

## ABSTRACT

MIRAMONTES, EMILY HEATHER. Myogenic Response to Increasing Concentrations of Ammonia Differs Between Mammalian, Avian, and Fish Species. (Under the direction of Dr. Paul Mozdziak).

Fish skeletal muscle differs from mammalian and avian muscle in several ways. Most notably, fish skeletal muscle utilizes both hypertrophy and hyperplasia in post-natal growth, while mammals and avians exclusively utilize hypertrophy after embryonic development. Fish skeletal muscle is made of three types of fibers: red, white, and pink muscle fibers. Pink muscle fibers are an intermediate muscle fiber with physiological and functional properties that are between red and white fibers. Not all fish have pink muscle fibers, and the presence of pink fibers largely depends on the swimming needs and size of the fish species. The molecular regulation of skeletal muscle growth is largely conserved between birds, mammals, and fish. Myostatin, a negative regulator of muscle growth, is more diffusely expressed and has multiple isoforms in fish, indicating a potential for additional functions of the gene for fish as compared to mammals and avians. High levels of ammonia are toxic to all three species and can lead to death. All three species utilize different methods of ammonia detoxification and excretion. Mammals are ureotelic and excrete ammonia as urea, avians are uricotelic and excrete ammonia as uric acid. Most fish are ammoniotelic and excrete ammonia across the gills into the water. Some fish that live in extreme environments utilize ureotelic methods of excretion in conjunction with ammoniotelic strategies, or solely as urea. In mammals and avians, hyperammonemia has been linked to changes in myostatin expression. Mammalian skeletal muscle responded negatively to increased myostatin, while avians responded positively at 10 mM of ammonia. The effects of ammonia on fish skeletal muscle and myostatin expression has not been previously studied.

This study examines how murine, avian, and fish cells respond to increasing levels of ammonia up to 50 mM. C2C12, primary chick, and primary tilapia myoblast cells were cultured and then exposed to 10 mM, 25 mM, and 50 mM ammonium acetate, sodium acetate, and an untreated control for 24 hours. High levels of ammonia were detrimental to the C2C12 cells, causing increased MSTN expression and decreased myotube diameters between 10 mM and 25 mM ( $P < 0.002$ ) ammonium acetate. Ammonia at 10 mM continued the positive myogenic response in the chick, with lower MSTN expression than the C2C12 cells and larger myotube diameters, but myotube diameter at 50 mM ammonium acetate was significantly smaller than those at 10 mM and 25 mM ( $P < 0.001$ ). However, chick myotubes at 50 mM were still significantly larger than the sodium acetate treated and untreated control ( $P < 0.001$ ). The tilapia cells showed no significant difference in MSTN expression or myotube diameter in response to increasing the concentrations of ammonia. Comparing the three species, C2C12 cells had higher expression of MSTN than the chick and tilapia cells, while the chick and tilapia cells showed no significant difference in MSTN expression ( $P < 0.004$ ). Overall, these results confirm that increasing concentrations of ammonia are detrimental to mammalian skeletal muscle, while chick cells responded positively at lower levels, but began to exhibit a negative response at higher levels, while the tilapia were able to metabolize ammonia and experience no detrimental effects to muscle. Future studies should examine the effects of ammonia on skeletal muscle and myostatin expression of different fish species, since fish differ in their skeletal muscle properties and myostatin expression patterns.

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Myogenic Response to Increasing Concentrations of Ammonia Differs Between Mammalian,  
Avian, and Fish Species

by  
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## **BIOGRAPHY**

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# CHAPTER 1

## LITERATURE REVIEW

### Introduction

Elevated levels of ammonia may lead to sarcopenia, or muscle wasting, and this has been linked to increased myostatin expression, a negative regulator of muscle growth. Most of the pre-existing research has focused on mammalian and avian models for examining the mechanism of ammonia toxicity, but many fish have a higher ammonia tolerance, such as the gulf toadfish, oyster toadfish, African sharptooth catfish, and multiple mudskippers (Wang & Walsh, 1999; Wee et al. 2007; Ip et al., 2005). Fish also utilize a different mechanism of ammonia excretion than that of mammals and avians. Understanding the underlying physiology of fish ammonia metabolism, and the effect of ammonia on fish skeletal muscle and other organ systems could lead to therapeutic approaches to hyperammonemia secondary to liver disease in human medicine. Since fish are an important source of food, understanding the impact of ammonia on skeletal muscle mass and quality is also important for optimizing feed and environment of fish used for food production.

### Skeletal Muscle Fibers

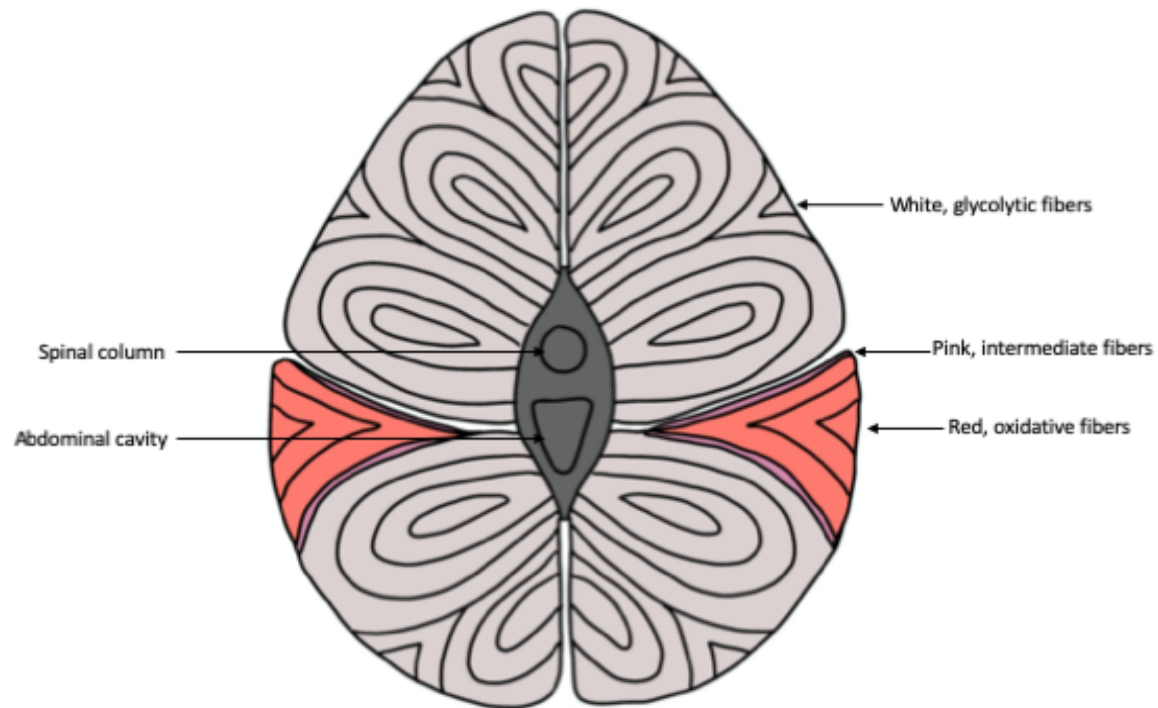
There are 3 main classifications of muscle fiber types in the fish: white, red, and pink muscle fibers (Fig. 1.1) (Sanger & Stoiber, 2001). White muscle composes approximately 70% or more of the myotome of most fish, and are typically the largest of the fibers, ranging from 50-100  $\mu\text{m}$  (Greer-Walker, 1975). White muscle runs the length of the fish, with the largest percentage of the muscle existing caudally (Zhang et al, 1996; Rome et al., 1993). Similar to mammals, white muscle is classified as such due to its low levels of myoglobin, vascularization, and mitochondria content (Bone, 1978). This type of muscle is typically recruited during faster

swimming states, and short burst swimming sessions (McKenzie, 2011; Jayne & Lauder, 1994). Due to the fast nature of this fiber type, it utilizes mostly anaerobic breakdown of muscle glycogen to supply energy (Driedzic & Hochachka, 1976).

Converse to white muscle, red muscle is categorized as slow-twitch muscle and is named for its high myoglobin content, and high vascularization (Bone, 1978). In fish, red muscle typically comprises at most 10% of the myotome and is only a thin section along the lateral line of the fish (Greer-Walker, 1975). In contrast to white muscle, the distribution of red muscle is higher anteriorly, and decreases caudally, and utilizes aerobic metabolism to sustain longer bouts of swimming, typically at lower speeds than white muscle (Videler, 1993; McKenzie, 2011; Jayne & Lauder, 1994). Studies done on scup found that red muscle is recruited differently at different points along the body for maximal power, indicating there could be some species differentiation in when and where red muscle is recruited for maximal power swimming (Rome et al., 1993; Zhang et al., 1996). The differences between species in muscle fiber recruitment strategies could be due to the large variation in body size and shape as well as differences in swimming type needs between species.

In fish there has been identified a third muscle fiber type, pink muscle, that is located in between the white and red muscle sections (Rowlerson et al., 1985). It is aptly named, as this muscle type is situated in between red and white muscle fibers in most all aspects of its physical appearance and physiological functioning (Johnston, Davison, & Goldspink, 1977; Rowlerson et al., 1985). Pink muscle fibers are typically recruited the most at intermediate swimming speeds as well as alongside red muscle fibers for sustained swimming at high speeds (Johnston, Davison, & Goldspink, 1977). The amount of this fiber type, if it exists in a particular species at all, varies and depends on the swimming needs of that particular species (Rowlerson et al.,

1985). Pink fibers can be identified histochemically through the fiber's stability during alkaline pre-incubation before myofibrillar ATPase staining (Johnston et al., 1974).



**Figure 1.1.** A diagram of a cross-section of a fish body showing the distribution of the fibers throughout the myotome. The thin section of pink muscle fibers in between the white and red muscle fibers is not present in every fish species.

Mammals and avians on the other hand classify muscle fibers slightly differently. Similar to the fish, mammalian muscle fibers are classified either red or white based on appearance during staining and their metabolic properties (Schiaffino & Reggiani, 2011). However, while fish have the intermediate pink muscle fibers, some red and white muscle fibers in mammals exhibit metabolic properties of either red or white fibers but have intermediate twitch speeds (Gerrard & Grant, 2003). On the other hand in avians, muscle fibers are primarily classified by their metabolic properties, using ATPase staining to identify fast and slow twitch muscles and SDH staining to identify their metabolic state (Nierobisz, Hentz, Felts, & Mozdziak, 2010; Wiskus et al. ,1976). Avians and mammals also differ from fish in their distribution of muscle

fibers. While fish have more distinct sections of red and white muscle, avian muscle is more heterogenous, and muscle groups have a mixture of all three types of muscle fiber in a single muscle (Pette & Staron, 2000).

