was again mixed prior to loading into sample racks. Standards were run at four points throughout sample runs from each time point to ensure accurate peak labeling, and all samples were run in duplicate. Concentrations of amino acids were normalized using area under the curve of internal standard of known concentration.

6.3.5 Plasma Glucose and Blood Urea Nitrogen Concentration

Plasma samples spun from blood collected at 1400 h on d1 and d12 in EDTA coated vacutainers were analyzed for glucose (Sigma Kit #GAGO-20, Sigma, Oakville, Canada) and blood urea nitrogen (Teco Kit # B549-50, Teco Diagnostics, Anaheim, CA).

6.3.6 RNA Isolation, cDNA Synthesis and Comparative Real-time PCR

Total RNA was isolated using the TRIzol method (Invitrogen, Burlington, Canada) as previously described (Greenwood et al., 2008) using approximately 400 mg of frozen tissue. Total RNA was quantified (NanoDrop® ND-1000, Thermo Scientific). A DNase treatment (Invitrogen) was applied to 500 ng total RNA prior to reverse transcription using Superscript II, performed as directed by the manufacturer (Invitrogen). All cDNA samples were frozen at -20°C until Real-time PCR was performed. Hepatic and renal tissue was examined for mRNA expression of enzymes relevant to glutamate/glutamine recycling (glutamine synthetase, glutaminase, glutamate dehydrogenase). Extensor digitorum longus and soleus muscle samples were analyzed for mRNA expression of components of the 26S proteasome (19S component subunit 19S11, and 20S component subunits C5 and C8), protein synthesis (P70S6K), and components involved in cross-linkage of these pathways (Caspase-3, PI3K). Glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) was used as the housekeeping gene, and was determined to be unaffected by treatment for all tissues ($P > 0.05$). Primer sequences and tissue types in which they were analyzed are listed in Table 6.2. Control animal Ct values were pooled within gene and used as the control values for corresponding targets according to Pfaffl (2001).

Real-time PCR samples were prepared using 10 μL Power SYBR green (Applied Biosystems, Foster City, CA), 0.2 μL each of 10 μM forward and reverse primer, 4.4 μL of water and 5 μL of cDNA. Standard curves using mRNA dilutions of 1:10, 1:20, 1:40, 1:80 were run on each plate, and efficiency ($E = 10^{-1/slope}$) was determined per plate for calculation of relative mRNA expression. Real-time PCR and dissociation curve was performed in triplicate using a Prism 7000 Real-time PCR Machine (Applied Biosystems).

6.3.7 Statistical Analysis

All data were analyzed using PROC MIXED of SAS (SAS Inst. Inc., Cary, NC). Blood gas, blood acid-base, urinary pH, and mRNA expression in tissues were analyzed using fixed effects of block, treatment and block x treatment interaction. Block was insignificant and removed from the model. Baseline values (d 9) were used as covariates for blood gas, blood acid-base and urine analyses. Plasma amino acid concentrations were analyzed using treatment, time, treatment x time and block as fixed effects. Block was insignificant and removed from the model. Pairwise comparisons were conducted using orthogonal contrasts for all parameters. Weighted coefficients for orthogonal contrasts were determined using PROC IML and calculated based on the maximum

anionic load offered to each treatment group. All data are represented as mean ± SEM and declared significant at $P < 0.05$.

6.4 RESULTS

6.4.1 Verification of Successful Induction of Metabolic Acidosis

In the context of blood gas, blood acid-base and urinary pH, metabolic acidosis was successfully induced in a linear manner as a result of anionic load (Table 6.3). The lack of response of pCO_2 ($P = 0.6$) indicates a metabolic rather than respiratory response (Kellum, 2000). Metabolic acidosis is further characterized by the significant decline in blood pH ($P < 0.0001$), urinary pH ($P < 0.0001$), blood bicarbonate ($P < 0.0001$) and blood TCO_2 ($P < 0.0001$), all parameters previously validated as indicators of acidosis (Constable, 2000; Kellum, 2000; Fidkowski and Helstrom, 2009). Calculation of base excess, both blood ($P < 0.0001$) and extra cellular fluid ($P < 0.0001$), also demark a significant linear induction of metabolic acidosis dependent on dietary anionic load.

6.4.2 Glutamate/Glutamine Response to Metabolic Acidosis

The current study observed a significant linear increase in plasma glutamine ($P = 0.05$; Fig. 6.1) in response to dietary anionic load, with no change in plasma glutamate ($P = 0.16$; Fig. 6.2). A significant linear increase in hepatic glutamine synthetase mRNA expression ($P = 0.02$; Fig. 6.3) also occurred as a result of anionic load. A significant linear increase in renal glutamate dehydrogenase mRNA expression ($P = 0.02$; Fig. 6.4) due to treatment also occurred. However, no observed regulation of renal ($P = 0.99$) or hepatic ($P = 0.88$) glutaminase mRNA expression occurred as a result of treatment.

Relative mRNA expression of alanine aminotransferase in EDL muscle ($P = 0.13$; Fig. 6.5) and soleus muscle (P = 0.78) were not different across treatments.

6.4.3 Additional Plasma Responses

Significant time effects were observed for concentrations of all plasma amino acids measured ($P < 0.05$). A significant time x treatment effect was observed only for ornithine ($P = 0.03$), where treatment means converged at the 1400 h sample period. The plasma concentrations of glucogenic amino acids cysteine ($P = 0.06$; Fig. 6.6), threonine ($P = 0.06$; Fig. 6.7) and tryptophan ($P = 0.07$; Fig. 6.8) had quadratic decreases in plasma concentration due to acidosis while no change in plasma glucose was observed due to treatment ($P = 0.48$; Table 6.3). Plasma glycine ($P = 0.02$; Fig. 6.9), plasma asparagine (P = 0.06, Fig. 6.10), plasma histidine ($P = 0.0002$; Fig. 6.11), and plasma arginine ($P < 0.0001$; Fig. 6.12) increased linearly due to acidosis treatment. No change in plasma alanine, aspartate, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, tyrosine, or valine ($P > 0.1$) was observed as result of treatment (data not shown).

6.4.4 Urea Cycle

Though, as stated above, a significant increase in plasma arginine was observed, as well as a significant linear increase in blood urea nitrogen ($P = 0.004$; Table 6.3) no significant changes in plasma citrulline ($P = 0.13$), plasma ornithine ($P = 0.16$), plasma aspartate ($P = 0.43$) or hepatic CPS1 mRNA expression ($P = 0.59$) were observed as a result of treatment (data not shown).

6.4.5 mRNA Expression of Components Related to Protein Turnover in Slow- and Fast- Twitch Muscle

No significant difference was observed in any of the UPP components examined ($P > 0.51$; Fig. 6.13 and 6.14) as a result of anionic load in either muscle type. In addition, no regulation of mRNA expression of phosphatidyl inositol-3-kinase (PI3K) occurred ($P > 0.47$; Fig. 6.15), nor was any regulation of the mTOR factor P70S6K ($P > 0.31$; Fig. 6.16) observed in soleus or EDL muscle. Despite no regulation of PI3K at the mRNA level in either muscle type, a significant decrease in Caspase-3 mRNA expression was observed in soleus muscle ($P = 0.01$; Fig. 6.17) while no significant regulation was observed in EDL muscle ($P = 0.45$).

6.5 DISCUSSION

In the current study we have examined the physiological buffering capacity of sheep during severe metabolic acidosis in terms of plasma amino acid response, mRNA expression of enzymes related to metabolism of glutamine/glutamate and mRNA expression of components related to protein turnover in muscle. We have previously examined models of metabolic acidosis in growing sheep using dietary inclusion of HCl-treated canola meal (Las et al., 2007; Odongo et al., 2009). In all previous models, we have been unable to elicit proteolysis when creating a modest depression in blood pH (Greenwood et al., 2008; 2009). Here, a moderate anionic load (-32.0 mEq/100 g DM) was used to equal the maximum anionic load previously used in this laboratory (Odongo et al., 2009) and is comparable to other ruminant research (Heitmann and Bergman, 1978; Lobley et al., 1995), while a more severe anionic load (-41.7 mEq/100 g DM) was offered

to the high treatment group in an attempt to induce a compensatory response in terms of protein and amino acid metabolism.

We investigated the peripheral amino acid and protein response in order to gain understanding of the adaptation of excess H^+ and NH_4^+ buffering during a more severe metabolic acidosis in growing sheep. In terms of ammoniagenic amino acids, though glutamine is the primary source of renal ammonia for urinary excretion, previous research in dogs has estimated that approximately 5% of renal ammonia is provided by glycine (Pitts et al., 1965) and is used by the renal glycine cleavage enzyme complex (Lowry et al., 1985). In agreement with this research and previous ruminant research (Heitmann and Bergman, 1978; Lobley et al., 1995), an increase in plasma glycine was observed due to acidosis treatment in the current study.

