The effects of in ovo injected Nicotinamide Riboside on avian myogenesis

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Abstract

The objective of this study was to determine the effects of *in ovo* injection of nicotinamide riboside (**NR**) on avian embryonic myogenesis. In experiment one, fertilized broiler eggs (*n*=156; Cobb 500) were randomly assigned to 1 of 4 treatments in a completely randomized design with a 2 × 2 factorial arrangement. Factor 1 consisted of treatment (**TRT**), with eggs injected with 100 µl of 0.0 (**-**) or 2.5 mM (**+**) NR in sterile saline. Factor 2 was injection location (**LOC**), with either treatment injected into the yolk (**YO**) or albumin (**AL**). Post-hatch, chicks were euthanized and body and *pectoralis major* (**PM**) measures were examined. The left PM was kept for muscle fiber analysis. There were no TRT×LOC interactions for all body and PM measures ($P \geq 0.07$), except PM weight and length ($P < 0.01$). *Pectoralis major* muscles from the YO+ treatment were heavier ($P < 0.01$) than all other treatments. Length of PM from AL+ were longer than control chicks injected into either the yolk or albumen ($P < 0.04$), but was shorter ($P < 0.01$) than YO+ chicks. There was a TRT×LOC interaction for muscle fibers per 1000µm^2 with YO+ muscles having the largest number of fibers ($P < 0.01$) compared to all other treatments, which did not differ ($P \geq 0.16$) from each other. There were no TRT×LOC ($P \geq 0.43$) interactions for muscle fiber cross sectional area. Injected NR increased PM muscle development and greater effects were seen when NR is injected into the yolk. In experiment two, the effect of increasing *in ovo* NR concentration on avian myogenesis was examined. Fertilized broiler eggs (*n*=420; Cobb 500) were randomly assigned to 1 of 4 treatments: 0.0, 2.5, 5.0, or 10.0 m*M* NR made in 0.9% sterile saline. At d10 of incubation, 100µl of allotted treatment was injected only into the egg yolk. After euthanasia, body measures, PM measures, leg area, and muscle fiber characteristics were analyzed for broiler chicks. For d21 chicks, there were no treatment differences for body measures ($P \geq 0.08$), excluding head length and chest

width, which were larger ($P \geq 0.05$) from 2.5m*M* NR compared to the saline treatment. The weight and width of chick PM were increased ($P < 0.01$) at every NR level. Chicks given 2.5 and 5mM NR solutions had longer PM measurements ($P \leq 0.01$) than saline chicks. Injection of 5m*M* NR increased PM depth ($P \leq 0.04$) from d21 chicks. Treatment did not affect leg muscle area or muscle fiber CSA ($P \geq 0.20$). All NR injections increased muscle fiber number ($P < 0.01$); though 10mM injection led to the largest increase in fibers ($P < 0.01$). Injecting up to 5.0 mM NR in the yolk of the developing embryo did not affect body weight but increased PM measures, indicating NR influenced avian myogenesis. **Key Words:** broiler, *in ovo* injection, nicotinamide riboside, muscle fiber, *pectoralis major*

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Dedication

To my husband, parents, and friends who supported me throughout the last two years. I would not have made it where I am today without your encouragement.

Chapter 1 - Introduction

Food security, or the state of having access to sufficient, affordable, nutritious food, has become a crucial topic for scientists around the world. With the world population increasing from 7.5 billion in 2018 to a projected 9.4 billion in 2050 (U.S. Census Bureau, 2018), providing enough food for the world's inhabitants is critical.

In the United States, meat from beef, pork, and poultry is the number one source of protein for the vast majority of people (Phillips et al., 2015). One focus of the agricultural industry is to meet the demands of the growing population for meat as a protein. Consumers preferences for meat have changed in past years. Historically, per capita consumption of red meats had more than doubled chicken consumption; however, in 1993, chicken consumption had overtaken both pork and beef. In the past 60 years, per capita consumption of chicken has increased by 229% (USDA, 2018). With chicken consumption in the U.S. continually on the rise, researchers are investigating ways to maximize the availability of this protein.

Several methods could be utilized to meet the demands of the consumers: increasing production of broilers, increasing growth rate of broilers, or increasing the amount of muscle found on each broiler. To achieve improved growth rate or muscle accumulation, one potential approach could be to alter myogenesis during development of broilers. Myogenesis is the formation of muscle and occurs during development of the broiler in the egg. Overall muscle mass is determined by gestational hyperplasia, and increase in muscle fiber number, and postnatal hypertrophy, an increase in fiber size (Lawrence et al., 2012). To increase the quantity of meat harvested from broilers, either fiber number or fiber size within the skeletal muscle must be altered.

Nicotinamide riboside **(NR)**, a vitamin B3 analogue and NAD+ precursor (Chi and Sauve, 2013), has positive effects on skeletal muscle qualities in mice. Canto et al. (2013), found NR increased NAD^+ levels in muscle mitochondria. In skeletal muscle, NAD+ acts as an electron transfer molecule and plays a role in energy production (Nelson and Cox, 2007). Increasing levels of NAD+ via NR supplementation may shield skeletal muscle from decline due to aging in mice (Guarente et al., 2016; Fletcher et al., 2017). Nicotinamide riboside has not only shown to increase NAD+ levels in the muscle mitochondria, it also has increased mitochondrial biogenesis in mice as well (Khan et al., 2014). Mitochondria has many critical roles in skeletal muscle, including energy provision and cellular metabolism. With an increase in mitochondria due to NR supplementation, ATP production is also amplified (Khan et al., 2014; Ryu et al., 2016; Frederick et al., 2016; Zhang et al., 2016). Though results have not been consistent in all studies, some experiments show positive influences on both skeletal muscle growth as well as muscle fiber characteristics (Khan et al., 2014; Frederick et al., 2016; Ryu et al, 2016). Due to the changes made to the skeletal muscle physiology, altered muscle performance and endurance has been observed (Canto et al., 2012; Frederick et al., 2016; Kourtzidis et al., 2016; Ryu et al., 2016; Zhang et al., 2016).

