ABSTRACT

STERN, RACHEL ALLYSA. The Effect of Hyperammonemia on Myogenesis in Broiler Embryos. (Under the direction of Paul Mozdziak).

The embryonic environment and regulation of myogenesis is of particular interest in muscle biology because muscle fiber number is determined during embryonic growth. During embryonic myogenesis, growth is accomplished by the development of muscle fibers from progenitor cells of the somites. Myogenic regulatory factors are largely responsible for the specification and differentiation of somatic cells to become mature myocytes. Post-natal and post-hatch muscle growth is a result, exclusively, of increased muscle fiber size. Muscle fiber hypertrophy is dependent upon the addition of new myonuclei by satellite cell fusion, as the existing myonuclei are post-mitotic. Myogenesis and post-natal growth are intricately orchestrated by the myogenic regulatory factors, and satellite cell activity, all of which are inhibited by myostatin, a powerful negative regulator of muscle growth. Myostatin has been demonstrated to inhibit both myogenesis and post-natal growth, as myostatin-mutants reveal a severe hyperplasic and hypertrophic phenotype. Additionally, increased myostatin expression has been determined to be a contributor to severe muscle wasting, or sarcopenia, seen as a secondary effect of many diseases. Previous studies aimed to determine the cause of sarcopenia have revealed convincing data suggesting that hyperammonemia, as seen in patients with cirrhosis and in animal models of liver failure, causes an increase in myostatin expression. The objective of the present study was to determine the effect of inducing hyperammonemia in broiler embryos on the expression of myostatin and myogenic regulatory factors during myogenesis, where muscle fiber number is determined. To accomplish hyperammonemia, an ammonium acetate solution (50 mmol/kg body weight)

was administered to the amniotic fluid of broiler eggs four times over 48 hours, beginning on embryonic day (ED) 15 or 17. Twelve hours after the last injection, serum and pectoralis muscle samples were obtained for analysis. The ammonium acetate administration protocol was successful in increasing serum ammonia concentration, more than four times higher than control samples, ($P < 0.05$) for both ED17 and ED19 collected samples. Pectoralis major samples were assessed for mRNA expression, determined by real-time PCR, of myostatin (MSTN), and myogenic regulatory factors, myogenic factor 5 (MyF5), myogenic determination factor 1 (MyoD), myogenin (MYOG), and myogenic regulatory factor 4 (MRF4) was evaluated for experimental and sham-injected controls. A highly significant reduction (54% and 77%, respectively) ($P < 0.01$) in MSTN expression was observed in both ED17 and ED19 collected samples with increased serum ammonia concentrations. MyF5 expression was increased more than 100% ($P < 0.05$) in ED17 samples, which supports an increase in myoblast proliferation. Additionally, MRF4, which is expressed in mature myocytes, was decreased by more than 30% in both ED17 and ED19 samples ($P \leq 0.05$), further suggesting that myoblast proliferation was prolonged. No significant difference was observed in the expression of MyoD or MYOG. MSTN downregulation was confirmed by Western blot analysis ($P < 0.05$). These data suggest that increasing serum ammonia concentration in late-stage broiler embryos inhibits MSTN expression, creating an embryonic environment that supports an increase in muscle fiber number, which improves the potential for post-hatch muscle growth, and could positively impact ultimate meat yield. To fully understand the potential impact of the current results, the mechanistic pathway by which hyperammonemia inhibits the expression of myostatin must be investigated.

The Effect of Hyperammonemia on Myogenesis in Broiler Embryos

by
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DEDICATION

This thesis is dedicated to my family, because without their support and encouragement I wouldn't be here today. To my parents, thank you for instilling in me a sense of responsibility and pride in always doing my best. To my grandfathers, who have always encouraged higher education and set examples of career achievement that I can only hope to attain one day, and my grandmothers, who put up with them, and have their own inventory of career successes while being the glue that holds everything together. I only wish that all of you were here to read this. To my fiancé, brother, sister, and those I choose to call family for the constant support and inspiration. I love you all.

BIOGRAPHY

Rachel Allysa Stern was born and raised in Wilmington, North Carolina. From a young age, she had a passion for animals, and particularly, horses. Most of her childhood memories involve the family dog or her horses. From a very young age, she had dreams of becoming a large animal veterinarian and attended North Carolina State University for a Bachelor of Science in Animal Science. It was there that her interest in pursuing a research-based career was sparked while performing undergraduate research under the guidance of Dr. Shannon Pratt-Phillips. After graduating in 2010, she took time off to further consider a career in large animal veterinary medicine before choosing to come back to North Carolina State University for a Master's degree in Physiology, where she would perform research under the guidance of Dr. Paul Mozdziak.

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CHAPTER I

LITERATURE REVIEW

Introduction

The chick embryo has been a model in developmental biology for many years, due to the solitary nature of their development. The avian model also provides a unique opportunity to observe effects of a manipulated embryonic environment on normal development that is difficult to achieve in mammalian species. While much of the focus in poultry production has focused on post-hatch strategies of optimizing meat production, there may be greater potential for improving muscle growth during embryonic myogenesis. Manipulation of growth and differentiation factors responsible for regulating muscle development is a logical strategy for improving embryonic myogenesis and may provide an opportunity to optimize meat production and quality that has yet to be considered.

Skeletal Muscle Growth

Skeletal muscle growth is characterized by two distinct phases: hyperplasia, an increase in cell number, and hypertrophy, an increase in cell size. Embryonic muscle hyperplasia is determined by genes regulating progenitor cell specification, proliferation, and differentiation, growth factors, nutrition, and genes inhibiting growth, such as myostatin. It is well established that muscle fiber number is determined during embryonic development while post-natal growth largely relies on hypertrophy of existing fibers (Wigmore et al., 1983; Remignon et al., 1995; Mozdziak et al., 1997). Myonuclear accretion, protein

synthesis, and external factors, such as nutrition and mechanical load are responsible for post-natal muscle growth (Mozdziak et al., 1997; Schiaffino et al., 2013). The embryonic environment is crucial to muscle development and ultimate meat yield, as it establishes the framework for post-natal muscle growth.

Myogenesis and Myogenic Regulatory Factors

During embryogenesis, the mesoderm is the only germ layer that gives rise to skeletal muscle. More specifically, progenitor cells of the dermomyotome, a partition of epithelial somites from the paraxial mesoderm, are the precursor cells to myoblast cells (Pownall et al., 2002; Yusuf and Brand-Saberi, 2012). Somites can be identified by the expression of Pax genes, a family of developmentally regulated genes. *Pax-3*, a mammalian homolog of the Drosophila *paired* gene, is expressed in the unsegmented paraxial mesoderm and epithelial somite (Tajbakhsh et al., 1997). *Pax-3* is expressed upstream of myogenic regulatory factors and is necessary for the migration of muscle precursor cells from the somites to developing limb buds, as murine *Pax-3* mutants do not develop limb muscles (Tajbakhsh et al., 1997; Yusuf and Brand-Saberi, 2012). *Pax-7* is expressed later than *Pax-3* in the central dermomyotome of somites (Mansouri et al., 1996; Tajbakhsh et al., 1997). *Pax-7* has been observed to influence skeletal formation of the head in *Pax-7* mutant mice (Mansouri et al., 1996). Further, *Pax-7* is required for the specification of myogenic satellite cells (Seale et al., 2000). A group of genes, termed myogenic regulatory factors (**MRFs**) are responsible for the specification and differentiation of cells from the somites to become myocytes

(Sabourin and Rudnicki, 2000; Pownall et al., 2002; Yusuf and Brand-Saberi, 2012). The MRFs are a subfamily of the basic helix-loop-helix superfamily of transcription factors.

The commitment of multipotent somite cells to myoblast cells is the first step in myogenesis and the major role of the primary MRFs. Myogenic factor 5 (**MyF5**) and myogenic determination factor1 (**MyoD**) are activated in all somite-derived muscle progenitor lineages (Sabourin and Rudnicki, 2000; Pownall et al., 2002). In avian embryos, MyF5 and MyoD are activated in progenitor cells, while murine embryos express MyF5 in progenitor cells and MyoD in differentiated myotomes (Sassoon et al., 1989; Pownall et al., 2002). In the chick embryo, low levels of MyF5 and MyoD mRNA expression, without protein expression, have been detected prior to somatogenesis in the epiblast layer of the blastoderm, suggesting that initial regulation of myogenesis occurs many stages before terminal myoblast differentiation (Kiefer and Hauschka, 2001; Gerhart et al., 2007).

MyoD cDNA was initially identified by its ability to convert fibroblasts into myogenic cells (Sabourin and Rudnicki, 2000). Gerhart et al. (2007) found that MyoD expressing cells from the early epiblast were committed to skeletal muscle lineage, and are capable of self-renewal and differentiation when grown in culture. Targeted inactivation studies performed in mice show that there is some redundancy in the function of these two genes. When both *MyF5* and *MyoD* are inactivated in murine embryos, the embryos do not survive and lack skeletal myoblasts entirely (Rudnicki et al., 1993). The lack of myoblasts in *MyF5* and *MyoD* knock-outs is direct evidence that MyF5 and MyoD are responsible for the determination of somite cells to become myoblasts, and myoblast proliferation. When *MyoD* is inactivated, murine embryos have a normal muscular phenotype, but there is a four-fold

increase in *MyF5* expression when compared to wild-type embryos (Rudnicki et al., 1992). While myoblast determination still occurs, further developmental investigations produced evidence that mice null for either *MyF5* or *MyoD* exhibit delays in development of different muscle types, suggesting that they only play partially redundant roles and remain unique in their functions during normal development (Kablar et al., 1997). In addition to its role in myoblast specification, cell culture studies suggest that MyoD plays a role in preparing myoblasts for differentiation into myocytes. Ectopic MyoD causes inhibition of the cell cycle prior to the S-phase independently of induction of cellular differentiation (Crescenzi et al., 1990; Sorrentino et al., 1990).