Further characterization of the amino acid response demonstrates the biological differences between ruminants and monogastrics in terms of compensatory mechanisms to this metabolic state. Depressed plasma glutamine as a result of metabolic acidosis is well documented in monogastrics (Taylor and Curthoys, 2004), where hepatic glutamine synthetase is typically increased with a concurrent increase in renal glutaminase expression and activity to produce 2 NH_4^+ and 2 HCO_3^- for buffering (Newsholme et al., 2003). We have previously examined glutamine and glutamine-related enzyme response to a mild systemic acidosis in lambs, and have observed depressed renal glutamine synthetase expression (Xue et al., 2009) with little (Greenwood et al., 2008) or no (Odongo et al., 2009) change in plasma glutamine due to acidosis. However, the current study was able to induce a significant increase in plasma glutamine in response to dietary anionic load, typically suggestive of an acutely induced metabolic acidosis (Schröck and

Goldstein, 1981; Hughey et al., 1980). Upregulation of hepatic glutamine synthetase and renal glutamate dehydrogenase mRNA expression as a result of anionic load demonstrate both an increased need for glutamate derived production of glutamine for N transport and glutamate usage.

The numerically lower plasma glutamate observed in acidotic lambs further indicates a large shift in inter-organ N transport. Typically under conditions of a chronic metabolic acidosis in monogastrics, both renal glutaminase and glutamate dehydrogenase mRNA expression are upregulated, ultimately increasing NH_4^+ urinary excretion, increased bicarbonate production for release in the blood, and increased gluconeogenesis (Taylor and Curthoys, 2004). However, we were unable to detect any changes in hepatic or renal glutaminase expression, which is in agreement with our previous research (Xue et al., 2009). When the present work is put into context, our research does suggest perivenous hepatocyte sequestration of glutamate for glutamine production, with increased renal utilization of glutamate for α-ketoglutarate production, but not necessarily for gluconeogenesis. Similarly, Heitmann and Bergman (1978) observed no change in gluconeogenesis as a result of acidosis in sheep.

Examination of other amino acids and enzymes can also aid in better understanding our observation of increased glutamate demand and usage. The increased plasma histidine and plasma arginine concentrations demonstrate shifts in catabolic pathways involved in glutamate production, as both arginine and histidine can be used as precursors for glutamate formation (Cedrangolo et al., 1979; Cynober et al., 1995). While elevated arginine could also contribute to ureagenesis for the increased appearance of blood urea nitrogen, examination of amino acids and enzymes that participate in urea

cycle function further suggest that nutritional induction of metabolic acidosis using NH_4Cl treated DDG did not elicite any significant changes in urea cycle activity, despite arginine availability for participation in the urea cycle and potential activation of N-acetylglutamate synthase for N-acetylglutamate formation (Cynober et al., 1995). The lack of response in plasma citrulline, plasma ornithine, plasma aspartate or hepatic CPS1 mRNA expression are indicative of no changes in the urea cycle response to dietary anionic load.

Provision of amino acids for increased buffering in monogastrics is accomplished via several mechanisms, including increased ubiquitin-mediated proteolysis of skeletal muscle (Lecker et al., 1999). The current research examined mRNA expression of three components of the 26S proteasome, including the 19S cap, the α-20S C8 subunit, and the β-20S C5 subunit, to identify any participation of the UPP in contributing to the increasing pool of plasma glutamine. Previous identification of muscle type specific regulation of the UPP in monogastrics during sepsis has also suggested that fast-twitch muscle may be more readily sacrificed during disease mediated proteolysis (Hobbler et al., 1999). We examined this possibility in ruminant fast- and slow- twitch muscle, as we have previously used a mixed muscle for examination of proteolytic components (Greenwood et al., 2008; 2009). However, despite induction of a severe metabolic acidosis, no regulation of UPP components, protein synthesis component, or PI3K was detected. Again, this is contrary to monogastrics, where fractional synthesis rates are significantly lower in skeletal muscle of acidotic rats (Caso et al., 2004), and PI3K is a key regulator of both apoptotic and UPP mediated proteolysis (Lee et al., 2004).

However, our observation of significant downregulation of Caspase-3 mRNA expression in soleus muscle but not in EDL muscle is the first research to demonstrate differential caspase-3 regulation as a result of metabolic acidosis in ruminants. Caspase-3 is considered dual purpose in nature in regards to its involvement in proteolysis. Firstly, under catabolic states, caspase-3 first degrades actomyosin into fragments (Du et al., 2004). It is only after caspase-3 has created these fragments that the UPP can then further degrade actin and myosin into oligopeptides for amino acid recycling and antigen presentation. In the current study, the observed downregulation of caspase-3 suggests that slow-twitch muscle fiber is being sheltered from initial actomyosin degradation. Not only does this potentiate a muscle specific protective mechanism against proteolysis, but also could provide an explanation as to why no regulation in UPP components occurs. Rodent research has previously demonstrated unresponsiveness of the UPP to proteolytic stimuli if caspase-3 is first inhibited (Du et al., 2004). The second function of caspase-3 in proteolysis is directly due its involvement in apoptosis. The potential of caspase-3 regulation as a result of apoptosis could also play a role in response to acidosis. Previous research has demonstrated that insulin suppresses caspase-3 activity (Gao et al., 2008), and this would coincide with the observed glucogenic amino acid shifts in this study. Caspase-3 regulation also appears to be dependent on the severity of acidosis, as our previous examination of Caspase-3 in response to a moderate metabolic acidosis in sheep yielded no demonstrable change in mRNA expression (Greenwood et al., 2009).

In conclusion, we have demonstrated that acidotic growing sheep do not increase UPP proteolysis. In addition, there is no indication of a differentiated increased UPP-mediated proteolysis of slow- or fast- twitch muscle in response to a more severe

metabolic acidosis. Instead, Caspase-3 mRNA is differentially downregulated in slow-twitch muscle with no observable regulation in fast-twitch muscle, suggesting suppression of proteolysis in a muscle specific manner.

Table 6.1. Ingredients and chemical compositions of the control, moderate and high diets at their maximum anionic load. Level of ammonium chloride (NH_4Cl) treated dried distillers grain (DDG) was offered in increasing amounts in a stepwise pattern at 25% increase every 3 d until the listed anionic load was achieved.

	Control diet	**Moderate diet**	**High diet**
Ingredients		% DM	
Dehydrated alfalfa pellet, %	59.6	59.8	59.8
Grain pellet[1], %	28.0	28.1	28.1
Dried corn distiller's grain (DDG), %	12.5	6.9	5.4
NH_4Cl treated DDG, %	0	5.3	6.6
Chemical Composition		% DM	
DM, %	89.9	89.6	89.5
CP, % (N x 6.25)	17.3	17.8	17.9
ADF, %	23.6	23.5	23.5
NDF, %	34.1	34.2	34.2
DCAD (mEq/100g DM)[2]	5.2	-32.0	-41.7

[1]Grain pellet is composed of 40% wheat, 40% barley, 20% corn mixture

[2]Dietary cation: anion difference, DCAD; DCAD = (Na + K) – (Cl + S)

Table 6.2. Primer sequences used to determine Relative mRNA expression of genes of interest. Sheep tissues in which specific genes of interest were examined are listed.

Gene	Forward Primer	Reverse Primer	Tissues
19S11[1]	CCATCGTGAAGCGTGACATT	CTTGACCTGCACTGCCTCC	Muscles
Alanine Aminotransferase	AGGTCATCCGTGCCAACATC	TGCCATTACCTGTCGCAGG	Muscles
C5[2]	CACTGCCAATGCTCTCGC	CCCCTGGTTCTGCAATGG	Muscles
C8[2]	GAAGAAGGTTCCAACAAACGACTTT	ACGAGCGTCTGCCAACAAA	Muscles
Caspase-3	AGAAGTCTGACTGGAAAACCCAAAC	GTCTCAATACCACAGTCCAGTTCTG	Muscles
CPS1[3]	ACACTGGCTGCAGAATACCC	TTCTTGCCAAGCTGACGCAA	Liver
GAPDH[4]	CGGCACAGTCAAGGCAGAG	CTCGCTCCTGGAAGATGGTG	All
Glutamate Dehydrogenase	TTGCAACATGGAACAATCCTG	TCACAGTCAACCTCCAAGATGC	Kidney, Liver
Glutaminase	GCACAGACATGGTTGGTATATTGGA	CACACTAGCTGACTCACAAGTTACT	Kidney, Liver
Glutamine Synthetase	GGTCATGCCTGCACAGTGG	ATGATCGCCCATGTCGATTC	Kidney, Liver
P70S6K	TGAACTTGGCATGGAACATTG	TCTGGCCCTCTGTTCACACTAG	Muscles
Phosphoinositol-3-kinase	CCCATGCAGGACTGAGTAACAG	CGTGTACAAATTGCTCGGAGC	Muscles

[1]Isoform of the 19S component of the 26S proteasome

[2]Isoform of the 20S component of the 26S proteasome

[3]Carbamoyl-phosphate synthetase 1

[4]Glyceraldehyde-3-phosphate dehydrogenase

Table 6.3. Blood acid-base, blood gas, and urinary pH results of sheep (n=30) receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diets.