Administration of NR has varied among studies. For the majority, supplementation has occurred via oral dosage in either feed or water supply (Canto et al., 2013; Khan et al., 2014; Guarente et al., 2016; Fletcher et al., 2017). One potential technique to deliver NR to growing broiler embryos, is to inject supplementation into the egg. While previous studies have used *in ovo* injection of other supplements such as amino acids, carbohydrates, hormones, probiotics, and immunostimulants (Roto et al., 2016), no study has utilized *in ovo* injection of NR.

While there are many studies examining the effects of NR supplementation on skeletal muscle in mice, none have been observed in broilers. Therefore, the objective of this study was to observe the effects of *in ovo* injection of nicotinamide riboside on whole-body growth measurements, NAD+ content, mitochondrial quantity, and myogenesis in broilers.

Chapter 2 - General review of literature

BROILER EMBRYONIC MYOGENESIS

Origin of skeletal muscle

Skeletal muscle is vital for both movement and support of the body. Myogenesis is the creation and formation of skeletal muscle, which only occurs during embryo development. During the embryonic phase of development, skeletal muscle originates from the mesoderm, one of three layers of the trilaminar disk that forms during gastrulation which occurs. The mesoderm later differentiates into muscle, bone, and some organs. As part of gastrulation, somitogenesis occurs where somites are initially formed. Somites are cuboidal, fluid filled clusters of mesoderm stem cells that form along the neurotube of the embryo and have a predetermined outcome. From the somites, after activation with the myogenic factor Myf5, myogenesis transpires.

Myogenesis occurs in two waves. The timing of myogenesis is different for each species. In poultry, primary myogenesis initiates from Pax3+ and Pax7+ progenitor cells, or muscle stem cells. This first waves forms primary myofibers, which act as a foundation where additional muscle fibers will later be established. This takes place between days 3 and 7 of development of a broiler embryo (Chal and Pourquie, 2017). Secondary myogenesis, or the second wave of muscle formation, then adds more myofibers around the primary fibers. This wave starts after primary myogenesis has ended, beginning at day 8 of embryogenesis (Chal and Pourquie, 2017). These primary and secondary fibers fuse together to create muscle bundles, which together establish muscle. Pax7+ progenitor cells will produce adult muscle stem cells, also known as satellite cells, which are capable of repairing damaged muscle later on.

During myogenesis, hyperplasia is occurring, which is the proliferation of muscle cells. An increase in muscle fibers only occur at this point in time, during myogenesis of a growing embryo. Once myogenesis is complete and after the animal is born, expansion of muscle relies on only hypertrophy, or the enlargement of the muscle fibers due to added protein. Scientists have been able to create larger muscles across many species only by increasing the overall size of muscle fibers. Though much research has been conducted regarding broiler myogenesis, no studies have examined the effect of nicotinamide riboside and its effects on prenatal and postnatal muscle growth in broiler embryos.

Cyclins D1, D2, and D3 are important regulators of the cell cycle both prenatally and postnatally. These D cyclins are factors that drive cells to go through the G1 phase of the cell cycle (Giannattasio et al., 2018). Cyclin D's will subsequently activate Cyclin E, which transitions cells from G1 to the synthesis phase (Sherr, 1995). D-type cyclins can be activated by many environmental influencers such as stress, nutrients, and hormones (Fajas, 2013). Each D cyclin has shown to have different effects on skeletal muscle. Cyclin D1 has been shown to inhibit mitochondrial function (Fu et al., 2005). However, higher levels of Cyclin D1 corresponds to an onset of myogenesis (Zhang et al., 1999). Lower levels of Cyclin D3 leads to a muscle fiber type shift in skeletal muscle towards more oxidative fibers (Giannattasio et al., 2018). High levels of Cyclin D3 are found in differentiated myotubes (Ciemerych et al., 2002). There have been no studies examining the effect of nicotinamide riboside of Cyclin D levels in skeletal muscle.

NICOTINAMIDE RIBOSIDE

Nicotinamide riboside compound

Nicotinamide riboside (**NR**) is a nucleoside, which combines both the nicotinamide and riboside molecules into one. Nicotinamide riboside is a form of vitamin B3 and is only found in trace amounts in few foods, including products made with yeast as well as milk-derived foods. Although naturally occurring, the quantity of NR found in these foods are very low (Chi and Sauve, 2013). In recent years, new ways to synthesize NR have become available. One company, Chromadex, has created a process of manufacturing NR in yeast, making large quantities of the vitamin obtainable. Their brand of NR supplements specifically made for human use, called NIAGEN, is now readily available to the public (Chromadex Incorporated, Irvine, California, USA).