Myoblast differentiation is marked by the fusion of multiple myoblasts into myocytes, which are the functional cellular units of mature muscle fibers. Myogenin, which is expressed downstream of genes responsible for progenitor cell determination, is the major determinant of myoblast differentiation. In similar gene inactivation studies, mice lacking myogenin were observed to have a normal number of myoblasts, but completely lack myofibers, and therefore die at birth (Hasty et al., 1993; Nabeshima et al., 1993). Additionally, myogenin was found to compensate for inactivation of myogenic regulatory factor 4 (**MRF4**), also known as herculin or MyF6, resulting in a normal muscle phenotype but abnormalities in rib formation (Zhang et al., 1995). Differently, in myogenin inactivated mice, MRF4 expression is low and normal myoblast differentiation does not occur, suggesting that MRF4 plays a downstream role in the maturation of differentiated myocytes (Zhang et al., 1995; Olson et al., 1996). Though, MRF4 is normally the most highly expressed MRF in postnatal skeletal muscle, in MRF4 inactivated mice, myogenin was

increased four-fold in adult muscles, further demonstrating its ability to compensate for MRF4 in muscular growth and maturation (Zhang et al., 1995). While there is some evidence that myogenin can compensate for reduced or inactivated MRF4 expression, similar to the primary MRFs, the secondary MRFs maintain their individual importance in embryonic development.

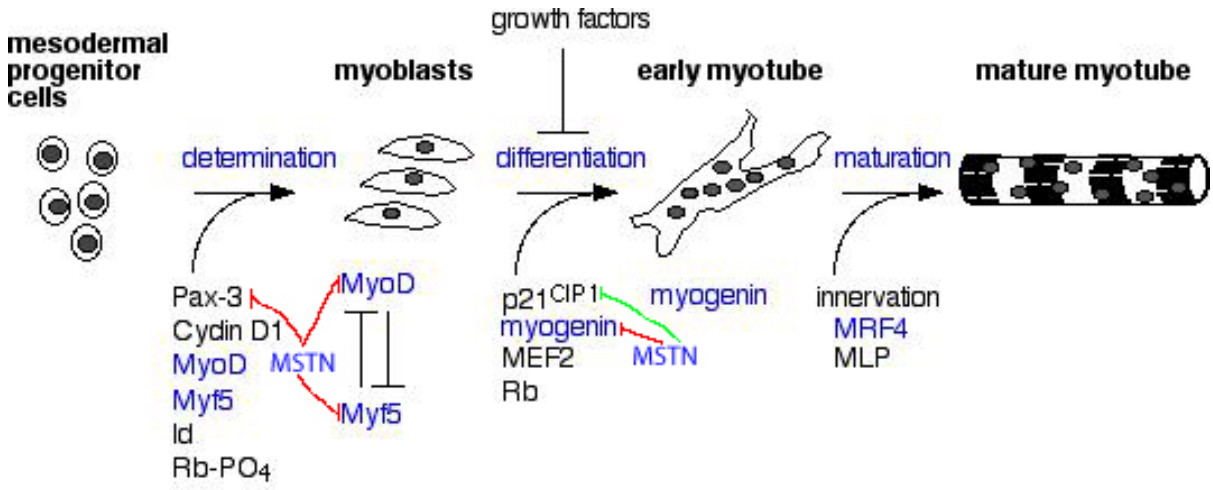

Figure 1.1 Role of myogenic regulatory factors in embryonic myogenesis. (edited from http://www.faculty.virginia.edu/mammgenetics/myogenesis.html)

Myostatin and its Role in Myogenesis and Muscle Growth

Myostatin, also called growth differentiation factor 8, is a member of the transforming growth factor-β (**TGF-β**) superfamily. Unlike other TGF-β family members, myostatin is predominantly expressed in skeletal muscle. Myostatin is a powerful inhibitor

of muscle growth and is expressed in embryonic and adult muscles. The inhibitory role of myostatin was first discovered, by McPherron, Lawler, and Lee, in *MSTN* knock-out mice where an extreme hyperplasic and hypertrophic phenotype was observed (McPherron et al., 1997) (Figure 1.2a). Later, the *MSTN* mutation was identified in naturally occurring hypermuscular Belgian Blue and Piedmontese cattle, suggesting the role of myostatin is highly conserved across species (McPherron and Lee, 1997) (Figure 1.2b). In particular, the biologically active C-terminal region of the predicted myostatin protein is identical in humans, mice, rats, pigs, chickens, and turkeys (McPherron and Lee, 1997).

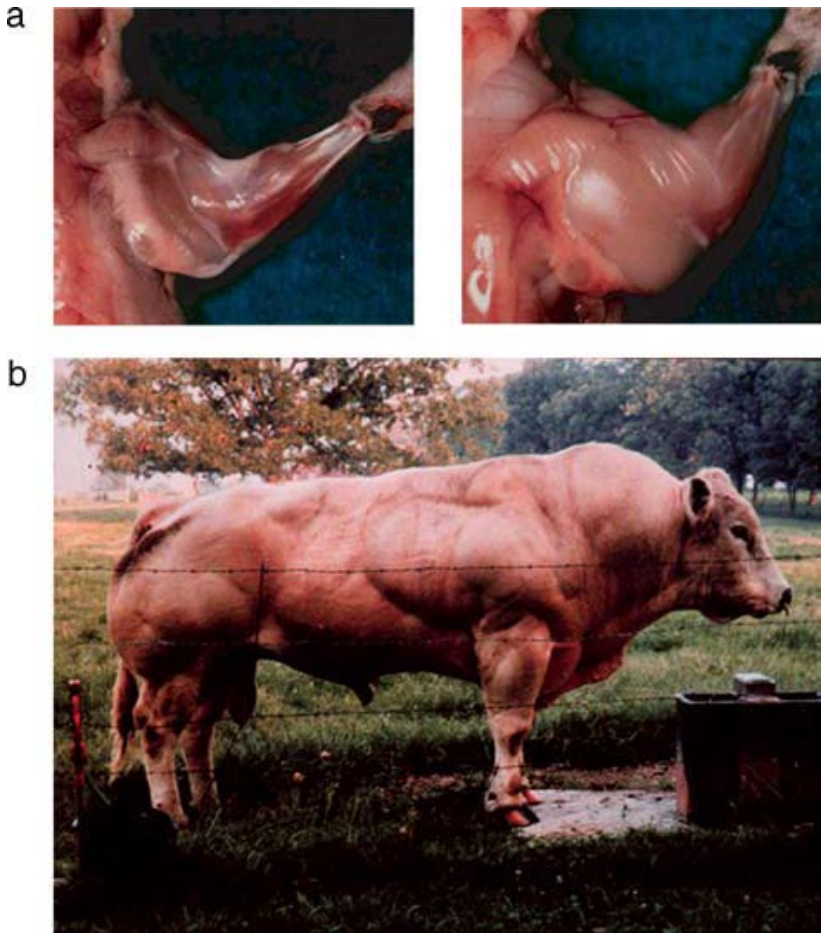

Figure 1.2 Myostatin mutations are highly conserved across species. (a) the extreme hyperplasic and hypertrophic phenotype in the forelimb of a *MSTN* knock-out mouse (right) compared to a wild-type mouse (left) (b) Belgian Blue bull demonstrating the double muscling phenotype (Lee, 2004).

Myostatin is transcriptionally regulated by various pathways, including the NF-κ-B pathway, and has also been shown to have an auto-regulatory feedback mechanism, utilizing the Smad7 pathway, during normal development (Forbes et al., 2006; Qiu et al., 2013). The myostatin protein is produced as a prepropeptide and has been shown to exist in a dimerized latent form in the blood and other tissues (Lee, 2008). To become active, the propeptide is cleaved at the C-terminal end by the BMP-1/TLD family of proteases, or by free radical

action or a decrease in pH releasing the active form of the myostatin protein (McPherron et al., 1997; Lee, 2008; Han and Mitch, 2012) (Figure 1.3). This suggests that the propeptide plays a regulatory role in the activity of myostatin.

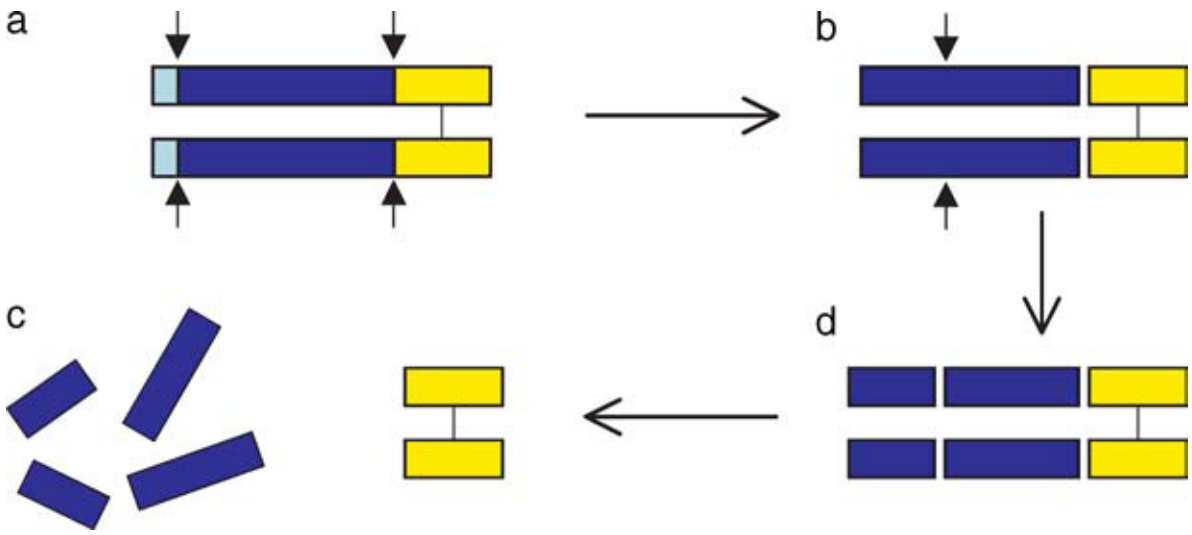

Figure 1.3 Processing of myostatin protein. (a) Myostatin precursor (approx. 52 kDa) which must undergo two proteolytic events (b) Latent complex of myostatin (approx. 40 kDa) following the proteolytic removal of the N-terminal signal sequence, the propeptide (blue) and the disulfide linked C-terminal dimer (yellow) remain bound (d) the activation of the latent myostatin dimer occurs by proteolytic cleavage leaving (c) the myostatin dimer (approx. 26kDa) and dissociated propeptide fragments (Thomas et al., 2000; Lee, 2004).