	Treatment						P value	
	Control	SE	Moderate	SE	High	SE	Linear	Quadratic
Blood pH	7.44[a]	0.02	7.35[b]	0.01	7.31[b]	0.01	<0.0001	0.76
Partial Pressure of CO_2, mmHg	45.43	1.76	47.91	1.73	46.49	1.79	0.51	0.48
Partial Pressure of O_2, mmHg	37.03	1.31	40.14	1.33	39.35	1.32	0.13	0.47
Hematocrit, %	27.76	0.69	27.10	0.68	25.36	0.70	0.05	0.18
Sodium, mM	148.98	0.62	151.00	0.65	151.12	0.64	0.0139	0.71
Potassium, mM	4.63	0.12	4.90	0.12	4.93	0.12	0.07	0.81
Intracellular Ca^3, mM	1.35[a]	0.01	1.40[b]	0.01	1.45[c]	0.01	0.0001	0.10
Bicarbonate, mM	30.34[a]	0.67	25.60[b]	0.68	23.34[b]	0.67	<0.0001	0.40
Total CO_2, mM	31.72[a]	0.69	27.09[b]	0.70	24.77[b]	0.70	<0.0001	0.37
Base Excess (Blood), mM	5.38[a]	0.68	.-0.66[b]	0.67	-3.22[c]	0.66	<0.0001	0.41
Base Excess (Extracellular Fluid), mM	6.23[a]	0.75	.-0.11[b]	0.75	-2.88[c]	0.74	<0.0001	0.40
O_2 Saturation, %	71.02	2.19	69.92	2.15	65.98	2.23	0.20	0.32
Total Hemoglobin, g/dL	9.44	0.23	9.20	0.23	8.63	0.24	0.05	0.20
Plasma Glucose, mM	4.03	0.21	3.71	0.20	4.01	0.21	0.66	0.27
Plasma Blood Urea Nitrogen, mM	7.69[a]	0.25	9.08[b]	0.24	8.55[b]	0.24	0.0043	0.032
Urine pH	7.90[a]	0.14	5.72[b]	0.13	5.40[b]	0.14	<0.0001	0.25
Chloride, mM	102.93[a]	1.76	109.23[a]	1.85	114.74[b]	1.82	0.0003	0.24

[a,b,c] Means within a row with different superscripts differ ($P < 0.05$)

Figure 6.1. Plasma glutamine concentrations (*P* linear = 0.05) of sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.

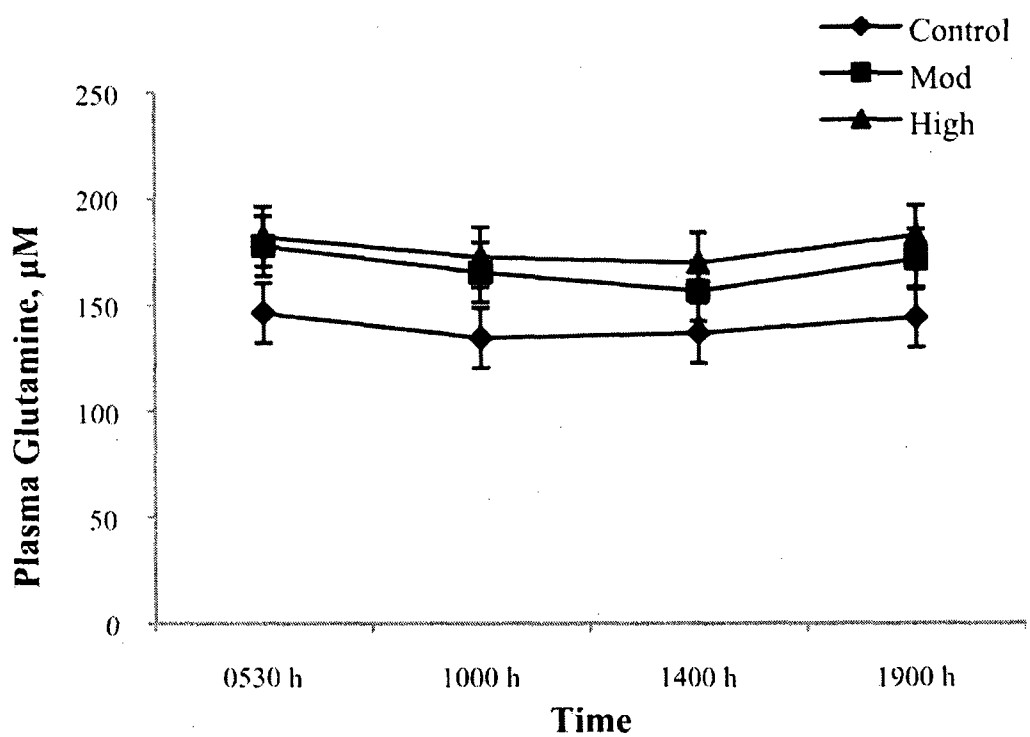


Figure 6.2. Plasma glutamate concentration (*P* linear = 0.16) of sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.

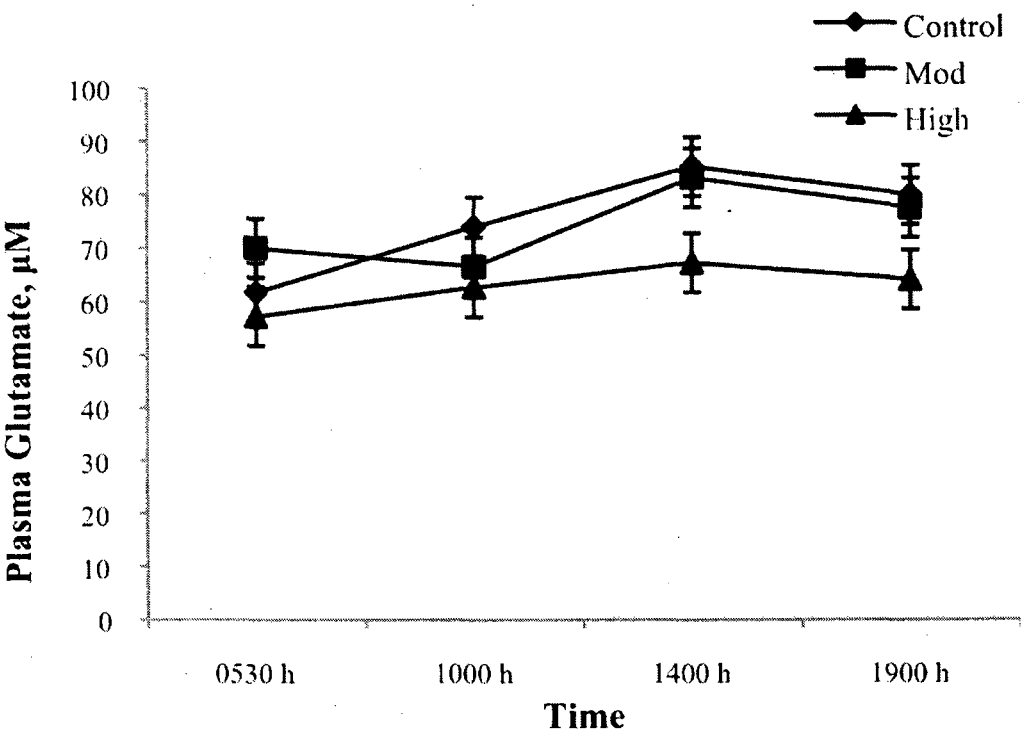


Figure 6.3. Hepatic mRNA expression of glutamine related enzymes (Hepatic glutamine synthetase *P* linear = 0.02) of sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.

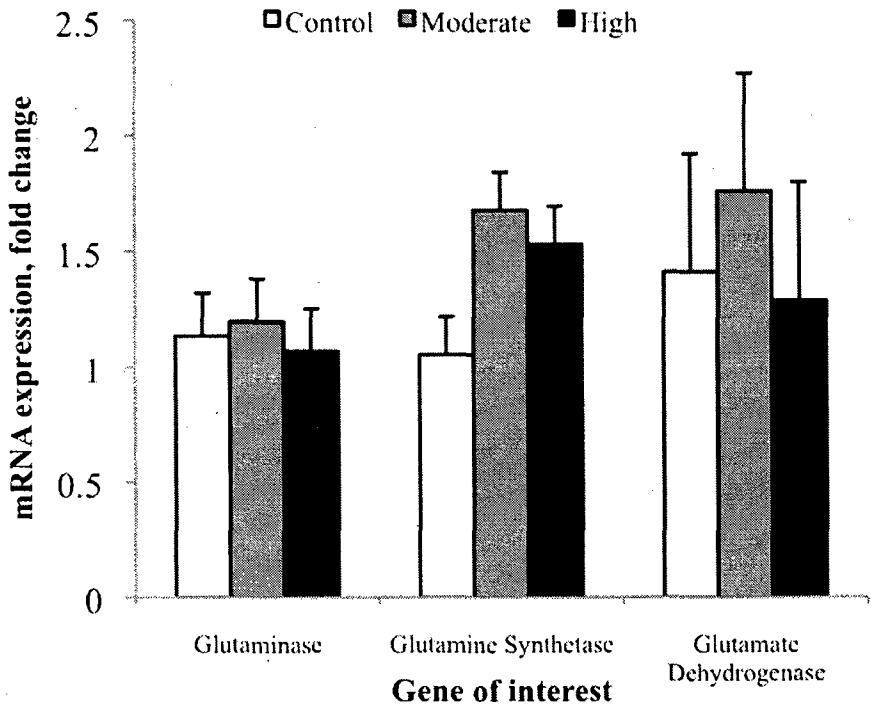


Figure 6.4. Renal mRNA expression of glutamine related enzymes (Renal glutamate dehydrogenase *P* linear = 0.02) of sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.