Nicotinamide riboside is a nicotinamide adenine dinucleotide **(NAD+)** precursor and multiple studies demonstrated NR increased NAD+ levels in many species including yeast, rodents, and humans (Belenky et al., 2007; Khan et al., 2014; Martens et al., 2018). Nicotinamide adenine dinucleotide is essential for many metabolic processes in the body, but levels of NAD+ decline as age increases (Yoshino et al., 2011). With the augmented levels of NAD+ observed with supplementation of this compound, NR is hypothesized to improve metabolic function. Other forms of vitamin B3, including Nicotinic Acid (**NA**) and nicotinamide mononucleotide (**NMN**), are also NAD+ precursors but have shown to not be as effective as NR (Bitterman et al., 2002). Without proven adverse side effects of NR and with an increase in NAD+ levels and metabolic functions, nicotinamide riboside may be widely adopted for maintaining health in both humans and animals.

Administration and dosage of NR

The administration of NR has varied between each study. For cell culture studies, NR was supplemented directly into the culture itself at levels spanning 0.1m*M* to 1m*M*. (Canto et al., 2012; Frederick et al., 2016; Agerholm et al., 2017; Fletcher et al., 2017). In live trials for mice and rats, the majority of studies mixed NR into the feed at amounts of 300-400mg/kg/day (Canto et al., 2012, Khan et al., 2014; Ryu et al., 2016; Zhang et al., 2016); however, Canto et al. (2012) administered NR through feed and water with 10m*M* NR. In a human study, NR was supplemented as pills with a tested level of 500mg (Martens et al., 2018).

Effect on NAD+ concentration

Nicotinamide Adenine Dinucleotide is a compound essential for the body and partakes in helping to transfer energy from nutrients. Nicotinamide Adenine Dinucleotide also plays a large role in aging by activating proteins called sirtuins, which are important for mitochondrial function. Many studies demonstrate NAD+ levels have an inverse relationship with age, with the highest quantity found in newborns (Yoshino et al., 2011; Massudi et al., 2012). Therefore, it is important to provide alternative ways to stimulate NAD+ quantity improvements. Nicotinamide riboside is able to be converted to NAD+ in two different ways, as covered by Chi and Sauve (2013) and Frederick et al. (2016). Before the conversion takes place, NR must be transferred into the cells. One path starts with NR converting to nicotinamide **(NAM)**, which is then converted to NMN. In the second pathway, NR is immediately converted to NMN. In both pathways, NMN is phosphorylated to NAD+. By increasing levels of NR available to cells, more NAD+ can be created for utilization.

As mentioned previously, supplementation of NR increased levels of NAD+ in the majority of studies. This result has been seen across several species. Originally, research focused

on yeast. In wild-type yeast strains, cells were given 10mM NR in culture and saw a positive increase of about 150% in the amount of NAD+ over time (Belenky et al., 2007). Many studies show similar effects of increased NAD+ levels in murine cell lines. Cell lines from mice were utilized for NR supplementation at different levels and exhibited enhanced levels of NAD+ (Canto et al., 2012; Frederick et al., 2016; Agerholm et al., 2017; Fletcher et al., 2017). Both Canto et al. (2012) and Flethcher et al. (2017) found 0.5mM of NR in culture with mice cell lines had improved levels of NAD+ over that of controls, while Canto et al. (2012) and Frederick et al. (2016) also saw similar increases in NAD+ with a larger concentration of 1mM NR in culture. Though lower amounts of NR were used including 0.05m*M*, 0.1m*M*, and 0.2m*M* in the Canto et al. (2012) study, maximum NAD+ enhancement occurred at the higher levels of 0.5m*M* and 1m*M* of NR. A study done by Yang et al. (2007), added different levels of NR from 0m*M* to 0.6m*M* to human embryonic kidney cells in culture and found up to a 157% in NAD content increase compared to controls when using 0.6mM of NR. Agerholm et al. (2017) utilized cell lines from nicotinamide phosphoribosyltransferase **(NAMPT)** knockout mice, a mutation characteristic of a decline in NAD+. With supplementation of NR, NAD+ concentration was restored to normal levels equivalent to that of the controls.

Aside from cell culture, NR's effect on NAD+ concentration is also consistent when provided as an oral supplement in mice. Studies from Canto et al. (2012), Khan et al. (2014), and Ryu et al. (2016) all utilized a dosage of 400mg/kg/day of NR as a feed additive and found amplified quantities of NAD+ in skeletal muscle compared to the controls, though supplementation took place at different ages and had diverse lengths of supplementation. In one study multiple tissues were tested for NAD+ content after oral supplementation, and NR was found to have increased NAD+ levels in the skeletal muscle and liver tissues of these mice by

60% and 40% respectively. There were no differences found in NAD+ content in the tissues from the brain or white adipose tissue. (Canto et al., 2012).

Age at NR feeding does not affect the ability of NR to improve NAD quantity. Zhang et al. (2016) fed NR to mice aged 1 month as well as to mice aged 22-24 months for six weeks. At both ages, NR boosted the amount of NAD+ found within muscle stem cells. For the young mice, NAD+ was increased by 70% while in old mice NAD+ was boosted by about 140% (Zhang et al., 2016). Cellular mutation in mice also does not hinder the consistency of results when adding NR to the diet. Similar to the Agerholm et al. (2017) study with the NAMPT knockout mice cell lines, Frederick et al. (2016) tested mice with the same mutation and found consistent results and increased NAD+ concentration within the skeletal muscle.

Only one study to date have examined the effect of NR on human subjects and levels of NAD+. In humans with an average age of 65, taking 500 mg of NR twice a day for six weeks resulted in a 60% increase in NAD+ compared to those who received the placebo (Martens et al., 2018). The results shown through many studies across species are in agreement and show conclusive evidence that Nicotinamide riboside can be an effective additive to increase levels of NAD+ in the body.