As myostatin-null individuals illustrate both a hyperplasic and hypertrophic response, it can be assimilated that myostatin has inhibitory effects during embryonic myogenesis and post-natal muscle growth. During mouse embryogenesis, myostatin is expressed in developing somites as early as embryonic day 9.5 and continues to be expressed throughout embryonic development, suggesting it plays a regulatory role in normal muscle development and growth (McPherron et al., 1997; Lee and McPherron, 1999). A number of in vitro

studies have shown that myostatin inhibits both myoblast proliferation and differentiation in C2C12 myoblasts (Thomas et al., 2000; Langley et al., 2002). Similar results have been noted in fetal bovine myoblasts and in chick limb bud development (Thomas et al., 2000; Amthor et al., 2002; Amthor et al., 2004). Specifically, myostatin has been found to downregulate MyF5, and Pax3, genes associated with proliferation, and increase the expression of p21, a cyclin-dependent kinase inhibitor, which has a negative effect on proliferation (Thomas et al., 2000; Amthor et al., 2002). Cyclin-dependent kinases (**Cdks**) catalyze transitions in the cell cycle. The p21 family inhibits all Cdks involved in G_1/S phase transition, which has been observed in the growth of myostatin treated C2C12 myoblasts (Thomas et al., 2000). The negative effect of myostatin on myoblast differentiation can be attributed to the downregulation of MyoD and myogenin (Langley et al., 2002; Rios et al., 2002). As previously mentioned, MyoD expression has been observed to cause growth arrest in cultured cells, and has been shown to inhibit the G_0/S phase transition in quiescent NIH 3T3 cells stimulated to re-enter the cell cycle (Crescenzi et al., 1990; Sorrentino et al., 1990). By downregulating MyoD expression, myostatin has a negative effect on the differentiation of myoblasts by inhibiting cell cycle arrest (Langley et al., 2002).

Observed hypertrophy in myostatin-null individuals is evidence of its regulatory role in post-natal muscle growth. Satellite cells are mononucleated cells that lie quiescent between the basal lamina and sarcolemma of muscle fibers in non-growing muscle. Their role in post-natal growth is to fuse with growing myofibers to provide nuclei (DNA) to growing myofibers, as the myofibril nuclei are post-mitotic (Schultz, 1996; Mozdziak et al.,

1997). During normal muscle growth, satellite cells re-enter the cell cycle and proliferate to maintain a population of satellite cells available for myonuclear donation (Schultz, 1996).

Several studies, both in vivo and in vitro, suggest that increased myostatin inhibits satellite cell function by upregulating p21, which negatively regulates the cell cycle and prevents satellite cell mitosis (McCroskery et al., 2003; Dasarathy et al., 2004; Akita et al., 2013). Additionally, myostatin has been shown to downregulate MRFs, which are involved in muscle growth and serve as indicators of satellite cell proliferation and differentiation (Muroya et al., 2002; Dasarathy et al., 2004). Satellite cell activity is often assessed by administration of 5-bromo2-deoxyuridine (**BrdU**), a synthetic analog of thymidine that substitutes during DNA replication, which can then be detected by antibody staining to identify satellite cells that are actively undergoing mitosis. McCroskery et al. (2003) found that at multiple stages of growth (4 wk, 8 wk, and 6 mo) myostatin knockout mice had 33% more satellite cells in the S-phase of mitosis than wild-type counterparts after BrdU labeling, demonstrating that the presence of myostatin regulates satellite cell activation in normal growth. Further, a significantly higher amount of steady-state satellite cells per muscle fiber unit was observed in myostatin-null adult mice when compared to controls (McCroskery et al., 2003). Similarly to the regulatory role of myostatin in embryonic myogenesis, this evidence suggests that in post-natal muscle growth and maintenance of adult muscle, myostatin serves to suppress satellite cell function, until growth or regeneration is necessary.

Myostatin Mutations in Livestock and Meat Production

The hyperplasic and hypertrophic phenotype observed in myostatin mutated animals has been of particular interest in agricultural research, as efficiency of meat production and higher meat yield are constant goals of the industry. The higher percentage of muscle to carcass weight observed in myostatin mutants is desirable in meat-producing livestock. Additionally, myostatin mutant animals have been observed to have a lesser amount of connective tissue, which has been shown to positively impact meat tenderness (Arthur, 1995; Hope et al., 2013). However, there are many disadvantages that are associated with myostatin mutations that have prevented commercial agricultural practices from employing this gene manipulation as a strategy for increased muscle yield. Firstly, there are discrepancies on the impact of the myostatin mutation on meat quality in reference to flavor. McPherron and Lee (2002) found that the deletion of myostatin partially suppressed fat accumulation in mice. Similarly, reduced intramuscular fat content was observed in cattle and sheep with the myostatin mutation (Wiener et al., 2009; Masri et al., 2011; Hope et al., 2013). In these studies, there were varying reports of taste panel flavor scores. More importantly, and consistent, is the increased incidence of dystocia, or obstructed labor, due to large fetus size, and poor reproductive performance in myostatin mutant livestock, which presents a management challenge for large commercial operations. Several studies suggest that selective breeding for individuals heterozygous for myostatin mutations may be a more realistic approach to utilizing the benefits of the myostatin mutation in commercial meat production (Wiener et al., 2009; Hope et al., 2013).

Myostatin in Muscle Wasting Diseases

In addition to muscular dystrophy, severe muscle wasting is a serious side effect that has been observed to accompany many diseases, including cancer, congestive heart failure, chronic kidney disease, diabetes, AIDS, chronic obstructive pulmonary disease (**COPD**), and liver diseases. In all of these disorders, regulation of myostatin expression is a targeted therapy for sarcopenia and cachexia (Dasarathy et al., 2004; Zhou et al., 2010; Han and Mitch, 2012). Clinical studies have shown that patients with COPD have increased serum myostatin concentrations compared to healthy age matched individuals (Ju and Chen et al., 2012). Using a murine model of cancer, Zhou et al. (2010) were able to prevent and reverse muscle wasting, even in animals whose tumor growth was not inhibited, by pharmacologically blocking the myostatin signaling pathway. Similarly, a study of chronic kidney disease (**CKD**) revealed that infusion of a myostatin agonist into CKD mice suppressed myostatin in the muscle, and both muscle weight and body weight were increased compared to CKD mice given sham infusions (Zhang et al., 2011). In cirrhosis, which occurs in advanced liver diseases, sarcopenia is common and causes significant complications. Dasarathy et al. (2004) found in portacaval anastamosis (**PCA**) rats, which mirror conditions of cirrhosis and demonstrate significant loss of skeletal muscle protein, that myostatin, and its downstream targets like p21, were dramatically increased. In this model, and other murine models, the induction of hyperammonemia has been directly correlated with increased myostatin expression and enhanced muscle autophagy (Dasarathy et al., 2004; Qiu et al., 2012; Qiu et al., 2013).

Hyperammonemia and Muscle Function

Hyperammonemia, or excess ammonia in the blood as a result of a metabolic disruption or insufficiency, has been observed in numerous diseases. Acute ammonia toxicity is marked by symptoms of neurophysiologic effects on the central nervous system. At high doses, hyperammonemia (1-5 mM) can produce variable shifts between excitatory and inhibitory neurotransmission causing seizures, coma, and death (Albrecht, 1998). Animal models of ammonia toxicity demonstrate similar responses to rapid increases of ammonia in the blood, though species differences have been reported. Wilson et al. (1968) examined the differences in toxicity of exogenous ammonia between uricotelic species and ureotelic species, using young chicks and mice. They found that through intravenous injection, chicks were more susceptible to ammonia toxicity when compared to mice (LD_{50} of 2.72 mmol/kg and 5.64 mmol/kg, for chicks and mice, respectively) (Wilson et al., 1968). Intraperitoneally, there was no significant difference in ammonia toxicities between chicks and mice (LD_{50} of 10.44 mmol/kg and 10.84 mmol/kg, for chicks and mice, respectively) (Wilson et al., 1968).

Hyperammonemia is a trademark of liver disease, as nearly all ammonia detoxification occurs in the liver via the urea cycle. Clinical observations and research models of cirrhosis have found strong correlations suggesting hyperammonemia contributes to sarcopenia (Dasarathy et al., 2004; Qiu et al., 2012; Qiu et al., 2013). The previously mentioned increase in myostatin is one explanation of this phenomenon. Furthermore, it has been suggested that skeletal muscle becomes a site of ammonia detoxification, via synthesis of amino acids such as, glutamate and glutamine, in chronic conditions where liver

detoxification is inadequate (Holecek et al., 2000; Olde Damink et al., 2002). Specifically, significant ammonia uptake in the muscle is observed in patients with decompensated cirrhosis and muscle wasting when compared to cirrhotic patients with normal compensation (Olde Damink et al., 2002). This further suggests the correlation between hyperammonemia and sarcopenia.

Other diseases that have been associated with hyperammonemia suggest that excess ammonia is released from the muscle during metabolic stress. For example, exercise-induced hyperammonemia has been observed in patients with COPD and chronic heart failure. In these studies, hyperammonemia was exacerbated by exercise and was used as indicator of metabolic stress and skeletal muscle ATP depletion (Andrews at al., 1997; Calvert et al., 2010). It is well understood that hyperammonemia during exercise, specifically, can be dominantly attributed to activation of the purine nucleotide cycle, in which deamination of adenosine monophosphate occurs, likely as a response to depleted ATP or lack of ATP resynthesis (Graham et al., 1992). In this case, ammonia is a byproduct of the purine nucleotide cycle, which generates fumarate, to increase the availability of this intermediate of the citric acid cycle, in order to generate more NADH for ATP production via the electron transport chain. These clinical and experimental observations suggest that hyperammonemia and muscle metabolic function are intricately associated, and apply to a variety of diseases and disorders.