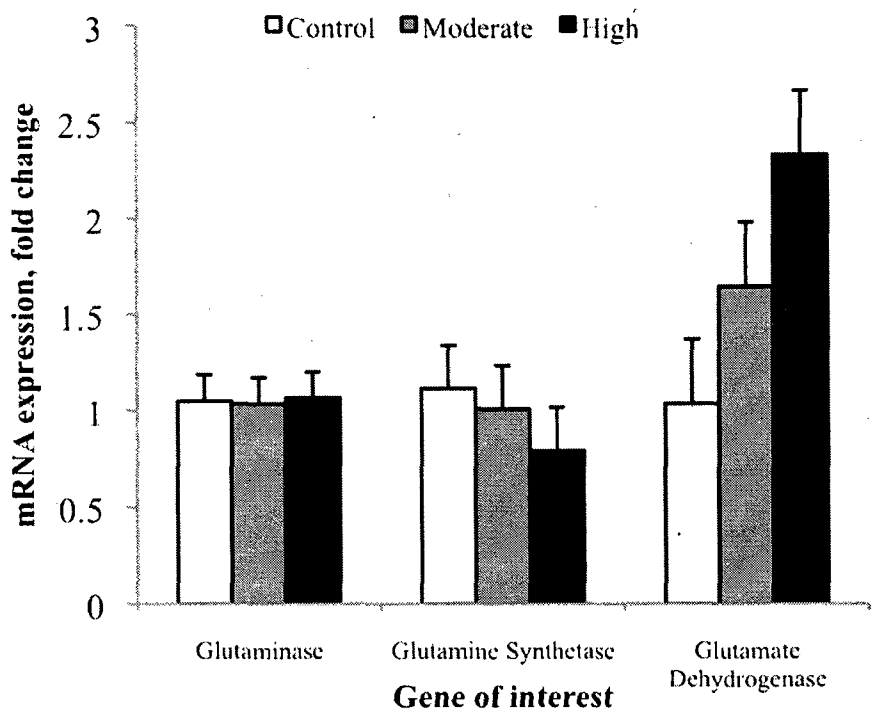


Figure 6.5. Skeletal muscle mRNA expression of alanine aminotransferase ($P > 0.05$) of sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.

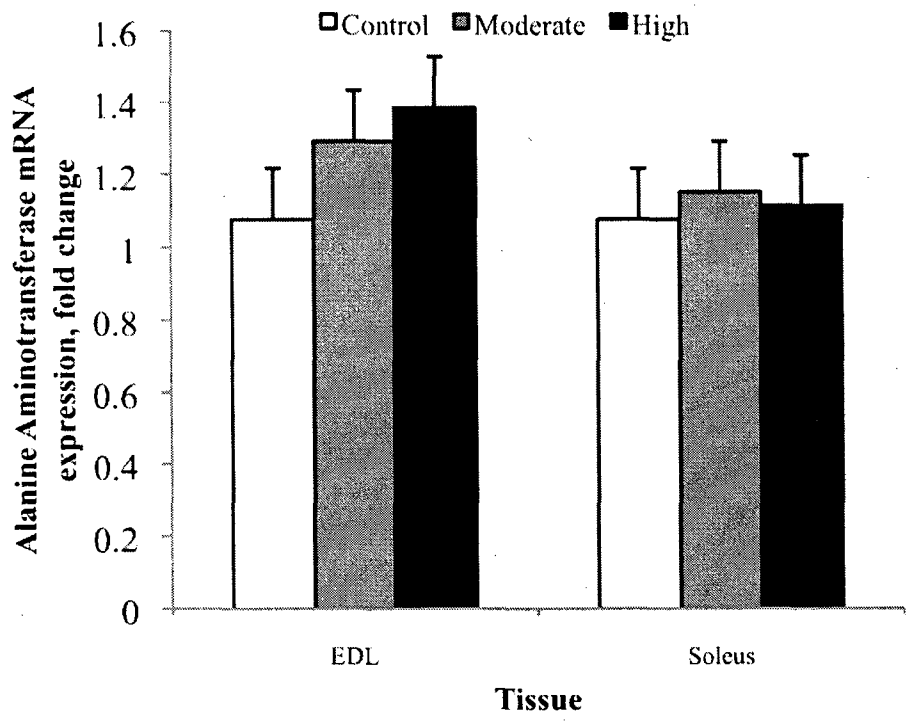


Figure 6.6. Plasma concentrations of cysteine (*P* quadratic = 0.06) of sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.

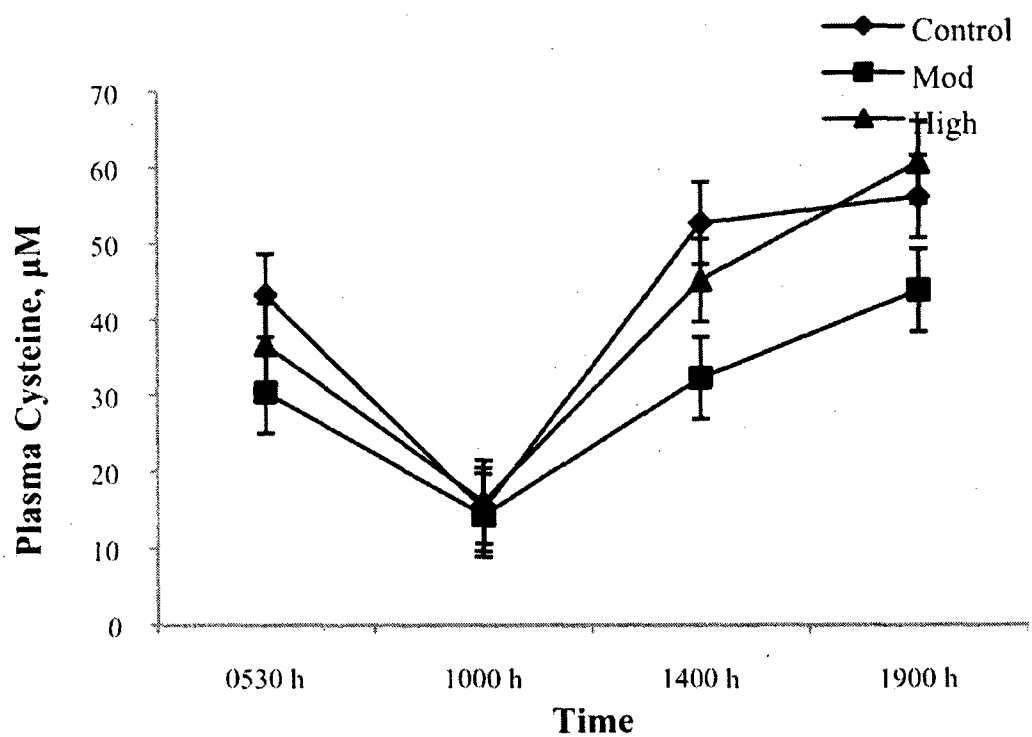


Figure 6.7. Plasma concentrations of threonine (*P* quadratic = 0.06) of sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.

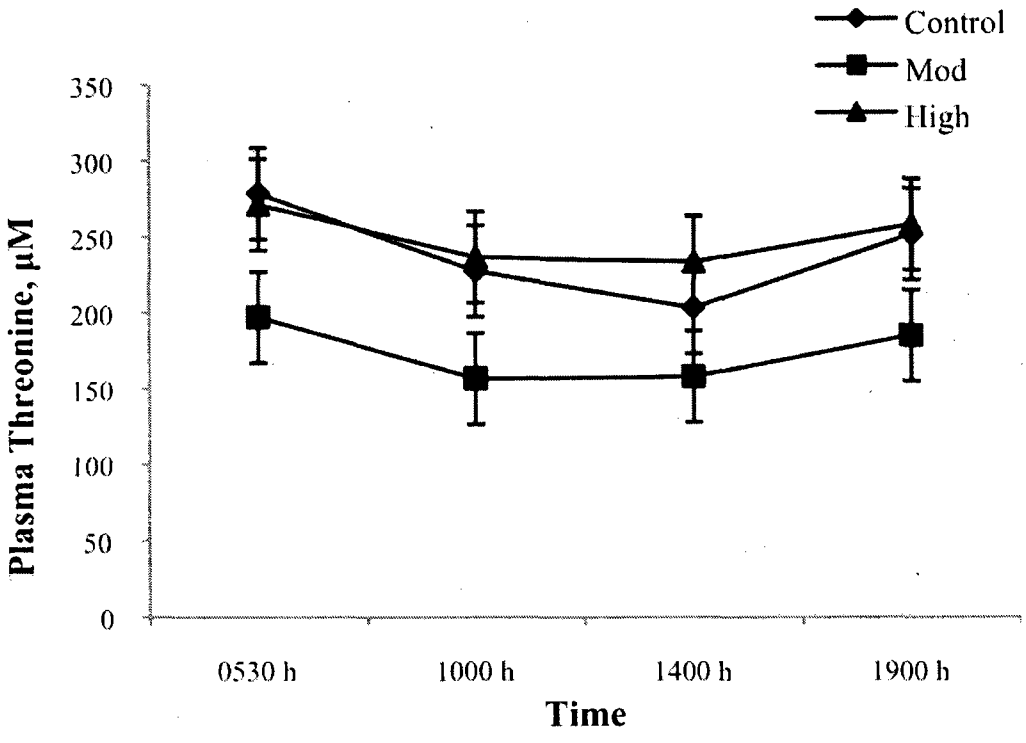


Figure 6.8. Plasma concentrations of tryptophan (*P* quadratic = 0.07) of sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.