Effect on mitochondria

Nicotinamide riboside has also affected metabolic processes by modifying mitochondria content. Mitochondria are commonly known as the powerhouses of cells due to their participation in energy production. Mitochondria take oxygen and nutrients and convert this into Adenosine Triphosphate **(ATP)** during the Tricarboxylic Acid **(TCA)** cycle. Mitochondria are indirectly affected by NAD+ quantity due to the fact that NAD+ activates sirtuin activity. Greater amounts on NAD+ will amplify sirtuin activity. Sirtuins are proteins imperative for

cellular processes including cell stress resistance, cellular aging and apoptosis, and mitochondria biogenesis (Mendelsohn and Larrick et al., 2017). If NAD+ concentration is too low, sirtuin activity decreases and mitochondrial function deteriorates.

Quantity as well as quality of mitochondria are important for metabolic function. Amplifying the number of overall mitochondria improves cellular function and oxidative phosphorylation due to enhancement of the rate at which mitochondria can produce energy. The condition of mitochondria is also an essential characteristic for metabolic processes. Having the correct mitochondrial structure and high amounts of cristae also enhances cellular function. Cristae are membranes that increase the surface area inside of the mitochondria, allowing more space for oxidative phosphorylation to occur and improving energy production (Rampelt et al., 2017).

Changes in mitochondria numbers can be attributed to NR due to the modifications made in NAD+ levels. These mitochondrial alterations have been represented in many scientific studies (Canto et al., 2012; Khan et al., 2014; Ryu et al., 2016; Zhang et al., 2016). In healthy mice, Canto et al. (2012) found that the NR-fed treatment group, compared to the controls, had an increased mitochondrial protein and mitochondrial DNA in the skeletal muscles. Nicotinamide riboside supplementation had similar improvements in mice with mitochondrial and muscular diseases. In deletor mice, or mice with mitochondrial myopathies, daily addition of NR to feedings raised the volume of mitochondria (Khan et al., 2014). Ryu et al. (2016) examined myoblasts from mice with muscular dystrophy. After only 12 hours of administration of NR, the mutated myoblasts had increased mitochondrial protein, mitochondrial DNA, and oxygen consumption rate, which is indicative of amplified mitochondrial function and increased mitochondria amount. In aged mice, Zhang et al. (2016) also reported increased mitochondrial

function by means of enlargement of mitochondrial membrane potential, which corresponded to a greater energy capacity in the mitochondria and a greater synthesis of ATP (Zorova et al., 2017). As shown by many studies, supplementation of NR in both culture settings for *in vitro* studies as well as in diets for *in vivo* studies has shown positive effects on mitochondrial magnitudes.

Along with NR affecting mitochondrial quantities, the quality of the existing mitochondrial is positively affect as well (Canto et al., 2012; Khan et al., 2014). A mitochondrial myopathy in deletor mice instigated abnormal, swollen mitochondria, but with the addition of NR, the number of cristae increased and the irregular structural impairments were restored to normal shape and size (Khan et al., 2014). Similarly, Canto et al. (2012) also depicted and increase in mitochondrial cristae with NR by about 400%. These improvements in mitochondrial structure enhance mitochondrial ability.

Effect on Adenosine Triphosphate

Due to the fact that nicotinamide riboside has proven advantageous for mitochondrial function and that mitochondria are important for the production of energy, we would expect to see an effect on ATP. This has been confirmed in several scientific trials (Khan et al., 2014; Ryu et al., 2016; Frederick et al., 2016; Zhang et al., 2016). In both young and aged mice, NR increased ATP content in muscle cells. Though younger mice have overall higher ATP levels in general, the addition of NR to aged mice resulted in ATP levels that mimic healthy young mice (Zhang et al., 2016). In both mitochondrial myopathy mice as well as NAMPT knock-out mice, ATP levels are smaller than that of control mice. With the supplementation of NR, two studies showed ATP concentration was transformed to match the ATP concentration of the control mice without mutations (Khan et al., 2014; Frederick et al., 2016). Similarly, muscular dystrophy mice

have significantly lower levels of ATP than customary, but analysis post-supplementation with NR, show normal and affected mice have identical levels (Ryu et al., 2016).

Effect on overall body weight

Nicotinamide riboside has proven to have many positive effects at the cellular level. On a larger scale, only one study has examined the effect on overall body weight as well as body weight gain (Canto et al., 2012). NR did not affect overall body weight of mice in experiments on a regular chow diet with or without the addition of NR. However, when mice were given a high fat diet in an obesity experiment, Canto et al. (2012) discovered that NR mice did have a decreased body weight compared to controls. This reduction in body weight was due to a significantly lower fat mass compared to mice who were not fed NR on a high fat diet.

Effect on skeletal muscle

With increased levels of NAD+, mitochondria, and ATP found in muscle, some studies have also examined the effects on the overall skeletal muscle growth though results have not been consistent. (Canto et al., 2014; Frederick et al., 2016; Ryu et al, 2016). With the addition of NR, healthy mice displayed similar muscle mass as compared to the controls who were not fed the supplement (Canto et al., 2014). Different results were observed in mice with muscular dystrophy with low muscle tone. Once fed NR for 12 weeks, there were no differences in overall lean mass in muscular dystrophy mice and the controls (Rye et al. 2016). In NAMPT knock-out mice, Frederick et al. (2016) exhibited that at 3 months of age, these mice showed no differences in muscle mass in the *gastrocnemius* and *quadriceps* muscles, but by 7 months of age these mutated mice had significantly less muscle mass. By supplementing NR in the feed for 6 weeks, these two muscles in the NAMPT knock-out mice were no longer smaller than controls.