Hyperammonemia and Embryogenesis

Ammonia is generated in vivo during catabolism of amino acids and is generally considered a toxic waste product. This toxicity has been previously discussed in reference to adults. There is evidence that supports that ammonia also has negative effects on embryogenesis in numerous in vitro studies. He et al. (2007) investigated effects on murine embryogenesis in glutamine synthetase (**GS**), the enzyme required for glutamine production from ammonia and glutamate, knock-out embryos in culture with and without supplementation of glutamine to the culture media. GS mutant embryos die due to the lack of GS metabolism, and presumably ammonia toxicity (He et al., 2007). Similarly, treatment of amphibian and echinoderm embryos in culture with ammonia resulted in abnormal or incomplete cell division at multiple stages (Webb and Charbonneua, 1987). Specifically, it was reported that ammonia prevented ribosomal RNA transcription in Xenopus embryos (Shiokawa et al., 1986; Shiokawa et al., 2010). Several studies have shown that increased plasma ammonia concentrations in ewes, due to urea supplementation of feed, had negative impacts on embryo development when compared to previous control observations of each animal (Bishonga et al., 1996; McEvoy et al., 1997). However, Hammon et al. (2000) found no significant difference in morula or blastocyst development when bovine embryos were cultured in media with varying concentrations of ammonia. Thus, the evidence of the effect of ammonia on embryonic development is somewhat inconsistent.

Ammonia Detoxification and Excretion: Urea vs. Uric Acid

Aside from its role in glucose synthesis and storage, the liver controls the availability of amino acids in the blood. Unlike glucose, amino acids cannot be stored and, therefore, must be used immediately for protein synthesis, or be broken down into α-keto acids and used for energy metabolism. The deamination of amino acids produces free ammonium ions, which as previously discussed are toxic at high concentrations in the blood. In bacteria and most mammalian species, ammonia is detoxified in hepatocytes, in a series of enzymatic processes termed the urea cycle, which produces non-toxic urea from ammonia and bicarbonate (Figure 1.4). Urea that enters the blood from the liver is excreted by the kidneys. Animals that excrete excess nitrogen in the form of urea are termed ureotelic animals.

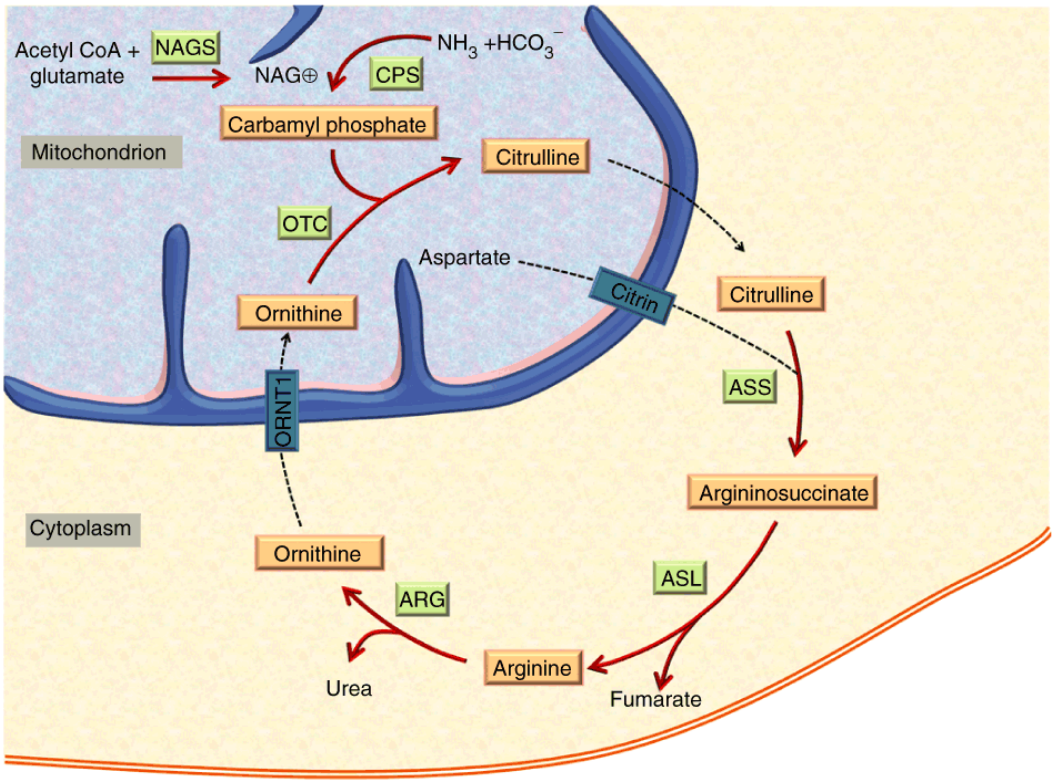

Figure 1.4 The urea cycle. Through a series of enzymatic reactions (green boxes), excess ammonia is converted to urea in the hepatocytes. ARG, arginase 1; ASL, argininosuccinate lyase; ASS, argininosuccinate synthase; CPS1, carbamoyl phosphate synthase 1; NAG, N-acetylglutamate; NAGS, N-acetylglutamate synthase; ORNT1, ornithine transporter; OTC, ornithine transcarbamoylase (Nagamani et al., 2012).

Reptiles and avian species are uricotelic animals, meaning they excrete excess nitrogen in the form of uric acid. It is has been conceded that this mode of nitrogen elimination was an evolutionary adaptation to water conservation, as uric acid requires less water for excretion when compared to urea. Functionally, avian species lack activity of some enzymes in the liver that are involved in the urea cycle. Mora et al. (1965) measured liver enzyme activity in numerous species, and found that in White Leghorn chickens carbamoyl phosphate synthetase 1 (**CPS1**), ornithine transcarbamoylase (**OTC**), and argininosuccinate

synthetase (**ASS**) were below the limit of detection. However, OTC and ASS activity have been reported in the kidney of chickens (Tamir and Ratner, 1960). Though their activity is not measurable in the liver, a genome mapping study identified all three of these enzymes, including CPS1, in the chicken genome which suggests that they may be physiologically relevant in some other metabolic process (Shimogiri et al., 2004).

Research Objectives

The overall objective of the current thesis research was to determine the effect of hyperammonemia on embryonic myogenesis. There doesn't appear to be any data on ammonia toxicity in broiler embryogenesis. The primary objective was to induce hyperammonemia in the developing embryo via injection of ammonium acetate solution into the amniotic fluid of the egg. Once hyperammonemia was achieved, the downstream effect on myostatin and myogenic regulatory factor expression was examined. Based on previous studies of hyperammonemia in PCA rats and other murine models (Dasarathy et al., 2004; Qiu et al., 2012), it was expected that induced hyperammonemia would increase expression of myostatin, decrease myogenic regulatory factor expression, and have a negative impact on embryonic muscle development.

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CHAPTER II

THE EFFECT OF HYPERAMMONEMIA ON MYOSTATIN AND MYOGENIC REGULATORY FACTOR GENE EXPRESSION IN BROILER EMBRYOS

ABSTRACT

Myogenesis is facilitated by four myogenic regulatory factors (**MRFs**) and is significantly inhibited by myostatin (**MSTN**). The objective of the current study was to examine embryonic gene regulation of MSTN/MRFs, and subsequent manipulations of protein synthesis, in broiler embryos under induced hyperammonemia. Broiler eggs were injected with ammonium acetate solution (50 mmol/kg body weight) four times over 48 hours beginning on either embryonic day (**ED**) 15 or 17. Serum ammonia concentration was significantly increased, more than four-fold, ($P < 0.05$) in ammonium acetate injected embryos for both ED17 and ED19 collected samples when compared to sham-injected controls. Expression of mRNA, extracted from pectoralis major of experimental and control embryos, was measured using real-time quantitative PCR for MSTN, and MRFs myogenic factor 5 (**MyF5**), myogenic determination factor 1(**MyoD**), myogenin (**MYOG**), and myogenic regulatory factor 4 (**MRF4**). A significant reduction (54% and 77%, respectively) ($P < 0.01$) in MSTN expression accompanied increased serum ammonia concentration in both ED17 and ED19 collected samples. There was a 100% increase in MyF5 expression ($P < 0.05$) in ED17 collected samples, supporting increased myoblast proliferation. In both ED17 and ED19 collected samples, MRF4 was decreased by more than 30% ($P \leq 0.05$) in ammonium acetate injected embryos suggesting increased myoblast proliferative activity.

No significant difference was seen in MyoD or MYOG expression for either age group. MSTN protein levels were evaluated by Western blot analysis, and also showed decreased myostatin expression ($P < 0.05$). Overall, it appears possible to inhibit myostatin expression through hyperammonemia, which is expected to increase embryonic myogenesis and postnatal muscle growth.

INTRODUCTION

It is widely accepted in both agricultural and human research that embryonic and immediate post-natal environment plays an important role in muscle development. In poultry, it is well established that muscle fiber number is determined during embryonic development and that post hatch growth is dependent on hypertrophy of the existing fibers (Remignon et al., 1995; Mozdziak et al., 1997). Embryonic muscle hyperplasia, or increase in myoblast proliferation, is controlled by a group of four basic helix-loop-helix transcription factors called myogenic regulatory factors (**MRFs**).

Two of the four MRFs, myogenic determination factor 1 (**MyoD**), and myogenic factor 5 (**MyF5**), are regulators of myogenic progenitor specification. These transcription factors are evident early in embryonic development supporting that they play a crucial role in the determination of embryonic stem cells to become committed myogenic cells. Research in MyoD and MyF5 knockout mice has demonstrated the importance of these regulatory factors on myoblast determination. Mice that lack MyoD or MyF5 develop normally, suggesting that there is redundancy in the role of these genes, while mutant mice for both MyoD and MyF5 completely lack skeletal muscle and do not survive (Rudnicki et al., 1992; Rudnicki et

al., 1993). Myogenin (**MYOG**), and myogenic regulatory factor 4 (**MRF4**), also known as MyF6 or herculin, are expressed later in embryonic development. MYOG is the major determinant of myoblast differentiation, while MRF4 is expressed in mature myocytes (Nabeshima et al., 1993).

Myostatin (**MSTN**), a transforming growth factor-β (**TGF-β**) family member, is the most powerful negative regulator of myogenesis, but is also expressed in adult muscles, suggesting it also inhibits postnatal muscle growth (McPherron et al., 1997; Lee and McPherron, 2001; Amthor et al., 2004). MSTN knockout mice display an extreme hyperplasic and hypertrophic phenotype termed double muscling (McPherron and Lee 1997). Mutations in the MSTN gene were deemed responsible for the same phenotype observed, as a result of genetic selection for muscle growth, in Belgian Blue and Piedmontese cattle proving that the role of the MSTN gene is highly conserved across species (McPherron and Lee, 1997). MSTN has been shown to inhibit myogenesis by downregulating expression of the crucial growth factors MyoD, MyF5, and MYOG (Langley et al., 2002; Amthor et al., 2004; Dasarathy et al., 2004).