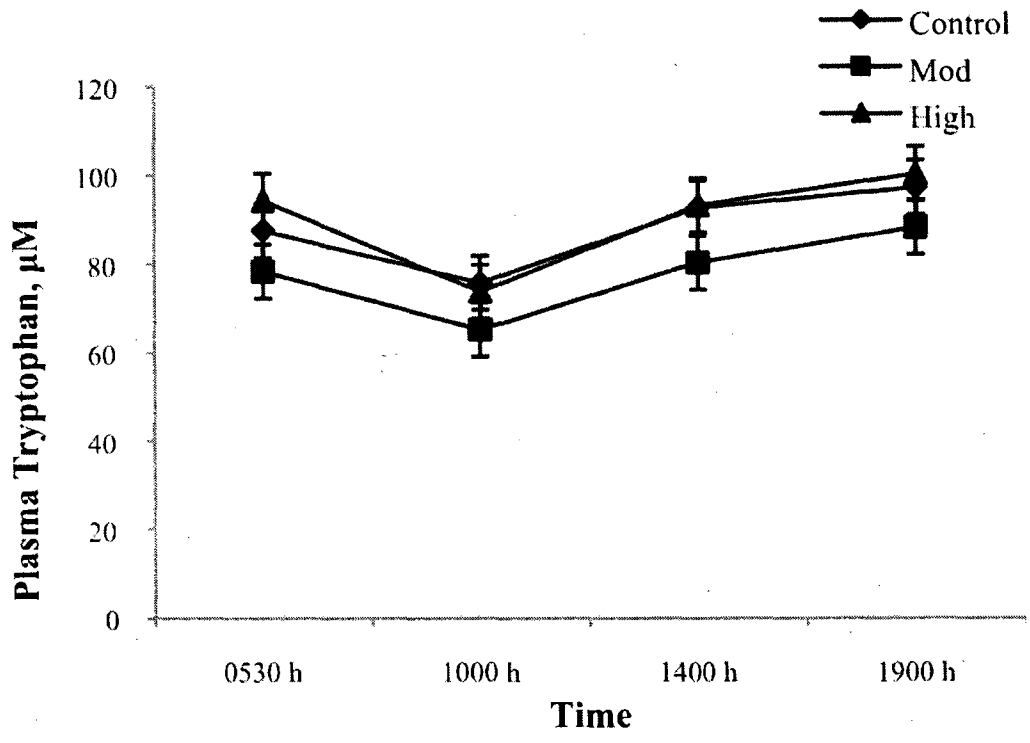


Figure 6.9. Plasma concentrations of glycine (*P* linear = 0.02) of sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.

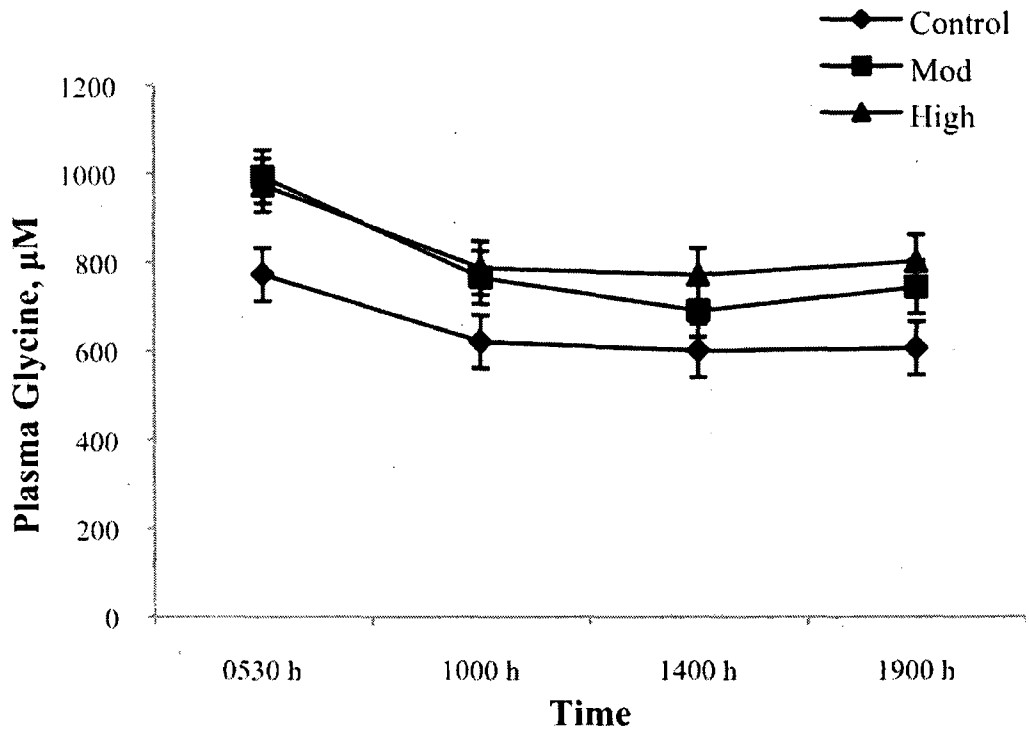


Figure 6.10. Plasma concentrations of asparagine (*P* linear = 0.06) of sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.

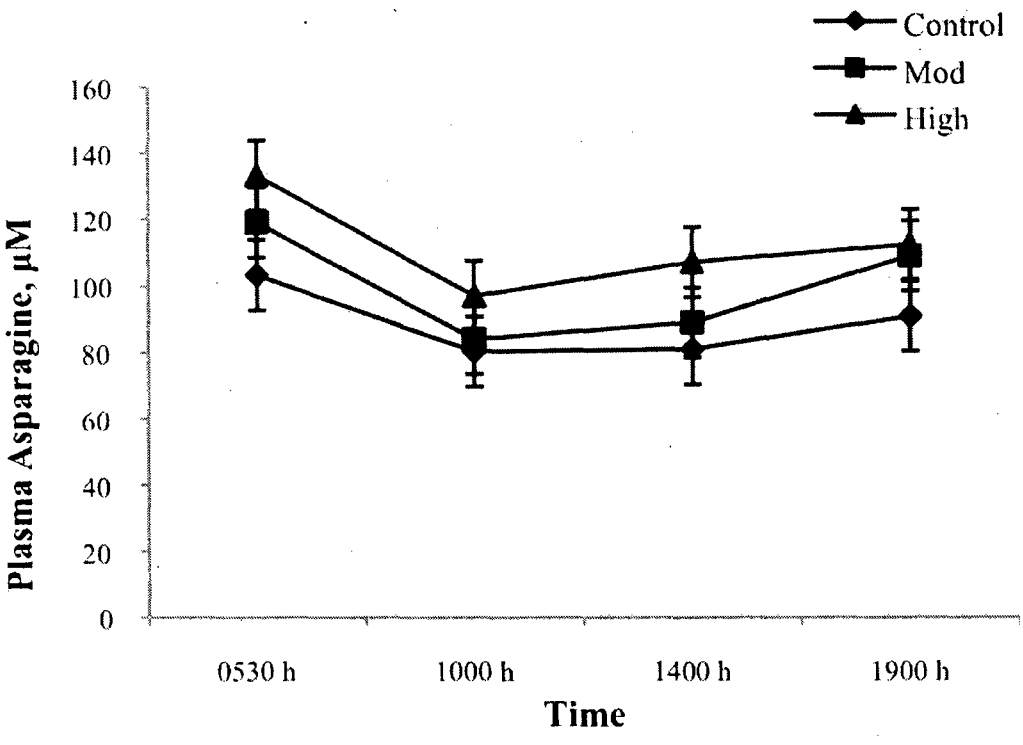


Figure 6.11. Plasma concentrations of histidine (*P* linear = 0.0002) of sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.