Different circumstances may trigger different reactions in overall skeletal muscle growth from NR supplementation.

Nicotinamide riboside has the potential to influence skeletal muscle growth in some instances, and these same studies examine the effects on muscle fiber transformations (Canto et al., 2012; Frederick et al., 2016; Ryu et al, 2016; Zhang et al., 2016). Muscle fiber oxidative property, fiber size, and fiber number are all indicative of skeletal growth and function. Canto et al. (2012) completed a Succinate dehydrogenase **(SDH)** stain on skeletal muscle fibers from the *gastrocnemius* muscle. The SDH staining procedure stains for fiber types based off of oxidative capacity. Other studies have focused on fiber size alterations after NR, though none have looked at muscle fibers from healthy mice. Two studies by Frederick et al. (2016) and Ryu et al. (2016) analyzed fiber sizes in unusual mice models. In NAMPT knock-out mice, skeletal muscle fiber size is significantly smaller than that of healthy mice. With the addition of NR to feed, fiber size of these affected mice is enlarged, though not to the size of normal mice (Frederick et al., 2016). In mice with muscular dystrophy, NR supplementation initiates both an increase in fiber cross sectional area **(CSA)** and the minimal muscle fiber diameter, compared to mice who were not given NR (Ryu et al., 2016). Only one study utilized NR to look at repair of fibers after muscle damage. Zhang et al. (2016) reported significantly more regenerating muscle fibers from mice fed NR than mice who were not. They attribute improvement in regeneration to the fact that supplementation with NR nearly doubled the amount of muscle stem cells in aged mice (Zhang et al., 2016).

Effect on skeletal muscle performance and aging

With all of the positive effects nicotinamide riboside has on the biological processes and growth of skeletal muscle, few studies have examined how this can affect performance and

endurance of the muscles (Canto et al., 2012; Frederick et al., 2016; Kourtzidis et al., 2016; Ryu et al., 2016; Zhang et al., 2016). Canto et al. (2012) tested healthy mice muscle performance using a treadmill test and found that NR supplementation significantly increased distance run by over 200 meters compared to mice who were not given the vitamin. Nicotinamide riboside lengthened the amount of time and distance ran in aged mice as well as improved grip strength in aged mice (Zhang et al., 2016). Ryu et al. (2016) also looked at skeletal muscle function in muscular dystrophy mice and saw that when fed NR for 7 weeks, mice had an improved grip strength by about 33% compared to muscular dystrophy mice who were not given NR. In the NAMPT knock-out mice model studied by Frederick et al. (2016), NR was able to restore peak twitch force of muscle to the same level as mice without the defect. Due to beneficial effects of NR on other skeletal muscle aspects such as NAD+ levels, mitochondrial biogenesis, and ATP production, most journals speculate that nicotinamide riboside would have positive influences on muscle performance. Contradictory to this, only one opposing study by Kourtzidis et al. (2016) demonstrated a negative result from NR. Treatment consisted of 300mg/kg/day for 21 days and was given to male rats. After this supplementation period, a tendency for these rats to have a worse performance in a swimming endurance test (Kourtzidis et al., 2016). Though NR affected rats in this study negatively, in other experiments, NR had a beneficial outcome.

Other studies focused on if aging of skeletal can be delayed with the use of NR (Frederick et al., 2016 Zhang et al., 2016). Zhang et al. (2016) determined reversal of muscle damage due to aging is possible with administration of NR. After muscle damage, significantly more fibers regenerated. This study speculates that repairing of muscle was due to a significant increase in muscle stems cells after supplementation. Zhang et al. (2016) also examined aged mice, 22-24 months, as compared to young mice at 1 month of age. As mentioned previously,

certain biological levels and functions decrease with aging. But, when using NR as a feed additive, the levels of ATP, number of stem cells, and muscle performance were all brought back to levels of 1month old mice. Aged mice even had higher levels of NAD+ than that of young mice and, accordingly, it is believed that aging could be slowed, or reversed, with NR. In concurrence, Frederick et al (2016) saw that muscle deterioration was reversible with the application of NR, leading to the belief that this b3 vitamin can help slow the aging process of skeletal muscle.

Effect on cardiac muscle

Though many studies utilizing NR have also looked at the effects of skeletal muscle, only one study to date has also examined the impact of NR on cardiac muscle. In mice with muscular dystrophy, Ryu et al. (2016) observed both skeletal and cardiac muscle post NR administration. In the case of muscular dystrophy, often deaths eventually occur due to heart failure (Moat et al., 2013). Feeding 400mg/kg/day of NR for a duration of 12 weeks, resulted in a reduction of cardiac fibrosis, or the thickening and scarring of the connective tissue in the heart. Addition of NR to the diet also decreased the amount of necrosis of cardiac muscle cells and reduced inflammatory cell infiltration in the heart of muscular dystrophy mice (Ryu et al., 2016). This study demonstrates a possibility of NR to improve both skeletal and cardiac muscle.