In agricultural research, the importance of MSTN and MRF expression in early stages of development is well understood to impact meat quality and ultimate meat yield. Though MSTN mutant livestock have significantly increased muscle yield, MSTN mutations also presents disadvantages. Increased calving difficulty and reduced reproductive performance are associated with MSTN mutant hypermuscular animals preventing the selection for this phenotype in commercial practice (Wiener et al., 2009). There is evidence that performing selective breeding for livestock that carry only one mutant MSTN allele may be a more

realistic opportunity to benefit from increased hyperplasia and hypertrophy observed with MSTN mutations (Wiener et al., 2009; Hope et al., 2013). It is therefore of particular interest for agricultural advancement to metabolically adjust the expression of MSTN, and subsequently MRFs, without completely eliminating its role in normal regulation of muscle growth.

Increased MSTN expression has been noted in the investigation of diseases, such as cancer, heart and kidney failure, and cirrhosis, where muscle wasting is frequently a secondary, but serious, side effect (Dasarathy et al., 2004; Han and Mitch, 2012; Qiu et al., 2013). Regulation of MSTN expression is targeted as a potential therapy for sarcopenia and cachexia (Dasarathy et al., 2004, Han and Mitch, 2012). In a recent study, Qiu et al. (2013), observed increased MSTN expression under induced hyperammonemia using rats as a model for cirrhosis. Additionally, hyperammonemia has been observed in other diseases including chronic obstructive lung disease and heart failure (Andrews et al., 1997; Calvert et al., 2010). These clinical and mechanistic observations suggest that hyperammonemia has an adverse effect on muscle structure and function in a broad range of disorders.

Ammonia is generated in the developing embryos when amino acids are catabolized to generate energy (Terjesen et al., 2002; He et al., 2007). Ewe and murine embryos are adversely affected by hyperammonemia, while bovine embryos have been reported resistant to increased ammonia concentrations in vitro (Bishonga et al., 1996; McEvoy et al., 1997; Hammon et al., 2000). Similar results of the adverse effects of ammonia have been reported in amphibians and echinoderms (Webb and Charbonneua, 1987). Further, previous studies have reported that ammonia inhibits transcription of ribosomal RNA in Xenopus embryos,

therefore inhibiting protein synthesis (Shiokawa et al., 1986; Shiokawa et al., 2010). Thus, the adverse effects of ammonia on the growing embryo are inconsistent in contrast to the consistent negative effects in adults. There is no data on the role of ammonia toxicity in the developing embryo of broilers. The objective of the present study was to investigate chick embryonic gene manipulation of MSTN and MRFs under induced hyperammonemia, and the effect of the gene manipulation on protein synthesis and growth.

MATERIALS AND METHODS

Injection of Eggs

Fertilized broiler eggs (Ross 708 x Ross 344) were incubated at 37^{o}C to embryonic day (ED) 15 or 17. In late stage broiler embryos, secondary muscle fiber formation is occurring and a population of adult myoblasts, or satellite cells, is being established, providing a great opportunity for growth enhancement between ED15 and ED19 (Stockdale et al., 1981; Maier, 1993). Ammonium Acetate (Sigma Life Science, St. Louis, MO) was diluted in Hanks' Balanced Salt Solution and blue food coloring (FD&C Blue 1; Food Lion, LLC, Salisbury, NC) was added to the solution (2%). In preparation for injections, the shell surface was disinfected using ethyl alcohol. The amnion was identified by candling, and ammonium acetate injections were delivered using a 25-gauge needle. A total of 200 mg (50 mg per dose) of ammonium acetate was delivered into the amniotic sac, via injection of 0.2 mL (250 mg/mL) every 12 hours, over 2 days. Control eggs were injected with a solution consisting of Hanks' Balanced Salt Solution and food coloring only. After each injection, entry holes in the shell were sealed using cellophane tape and eggs were immediately

returned to the incubator. Blood and tissue samples were collected 12 hours after the last injection (i.e. for groups beginning injections in ED15, samples were harvested on ED17, and those beginning on ED17, harvesting was performed on ED19). There was no observed toxicity, or marked difference in health, in experimental embryos, as compared to controls, at the time of sampling. Additionally, upon sampling from the ED19 group, internal pipping was noted in both the experimental and control groups, indicating that chicks in each group were preparing for hatch.

Collection of samples

Blood samples were drawn directly from blood vessels using beveled glass capillary tubes and placed in 0.5 mM EDTA treated tubes. Samples were centrifuged at 12,000 rpm for 10 min, the serum was separated, and placed in fresh tubes for storage at -80^{o}C. The embryos were removed from the shell, killed via decapitation and total body weight was recorded. Pectoralis major tissue was dissected and stored in RNA*later* (Ambion Inc., Grand Island, NY) at -20^{o}C for total RNA extraction, or snap frozen in liquid nitrogen and stored at -80^{o}C for total protein extraction.

Serum ammonia analysis

Serum samples were analyzed in duplicate for ammonia concentration using an ammonia assay kit (AA0100; Sigma Aldrich, St. Louis, MO), which determines the amount of ammonium ions present in a sample based on a relative decrease in absorbance after an enzymatic reaction. The enzyme, L-Glutamate dehydrogenase, was diluted 1:4 with 0.1 M Phosphate buffer (KH_2PO_4; pH 7.5) as needed prior to each assay. The working volume of reagent blank, test, and standard reactions were reduced by a factor of 5 to accommodate the

working volume of a 96-well plate. Absorbance was measured at 340 nm 5 minutes after the reagent was mixed with standard, sample, or water. The diluted enzyme was mixed (10 µL) into each well and allowed to incubate at room temperature for 5 minutes. Absorbance readings (340 nm) were taken in 1 minute intervals for 6 minutes. Change in absorbance was calculated based on the manufacturers' guidelines and ammonia concentration was calculated using the following equation provided for the ammonia assay kit (Sigma Aldrich, St. Louis, MO):

$$\Delta(\Delta A340)\text{Test or Standard} = \Delta A340(\text{Test or Standard}) - \Delta A340(\text{Blank})$$

$$\text{mg of } NH_3\text{/ml of original sample} = \frac{(\Delta A)\,(\text{Total Assay Volume})\,(\text{Sample Dilution Factor})}{(\text{Sample Volume})} \times 0.00273$$

RNA extraction, reverse transcription, and quantitative real time-PCR

Total RNA was isolated using the RNeasy Mini Kit protocol (Qiagen, Venlo, Limburg). Approximately 30 g of RNA*later* (Ambion Inc., Grand Island, NY) preserved pectoralis muscle was placed in 600 µL of the provided buffer for homogenization using a Mini-Beadbeater-1 (BioSpec Products, Bartlesville, OK). Total RNA concentration was determined by measuring absorbance at 260 nm. RNA quality was assessed using agarose gel electrophoresis. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Grand Island, NY) using the reverse transcriptase from the murine leukemia virus and random hexamers. After the reverse transcription reaction was complete, cDNA was diluted 1:20 before qPCR.

Five samples for each treatment (ED17 AA, ED17 C, ED19 AA, ED19 C) were prepared for qPCR analysis of MSTN, MyoD, MYOG, MyF5 and MRF4 genes. These genes

were chosen because they are known to play a critical regulatory role in muscle development and differentiation. β-actin was used as an internal control for normalization of each sample because our preliminary data showed no change in expression under our experimental conditions (data not shown). Primers were designed using Primer-BLAST (NCBI) which utilizes Primer3 software (Table 2.1). qPCR was performed at a volume of 20 µL, consisting of 1 µL diluted cDNA, 1 µL diluted primer (400 µM), 10 µL power SYBR Green PCR Master Mix (Applied Biosystems Inc., Grand Island, NY), and 8 µL ultra pure H_2O. For each reaction, the thermocycler (Applied Biosystems Inc., Grand Island, NY) was set to the following cycle, based upon optimum temperatures identified by a gradient reaction: 95^{o}C for 10 min, then 40 cycles of 95^{o}C for 30 sec denaturing, 62^{o}C for 30 sec annealing, 72^{o}C for 5 min extension. Standard curves for amplification efficiency were produced for each set of primers by performing serial dilutions of pooled cDNA (1:5, 1:25, 1:125, 1:625). At each extension step, fluorescence was measured and the cycle threshold (Ct) was calculated by the StepOne software (version 2.1, Applied Biosystems Inc., Grand Island, NY). All experiments were run in triplicate. Fold changes were calculated by method described by Pfaffl (2001).

Table 2.1 Primer sequences for real-time qPCR

Primer[1]	Sequence	% Eff	bp size	Spans an Exon/Exon Boundary
MSTN	F 5'-CGGAGAATGCGAATTTGTGTT-3'	102	110	NO
	R 5'-GGGACATCTTGGTGGGTGTG-3'			
MyoD	F 5'-CGCAGGAGAAACAGCTACGA-3'	103	104	YES
	R 5'-ATGCTTGAGAGGCAGTCGAG-3'			
MyF5	F 5'-TGAGGGAACAGGTGGAGAACT-3'	115	185	YES
	R 5'-ACTCTGCTCCGTCGCGTA-3'			
MYOG	F 5'-CAGCCTCAACCAGCAGGAG-3'	96	166	YES
	R 5'-ACTGCTCAGGAGGTGATCTG-3'			
MRF4	F 5'-AGGCTGGATCAGCAGGACAAAA-3'	102	139	YES
	R 5'-CGCGGGAATGGTCGGAAG-3'			
β-ACT	F 5'-GTCCACCTTCCAGCAGATGT-3'	94	168	NO
	R 5'-TAAAGCCATGCCAATCTCG-3'			

[1]Primers were designed using Primer-BLAST for myostatin (MSTN), myogenic determination factor 1 (MyoD), myogenic factor 5 (MyF5), myogenin (MYOG), myogenic regulatory factor 4 (MRF4), and β-actin (β-ACT).