### **Skeletal Muscle Growth**

Skeletal muscle accounts for approximately 60% of total body mass in fish (Sanger & Stoiber, 2001). Alongside its main role of facilitating movement, skeletal muscle also plays a role in metabolism, hormonal control, and blood sugar control. Fish muscle also plays an important role in food production and the growth and quality of fish skeletal muscle is an interest of the aquaculture industry (Vélez et al., 2017). Muscle growth and development in fish species is unique from that of mammals; most notably is the difference in postnatal growth between fish and mammals. Two types of growth are noted: hyperplasia and hypertrophy. Hyperplasia is defined as an increase in cell number, and hypertrophy, an increase in cell size. In fish, both hyperplasia and hypertrophy persist throughout post-natal growth in to adulthood (Mommensen, 2001; Johnston et al., 2001), while mammals exhibit hyperplasia in embryonic skeletal muscle growth, and hypertrophy in post-natal growth (Wigmore & Stickland, 1983; Bodine et al., 2001). Studies done in rainbow trout and carp revealed the persistence of hyperplasia in to adulthood, with slight differences in which type of growth predominates based on body length, fiber type, and the location of muscle sample studied (Stickland, 1983; Koumans et al., 1993a; Koumans et al., 1994).

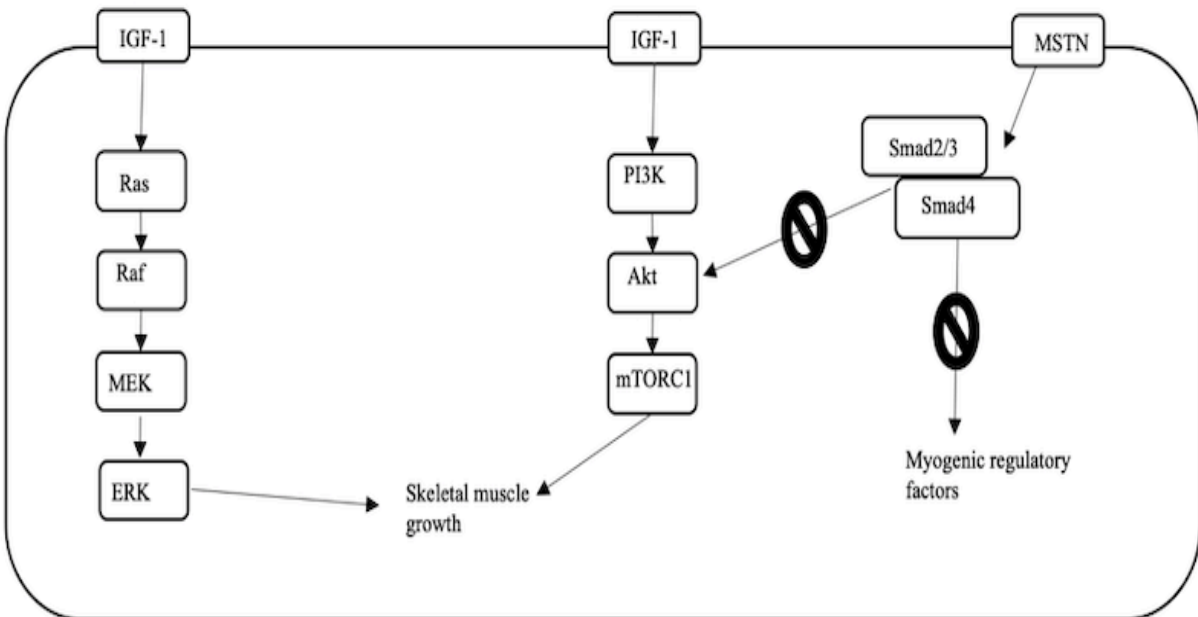
Different phases of hyperplasia have been identified in the fish and the timing of when these phases begin, and end vary between species (Rowlerson & Veggetti, 2001). In early development, during the larval stage, hyperplasia dominates growth, and occurs in the proliferative zones found in embryonic muscle growth (Rowlerson et al., 1995). Later in life,

during juvenile and adult growth, hypertrophy tends to dominate (Veggetti et al., 1993, Rowlerson et al., 1995). However, it has been found that some fish, especially those with larger terminal body sizes, have a secondary wave of hyperplasia that persists in to adulthood, giving the muscle a mosaic appearance in transverse sections with an intermingling of small and large fibers in the muscle cross-section (Carpenè and Veggetti, 1981, Rowlerson et al., 1995, Romanello et al., 1987). Fish that are relatively small will either have this phase greatly reduced or absent entirely during muscle growth (Patterson et al., 2008). The interplay between hyperplasia and hypertrophy throughout the lifespan of fish causes indeterminate growth, which causes constant growth in body length and mass, as well as the wide range of terminal body sizes across different species (Johnston et al., 2001; Froehlich et al., 2013; Ahammad et al., 2015).

Hypertrophy and hyperplasia in fish have been shown to be due to the recruitment of satellite cells (Koumans & Akster, 1995). Satellite cells are quiescent cells that exist in differentiated muscle tissues between the sarcolemma and basal lamina. These cells in adult muscle express paired box 7, or PAX7, and this allows the pluripotent stem cells existing under the basal lamina of muscle to be specified as muscle satellite cells (Seale, 2000; Wang & Rudnicki, 2012). Through studies in mammals, the activation of satellite cells is found to be achieved through a basic helix-loop-helix subfamily of proteins called myogenic regulatory factors (Wang & Rudnicki, 2012). Like embryonic myoblasts, muscle satellite cells express myogenic regulatory factor 5 (Myf5), which commits the cell to a muscle cell fate (Kablar et al., 1997; Rudnicki et al., 1993). Myogenic determination factor 1 (MyoD) is expressed in the cells shortly after Myf5, and together, these two transcription factors initiate the cell's determination in to myoblasts (Tajbakhsh & Cossu, 1997; Wang & Rudnicki, 2012). Differentiation of the myoblasts in to multinucleated myotubes is then initiated and regulated by myogenin and

myogenic factor 4 (MRF4) (Hasty et al. 1993; Nabeshima et al., 1993; Hinitis et al., 2007). The pattern of MRF expression previously described is also found in fish, as seen in zebrafish (Weinburg et al., 1996), carp (Cole et al., 2004), rainbow trout (Xie et al. 2001), and brown trout (Steinbaucher et al., 2007). There is potential for slight variations in gene expression across species due to the differences in the types of growth each species will utilize, especially since larger fish have shown to have hyperplastic growth persist for a longer period of time, as compared to smaller fish.

There are two main regulatory pathways for muscle growth (Fig. 1.1). First, the insulin-like growth factor 1 (IGF-1) pathway is a positive regulator for muscle growth, and promotes the proliferation of myocytes (Mommsen, 2001; Schiaffino et al., 2013). IGF-1 regulates muscle growth through the Akt /Protein Kinase B and rapamycin target pathway (mTOR) (Barclay et al., 2019). IGF-1 also induces muscle cell proliferation through the mitogen-activated kinase and extracellular signal-regulated kinase (MAPK/ERK) pathway (Tortorella et al., 2001; Li & Johnson, 2006). Studies done in gilthead seabream and salmon have shown a correlation between higher levels of plasma IGF-1 and increased growth, similar to what has been observed mammals (Perez-Sanchez et al., 1995; Beckman et al., 1998). Mammalian IGF-1 injected in to coho salmon had a positive effect on growth, exhibiting a certain level of conservation of the IGF-1 regulation pathway between fish and mammals (McCormick et al., 1992). Differences in IGF-1 levels and consequently, differences in growth, were observed in fast-growing and slow-growing fish, as well as warm-water and cold-water fishes, indicating some environmental and species variances in muscle growth across different fish (Beckman et al., 1998). The second regulatory pathway is the myostatin/Smad pathway, a negative regulator of muscle growth.



**Figure 1.2.** The regulation of skeletal muscle growth by the IGF-1 and myostatin pathways.

### Myostatin

Myostatin is a member of the transforming growth factor (TGF)- $\beta$  superfamily and contributes to the regulation of muscle growth and development (Lee & McPherron, 1999). Myostatin negatively regulates muscle growth through the activation of the Mstn/Smad pathway, inhibiting the transcription of the MRFs that regulate muscle cell differentiation and proliferation (Thomas et al., 2000; Zhu et al., 2004). Myostatin also negatively regulates muscle growth through inhibiting the Akt/mTOR pathway discussed with the IGF-1 pathway, and thereby slowing protein synthesis (Trendelenburg et al., 2009; McFarlane et al., 2006). In mammals, the double-muscle phenotype, a type of muscular hypertrophy, has been observed mice (McPerron, Lawler, & Lee, 1997) and cattle (Kambadur et al., 1997; McPherron & Lee, 1997) that are myostatin null. This double-muscle phenotype has also been seen in zebrafish (Acosta et al., 2005; Fuentes et al., 2013), medaka (Sawatari et al., 2010) and trout (Lee et al., 2010) which lack

myostatin expression, suggesting a similar function of myostatin during muscle growth and development in fish and mammals.

Multiple myostatin isoforms have been isolated from different fish species, while mammals and avians only have one protein for myostatin. Studies in trout (Rescan et al., 2001), Atlantic salmon (Ostbye et al., 2001), brook trout (Roberts & Goetz, 2001), zebrafish (Wang et al., 2018), and gilthead seabream (Maccatrazzo et al., 2001) have identified two different isoforms of myostatin. The different myostatin isoforms also were found to each have differential expression of each isoform across various tissues depending on the species. Fish had duplication of their genomes during evolution, and genes often evolved independently from one another to have differential expression and slightly different cDNA sequences (Leggatt & Iwama, 2003). The mature myostatin sequence in fish is well conserved as compared to mammals, with about 90% similarity, despite only about 60% similarity in the propeptide (Gabillard et al., 2013). The differences between fish and mammal prodomains and promoter sequences could be the reason for differential expression of myostatin in the body between fish and mammals (Gabillard et al., 2013).

In mammals, studies done on mice have isolated high myostatin expression in the muscle (McPherron, Lawler, & Lee, 1997), with weak expression in the adipose tissues (McPherron, Lawler, & Lee, 1997), mammary tissues (Ji et al., 1998), and cardiomyocytes (Sharma et al., 1999). Fish have also been found to express myostatin in muscle tissue, cardiomyocytes, and adipose. Studies done in seabream showed expression in those locations, as well as the brain, eye, intestines, and kidneys (Maccatrazzo et al., 2001). Rodgers et al. (2001) found tilapia and white bass expressed myostatin in the muscle, as well as eyes, ovaries, gut, brain, and heart. In zebrafish and sea bream, myostatin was localized to the brain, skeletal muscle, gills, kidneys,

intestines, and liver (Radaelli et al., 2003). Since fish express myostatin across multiple tissues, as opposed to the more restrictive expression seen in mammals, it could indicate a wider range of function of the protein in the fish.