IN OVO INJECTION IN BROILERS

Injection location and day of administration

Currently in the poultry industry, most commercial broiler hatcheries employ *in ovo* technology to vaccinate embryos at day 17 to 19 of incubation to prevent Marek's disease, Newcastle disease, and Infectious Bursal disease (Merck Veterinary Manual, 2018). Many studies in the literature were completed to determine the most effective site of vaccination.

Throughout the incubation period, there are several possible locations for *in ovo* injections to occur including the air cell, allantoic membrane, amniotic fluid, the yolk, the albumen, and in very late stages of development, the embryo body (Roto et al., 2016). Avakian et al. (2002) focused specifically on a Marek's disease vaccination and injected into four locations, the air cell, allantoic sac, amniotic fluid, and the embryo at d18 of incubation. The protective index, a percentage of effective immunity, for these injection sites were scored as 0, 26.3, 94.4, and 93.9, respectively, demonstrating that *in ovo* injection for vaccinations was most effective in the amniotic fluid and into the embryo itself. While these vaccinations occurred in late term embryogenesis, many studies observed the subsequent results of injections at multiple locations and different developmental time points.

Though various studies have applied *in ovo* technology, only few have monitored its effects on overall body and skeletal muscle growth. These same studies have developed different strategies for *in ovo* treatment and for injection positioning. According to Roto et al. (2016), the most common injection sites are into the air cell and the yolk sac of a broiler egg. Only two studies examining the overall body growth or skeletal muscle growth applied the air cell injection technique (Bhanja et al., 2015; Bednarczyk et al., 2016). The purpose of an air cell in an egg is to provide oxygen for the chick when it is ready to hatch. The contents placed into the air cell will not be directly incorporated into the embryo like other injection sites. Similarly, in two other studies, injection of treatment took place into the yolk of the egg (Kim et al., 2007; Macalintal et al., 2012). The yolk in a fertilized egg becomes the nutrition that the embryo will ingest and by the end of embryogenesis will be absorbed into the chick. Post-hatch, the contents of the remaining yolk will be absorbed into the intestines. (Speier et al., 2012). Both McReynolds et al. (2000) and Araujo et al. (2018) placed *in ovo* injections into the amnion of the

developing embryo, which is the sac directly surrounding the broiler embryo and is thought to be a form of protection for the maturing embryo. Several studies have observed injections into the albumen of the egg (Kocamis et al., 1998; Kim et al., 2007; Liu et al., 2012). The albumen of the egg provides water and some nutrients to the developing embryo (Willems et al., 2014). Abousaad et al. (2017) applied *in ovo* technology in a manner similar to current vaccinations of broiler embryos, directly to the body. This ensures that the embryo itself will directly receive treatment. Not only does the location of injection affect how treatment is received by the embryo, time of injection during the gestational period also plays a very large role to how treatment may affect the body and its functions.

Researchers have varied times at which injection takes place to potentially affect growth of certain tissues. The day at which this takes place will affect certain developments, depending on what structures are being formed at that time point. By day three of embryogenesis, both the brain and heart can be discerned. On days 3-7, organs continue to develop, primary myogenesis occurs, and limb buds are evident. Around day 8, the brain is fully in place and secondary myogenesis begins. After this point, growth of the bird-like features and overall size increases occur. In the last few days before hatching, the yolk is absorbed into the embryo and the chick prepares for hatch (Warin et al., 2009; Chal and Pourquie, 2017).

Liu et al. (2012) and Bhanja et al. (2015) administer injections on day zero, before incubation took place. Other studies also performed injections in the early stages of development between the days of three and seven, with the potential to affect both organ and skeletal muscle growth (Kocamis et al., 1998; Kim et al., 2007). Some researchers have chosen to inject midway through the gestational period, on days 10-12. (Hargis et al., 1989; Macalintal et al., 2012; Bednarczyk et al., 2016). These treatments would not be able to affect the formation of the

organs or of primary myogenesis, but could theoretically affect secondary myofibers and the subsequent growth. The injections implemented by McReynolds et al. (2000) and Araujo et al. (2018) were given at days 18 and 17.5, respectively. Though growth of the embryo is almost complete at this point of gestation, treatments given at this time have the chance to be absorbed into the body and consequently impact post-hatch development.

Effect on hatchability

In the United States, the egg fertility rate in the commercial broiler industry is about 94% with a hatchability rate of 85% (Cobb-Vantress, 2018). *In ovo* injections sometimes cause hatchability of broiler chicks to decrease (Kocamis et al., 1998; Kim et al., 2007; Macalintal et al., 2012). This is due to either the injection technique, the compound that is injected into the egg, or a combination of both technique and compound. Treatments can be given to the embryo, but only at an acceptable concentration to prevent death of the embryo. When injecting insulin-like growth factor 1 **(IGF-1)** into the albumen of the egg in the early days of embryogenesis, the hatchability was only 76.4% compared to the control that was not injected with a hatchability of 92.7% (Kocamis et al., 1998). Similarly, Kim et al. (2007) injected an anti-myostatin antibody at day 3 of embryogenesis into the yolk, which resulted in a 15.5% decreased in hatchability compared to the eggs that did not receive an injection. Though in this study, overall hatchability was low, with the controls only hatching about 68% of its broiler chicks. In the Macalintal et al. (2012) study, injection of Selenium at day 10 of incubation into the yolk of the fertilized egg led to only a 66% hatchability rate of broiler chicks. Not all *in ovo* injections cause decreases in the rate of hatch (Araujo et al., 2018). Five levels of vitamin E **(VE)** injections at day 17.5 of incubation resulted in varying hatch rates, though all were greater than the expected 85% hatchability. The largest VE injection of 60IU resulted in the highest hatchability rate of 92%

and the injection containing no VE had the smallest hatch rate of 86% (Araujo et al., 2018). Based off the findings of these studies, negative effects on hatchability rate often come from *in ovo* techniques requiring injections during the first half of embryogenesis, while injections occurring close to the day of hatch have no effect on hatchability.