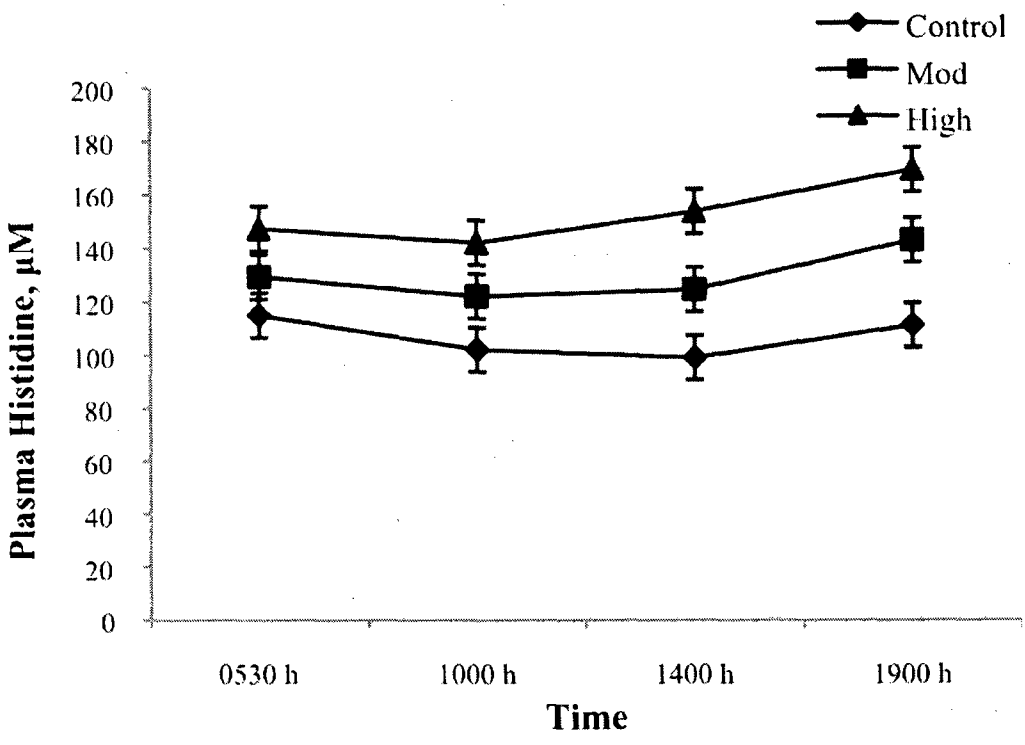


Figure 6.12. Plasma concentrations of arginine (*P* linear < 0.0001) of sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.

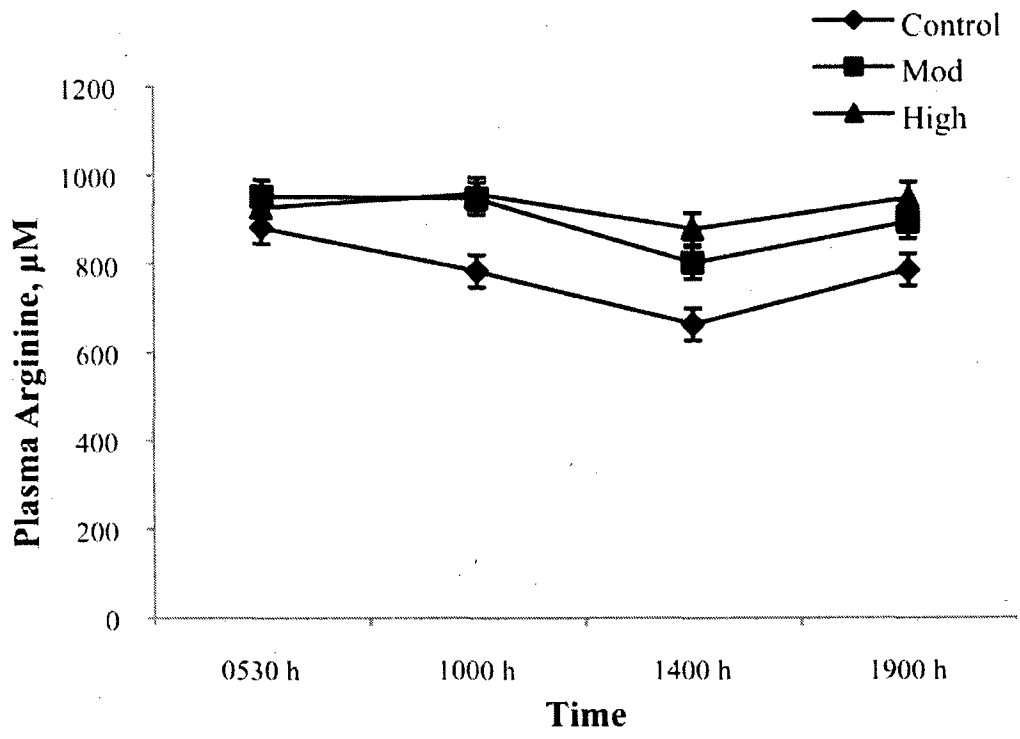


Figure 6.13. Relative mRNA expression of components of the 26S proteasome in extensor digitorum longus (EDL) muscle ($P > 0.05$) collected from sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.

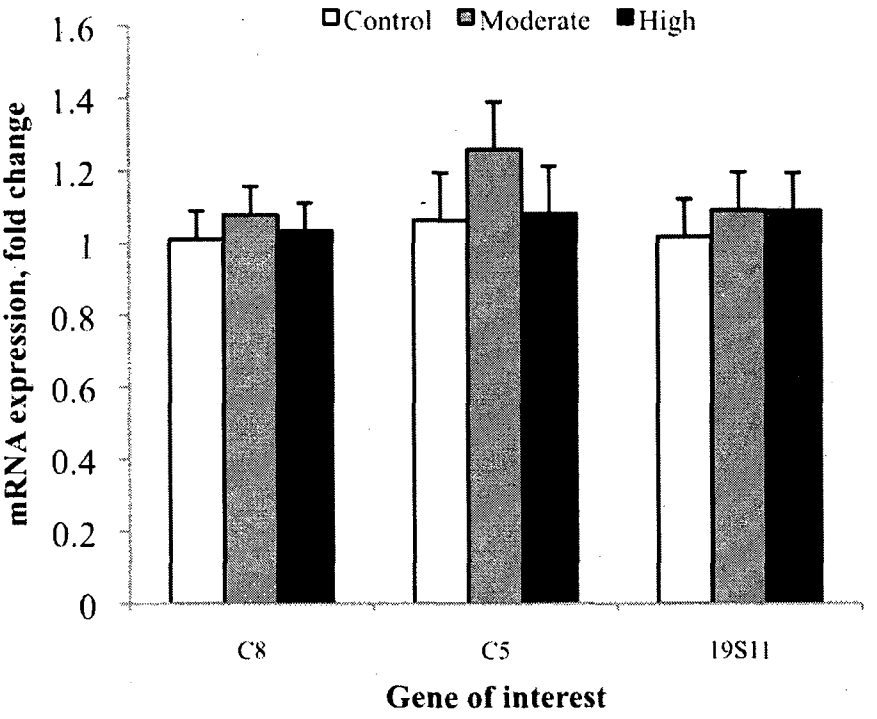


Figure 6.14. Relative mRNA expression of components of the 26S proteasome in soleus muscle ($P > 0.05$) collected from sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.

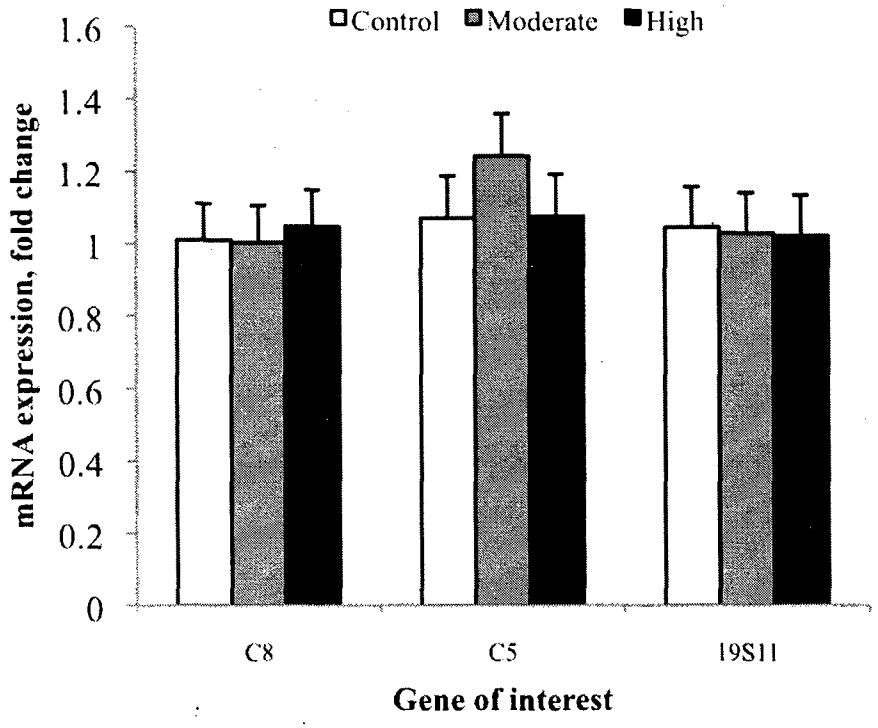


Figure 6.15. Relative mRNA expression of phosphatidyl-inositol-3-kinase in muscle ($P > 0.05$) collected from sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.