Protein extraction, SDS-PAGE, and Western blot analysis

Total protein was extracted using a modification of methods previously described (Dasarathy et al., 2004). Muscle samples (25 mg) were homogenized in 250 µL cold lysis buffer (20 mM Tris-HCL pH 7.5, 10 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$) using a bead beater and immediately placed on ice. The homogentate was centrifuged at 12,000 rpm for 4 min, returned to ice for 4 min, and centrifuged again for an additional 4 min to remove undissolved tissue debris. The supernatant was transferred to a new tube and placed on ice to prepare for the protein assay. In preparation for the protein assay, each sample was diluted 1:4 in lysis buffer to a volume of 25 µL for duplicate readings. Protein concentration was

measured using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories Inc., Richmond, CA), which produces an absorbance shift relative to the amount of solublized protein as Coomassie blue dye binds to protein, using Bovine Serum Albumin as a standard.

Loading volumes for each sample were calculated, considering a dilution factor of 5, to an amount of 20 µg protein per well. Samples were boiled 1:1 in Laemmli buffer (Bio-Rad Laboratories Inc., Richmond, CA) and separated by SDS-PAGE using a 10% Mini-PROTEAN TGX precast gel (Bio-Rad Laboratories Inc., Richmond, CA) with a 10-245 kDa Prism Ultra Protein Ladder (Abcam, Cambridge, MA). The protein was transferred to a polyvinylidene difluoride membrade (**PVDF**) via wet transfer, using a 0.02 M Tris/0.15 M glycine buffer with 18% methanol, at 400 mA for 1 hour. The membrane was Ponceau S stained to observe loading and transfer accuracy, then destained using ultra pure water.

The membrane was blocked with 5% goat serum (Life Technologies Corporation, Carlsbad, CA) in TBS-T (50mM Tris HCL, 200 mM NaCl, pH 7.5, 0.5% Tween-20) for 1.5 hrs on an orbital shaker at room temperature. The primary antibody for MSTN (Abcam, Cambridge, MA) is a rabbit polyclonal to human MSTN C-terminal peptide. The MSTN primary antibody was diluted 1:250 in 5% goat serum and incubated at 4^{o}C overnight. Three washes with TBS-T, 10 min each, followed primary antibody incubation. The secondary antibody used to detect the MSTN primary antibody is a goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP; Abcam, Cambridge, MA). The secondary antibody was diluted 1:500 in 5% goat serum and incubated at room temperature on an orbital shaker for 1.5 hrs. The membrane was washed three times with TBS-T, 10 minutes each wash. HRP

activity was detected using chemiluminescent reagent (Bio-Rad Laboratories Inc., Richmond, CA). The membrane was stripped of the MSTN antibodies using an acidic glycine buffer, as outlined in the Abcam mild stripping protocol (Abcam, Cambridge, MA). Chemiluminescent reagent was used to confirm the antibodies had been removed from the membrane.

After the PVDF membrane was stripped of the antibodies detecting myostatin, the membrane was re-blocked with 5% goat serum, washed, and re-probed with β-actin for a loading control. The primary antibody for β-actin (Thermo Scientific, Rockford, IL) is a mouse monoclonal to β-actin N-terminal peptide. The β-actin antibody was diluted 1:1000 in 5% goat serum and incubated overnight at 4^{o}C. Following primary antibody incubation, the membrane was washed three times with TBS-T, then probed with goat anti-mouse IgG secondary antibody conjugated with HRP (SouthernBiotech, Brimingham, AL), diluted 1:10,000 in 5% goat serum, for 1.5 hours on an orbital shaker at room temperature. Three washes with TBS-T were performed and HRP was detected using Chemiluminescent reagent. Semi-quantitative analysis of protein was determined by band intensity using Kodak 1D Scientific Imaging Systems (v.3.6.2, New Haven, CT). MSTN intensity of each band was normalized to β-actin prior to comparing experimental and control samples.

Statistical Analysis

Statistical analysis was performed using JMP Pro (v.10.0.0, SAS Institute Inc., Cary, NC) and SAS (v. 9.4, SAS Institute Inc., Cary, NC). All experiments were performed in triplicate and data is presented as mean ± SE. Significance ($P < 0.05$) was determined by oneway ANOVA. PCR results of mRNA content are expressed as mean fold-changes relative to the internal control, β-actin, ± SE.

RESULTS

Ammonia assay

Eggs injected with ammonium acetate had a higher serum ammonia level in both the ED17 and ED19 groups when compared to the controls. The ED17 collected samples (n=6 per treatment) were found to have more than four-fold increased ($P < 0.0001$) serum ammonia concentration in ammonium acetate administered embryos compared to controls (Figure 2.1A). Similarly, the ED19 group (n=5 per treatment) had a ten-fold increase in serum ammonia concentrations ($P < 0.05$) when comparing ammonium acetate administered embryos to control injected embryos (Figure 2.1B).

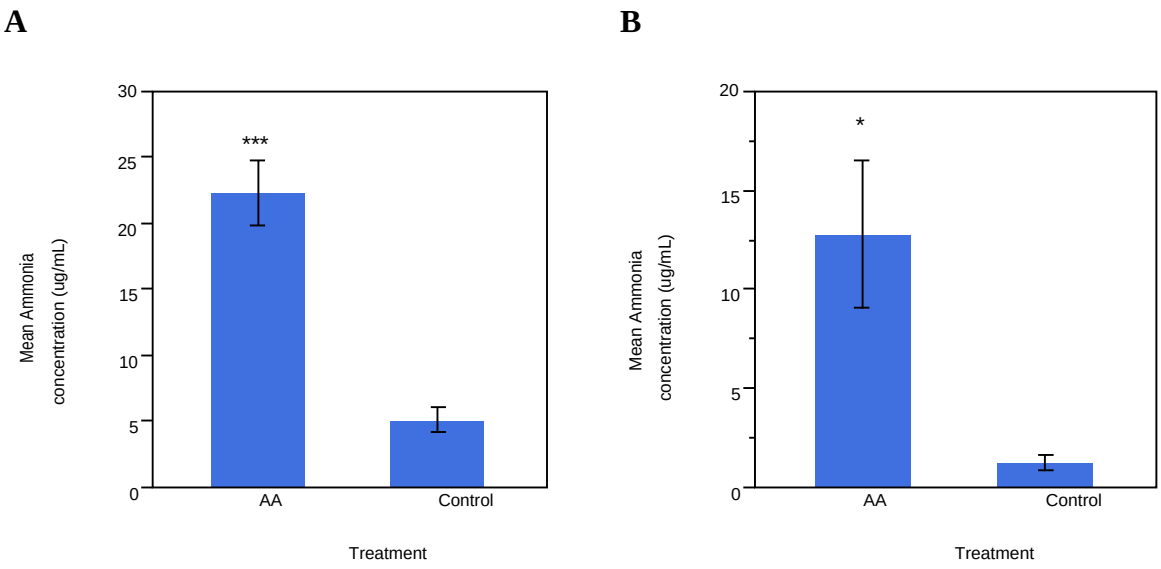


Figure 2.1 Serum ammonia concentrations. Serum ammonia concentrations taken from ammonium acetate and control injected embryos 12 hours after the last injection on A) ED17 or B) ED19. Bars represent mean ammonia concentration (µg/mL) ± SE. *** Indicates significance of ($P < 0.0001$); * Indicates significance of ($P < 0.05$).

qPCR

In both groups, the increase in serum ammonia concentration was accompanied by a highly significant reduction in MSTN expression (ED17 $P < 0.01$; E19 $P < 0.0001$). Figure 2.2 summarizes the fold changes of mRNA expression of each gene. ED17 collected samples showed a significant increase, in MyF5 expression ($P < 0.05$); while there was no significant difference in MyF5 expression in ED19 collected samples. In both groups, MRF4 expression was decreased with increased serum ammonia concentration (ED17 $P < 0.05$; ED19 $P = 0.05$). No significant difference was found in MyoD or MYOG expression in either sample population.

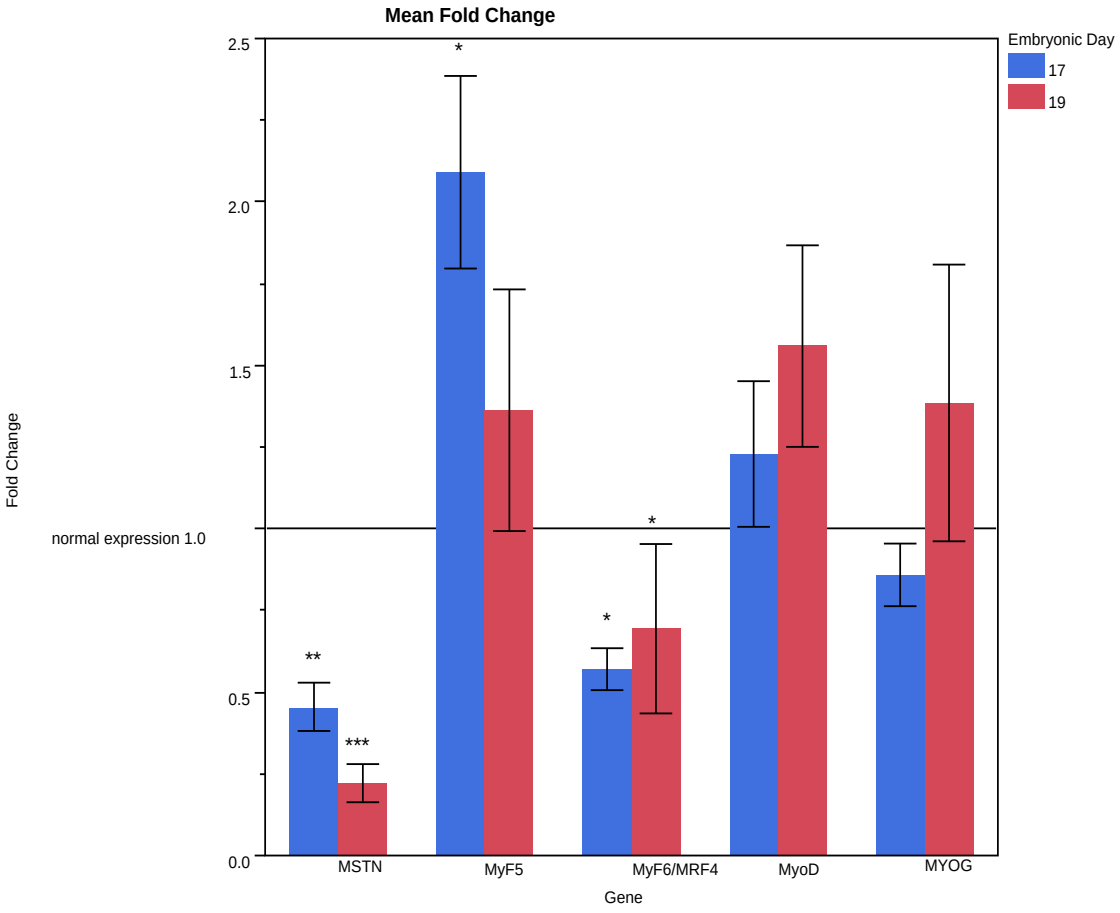
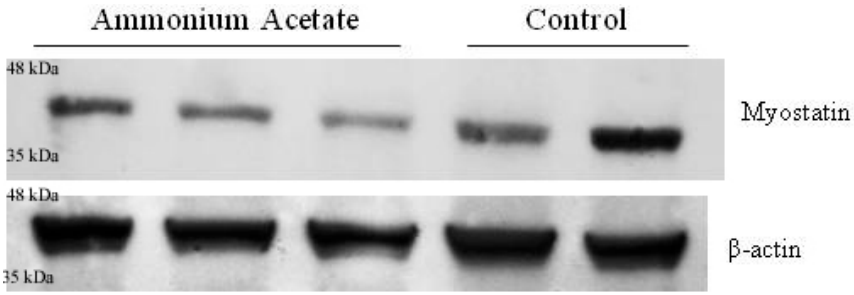