Effect of in ovo treatment on overall weight

Several studies have used *in ovo* treatments to impact the overall growth and weight of the bird. Those that have, have incorporated different compounds into the injection solutions. None of these practices have integrated the use of nicotinamide riboside and *in ovo* injection techniques. Injection solutions including Leptin, Selenium, Probiotics, and Casein during embryogenesis did not result in an increase in body weight compared to the controls throughout the entire experiments (Liu et al., 2012; Macalintal et el., 2012; Bednarczyk et al., 2016; Abousaad et al., 2017). Two *in ovo* studies initially did not demonstrate increases in body weight, but over time examined changes between treated broilers and controls (Kocamis et al., 1998; Kim et al., 2007;). Kocamis et al. (1998) injected IGF-1 into fertilized broiler eggs during the first several days of incubation and saw no changes in body weight during weeks 0-5; but in the 6^{th} week, birds treated with IGF-1 had significantly greater weights than the untreated birds. The anti-myostatin antibody injections at day 3 of development resulted in similar findings. From days 1-14, no differences in body weight were observes; however, male broilers treated with the antibody had increased weights (Kim et al., 2007). Some compounds used *in ovo* technology have triggered significant differences in body weight (Hargis et al., 1989; Araujo et al., 2018). Hargis et al. (1998) injected a growth hormone into developing embryos and exhibited and increase in overall body weight of broiler chicks. Likewise, the injection of VE at day 17.5 of incubation indicated that the largest levels did lead to an increase in body weight of chicks at

hatch compared to chicks not given VE. Though while this difference was statistically significant, the largest VE treatment weights were only about one gram larger than the control (Araujo et al., 2018). *In ovo* injections have led to variable results in body weight of broilers due to the diverse treatments tested in these studies.

Effect on organ characteristics

Several studies that injected solutions within the first three days of development did consider the impact on organ development (Kocamis et al., 1998; Kim et al., 2007; Bhanja et al., 2015). An IGF-1 injection placed into the albumen produced an increase in heart weights from males, though no difference was observed for females from 42 day old broilers. This could be expected due to male birds being overall larger than females. Liver weights remained similar for IGF-1 treated birds versus controls (Kocamis et al., 1998). Kim et al. (2007) found concurrent results with an injection of an anti-myostatin antibody, and the hearts from male broilers were larger than females. The liver and spleen weights between treatments were similar (Kim et al., 2007). Twenty-eight days post-hatch, Bhanja et al. (2015) also presented data indicating similar weights for the heart, liver, and spleen after injection of nano-silver particles before incubation of fertilized broiler eggs. None were able to find biologically significant alterations to organs from *in ovo* injections of these compounds. Studies in which injection took place during mid to the end of embryogenesis did not examine organ growth and development, likely due to the fact that organs are developed early on in the gestation period.

Effect on skeletal muscle growth

The results are varied for studies evaluating the effect of *in ovo* technology on skeletal muscle growth. Both the injections of anti-myostatin antibody and silver nano-particles at days three and zero respectively, did not change the weight of the breast muscles in resulting birds

(Kim et al., 2007; Bhanja et al., 2015). Though Kim et al. (2007) did not observe changes to the breast weight of broilers 28 days post-hatch, the thigh and leg weight of the birds given silver nano-particles into the yolk were smaller than that of the controls and the bird given the injection into the albumen. Some researchers have reported positive effects on skeletal growth with the in ovo injections of growth hormones, IGF-1, and leptin (Hargis et al., 1998; Kocamis et al., 1998; Liu et al., 2012). Kocamis et al. (1998) observed increases in both the leg and breast weight of 42 day old broilers that were treated with IGF-1 in the albumen on day three of development. Likewise, levels of leptin in the albumen given to fertilized eggs before incubation led to an increase in the *gastrocnemius* muscle of both male and female broilers (Liu et al., 2012).

Other aspects of skeletal muscle quality from the breast muscle have been studied after the use of *in ovo* technologies (Kocamis et al., 1998; Macalintal et al., 2012; Araujo et al., 2018). Between the untreated broilers and the IGF-1 injected broilers, the percentage of moisture, fat, protein, and ash from the breast muscle was similar (Kocamis et al., 1998). Contrary to IGF-1 injections, all levels of VE injected into the amniotic cavity resulted in an increase of protein found in the breast muscle of newly hatched chicks (Araujo et al., 2018). Macalintal et al. (2012) observed decreased oxidation in the breast muscle from broilers treated with selenium in the yolk midway through the gestational period. The outcomes of the muscle growth and quality examined in these studies are quite variable due to the differences in techniques and compounds injected into the developing embryonic environment.