In mammals, myostatin expression have only been found expressed in fast-twitch muscle fibers (Carlson et al., 1999). However, studies on different species of fish have found that myostatin is expressed differently across the different muscle fibers (Roberts and Goetz, 2001). Roberts and Goetz (2001) found in brook trout, king mackerel, and yellow perch myostatin expression localized to red muscle, while little tunny had expression in white muscle, and mahi mahi had expression in both red and white muscle. Rescan et al. (2001) found that myostatin 1 in the trout was expressed in slow and fast twitch muscle, and myostatin 2 was expressed only in slow twitch muscle. This difference in myostatin expression between muscle fiber types could be due to the wider range of muscle fiber type ratios and locomotion needs of different fish species (Roberts & Goetz, 2001).

### **Ammonia**

Ammonia is mainly produced through the catabolism of proteins from the diet, and specifically through the breakdown of the amino acids from those proteins. Although the gastrointestinal tract is the primary source of amino acids, many different organs and organ systems can produce ammonia (Graham & MacLean, 1990; Campbell et al., 1983). For fish, the  $pK_a$  of ammonia is approximately 1-2 units above the pH of the blood and intracellular fluid, so about 95% of the ammonia in the body exists as  $NH_4^+$  (Ip & Chew, 2010). Since excess ammonia cannot be stored in the body, any excess ammonia has to be excreted in a way that is not toxic to the body. Mammals are a ureotelic species, meaning nitrogenous waste is excreted

primarily as urea, while avian are uricotelic, and excrete primarily uric acid. Conversely, many fish species are ammoniotelic and excrete nitrogenous waste as ammonia.

In mammals, the majority of nitrogen is converted to the non-toxic compound urea through the urea cycle. The urea cycle primarily takes place in liver periportal hepatocytes, in both the mitochondria and the cytosol, using ammonia and bicarbonate to convert ammonia to urea through a series of enzyme-mediated reactions. Alongside the urea cycle, glutamine synthetase (GS) works to form glutamine from ammonia and glutamate, typically in tissues that lack urea cycle enzymes, such as the brain, skeletal muscle, gastrointestinal tract, and a small number of hepatocytes (Aoki et al., 1981; Cooper & Plum, 1987; James et al., 1998). Glutamate dehydrogenase (GDH) catalyzes a reversible reaction that converts  $\alpha$ -ketoglutarate to glutamate, using up free ammonia. GDH also works in the de-aminating direction and contributes to the balance of nitrogen levels in the body (Cooper, 2011). Avians, on the other hand, lack urea cycle enzymes and primarily rely on GS to form uric acid (Campbell & Vorhaben, 1976). GS in the bird is found diffusely in the liver, mimicking the expression pattern of urea cycle enzymes in the mammal (Smith & Campbell, 1988).

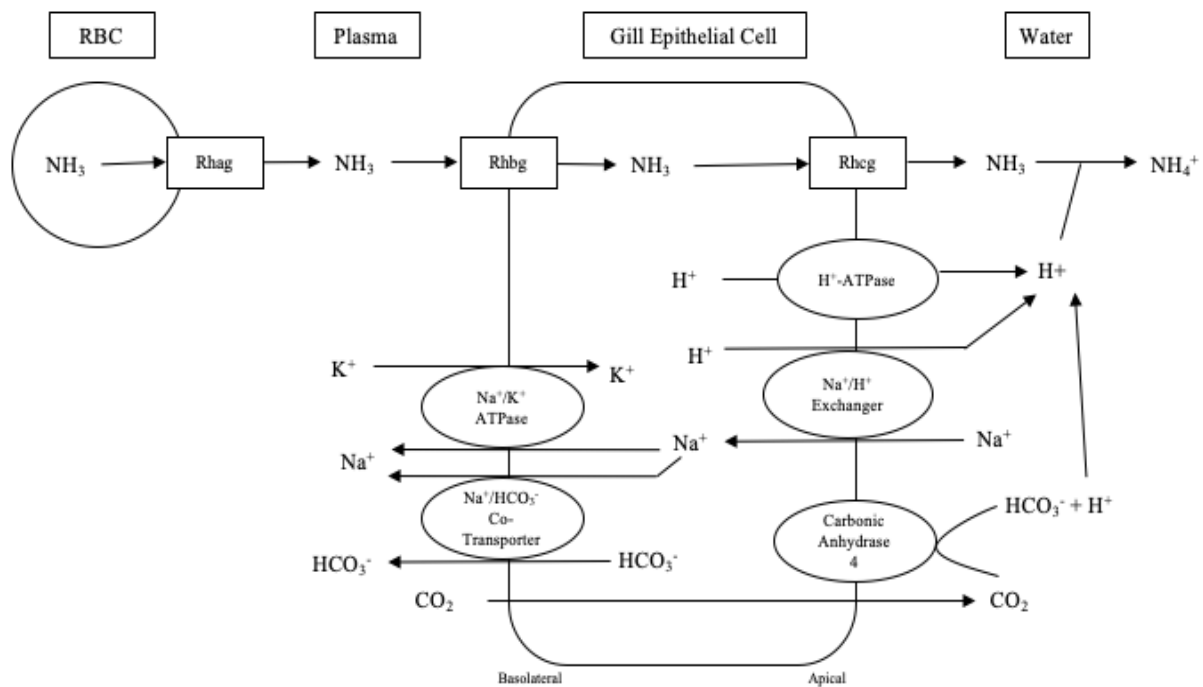
Fish display a wider variety of ammonia excretion between species, partly due to the large differences in environment that different species occupy. While most fish are ammoniotelic, excreting nitrogenous waste as ammonia, a select few fish are ureotelic, and, like mammals, excrete nitrogenous waste as primarily urea. For example, the Lake Magadi tilapia in Kenya lives in extremely alkaline conditions (pH 10-10.5) and has adapted to contain the full range of urea cycle enzymes in the liver and excretes almost all of its nitrogenous waste as urea, since ammonia excretion is impeded in such an alkaline environment (Randall et al., 1989; Lindley et al., 1999). The gulf toadfish is able to switch between ammoniotelic excretion and

ureotelic excretion. This fish changes its nitrogen excretion strategy when exposed to air or confined in small spaces, since it spends time on land as well as burrowed under the sand, both of which impede nitrogen excretion via ammonia (Walsh, Danulat, & Mommsen, 1990; Walsh & Milligan, 1995). However, not all air-breathing fish excrete ammonia as urea. The African sharptooth catfish is able to move on to land to avoid drying ponds and can live burrowed underground just as the gulf toadfish. However, these fish lack urea cycle enzymes in their livers, and after exposure to high external ammonia concentrations, did not have an accumulation of urea in the blood or ammonia in the body (Ip et al., 2004).

Despite some exceptions, the large majority of fish excrete nitrogenous waste as ammonia. The gills serve as the primary site of ammonia excretion due to it being the primary site of gas exchange and ion transport for the fish (Goldstein, Clairborne, & Evans, 1982; Evans et al., 2005; Blair, Wilkie, & Edwards, 2017). Ammonia is excreted down its concentration gradient, as  $\text{NH}_3$ , from the gills, since most aqueous environments have a relatively low concentration of ammonia (Danulat & Kempe, 1992). This favorable  $\text{NH}_3$  gradient is maintained via acid trapping at the gills, so that  $\text{NH}_3$  is converted to  $\text{NH}_4^+$  as it leaves the epithelium (Wright & Wood, 2009). Hydrogen ions at the apical membrane are pumped out via  $\text{H}^+$ -ATPase and  $\text{Na}^+/\text{H}^+$  exchanger proteins (Maetz, 1973; Wright & Wood, 2009). This layer of  $\text{H}^+$  ions at the apical membrane of gill epithelium is also generated by the hydration of  $\text{CO}_2$ , either catalyzed or uncatalyzed by carbonic anhydrase (Wright & Wood, 2009).

More recently, transport proteins have been found in the gills of fish that also participate in the movement of ammonia (Fig. 1.2). Rhesus (Rh) glycoproteins are a family of proteins known to be involved with ammonia transport in various tissues in mammals (Marini et al., 1997; Planelles, 2007). Studies done on pufferfish by Nakada et al. (2007) revealed orthologs to

the human Rh glycoprotein family that are localized to the gills of the fish. When these orthologs were used in *Xenopus* oocytes, ammonia uptake was enhanced as compared to the control (Nakada et al., 2007). Rh proteins have also been found to be localized to the gills in rainbow trout (Nawata et al., 2007), sea lamprey (Blair, Wilkie, & Edwards, 2017), largemouth bass (Egnew et al., 2019) and zebrafish (Braun et al., 2009). Wright & Wood (2009) proposed that the Rhag protein is involved in ammonia transport from erythrocytes to the plasma, followed by Rhbg transport across the basolateral membrane of gill epithelium. The ammonia is then excreted outside the gills by a metabolon on the apical membrane comprised of Rhcg, H<sup>+</sup>-ATPase, Na<sup>+</sup>/H<sup>+</sup> exchanger, and membrane Na<sup>+</sup> channels (Wright & Wood, 2009). Studies have found that Rh proteins are upregulated during exposure to high levels of ammonia in several species, indicating their involvement in ammonia transport (Nawata & Wood, 2009; Tsui et al., 2009; Chen et al., 2017). Braun et al. (2009) used selective gene knockout in the zebrafish to find that each Rh protein gene, when knocked out, reduced ammonia transport by about 50%, making all the Rh glycoprotein transporters necessary for maximal ammonia excretion in the zebrafish.



**Figure 1.3.** A model of how Rh glycoproteins are involved with ammonia excretion from the gills of fish. Rhag, Rhbfg, and Rbcg all participate in the movement of ammonia from the blood into the gill epithelium and out the basolateral side.

However, similar to mammals and avians, fish also utilize glutamine synthetase (GS) to detoxify ammonia, particularly in the brain (Singh & Singh, 1989; Iwata & Deguchi, 1995, Wang & Walsh, 2000). Studies in rainbow trout have isolated multiple GS genes, and found higher expression of GS in the brain than GDH (Wright et al., 2007). Rainbow trout also have increased glutamine concentrations in the brain and liver when exposed to ammonia, which corresponds to increased GS activity in the brain (Wicks & Randall, 2002). Banerjee et al. (2018) isolated three different isoforms of the glutamine synthetase gene in magur catfish, an air breathing-fish. After exposure to high levels of ammonia, the magur catfish had differential expression of the three copies of the gene, with each localizing to the liver, kidneys, gills, muscle, or brain (Banerjee et al., 2018). In the toadfish, it was also found that fish pre-treated

with methionine sulfoximine (MSO), an inhibitor of GS, were more susceptible to ammonia toxicity than control groups, further supporting the role of GS in detoxifying ammonia (Veauvy et al., 2005). The high levels of GS in the fish brain however would be thought to exert toxic effects during high levels of ammonia exposure, as glutamine can exert negative effects on astrocytes (Takahashi et al., 1991). However, the exact mechanisms of how fish are able to avoid the neurotoxic effects of glutamine accumulation are still unknown.