Figure 2.2 mRNA expression of MSTN and MRFs. RNA was isolated from ED17 and ED19 collected pectoralis major samples after 48 hours of induced hyperammonemia (four injections of [50 mmol/kg] ammonium acetate solution). mRNA expression was measured by quantitative real-time PCR and each sample was normalized using β-actin as an internal control. Bars represent the mean fold change ± SE. *** indicates significance of ($P <$ 0.001); ** indicates significance of ($P < 0.01$); * indicates significance of ($P < 0.05$).

Western blot analysis

MSTN protein expression was detected by Western blot and normalized to β-actin for relative quantification and statistical significance. The MSTN band assessed is approximately 43 kDa. ED17 ammonium acetate injected samples had a lower amount of MSTN expression when compared to the control injected samples (Figure 2.3A). Relative intensity measurements confirm the visual observation of decreased MSTN protein ($P < 0.05$) in experimental samples (Figure 2.3B). Similarly, the ED19 experimental samples showed a lower amount of MSTN expression, when the β-actin loading control showed consistent results between the two treatment groups (Figure 2.4A). The relative mean intensity of experimental samples was decreased ($P < 0.05$) as compared to controls (Figure 2.4B).

A



B

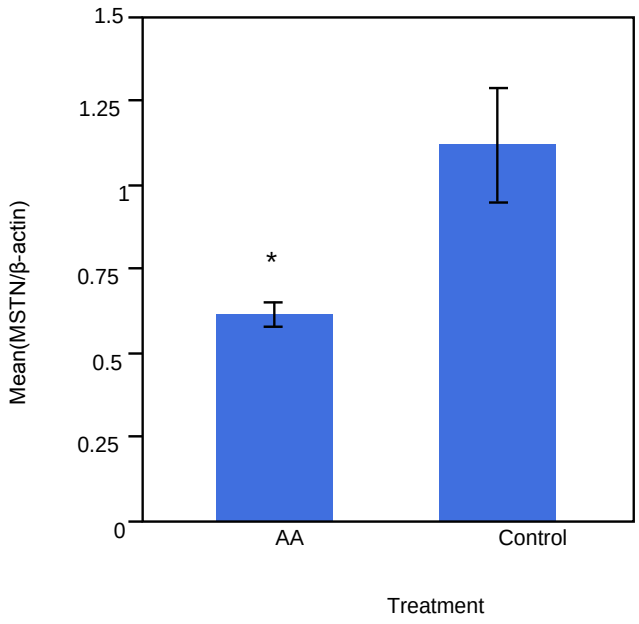
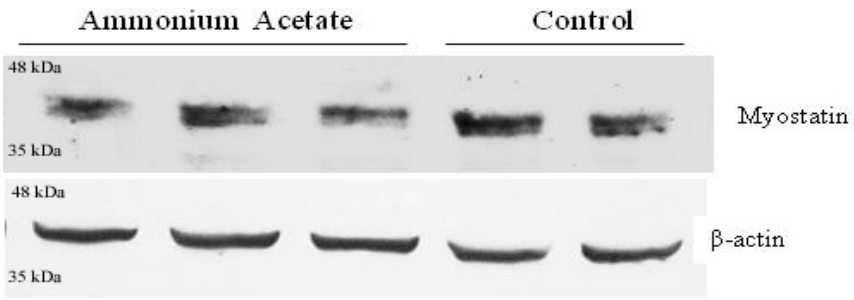


Figure 2.3 Western blot analysis of MSTN protein in ED17 collected samples. A) PVDF membrane images of MSTN and β-actin bands. B) Relative intensity measurements of PVDF membrane images. Bars represent the mean relative intensity, normalized to β-actin, ± SE. * indicates a significance of ($P < 0.05$).

A



B

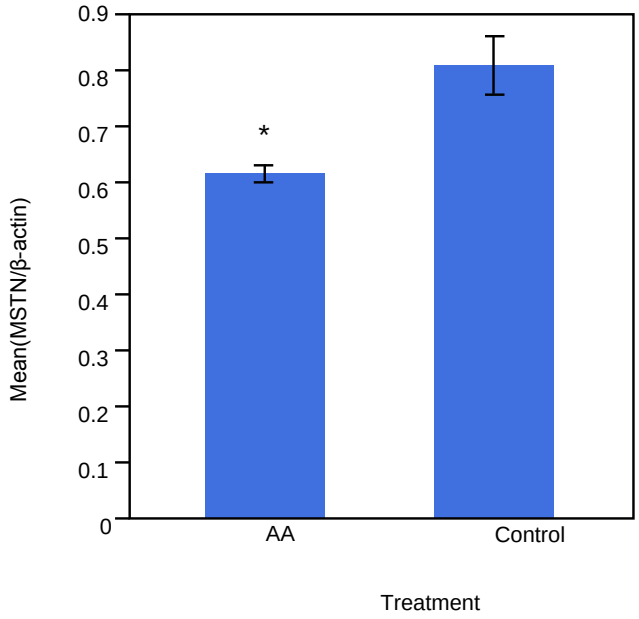


Figure 2.4 Western blot analysis of MSTN protein in ED19 collected samples. A) PVDF membrane images of MSTN and β-actin bands. B) Relative intensity measurements of PVDF membrane images. Bars represent the mean relative intensity, normalized to β-actin, ± SE. * indicates a significance of ($P < 0.05$).

DISCUSSION

The present study demonstrates that ammonia in broiler embryos may have an effect different from previous reports of murine models under hyperammonemic conditions because hyperammonemia in ovo resulted in a lower expression of MSTN (Dasarathy et al., 2004; Qiu et al., 2012; Qiu et al., 2013). Additionally, myogenic regulatory factor expression suggests an embryonic environment that supports myoblast proliferation and muscle growth. Agricultural and medical research greatly supports the importance of the embryonic environment and MRF expression on myogenesis and ultimate muscle growth. During embryonic development, MyoD, MyF5, MYOG, and MRF4 are crucial for determination, proliferation, and differentiation of myoblasts (Rudnicki et al., 1992; Rudnicki et al., 1993; Olson et al., 1996). Though redundancy has been noted in their specific functions during myogenesis, each MRF plays an important role in muscle development and regulation of transcription factor expression (Rudnicki et al., 1993; Olson et al., 1996). In post-natal and adult muscles, MRFs are responsible for muscle growth and serve as indicators of satellite cell activity, specifically satellite cell proliferation and differentiation (Muroya et al., 2002; Dasarathy et al., 2004).

Conversely, previous research has suggested that MSTN is a powerful negative regulator of myogenesis and is highly expressed in muscle wasting conditions (McPherron and Lee, 1997; Langley et al., 2002; Dasarathy et al., 2004; Han and Mitch, 2012). Livestock with MSTN mutations are of great interest due the double muscling phenotype characterized in MSTN-null individuals. However, due to disadvantages, like increased incidence of dystocia, as a result of large fetus size, insufficient reproductive capabilities, and

varying reports of impact on meat quality, selecting for MSTN-null animals for meat production has not become a commercially relevant practice (Wiener et al., 2009; Hope et al., 2013). Highly increased expression of MSTN has been identified in adult muscles of individuals exhibiting severe muscle atrophy (Dasarathy et al., 2004; Han and Mitch, 2012; Qiu et al., 2013). MSTN regulation, as it applies to medical research, could be a potential therapy for individuals suffering from sarcopenia and cachexia as a side effect of a number of diseases. Therefore, understanding the mechanisms that regulate MSTN expression is exceedingly valuable for both agricultural and medical research.

The aim of this study was to investigate changes in chick embryonic myogenesis under conditions of increased serum ammonia concentration, as seen in cirrhotic patients. Based upon previous studies reporting increases in plasma ammonia concentration negatively effecting satellite cell proliferation, via increased MSTN expression and reduced expression of proliferation factors, the present study aimed to understand the effects of hyperammonemia in late stage broiler embryos (Dasarathy et al., 2004; Qiu et al., 2012). During late chick embryonic development a transition from fetal myoblasts to adult myoblasts occurs. By ED18, adult myoblasts, also called satellite cells, become the predominant type of myoblasts, and are virtually exclusive at hatch (Hartley et al., 1992). Altering the proliferative activity of satellite cells, which provide essential nuclei to growing post-hatch myofibers, affects the reservoir of myogenic cells available for myonuclear donation and can have an impact post-hatch growth. The ammonium acetate dosage and frequency of administration was empirically derived, using non-toxic intraparitoneal dosages from murine methods (2.5 mmol/kg) and intraparitoneal LD_{50} toxicity observed in 4-week-

old chicks as guidelines for an initial low (2 mmol/kg) and high (10 mmol/kg) amount of ammonium acetate to be administered (Wilson et al., 1968; Yonden et al., 2010). The average body weight (12g) of ten ED15 embryos was used to calculate dosages for all injections. Empirically, it was determined that a dose of 50 mmol/kg every twelve hours imparts a significant increase in serum ammonia concentration without any adverse effects on the embryo.