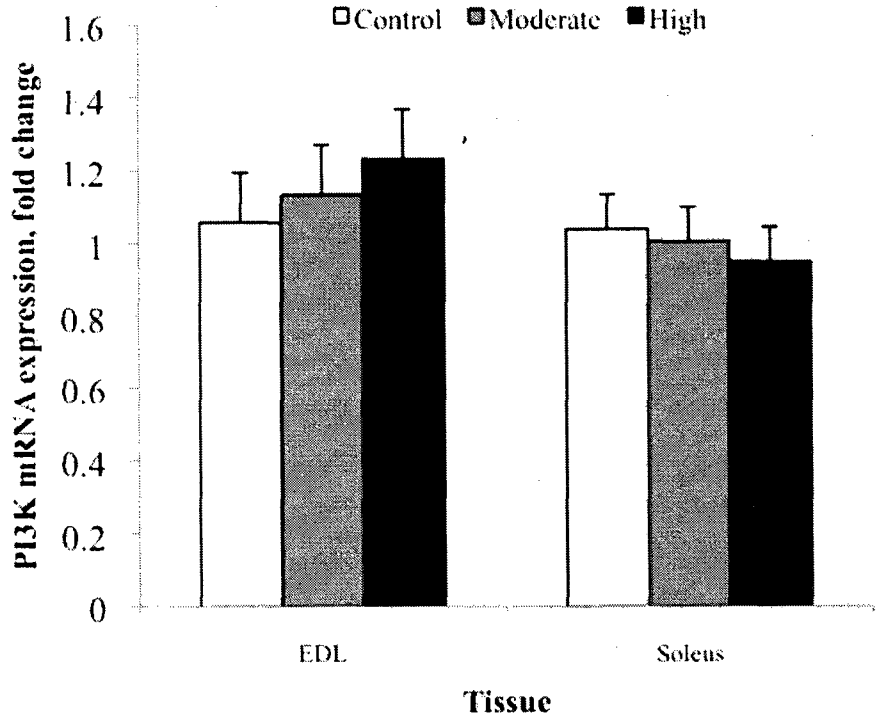


Figure 6.16. Relative mRNA expression of P70S6K in muscle ($P > 0.05$) collected from sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.

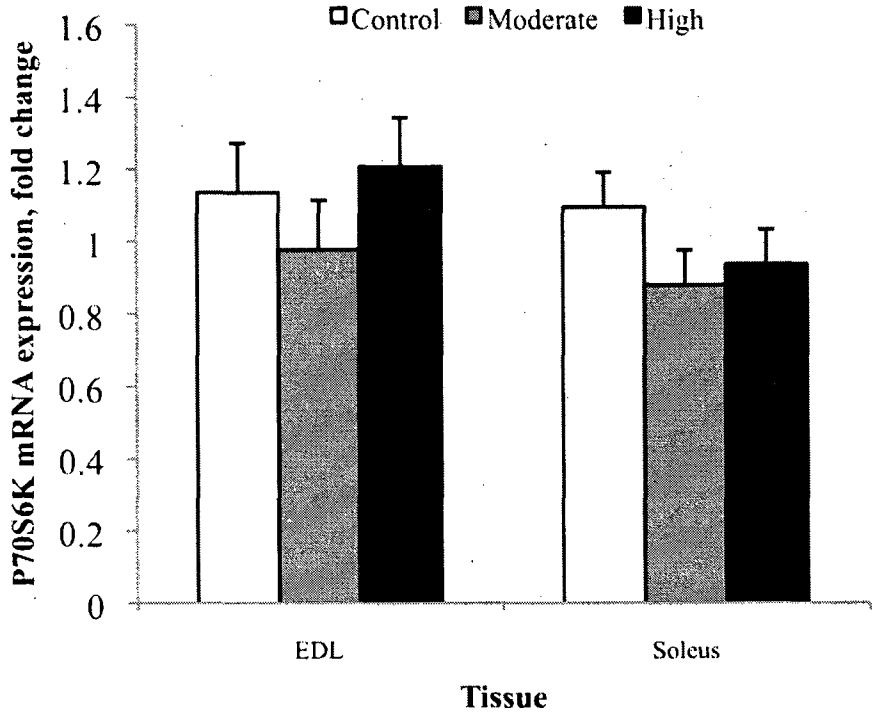
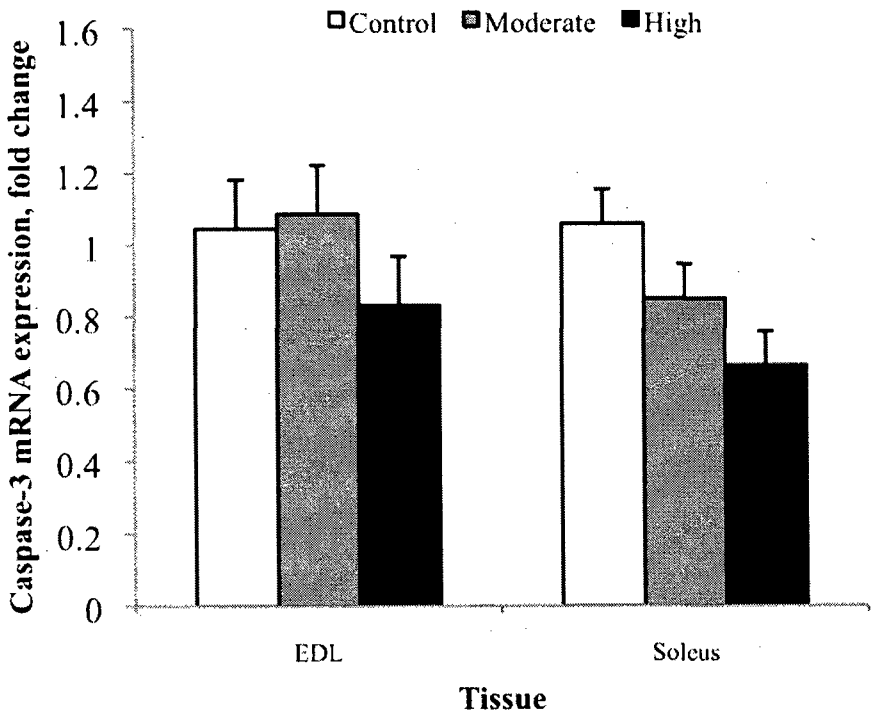


Figure 6.17. Relative mRNA expression of caspase-3 (Soleus muscle caspase-3 mRNA expression *P* linear = 0.01) mRNA expression in muscle collected from sheep receiving either control (+5 mEq/100g DM), moderate (-32.0 mEq/100g DM) or high (-41.7 mEq/100g DM) diet. Depicted as least square means ± SEM.



CHAPTER 7

General Discussion

The research presented in this thesis was performed with the focus of characterizing the effect of metabolic acidosis on protein turnover, with specific emphasis on the ubiquitin-mediated proteolytic pathway (UPP), in ruminants. In Chapter 3, the research indicated that lactation significantly regulates skeletal muscle mRNA expression of the UPP. Because of this interaction between lactation and the UPP, we needed to move to a non-lactating animal model to eliminate any potential interaction of lactation and isolate the effects of metabolic acidosis. The following chapters then outline research using non-lactating sheep as a model to characterize metabolic acidosis. Using this animal model, results demonstrated that a mild metabolic acidosis has no effect on mRNA expression of C8, E2, or ubiquitin, or ubiquitin protein expression (Chapter 4), that glutamine supplementation had no beneficial effects on UPP regulation in sheep with metabolic acidosis (Chapter 5), and that differential inhibition of caspase-3 in slow-twitch muscle could be prohibiting the UPP expression during metabolic acidosis in sheep (Chapter 6). With these results, the hypothesis that metabolic acidosis stimulates ubiquitin-mediated proteolysis in growing ruminants can be rejected.