### **Ammonia Toxicity**

In mammals, high levels of ammonia, or hyperammonemia, can be very toxic and has effects on many different organ systems in the body. Most notable is the effect of hyperammonemia on the brain, resulting in a condition called hepatic encephalopathy, common in patients with liver failure (Clemmesen et al., 1999). The increased absorption of ammonia in the brain by the astrocytes leads to increased glutamine production by GS causing osmotic dysregulation, ultimately leading to cellular swelling and metabolic dysfunction (Takahashi et al., 1991; Butterworth, 2014). Hyperammonemia also exerts a negative effect on skeletal muscle in mammals, leading to sarcopenia, or muscle wasting (Dasarathy, 2012). Skeletal muscle, like the brain, contain the enzyme GS, and during hepatic insufficiency, will uptake more ammonia than at basilar levels in an attempt to increase detoxification of ammonia (Ganda & Ruderman, 1976). Increased ammonia levels have also been connected to an increase in myostatin expression in mammals, which has been shown to be linked with sarcopenia (Dasarathay & Hatzoglou, 2018; Stern, Dasarathy, & Mozdziak, 2017). Avians have shown the adverse effects of exogenous ammonia as a gas on their nasal cavities and eyes due to the fact that many poultry houses can have high levels of ammonia gas as a result of poor ventilation, as well as chronic liver injury (Beker et al., 2004; Zhang et al., 2015). However, hyperammonia in chick embryos

has demonstrated potentially beneficial effects on embryonic skeletal muscle (Stern, Dasarathy, & Mozdziak, 2017).

The effects of hyperammonemia on the fish is not as extensively studied as it is in mammals. However, studies done on fish have shown similar effects of high levels of ammonia as found for mammals. Many symptoms of hepatic encephalopathy seen in mammals have also been observed in fish, such as hyperexcitability, convulsions, and hyperventilation (Smart, 1978; Lang et al., 1987; Randall & Tsui, 2002; Suski et al., 2006). Since fish live in aquatic environments, they are also potentially exposed to high levels of environmental ammonia. Fish exposed to high levels of exogenous ammonia also experienced gill hyperplasia (Smart, 1976; Robinette, 1976). Gill hyperplasia causes increased distance that ions must travel across the epithelium to diffuse out of the gills, potentially negatively affecting the efficiency of ammonia excretion in the fish (Daoust & Ferguson, 1984; Ferguson et al., 1992).

Nevertheless, the exact mechanism of ammonia toxicity is not as well understood in the fish as it is in the mammal. Studies in mudskippers (Ip et al., 2005) found that high levels ammonia induced high levels of glutamine in the brain, but not death, indicating that the mechanism for toxicity in the brain of fish could differ from that of mammals. Similar findings were reported for the African sharptooth catfish (Wee et al, 2007) and the swamp eel (Tng et al., 2009).  $\text{NH}_4^+$  has also been shown to be able to substitute for  $\text{K}^+$  in  $\text{K}^+$  ion channels in neurons, affecting the membrane potential and excitability of the neuron of the fish and mammals, and could account for the hyperexcitability and convulsions observed in hyperammonemic animals (Binstock & Lecar, 1969; Cooper & Plum 1987; Szerb & Butterworth, 1992).

Mammals have shown to respond negatively to high levels of ammonia, resulting in higher levels of myostatin expression and decreased myotube diameter (Stern, Dasarathy &

Mozdziak, 2017). Conversely, avians exhibited a positive myogenic environment in response to heightened levels of ammonia, with increased myotube diameter and a decrease in myostatin expression (Stern et al., 2015; Stern, Dasarathy & Mozdziak, 2017). Since myostatin appears to have similar functions in both the fish and the mammal, the effects hyperammonemia in mammalian muscle could be similar in fish muscle. It has been documented that high levels of ammonia negatively impacts growth in the fish (Dosdat et al., 2003; Lemarie et al., 2004; McKenzie et al., 2003). Dosdat et al. (2003) also found that after sea bass are removed from high ammonia environments, they exhibit compensatory growth, indicating ammonia exerted an inhibitory effect. However, this potential negative impact on the muscle of the fish has not been linked to increased myostatin expression as it has in mammals (Stern, Dasarathy, Mozdziak, 2017). The similarities of myostatin functioning between mammals and fish could potentially indicate a role of myostatin in the effect of ammonia toxicity on the growth of fish, but this has not been shown to date.

## **Conclusion**

Fish, mammals, and avians share some similarities in skeletal muscles and ammonia toxicity, but also have many differences. It is also well known that fish use a different strategy of ammonia excretion, and understanding the ammonia metabolic systems and proteins, such as the Rh proteins, that are involved is crucial for understanding how these fish are able to tolerate high ammonia levels. Some fish have even been found to have higher capacities for ammonia tolerance. Further investigation to examine how exactly these fish are able to be more tolerant of ammonia, and the strategies to effectively mitigate the toxic effects of ammonia could lead to new therapies for patients in liver failure. Examining the relationship between ammonia levels in the fish and myostatin expression, and the resultant effects on skeletal muscle will also increase

the current understanding of the role of this protein in the effects of hyperammonemia. Further studies of the fish on how different ammonia levels can affect skeletal muscle, can also contribute to the quality and quantity of production for the aquaculture industry.

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## CHAPTER 2

### MYOGENIC RESPONSE TO INCREASING CONCENTRATIONS OF AMMONIA DIFFERS BETWEEN MAMMALIAN, AVIAN, AND FISH SPECIES

#### Abstract

Studies in mammalian and avian species have linked hyperammonemia with increases in myostatin (MSTN) expression in mammals and decrease in MSTN expression in avians at a concentration of 10 mM of ammonium acetate. This study examines how murine, avian, and fish cells respond to increasing levels of ammonia up to 50 mM. C2C12, primary chick, and primary tilapia myoblast cells were cultured and then exposed to 10 mM, 25 mM, and 50 mM ammonium acetate, sodium acetate, and an untreated control for 24 hours. High levels of ammonia were detrimental to the C2C12 cells, causing increased MSTN expression and decreased myotube diameters between 10 mM and 25 mM ( $P < 0.002$ ). Ammonia at 10 mM continued the positive myogenic response in the chick with lower MSTN expression than the C2C12 cells and larger myotube diameters, but myotube diameter at 50 mM ammonium acetate was significantly smaller than those at 10 mM and 25 mM ( $P < 0.001$ ). However, chick myotubes at 50 mM were still significantly larger than the sodium acetate treated and untreated control ( $P < 0.001$ ). The tilapia cells showed no significant difference in MSTN expression or myotube diameter in response to increasing the concentrations of ammonia. Comparing the three species, C2C12 cells had higher expression of MSTN than the chick and tilapia cells, while the chick and tilapia cells showed no significant difference in MSTN expression ( $P < 0.004$ ). Overall, these results confirm that increasing concentrations of ammonia are detrimental to mammalian skeletal muscle, while chick cells responded positively at lower levels, but began to exhibit a negative response at higher levels. At the same time the tilapia was able to metabolize ammonia and experience no

detrimental effects to muscle.

## **Introduction**

Myostatin (MSTN) is an important negative regulator of embryonic and postnatal skeletal muscle growth in mammals, avians, and fish (Lee & McPherron, 1999; Amthor et al., 2004). Myostatin, a member of the TGF- $\beta$  super family, effects myogenesis by downregulating myogenic regulatory factor (MRF) expression and inhibits the Akt/mTOR pathway, which then causes a reduction in myoblast proliferation and differentiation (Thomas et al., 2000; Zhu et al., 2004). Low or absent myostatin expression results in increased levels of hyperplasia and hypertrophy resulting in the double-muscling phenotype, and high levels of myostatin causes muscle loss (McPherron, Lawler, & Lee, 1997; Kambadur et al., 1997; Acosta et al., 2005; Lee et al., 2010; Dasarathy et al., 2004). The function of myostatin in mammals and avians has been more extensively studied than in fish, but the presence of the double muscling phenotype in myostatin null fish indicates myostatin has a similar role in skeletal muscle regulation as it does in birds and mammals (Fuentes et al., 2013; Sawatari et al., 2010; Lee et al., 2010).

Despite similarities between mammalian and fish myostatin, there are also multiple differences that open up possibilities for new roles of myostatin in fish which are still unknown. Fish have a more extensive tissue expression of myostatin than what is seen in mammals. Myostatin in the fish has been isolated in the heart, eyes, kidney, intestines, and gills, as opposed to the stricter expression to muscle in mammals and avians (Maccatrazzo et al., 2001; Radaelli et al., 2003; Amthor et al., 2002). Some fish species have multiple isoforms of myostatin, with different patterns of expression for each isoform, unlike mammals with only one myostatin protein (Rescan et al., 2001; Wang et al., 2018; Maccatrazzo et al., 2001; Roberts & Goetz, 2001). The discrepancy between mammalian and fish myostatin makes it more difficult to

extrapolate the precise functions of myostatin in the fish solely on studies in mammals.

Previous studies in mammals and avians have linked hyperammonemia to changes in myostatin expression. In response to ammonia, mammals exhibit an increase in myostatin expression, and a resultant decrease in skeletal muscle (Qui et al. 2012; Stern, Dasarathy, & Mozdziak, 2017). Avians, however, had a positive myogenic response to hyperammonemia, with a decrease in myostatin expression (Stern et al., 2015; Stern et al., 2017). However, the effects of ammonia on myostatin expression in fish has not been previously studied. Studies have found that high levels of exogenous ammonia have been shown to negatively impact the overall growth of fish, but myostatin expression was not investigated in these studies (Dosdat et al., 2003; Lemarie et al., 2004).

Fish, mammals, and avians all differ in the primary method used to excrete ammonia. Mammals are ureotelic and utilize the urea-ornithine cycle (UOC) enzymes primarily found in the liver (Meijer, Lamers, & Chamuleau, 1990). Avians are uricotelic and excrete ammonia as uric acid, primarily depending on glutamine synthetase to detoxify ammonia (Campbell & Vorhaben, 1976). Most fish are ammoniotelic, and excrete ammonia as  $\text{NH}_3$ , primarily through the gills (Evans et al., 2005; Blair, Wilkie, & Edwards, 2017). However, there are a handful of fish that live in extreme environments and utilize the UOC enzymes to excrete ammonia or use both ammoniotelic and ureotelic excretion strategies (Randall et al., 1989; Walsh, Danulat, & Mommsen, 1990; Walsh & Milligan, 1995). Since avians and mammals differ in nitrogen excretion strategy and in the skeletal muscle response to hyperammonemia, it could indicate that fish muscle will also respond differently to high levels of ammonia (Stern et al., 2017). The main focus of this study was to examine the effects of increasing levels of ammonia on myogenesis and myostatin expression in mammalian and avian species, and to examine the myogenic

response to hyperammonemia in the fish.

## **Materials and Methods**

### *Cells and cell culture media*

C2C12 (ATCC) and broiler chick breast muscle cells (embryonic day 17) cells from the pectoralis major were recovered from frozen storage. The C2C12 cell line was used as this cell line respond similarly to ammonium as mammalian cells isolated from primary culture (Qiu et al., 2013). They were plated on 0.1% gelatin coated 6-well plates and cultured at 37 °C and 5% CO<sub>2</sub> in proliferation media consisting of 10% fetal bovine serum (Genesee Scientific), Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY), and 1% antibiotic-antimycotic solution (A5955, Sigma-Aldrich). The cells were grown to approximately 90-100% confluency, with media changed every 48 hours.