Previous studies, using portacaval anastamosis (**PCA**) rats as a model, investigating the mechanisms of muscle wasting secondary to cirrhosis have demonstrated a strong correlation between hyperammonemia and increased MSTN expression (Dasarathy et al., 2004; Qiu et. al., 2013). Therefore, it was expected that increasing serum ammonia in broiler embryos would upregulate MSTN expression and have a negative effect on muscle growth. The ammonium acetate administration protocol was successful in increasing serum ammonia concentration that is consistent with previous reports of inducing hyperammonemia in murine models (Kosenko et al., 2004; Qiu et al., 2013). These authors induced hyperammonemia in adult animals and reported adverse effects. However, the current results show a significant downregulation of MSTN mRNA expression in both ED17 and ED19 collected samples under induced hyperammonemia. Western blot analysis on MSTN protein content confirmed the downregulation of mRNA observed by qPCR in pectoralis samples obtained from embryos with increased serum ammonia concentration.

Potential reasons for suppression of MSTN by hyperammonemia include the relatively short term exposure to hyperammonemia compared to the previously reported PCA rat model with a long term hyperammonemic condition or the murine model with induced

hyperammonemia (Qiu et al. 2012). Also, the primary mechanism of excess ammonia excretion in mammals is producing urea to be eliminated as a urinary waste product. Avian species do not have a developed urea cycle and the principal mode of ammonia disposal is through uric acid generation (Wiggins et al., 1982). Administration of low levels of ammonium acetate to the embryonic environment may have provided substrate, acetic acid, for acetyl coenzyme A synthesis, which is oxidized via the citric acid cycle for energy production and increased nitrogen availability for protein synthesis. Additionally, expression of glutamine synthetase is high in avian tissue and the glutamine synthesized is used for purine and uric acid biosynthesis (Campbell and Vorhaben, 1976). Glutamine synthesis is driven by the reaction between ammonia and α-ketoglutarate from the tricarboxylic acid cycle to generate glutamate that combines with another molecule of ammonia to generate glutamine (Hod et al., 1982). Glutamine synthesis is potentially a major mechanism of skeletal muscle ammonium detoxification (Thompson and Wu, 1991; He et al., 2010). Glutamine also plays an important role in the metabolic activity of muscle and has been reported to inhibit MSTN expression (Hickson et al., 1995; Salehian et al., 2006; Bonetto et al., 2011). Thus increased skeletal muscle glutamine synthesis in response to hyperammonemia may be responsible for reduced MSTN expression in the broiler embryos. Furthermore, glutamine has been shown to upregulate gluconeogenesis via increased expression of phoshoenolpyruvate carboxykinase (**PEPCK**), a rate limiting enzyme of the gluconeogenesis pathway, providing energy in the form of glucose to the developing embryo (Lavoinne et al., 1996). This model needs to be evaluated further since it provides a potential mechanism of MSTN suppression and increased muscle yield in the avian system.

In addition to downregulating MSTN expression, a significant increase in MyF5 was observed in ED17 collected samples. Increased expression of MyF5 during myogenesis supports myoblast proliferation, which is determined during embryonic development. The downregulation of MRF4, which is expressed in mature myocytes, further suggests that ammonium acetate administration increases the time that myoblasts and satellite cells are active. Increasing myoblast activity, and ultimately increasing myofiber number, during development not only provides a greater potential for postnatal muscle growth, but may be a more efficient manner to increase ultimate meat yield in poultry. Given these interesting results, it would be necessary to examine the functional impact of these transcriptional changes induced by hyperammonemia on skeletal muscle mass, protein content, translational efficiency, rates of protein synthesis and breakdown in the embryos.

The results of the current study suggest that increased serum ammonia concentration in developing broiler embryos leads to a reduction of MSTN. Muscle mass is positively affected by reductions in MSTN expression. Therefore, it is possible that a transient reduction in MSTN expression in the embryo can increase post hatch muscle mass. An investigation of the downstream effects on growth, and an understanding of the biochemical pathway that leads to a transient downregulation of MSTN expression are needed to optimally employ these unanticipated findings as a strategy for improving muscle growth and ultimate meat yield in poultry.

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CHAPTER 3

SUMMARY

The embryonic environment is crucial to myogenesis and ultimately determines maximum potential skeletal muscle growth. The process of somatic progenitor cells becoming specified myoblasts, and subsequently differentiated myocytes is intricately controlled by *Pax* genes, myogenic regulatory factors, and inhibitors, such as myostatin. Initially expressed are the primary myogenic regulatory factors, myogenic factor 5 (**MyF5**) and myogenic determination factor 1 (**MyoD**), which are responsible for the commitment of somatic progenitor cells to become myoblasts (Rudnicki et al., 1993; Rudnicki et al., 1993). Without expression of Pax-3, the migration of somatic progenitor cells to the developing limb buds does not occur, and is therefore essential to skeletal muscle growth (Tajbakhsh et al., 1997). The secondary myogenic regulatory factors, myogenin and myogenic regulatory factor 4 (**MRF4**) are responsible for myoblast differentiation and maturation of myocytes, which are the functional units of mature muscles. This process, which only occurs in the developing embryo, determines the number of mature muscle fibers present in the adult animal. Post-natal muscle growth relies on an increase in myofiber size, or hypertrophy.

Post-natal hypertrophy occurs, largely, as a result of myonuclear accretion, or the increase in DNA units via satellite cell donation (Schultz, 1996; Mozdziak et al., 1997). Satellite cells, which become specified, in part, as a result of Pax-7 expression during embryonic myogenesis are mononucleated cells that, unlike mature myocytes, are mitotically active allowing them to maintain a population of cells for myonulear donation during muscle growth and regeneration (Mozdziak et al., 1997; Seale et al., 2000). Therefore, satellite cell

health and activity is as important to post-natal growth as myogenic regulatory factor and Pax expression is during myogenesis.

Myostatin, a gene predominantly expressed in skeletal muscle and a widely known inhibitor of muscle development and growth, has negative effects on myogenesis and post-natal growth via downregulation of myogenic regulatory factors and inhibition of satellite cell activity (McPherron et al., 1997; Thomas et al., 2000; McCroskery et al., 2003). Additionally, myostatin has been linked to muscle wasting conditions in a wide variety of diseases, including cancer, congestive heart failure, and cirrhosis (Dasarathy et al., 2004; Han and Mitch, 2012). In cirrhosis, specifically, a strong correlation has been drawn between hyperammonemia and sarcopenia, where increased myostatin expression has been observed, and is thought to be the cause (Dasarathy et al., 2004; Qui et al., 2012; Qiu et al., 2013). The effect of hyperammonemia on embryonic development has varying reports (Bishonga et al., 1996; McEvoy et al., 1997; Hammon et al., 2000; He et al., 2007).

The objective of the current thesis research was to observe the effects of inducing hyperammonemia in broiler embryos on myogenesis, particularly myostatin and myogenic regulatory factor expression. Contrary to previous studies of hyperammonemia, the current results suggest that inducing hyperammonemia in broiler embryos clearly downregulates myostatin expression. This finding, along with an increase in MyF5 expression, supports a hyperplasic embryonic environment, which could have a positive impact on post-natal muscle growth and ultimate meat yield. As these results were unexpected, based upon previous studies examining how hyperammonemia effects the expression of these genes, the

mechanism that results in decreased myostatin expression, by manipulation of the embryonic environment, deserves further investigation.

The concept of developmental programming of the fetus and neonate has been a topic of general knowledge, and has been highly targeted in both human disease prevention and the maximization of various agricultural practices for many years. An inadequate fetal environment, for even a short time during development, has the potential to permanently alter anatomical structure, physiology, and metabolism of the neonate through adulthood. Early ecological studies revealed that poor maternal nutrition is linked to high incidence of a variety of diseases in offspring, including coronary heart disease, diabetes, hypertension, and obesity (Langley-Evans, 2006). More recent studies have revealed that inadequate fetal environments can result in permanent changes in gene expression. For example, offspring of rats fed a low protein diets during gestation developed hypertension, which persisted through adulthood, due to reduced angiotensin II type 2 receptor expression (McMullen et al., 2004). Additionally, Markham et al. (2009) reports that increasing maternal feeding during designated times of fetal primary and secondary muscle fiber formation can change the proportions of fiber-type, resulting in increased oxidative capacity. These studies are testament to the theory that the embryonic environment provides opportunity to have both deleterious and beneficial impacts on the fetus throughout life.

The induction of hyperammonemia in broiler embryos during myogenesis has produced alterations in gene expression that have promising potential for a positive downstream effect on post-hatch muscle growth and ultimate meat yield. Therefore, determining the actual effect on post-hatch growth and meat yield at market age is of initial

importance. Additionally, it is imperative to reveal the mechanism that regulation of myogenesis is achieved, via inducing hyperammonemia, to develop a practical approach for benefiting from these findings. One proposed mechanism to investigate is whether or not increased glutamine production was a compensatory mechanism for eliminating increased concentrations of ammonia in the blood. Glutamine synthetase is high in avian tissues, as the production of glutamine is an intermediate of uric acid synthesis (Campbell and Vorhaben, 1976). Also, glutamine production has been previously shown to be a mechanism of ammonia detoxification in skeletal muscle (Thompson and Wu, 1991). Incidentally, glutamine administration has been reported to reverse muscle wasting, as seen with excess glucocorticoid administration, and was found to inhibit myostatin expression (Hickson et al., 1995; Salehian et al., 2006; Bonetto et al., 2011). In addition to positively impacting muscle growth via downregulating myostatin during myogenesis, increased glutamine production may increase gluconeogenesis (Lavoinne et al., 1996). This may be of particular importance during late stages of embryonic development, as investigated in this study, as increasing gluconeogenesis would provide glucose, an energy source, in preface to a time, immediately post-hatch, where fasting occurs in commercial production and metabolic energy requirements are only partially met by incorporation of the remaining yolk sac (Halevy et al., 2000). Future studies focusing on the mechanistic pathway by which this brief induction of hyperammonemia decreased myostatin expression, and the downstream effect of the transient decrease in myostatin expression on muscle growth are necessary to employ these findings to improve poultry production.

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