Chapters 4, 5 and 6 demonstrate the remarkable buffering capacity of ruminants. Upregulation of the UPP did not occur under conditions of mild (Chapter 4), moderate (Chapter 5) or a more severe (Chapter 6) metabolic acidosis. Ultimately, the sheep were able to buffer lower blood pH by increasing hepatic glutamine synthetase expression (Chapter 6) and increasing renal expression of glutaminase (Xue et al., 2009) and glutamate dehydrogenase (Chapter 6). Increased intestinal absorption of glutamine could

have also contributed to glutamine pools, making it unnecessary to upregulate the UPP, or perhaps activity of the UPP was increased without a concurrent increase in mRNA or protein expression; however, because of the strong correlation between expression and activity of the 20S core (Martin et al., 2002), it seems that the later is less likely. In Chapter 6, a significant decrease of caspase-3 mRNA expression was observed in soleus muscle, while no change in caspase-3 mRNA expression was observed in extensor digitorum longus (EDL) muscle. The muscle type specific regulation of caspase-3 could explain why no change in mRNA expression of UPP components was observed in Chapter 5, where a mixed muscle type was used, or this could be a result of increasing anionic load. The fact that caspase-3 was downregulated presents an interesting result, as this would depict a sparing effect, protecting the slow-twitch muscle type from sarcomeric cleavage for further breakdown by the UPP. When looking at other functions and interactions of caspase-3, perhaps integration of pathways can present a clearer understanding. The involvement of caspase-3 also potentiates a role for apoptosis in ruminants with metabolic acidosis, and the involvement of apoptosis in metabolic acidosis could result from interactions stemming from common hormonal stimulators. Previous research has demonstrated that insulin simultaneously suppresses caspase-3 activity and stimulates phosphorylation of the pro-apoptotic protein Bad (Gao et al., 2008). In its dephosphorylated state, Bad binds to other Bcl-2 family members and promotes apoptosis by allowing mitochondrial permeability for release of cytochrome C. The role of insulin presents an interesting prospect, as insulin has a significant effect on protein turnover, stimulating protein accretion and suppressing degradation. It also demonstrates that the regulation of caspase-3 mRNA expression in sheep with metabolic

acidosis could present an important finding for future application, as targeting caspase-3 for therapeutic use could be useful in situations where insulin participates in shifting apoptosis or protein turnover, such as lactation.

Though earlier research examining metabolic acidosis in lactating dairy cows demonstrated significant regulation of the UPP system as a result of acidosis (Mutsvangwa et al., 2004), it now seems apparent with the findings in this thesis that the physiological state of lactation potentiated the catabolic state of protein loss and the UPP induction. Induction of metabolic acidosis alone is not sufficient to trigger upregulation of the UPP. During lactation, increased GH secretion, decreased IGF-1 secretion and increased receptivity to catecholamines all aid in energy mobilization, while insulin resistance enables muted signaling by insulin in order to permit proteolysis and attenuate protein synthesis (Lomax et al., 1979; Bauman and Currie, 1980; Reist et al., 2003; Beerda et al., 2004). Acidosis in turn rapidly increases cortisol concentrations (Bereket et al., 1996), and inhibits insulin receptor substrate-1 associated phosphoinositol-3 kinase (PI3K) activity, ultimately blocking insulin signaling (Franch et al., 2004). Amplitude and mean mass of serum growth hormone (GH) pulses are significantly decreased by acidosis (Challa et al., 1993). Acidosis also blunts the ability of GH to stimulate insulin-like growth factor-1 (IGF-1) secretion (Green and Maor, 2000), ultimately decreasing mRNA, protein and serum concentrations of IGF-1 (Bereket et al., 1996; Green and Maor, 2000). If the use of a non-fiber carbohydrate rich diet at the onset of lactation induces acidosis in conjunction with insulin insensitivity associated with lactation, perhaps there is potential for use of amino acids, such as leucine, to reverse the adverse effects of acidosis in lactating dairy cows.

Plasma amino acid concentrations observed in Chapters 4 and 6 demonstrate that some compensation occurs in the amino acid pools. The observed increase in plasma glutamine concentrations in acidotic sheep in Chapter 6 further suggests that perhaps other amino acids could prove more benefit than glutamine during metabolic acidosis in ruminants. One amino acid of interest is the branched-chain amino acid (BCAA) leucine. Though leucine's mechanisms of action are not entirely clear, it is understood that leucine stimulates protein synthesis by at least two mechanisms. The first is by essentially bypassing phosphoinositol-3-kinase and Akt and directly activating mTOR complex 1 (mTORC1) for stimulation of protein synthesis (Herningtyas et al., 2008; Suryawan et al., 2008), or bypassing mTOR regulation and directly affecting phosphorylation of translation proteins (Kimball and Jefferson, 2006). The second mechanism is by increasing sensitivity to insulin in some animals (Garlick et al., 1998). Ultimately, leucine exerts its effects by increasing mTOR-mediated translation, and does not appear to alter transcription. Increases in translation can be executed via two mechanisms. The first relies on mTOR mediated phosphorylation of 4E binding protein (4EBP), which is bound to eukaryotic initiation factor (eIF) 4E (eIF4E) in the unphosphorylated state. eIF4E is then free to engage eIF4G, which bridges eIF4E to the ribosomal 40S subunit, along with eIF4A to create the eIF4F complex (Anthony et al., 2001). This complex binds the eIF2, which is bound to the methylated cap of the 43S ribosomal subunit, to tRNA and the 60S ribosomal subunit for translation of mRNA (Kimball and Jefferson, 2006). Bolster et al. (2004) suggested that leucine exerts its effects through increased eIF4G phosphorylation, not through changes in 4EBP phosphorylation; however, other research suggest that not only does leucine increase eIF4G phosphorylation, but also increases eIF4E availability

and stimulates hyperphosphorylation of 4EBP (Vary, 2007). The second mTOR-mediated pathway of translation initiation is via mTOR phosphorylation of S6 kinase (S6K), allowing for subsequent activation of the S6 protein (Nair and Short, 2005; Vary, 2007; Rhoads and Wu, 2009).

In examining how these pieces contribute to metabolic acidosis in ruminants, we can begin to re-evaluate some of the potential areas of interest in commercial situations of metabolic acidosis. Firstly, though we did not observe any shift in p70S6K mRNA expression in growing sheep as a result of acidosis (Chapter 6), the decreased gain observed over time in steers with acidosis (Muir et al., 1981) suggests that in fact protein deposition may be affected if acidosis persists, potentially affecting synthesis at the level of translation as suggested by Caso and Garlick (2005). Insulin and leucine have a synergistic effect on suppression of protein degradation, and both stimulate protein synthesis (Garlick et al., 1998; Sadiq et al., 2007). In circumstances of fasting, only high doses of insulin can stimulate protein synthesis. However, if BCAAs are infused concurrently with insulin, then there is an additional 2%/day increase in protein synthesis when compared to insulin infusion alone (Garlick and Grant, 1988). When Wester et al. (2004) infused fasted lambs with a mixture of BCAA, glucose or a combination of the two, no significant change in the rate of protein synthesis occurred. However, abomasal infusion of arginine, histidine, lysine, methionine, threonine and tryptophan in steers fed a high concentrate diet increased protein synthesis by 11% and increased protein degradation by only 9% (Wessels et al., 1997), demonstrating that amino acid supplementation can promote protein synthesis in ruminants. Duodenal infusions of 40 g/d of leucine also increased milk protein output in cows offered leucine deficient diets

(Rulquin and Pisulewski, 2006), demonstrating that leucine supplementation can affect mammary protein synthesis. Tesseraud et al. (1993) also demonstrated that the effect of insulin on leucine concentration and oxidation is dependent on the stage of lactation, potentially due to insulin insensitivity. Use of leucine as a supplement for increasing protein synthesis and suppressing protein degradation in lactating ruminants seems plausible. But what about ruminants with metabolic acidosis? This too seems possible. The ability of leucine to suppress proteolysis in disease states has also been found in some experiments. Examination of rat skeletal muscle cells has demonstrated that protein content of these cells can be increased by leucine during HCl-induced muscle wasting (Bevington et al., 2001), and some reports have observed the benefits of leucine provision for increased BCAA oxidation in patients with chronic renal failure (Cano et al., 2006). Formulation of fat protected amino acids has created complexes that resist microbial usage of the amino acids, instead allowing the amino acids to bypass the rumen and be absorbed in the small intestine. Development of bypass methionine and lysine are examples of this technology, and are used in dairy production systems to provide additional methionine and lysine for increased milk protein synthesis (Xu et al., 1998). Potential for this technology again exists during disease states where an amino acid, such as leucine, could be used to alter protein turnover.

Ultimately, though regulation of proteolysis during metabolic acidosis appears to be different between ruminants and monogastrics, potential still exists for use of strategic amino acid supplementation. The research presented in this thesis demonstrate that lactation does upregulate mRNA expression of C8, E2 and ubiquitin in a tissue specific manner; however, metabolic acidosis does not affect UPP components at the mRNA or

protein expression level in skeletal muscle but could be due to downregulation of caspase-3 in slow-twitch muscle. In addition, increased mRNA expression of hepatic glutamine synthetase and renal glutamate dehydrogenase, in conjunction with a significantly higher glutamine concentration as a result of metabolic acidosis, are indicative of shifts in inter-organ nitrogen transport. Upon examination of commercial systems, the combination of lactation and metabolic acidosis appears to upregulate the UPP, while use of metabolically acidotic sheep demonstrate that shifting amino acid concentrations provide a source of buffering. Though the current research indicates that glutamine may not be the key amino acid for attenuation of metabolic acidosis in ruminants, other amino acids may prove useful. The mechanisms of leucine are not entirely understood; however, it seems clear that this amino acid may have potent effects on both protein synthesis and degradation, and could provide beneficial effects for ruminants during periods of physiologic demand, such as lactation and/or metabolic acidosis.

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