Tilapia cells were isolated from epaxial skeletal muscle of commercially obtained adult tilapia. The tilapia species used was a hybrid between the Nile tilapia and the blue tilapia based on the sequencing results of the primer products. The fish was euthanized and then thoroughly cleansed with 70% EtOH and chlorohexidine. A small incision was made with sterile scissors, and 4 pieces of epaxial muscle were excised aseptically. The muscle samples were placed in sterile Hanks Balanced Salt Solution (pH 7.4, HBSS, Sigma-Aldrich, St. Louis, MO). Each muscle tissue sample was mechanically dissociated in the HBSS with sterile scissors and then enzymatically digested with 0.17% trypsin (Sigma-Aldrich) and 0.085% collagenase (Sigma-Aldrich) for 35 minutes at 37 °C, 5% CO<sub>2</sub>. After incubation, the muscle samples were centrifuged at 18,000 rpm for 5 minutes. After removing the trypsin-collagenase solution, the samples were washed twice by resuspending the pellet in tilapia proliferation media and centrifuged for 3 minutes. Proliferation media for the tilapia myoblast cells consisted of 20%

FBS (Genesee Scientific), Lebovitz-15 (L-15, Gibco, Grand Island, NY) media, 1% non-essential amino acids (NEAA, Gibco, Grand Island, NY), and 1% antibiotic-antimycotic solution. L-15 media has been formulated to function in non-CO<sub>2</sub> supplemented environments, and buffers through the use of phosphate buffers, higher levels of sodium pyruvate, and free-base amino acids. The cells were resuspended in 3 mL of tilapia proliferation media and passed through an 18-gauge needle, and then through a 70 µm cell strainer (Genesee Scientific). The cells were counted using a hemocytometer and plated on Matrigel (Corning) coated 6-well plates. The plates were covered with 500 µl of 4mg/ml Matrigel in DMEM and incubated at 37°C for 30 minutes before use. The cells were grown at 27°C, room air to approximately 90% confluency, with media changed every 24 hours.

#### *Differentiation and treatment media*

After reaching approximately 90% confluency, the media was changed to differentiation media. The C2C12 and chick differentiation media consisted of 10% horse serum (Gibco), DMEM (Gibco), and 1% antibiotic-antimycotic solution (A5955, Sigma-Aldrich). Tilapia differentiation media consisted of 5% FBS (Genesee Scientific), L-15 (Gibco), 1% NEAA (Gibco), and 1% antibiotic-antimycotic solution (A5955, Sigma-Aldrich). The cells were differentiated until they reached 80% differentiation. After reaching 80% differentiation, the 6 well plates were randomly chosen to be treated with 10 mM ammonium acetate (AA, Fisher Scientific, Fair Lawn, NJ), 25 mM AA, 50 mM AA, 10 mM sodium acetate (SA, Fisher Scientific, Fair Lawn, NJ), 25 mM SA, 50 mM SA, or a control of untreated differentiation media, with replicates of 8 for each treatment. The treatment media was incubated for 24 hours at 37°C, 5% CO<sub>2</sub> for the C2C12 and chick cells, and at 27°C, room air for the tilapia. After incubation for 24 hours, the cells were either fixed in the well for myotube analysis, collected for

RNA analysis, or collected for western blot analysis.

#### *Myotube diameter*

Cells used for myotube diameter analysis were fixed with 70% ethanol for 10 minutes. The ethanol was removed, and images of the plates were taken with light microscopy at 20x (Leica Microsystems, Buffalo Grove, IL) and SPOT camera (SPOT Imaging, Sterling Heights, MI). Images from 10 wells per treatment were taken with 10 randomly selected fields of vision per well. For each image taken, 1 myotube was measured at 3 points equidistant along the myotube, for an average of 100 myotubes measured per treatment for each species. Myotube diameters were measured using ImageJ software (<https://imagej.nih.gov/ij/>).

#### *RNA extraction and qPCR*

Cells collected for RNA analysis were first washed with 1 mL of HBSS (Sigma-Aldrich). After removing the HBSS, the cells were removed from the bottom of the plate using 0.5 mL of 0.25% trypsin-EDTA solution (Gibco) for approximately 1 minute. The trypsin-EDTA was neutralized by differentiation media, the cell suspension spun down, and the differentiation media taken off the cell pellet. The pellet was then resuspended in 500  $\mu$ L of RNeasy (Thermo Fisher Scientific, Vilnius, Lithuania) and stored at -20°C until RNA extraction occurred.

Total RNA was extracted from the cell samples following the protocol of the RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA was stored at -20°C in RNase free water. cDNA was then generated following the protocol of the cDNA Reverse Transcriptase Kit (Applied Biosystems, Vilnius, LT) and diluted to a concentration of 1 ng/ $\mu$ L. The diluted cDNA was stored at -20°C until qPCR was performed.

Primers for real time qPCR were designed using primer-BLAST (NCBI). DNA sequencing for tilapia primers was used to verify qPCR products for each gene (Eton Bioscience,

Durham, NC). The sequences of each primer for each gene can be seen in Table 1.

The qPCR was run using 1  $\mu$ l of 1 ng/ $\mu$ l of cDNA, 10  $\mu$ l of SYBR Green Mastermix (Applied Biosystems, Warrington, UK), 2  $\mu$ l of the appropriate primer (10  $\mu$ m forward and 10  $\mu$ m reverse primer mix), and 7  $\mu$ l of water. The qPCR analysis was run on five samples of each cell type and treatment level in triplicate for n=5 replicates. Relative fold changes for each gene was calculated using the Pfaffl method (Pfaffl, 2001).

### *Statistical analysis*

Statistical analysis was performed using JMP Pro 15 (SAS Institute Inc., Cary, NC). Myotube diameters were represented as the mean  $\pm$  SE for ammonium acetate, sodium acetate, and untreated samples. Quantitative real-time PCR was expressed as the mean relative fold change of ammonium acetate treated samples compared to the sodium acetate treated samples, relative to the housekeeping gene,  $\beta$ -actin  $\pm$  SE. All data sets were analyzed by one-way ANOVA and Tukey-Kramer HSD test for multiple comparisons ( $\alpha = 0.05$ ).

## **Results**

### *Myostatin response to ammonium acetate differs between species*

Quantitative real-time PCR was utilized to examine changes in gene expression of myogenic markers in response to increasing levels of ammonium acetate for the mouse, chicken, and tilapia. As seen in Fig. 2.1, C2C12 cells exhibited a higher MSTN gene expression as compared to the chick and tilapia cells for each increasing treatment ( $P < 0.004$ ). There was no significant difference between tilapia and chick MSTN expressions. The species individually showed different responses to increasing ammonium concentration. C2C12 cells at 25 mM and 50 mM had significantly higher MSTN expression than C2C12 cells at 10 mM ( $P < 0.002$ ), but the expression between 25 mM and 50 mM was not significantly different. Tilapia and chick

cells each had no significant difference in MSTN expression between increasing treatment levels.

The other myogenic regulatory factors analyzed, Myf5, Myf6, MyoD, MYOG, and PAX7, did not result in any significant difference within each species between each treatment at 10 mM, 25 mM, and 50 mM ammonium acetate or between the ammonium acetate and untreated or sodium acetate treatments (Fig. 2.2 A-E). There was also no significant difference in gene expression between the three species for each MRF analyzed.

#### *Myotube diameter changed in response to myostatin expression for mammals and avians*

Myotube diameters were measured to examine the myogenic response of the cells to increased ammonium acetate concentrations. As seen in Fig. 2.3, C2C12 myotubes treated with 10 mM, 25 mM, and 50 mM had significantly smaller diameters than untreated and sodium acetate treated ( $P < 0.0001$ ). The 25 mM and 50 mM C2C12 myotubes were also significantly smaller than the 10 mM treated myotubes ( $P < 0.008$ ), but there was no significant difference between 25 mM and 50 mM treatments. In chick myotubes, myoblasts treated with 50 mM ammonium acetate were significantly smaller than those treated with 10 mM and 25 mM ammonium acetate ( $P < 0.0001$ ), but there was no significant difference between 10 mM and 25 mM ammonium acetate. The ammonium acetate treated myotubes at 10 mM, 25 mM, and 50 mM for the chick overall had larger diameters as compared to the untreated and sodium acetate treated myotubes ( $P < 0.001$ ). For the tilapia, myotube diameters were not significantly different between ammonium acetate treated or untreated and sodium acetate treated, or between different treatment levels. Images of the myotubes for each species and treatment are represented in Figs 2.4-2.6.

## **Discussion**

Previous studies found that at 10 mM of ammonium acetate, C2C12 cells had a significant increase in MSTN expression compared to chick myoblast cells at the same level (Stern et al., 2017). The downregulation of MSTN in the avian cells also resulted in significantly larger myotube diameters, while C2C12 cells had significantly smaller myotube diameters (Stern et al., 2017). This study examined the effects of titrating the ammonia concentration up to higher levels than previously studied, and how this increase in ammonia affected myogenic gene expression avian, mammalian, and fish species. While the highest concentration examined in this study was 50 mM, 100 mM of ammonia acetate was also administered but resulted in the death of too many of the C2C12 and avian cells to be able to perform analysis, resulting in the upper level of ammonium used to be 50 mM.

The results starting at 10 mM were consistent with previous studies for both the C2C12 and the chick cells with increased MSTN expression for the C2C12 and lower expression in the chick. This observation was similar to the myotube diameter results reported by Stern et al., (2017). Increasing the concentration of ammonium acetate to 25 mM and 50 mM resulted in continued high expression of MSTN in the C2C12 and a further decrease in myotube diameter as compared to the untreated and sodium acetate treated C2C12 cells. Increased expression of myostatin has been previously linked with decreasing myotube diameter in both the C2C12 cell line and primary mouse myoblast cultures. (Trendelenburg et al., 2009; Stern et al., 2016; Qiu et al., 2013). Following previous studies on C2C12 cells, the C2C12 cells continued to exhibit the same pattern of change of myostatin expression and myotube diameter at 10 mM ammonium acetate. The higher the level of ammonium acetate the more detrimental ammonia was to skeletal muscle growth. This response is also seen in patients with liver failure experiencing muscle wasting due to the high levels of ammonia in the body (Dasarathy, 2012; Dasarathy &

Hatzoglou, 2018).

While the chick had no significant difference in MSTN expression between each treatment, at 25 mM and 50 mM ammonium acetate the change in chick MSTN expression was significantly lower than the change C2C12 MSTN expression, which allowed for larger myotubes in the chick. The myotube diameters for the chick were not significantly different when going from 10 mM to 25 mM but were significantly smaller at 50 mM. However, the chick myotubes at 50 mM were still significantly larger than untreated and sodium acetate treated myotubes. The decrease in chick myotube diameter as the concentration of ammonium increased could be evidence of the chick cells starting to respond to the other toxic effects ammonia can exert cells but are able to still regulate expression of myostatin, as myostatin expression did not increase significantly between 25 mM and 50 mM ammonium acetate.

The avian cells at lower levels exhibit a positive myogenic environment with increased myotube diameters in response to ammonium acetate. With increasing levels, there is potentially the beginnings of a negative response to ammonia in avians, as the myotubes at the highest concentration on ammonium acetate are smaller than at lower levels of ammonia. However, the myotubes were still larger than those not treated with ammonium acetate. Ammonia could potentially be starting to exert a negative effect on skeletal muscle growth in avians at higher concentrations outside of myostatin expression, such as osmotic dysregulation seen in astrocytes due to glutamine accumulation (Takahashi et al., 1991; Butterworth, 2014).

The fish showed no significant difference between the three treatments and between the experimental and control samples for MSTN gene expression or myotube diameter. Compared to the avian and murine gene expression, the tilapia had significantly lower changes in gene expression than the C2C12 cells but was not significantly different from the avian cells.

However, the tilapia cells did not exhibit the positive myogenic response to ammonium that the avian cells exhibited. While the avian cells had an increase in myotube diameter compared to the untreated and sodium acetate cells, the tilapia did not have any significant difference in the myotube diameters in response to ammonium acetate. Tilapia specifically are able to tolerate a large range of environmental conditions and have been shown to grow well in conditions otherwise toxic to other fish (El-Sayed, 2006; Redner & Stickney 1979). While not every fish has the tolerance observed in the tilapia, the differences in ammonia toxicity between the three species could point to mechanisms in the fish for ammonia detoxification that are different from those in the mammal and avian.

Fish and avian species have been found to have high levels of glutamine synthetase in the liver and skeletal muscle, and high levels of ammonia cause an increase in glutamine production (Singh & Singh, 1989; Iwata & Deguchi, 1995; Smith & Campbell, 1988; Stern et al., 2019). Glutamine is known to inhibit myostatin expression in mammalian and avian cells via decreasing expression of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) (Bonetto et al., 2001). In avians, hyperammonemia increased glutamine production in skeletal muscle suppressed myostatin expression as compared to that in C2C12 cells (Stern et al., 2019). If fish have high levels of GS activity during high ammonia states, the increased presence of glutamine in skeletal muscle could be the reason myostatin expression and myotube diameter did not change significantly.

While this study did not focus on glutamine's effect on the expression of myostatin in the three different species in response to the increasing ammonium levels, future studies should examine glutamine production as a mechanism for mediating ammonia toxicity in skeletal muscle, particularly in the fish. It should also be noted the limitation of only examining just the Nile and blue tilapia. With the wide variety between different fish species in terms of

environment, size, and diet, examining one species does not give a full picture of the effects of ammonia on fish species as a whole. Since many fish species also have multiple isoforms of myostatin, future studies on fish myostatin should also include the effects of multiple isoforms on myostatin response and the exact functions of multiple myostatin copies.

## **Conclusion**

In conclusion, increasing concentrations of ammonia continued to be detrimental to mammalian muscle cells, with smaller myotube diameters and increased myostatin expression. Avian cells showed a decrease in the positive myogenic response to increased levels of ammonia by exhibiting a decrease in myotube diameter when at 50 mM of ammonium acetate. This could indicate the chick cells are approaching a maximum amount of ammonia they can tolerate. Fish cells showed no difference in myogenic response to increased levels of ammonia, indicating the fish cells possess a way of mediating ammonia toxicity that is not present in avian and mammalian species.

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**Table 1.** Forward and Reverse Primers for RT-qPCR

Species	Primer	Sequence	Bp size
Tilapia	MSTN	F 5'-GCTGCGAATGAAGGAAGCTC-3' R 5'-CGGTGGTCACTTCTTCCGAT-3'	317
Tilapia	MyoD	F 5'- ACGGCATGACGGATTTTAACG-3' R 5'- CTTGGTAAATCAGGTTGGGGTC-3'	315
Tilapia	Myf5	F 5'- AATGCAAACACTACAGCAACGGC-3' R 5'- GACAGGCGGTCCACGATACT-3'	110
Tilapia	MYOG	F 5'- CAGCAGGGTTTGCTCTACCG-3' R 5'- CTGAACTGGGCTCGCTTGAC-3'	102
Tilapia	Myf6	F 5'- CCCAAGCGGGTCACGATAAT-3' R 5'- GCCTTACGTCTATCCGTGGG-3'	160
Tilapia	PAX7	F 5'- GACAGGCGGTCCACGATACT-3' R 5'- TGC GCCTCTGCTTCCTTTTA-3'	194
Tilapia	$\beta$ -Actin	F 5'-TGGTGGGTATGGGTCAGAAAG-3' R 5'-CTGTTGGCTTTGGGGTTCA-3'	217
Murine	MSTN	F 5'-TCACGCTACCACGGAAACAA-3' R 5'-AGGAGTCTTGACGGGTCTGA-3'	166
Murine	MyoD	F 5'-GCTCTGATGGCATGATGGATT-3' R 5'-CTATGCTGGACAGGCAGTCG-3'	150
Murine	Myf5	F 5'-AACTATTACAGCCTGCCGGG-3' R 5'-GCTGGACAAGCAATCCAAGC-3'	198
Murine	MYOG	F 5'-GTGCCCAGTGAATGCAACTC-3' R 5'-CGAGCAAATGATCTCCTGGGT-3'	94
Murine	Myf6	F 5'-AGAAATTCTTGAGGGTGCGG-3' R 5'-GCCCCTGGAATGATCCGAAA-3'	76
Murine	PAX7	F 5'-AGTTCGATTAGCCGAGTGCT-3' R 5'-CATCCAGACGGTTCCTTTGT-3'	142
Murine	$\beta$ -Actin	F 5'-AGATCAAGATCATTGCTCCTCC-3' R 5'-AGCTCAGTAACAGTCCGCCTA-3'	170

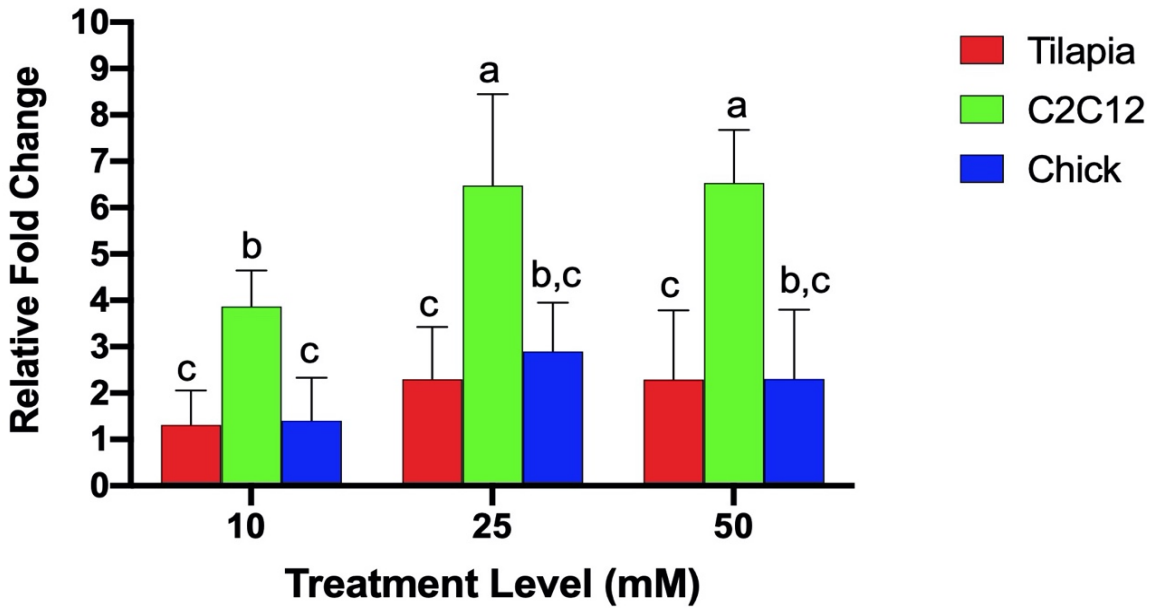
**Table 1** (Continued).

Avian	MSTN	F 5'-CGGAGAATGCGAATTTGTGTTTC-3' R 5'-GGGACATCTTGGTGGGTGTG-3'	110
Avian	MyoD	F 5'-CGCAGGAGAAACAGCTACGA-3' R 5'-ATGCTTGAGAGGCAGTCGAG-3'	104
Avian	Myf5	F 5'-TGAGGGAACAGGTGGAGAACT-3' R 5'-ACTCTGCTCCGTCGCGTA-3'	185
Avian	MYOG	F 5'-CAGCCTCAACCAGCAGGAG-3' R 5'-ACTGCTCAGGAGGTGATCTG-3'	166
Avian	Myf6	F 5'-AGGCTGGATCAGCAGGACAAA-3' R 5'-CGCGGGAATGGTCGGAAG-3'	139
Avian	PAX7	F 5'-GAAGGCCTTTGAGAGGACCC-3' R 5'-GGTTGAATGCTGCGAGTTGG-3'	158
Avian	$\beta$ -Actin	F 5'-GTCCACCTTCCAGCAGATGT-3' R 5'-TAAAGCCATGCCAATCTCG-3'	168

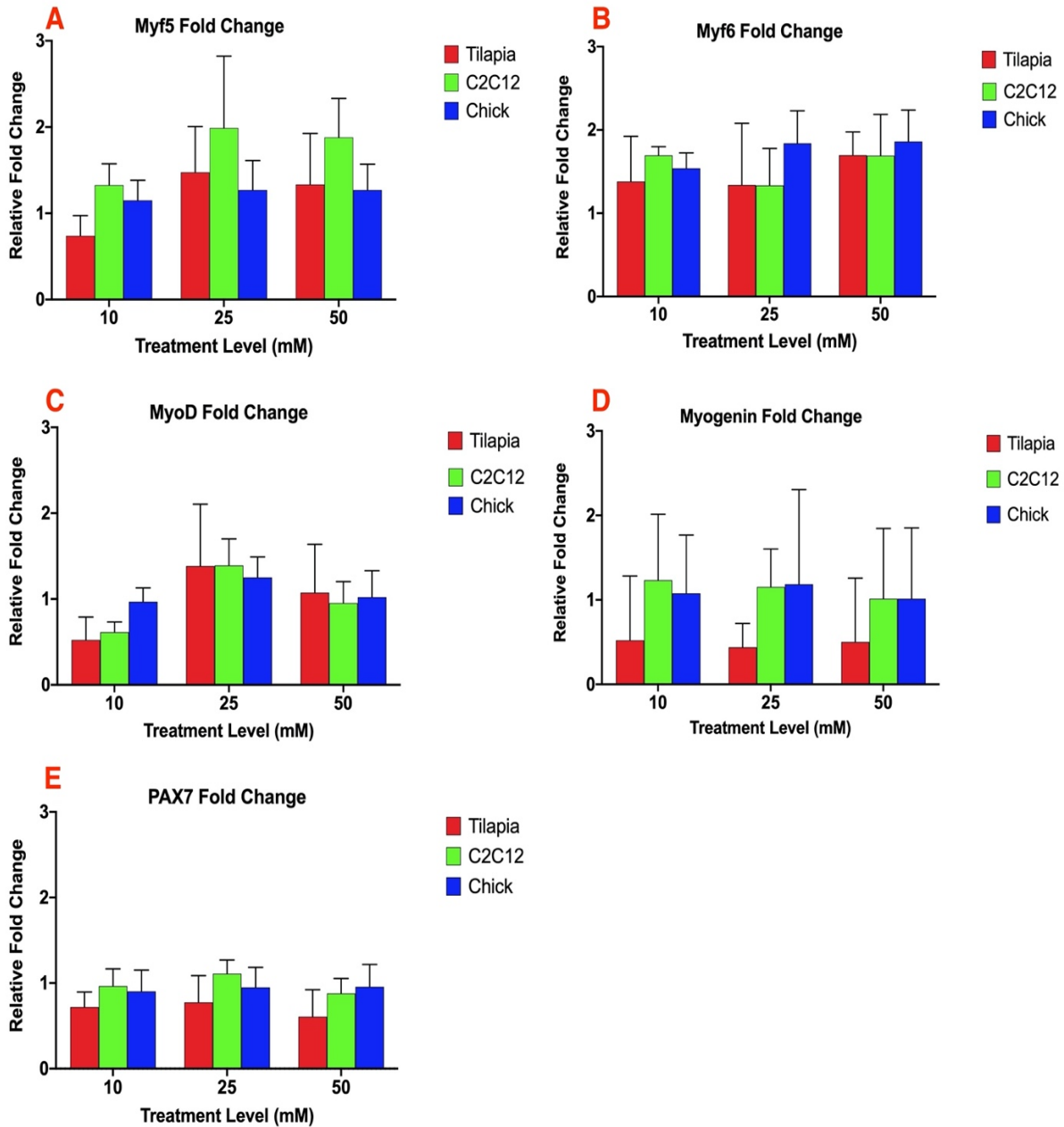
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## Myostatin Fold Change

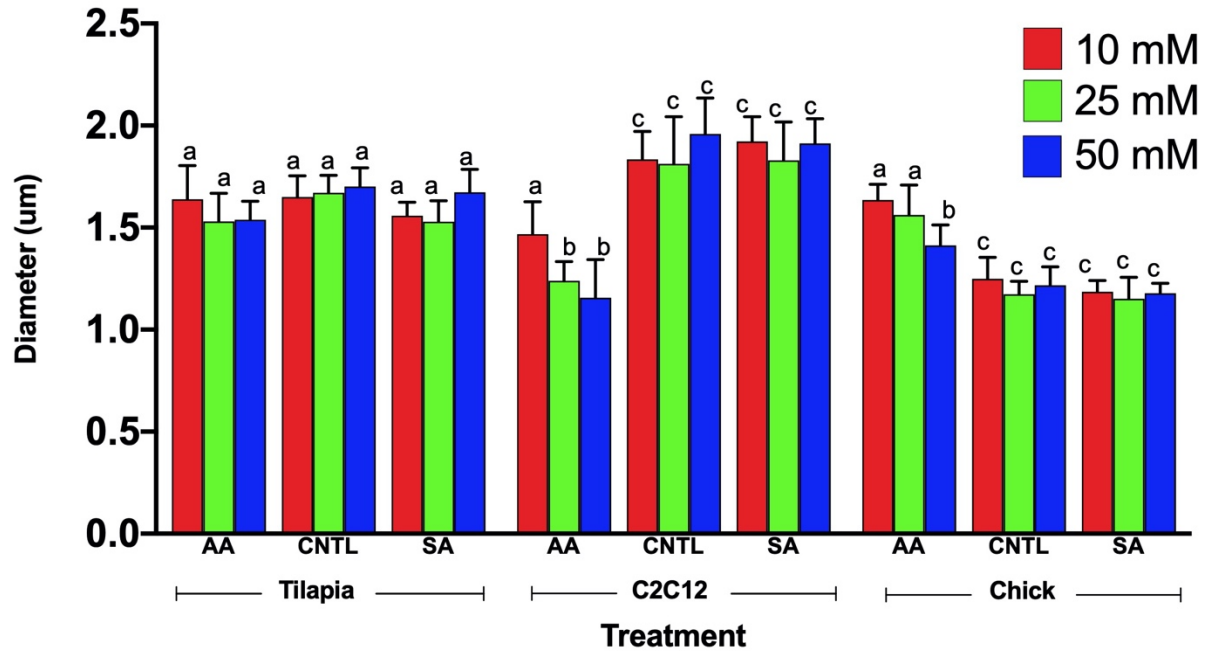


**Figure 2.1.** Relative MSTN gene expression of myotube cells treated with 10 mM, 25 mM, and 50 mM ammonium acetate compared to sodium acetate treated cells, relative to the housekeeping gene,  $\beta$ -actin, for the tilapia, C2C12, and chick myoblast cells. Letters indicate a significance difference between gene expression between treatments, treatment levels, and species ( $P < 0.05$ ).

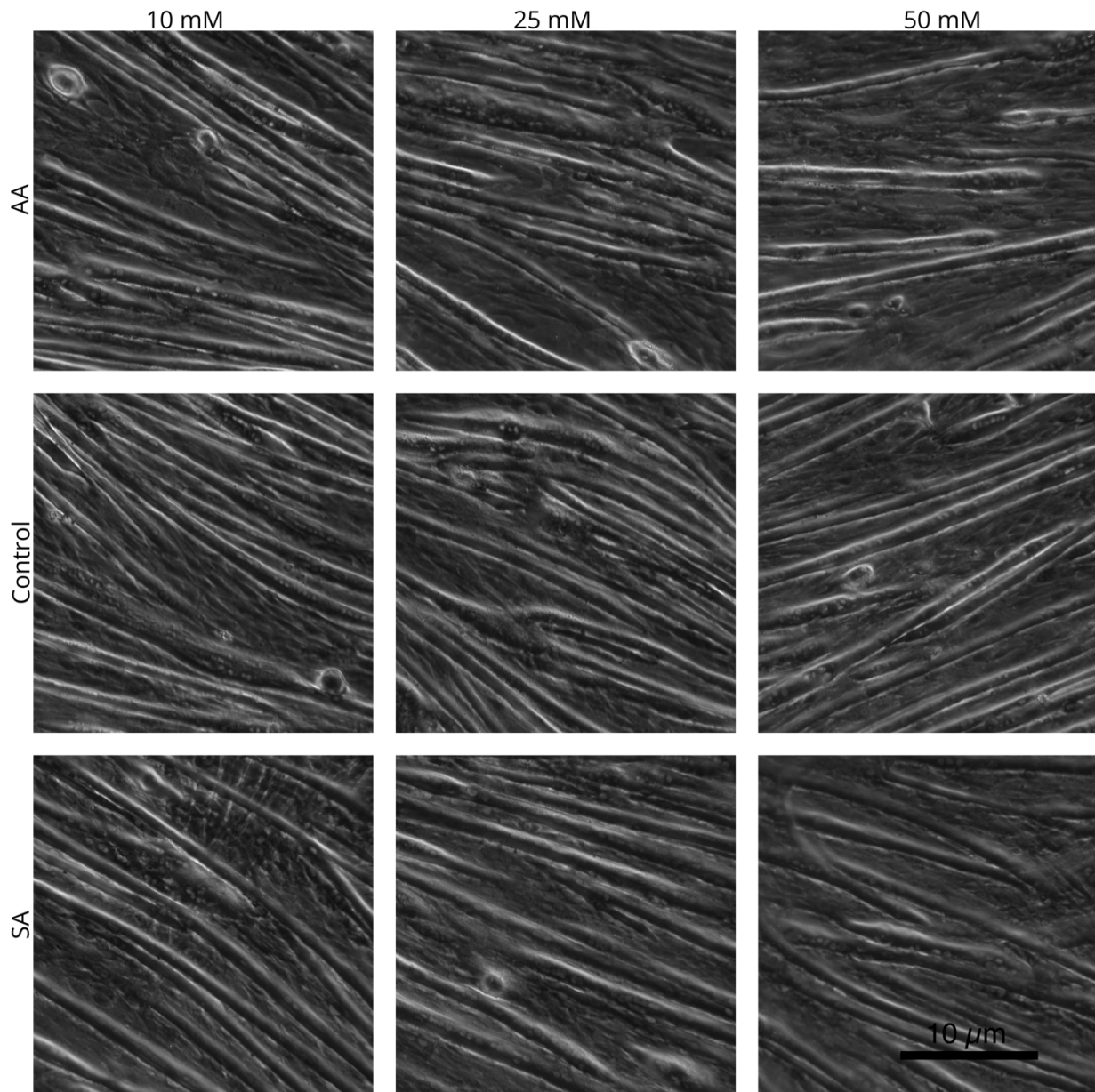


**Figure 2.2.** Relative gene expression of (A) Myf5, (B) Myf6, (C) MyoD, (D) MYOG, and (E) PAX7 for myotube cells treated with 10 mM, 25 mM, and 50 mM ammonium acetate compared to sodium acetate treated cells, relative to the housekeeping gene,  $\beta$ -actin, for the tilapia, C2C12, and chick myoblast cells. There was no significant difference in expression for each gene between each species or treatment level.

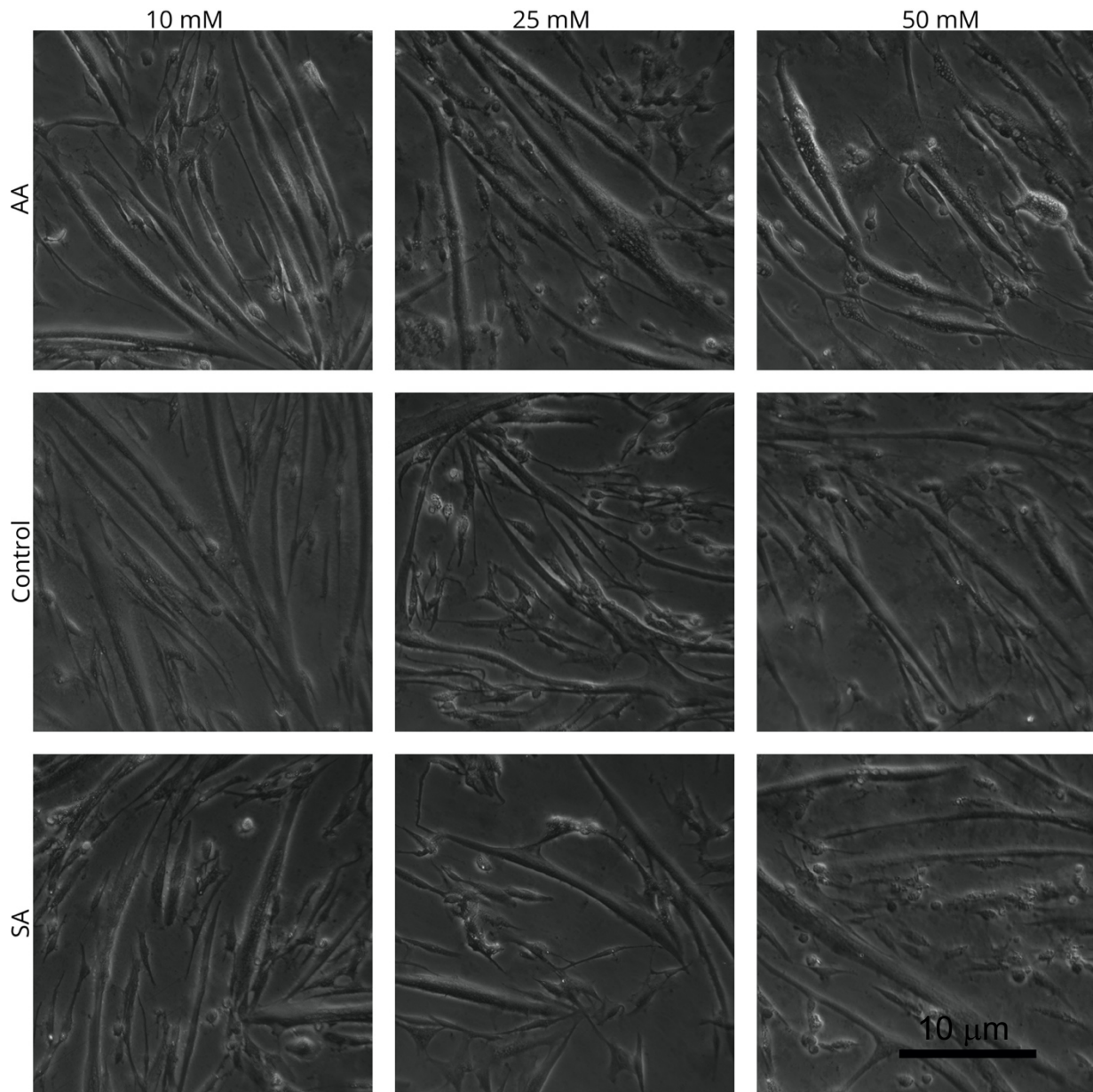
### Myotube Diameter



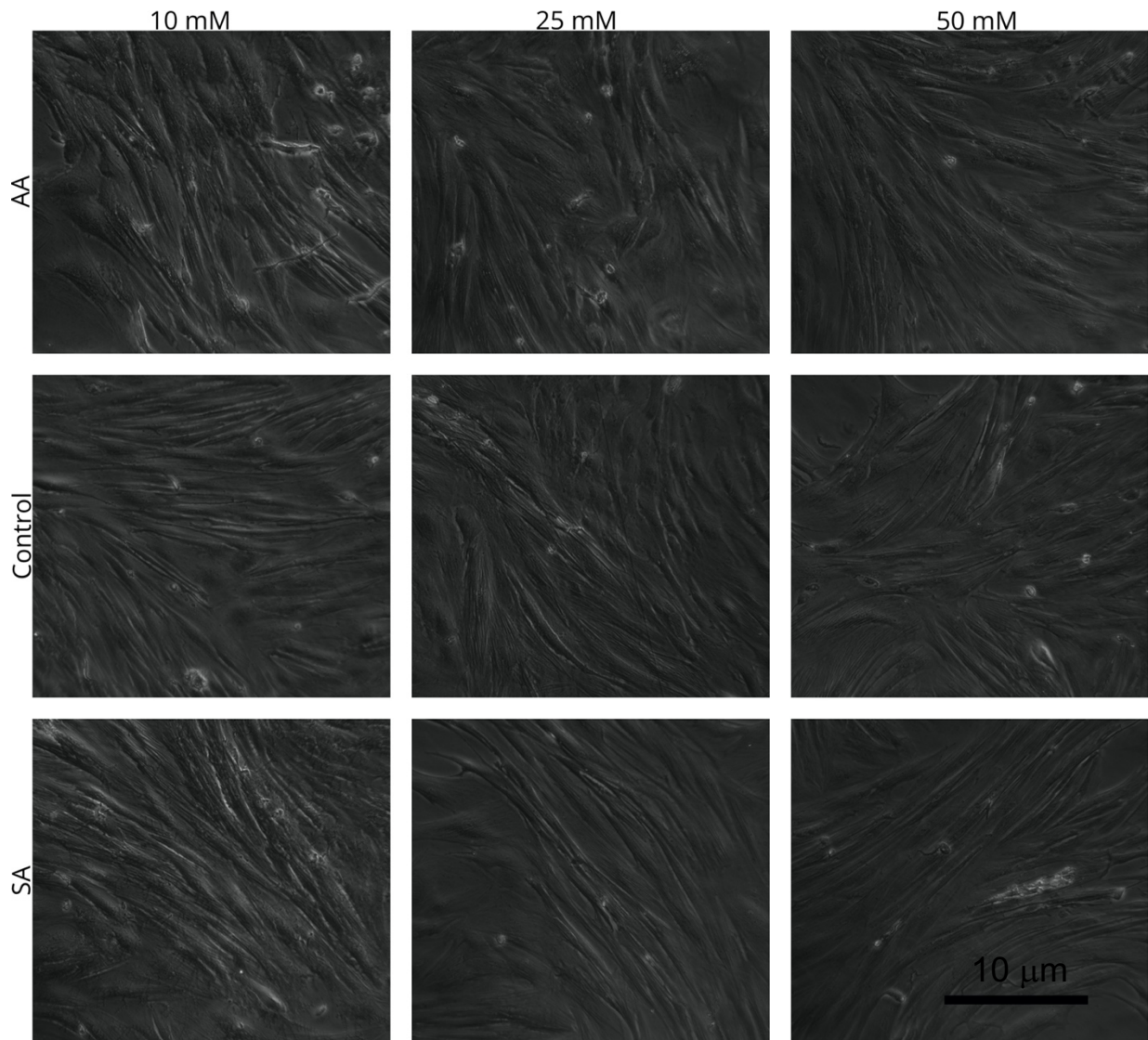
**Figure 2.3.** Average myotube diameters for 10 mM, 25 mM, and 50 mM ammonium acetate, sodium acetate, and untreated tilapia, C2C12, and chick myoblast cells. Letters indicate significant difference within each species ( $P < 0.05$ ).



**Figure 2.4.** Images of cultured myotubes for C2C12 cells after treatment with 10 mM, 25 mM, and 50 mM ammonium acetate, sodium acetate, and proliferation media treatments.



**Figure 2.5.** Images of cultured myotubes for chick cells after treatment with 10 mM, 25 mM, and 50 mM ammonium acetate, sodium acetate, and proliferation media treatments.



**Figure 2.6.** Images of cultured myotubes for tilapia cells after treatment with 10 mM, 25 mM, and 50 mM ammonium acetate, sodium acetate, and proliferation media treatments.

## CHAPTER 3

### SUMMARY

Fish skeletal muscle shares many similarities with mammalian and avian skeletal muscle, but with a few notable differences. While mammals utilize hypertrophy in post-natal skeletal muscle growth, many fish species experience both hypertrophy and hyperplasia post-natally (Wigmore & Stickland, 1983; Mommsen, 2001; Johnston et al., 2001). This contributes to the mosaic appearance of fish skeletal muscle, and the wide range of terminal body sizes between many fish species (Rowlerson & Veggetti, 2001; Romanello et al., 1987). Fish muscle fibers also are very homogenous in their distribution, with little intermingling of different fiber types, while avian and mammalian species have heterogenous distribution within muscles (Wiskus et al., 1976; Rowlerson et al., 1985). However, each species classifies muscle fiber types based on the functional and physiological properties of the fibers, albeit with different nomenclature (Johnston, Davison, & Goldspink, 1977; Schiaffino & Reggiani, 2011; Nierobisz et al., 2010). The regulation of skeletal muscle growth has been shown to be conserved across species, including the functioning of myostatin, a negative regulator of skeletal muscle growth (Weinburg et al., 1996; Schiaffino et al., 2011; Acosta et al., 2005; Fuentes et al., 2013).

Previous studies have documented the difference between avian and mammalian species myogenic response to hyperammonemia (Stern et al., 2015; Stern et al., 2017). In liver disease patients, sarcopenia, or muscle-wasting, is a common complication, and leads to poor patient outcomes. Ammonia applied to mammalian myotubes resulted an increase in myostatin expression, leading to decreased myotube diameters (Dasarathy et al., 2004; Qiu et al., 2012). In avians, hyperammonemia resulted in a positive myogenic response, with decreased myostatin expression and increased myotube diameters (Stern et al., 2015; Stern et al., 2017). The effect of

hyperammonemia on fish skeletal muscle has not been previously studied. Fish myostatin has a similar function to mammalian and avian myostatin, but a more diffuse expression pattern in the body and multiple isoforms could indicate myostatin has more functions in the fish than in mammals and avians (Rescan et al., 2001; Ostbye et al., 2001; Wang et al., 2018; Maccatrazzo et al., 2001; Rodgers et al., 2001). Some fish can even tolerate higher levels of ammonia than mammals and have mechanisms to metabolize ammonia at high levels to mitigate the toxic effects (Ip et al., 2005; Wee et al., 2007).

The objective of this research was to examine the effects of ammonia on fish skeletal muscle, and to observe what changes occur to mammalian, avian, and fish skeletal muscle in response to increasing concentrations of skeletal muscle. The outcomes of this research confirmed previous studies findings of detriment to mammalian skeletal muscle and a positive response in avians (Stern et al., 2015; Stern et al., 2017; Qiu et al., 2012). Increasing the ammonia concentration continued to be detrimental to the mammalian cells. While the avian cells experienced the positive myogenic response seen at the lower levels of ammonia, it did not continue to increase with each increasing ammonia concentration. At the highest level, the avian cells exhibited signs of toxicity, but still had a positive response as compared to the control samples. The fish muscle cells had no change in response to applied ammonia, with no change in myotube diameter and myostatin expression in response to ammonia or increasing ammonia concentrations.

These results emphasize the important of regulating the amount of ammonia exposure animals have in their diet as well as in their environment. Ammonia is highly toxic to all three species, and mitigating exposure to high levels of ammonia is beneficial. However, as seen in avians, a low level of ammonia exposure could be valuable for increasing muscle mass for food

production. Novel insights in to ways different species metabolize and excrete ammonia could also lead to new treatments for patients who suffer from muscle wasting due to liver disease and hyperammonemia. Further studies on the fish, especially expanding to include more species from different environments and of different body sizes, are necessary to fully understand how ammonia effects fish skeletal muscle.

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