Chapter 3 - The effect of in ovo injection of Nicotinamide Riboside on avian myogensis

ABSTRACT

The objective of this study was to determine the effects of *in ovo* injection of nicotinamide riboside (**NR**) on avian embryonic myogenesis. Before incubation, fertilized broiler eggs (*n*=156; Cobb 500) were randomly assigned to 1 of 4 treatments in a completely randomized design with a 2 × 2 factorial arrangement. Factor 1 was treatment (**TRT**), with eggs injected with 100 µl of 0.0 (-) or 2.5 mM (+) NR in sterile saline. Factor 2 consisted of injection location (**LOC**), with treatments injected into the yolk (**YO**) or albumin (**AL**). Injection took place at d 10 of incubation. Twenty-four hours post-hatch, chicks were euthanized. Measurements including chick weight, head and chest measures, and left *Pectoralis major* (**PM**) weight and dimensions were collected. The left PM was used for muscle fiber analysis. There were no TRT×LOC interactions for all body and *pectoralis majo*r measures ($P \geq 0.07$), except PM weight and length ($P < 0.01$). *Pectoralis major* muscles from the YO+ treatment were heavier ($P < 0.01$) than all other treatments. Length of PM from AL+ were longer than control chicks injected into either the yolk or albumen ($P < 0.04$), but was shorter ($P < 0.01$) than YO+ chicks. All body measures were not affected by the main effects of TRT or LOC ($P \geq 0.12$), with the exception of chest width, in which YO chicks has wider chests ($P = 0.01$) than AL chicks. There was a TRT×LOC interaction for muscle fibers per 1000µm^2 with YO+ muscles having the largest number of fibers ($P < 0.01$) compared to all other treatments, which did not differ ($P \geq 0.16$) from each other. There were no TRT×LOC ($P \geq 0.43$) interactions nor TRT or LOC main effects ($P = 0.06$) for muscle fiber cross sectional area. Treatment affected PM width

and depth ($P > 0.01$), with NR chicks having wider and thicker muscles

that control chicks. Injecting NR into the egg of the developing broiler embryo increases PM muscle development and data would indicate greater positive effects when NR is injected into the yolk.

Key Words: broiler, fiber cross-sectional area, *in ovo* injection, nicotinamide riboside, *pectoralis major*

INTRODUCTION

Obtaining food security for the world has been a pressing issue as the population continues to drastically increase. In the United States, poultry consumption over the past 60 years has also dramatically intensified by 229% (Nation Chicken Council, 2019). To meet the demands of escalating preferences for chicken, producers must either increase broiler production, increase their rate of growth, or increase the amount of muscle harvested from each bird. Myogenesis, or the formation of muscle, may be altered to potentially achieve an increase in rate of growth or skeletal muscle accumulation. Though overall body weight has increased and breast to feed conversion rate has decreased significantly in the past 60 years (Zuidhof et al., 2014), improvements could still be made to provide for the growing population.

Nicotinamide riboside **(NR)** is a vitamin B3 analogue and has induced positive effects on skeletal muscle physiology. One known outcome of NR supplementation is protection of skeletal muscle against aging (Guarente et al., 2016; Fletcher et al., 2017), which has been thought to be due to increases in muscle satellite cells with the addition of NR (Zhang et al., 2016). Other studies have proved that with the oral supplementation of NR, biogenesis of mitochondria is also amplified after the addition of NR to the diet (Canto et al., 2012; Ryu et al., 2016). As a result, NAD+ levels within muscle were augmented as seen in both murine models by increases of 34-142% (Khan et al., 2014; Zhang et al., 2016) and in the human model by about 60% (Martens et al., 2018). Biogenesis of mitochondria is also amplified after the addition of NR to the diet (Canto et al., 2012; Ryu et al., 2016). An increase in Adenosine Triphosphate **(ATP)** is also observed from NR treatment due to improvements to mitochondrial function (Khan et al., 2014;

Ryu et al., 2016; Frederick et al., 2016). Some studies also observed increased muscle mass and muscle performance (Frederick et al., 2016; Ryu et al., 2016).

One approach to deliver NR to developing broiler embryos is *in ovo* injections. This technology is currently utilized for vaccine administration in the commercial poultry industry. Researchers have used *in ovo* injection of other supplements such as amino acids, carbohydrates, hormones, probiotics, and immunostimulants, leading to varied results on skeletal muscle and overall growth (Roto et al., 2016). Both injections of growth hormone as well as IGF-1 led to increases in skeletal muscle growth in the breast of broilers (Hargis et al., 1989; Kocamis et al., 1998). While there are many studies examining the effects of NR supplementation on skeletal muscle in mice, none have been observed in broilers. Therefore, the objective of this study was to observe the effects of *in ovo* injection of nicotinamide riboside on overall growth measurements and skeletal muscle physiology.

MATERIALS AND METHODS

Egg Collection, Treatment Assignment, and Incubation

Fertilized broiler eggs (n = 156) with an average weight of 70.3 g (Cobb 500; Cobb Vantress, Siloam Springs, AR) were transported to Kansas State University (Manhattan, KS). Upon arrival, egg weights were recorded and eggs were ordered by weight. Within each 4 egg strata, eggs were randomly assigned to treatment within a 2 × 2 factorial arrangement. Factor 1 was NR treatment with eggs receiving 0.0m*M* (-) or 2.5m*M* (+) NR. Factor 2 consisted of injection location, with treatments injected into either the yolk **(YO)** or albumen **(AL)**. After treatment assignment, eggs were positioned with equal treatment representation onto trays and placed in an incubator (Sportsman 1502; GQF Manufacturing Company Inc., Savannah, GA) set to operate at a temperature of 37°C and a relative humidity of 40 ± 2% for the first 18 d of

incubation. The incubator rotated hourly to reposition eggs and the trays were rotated daily throughout the incubator to account for variation in temperature and humidity. Tray weights were recorded each day to determine egg weight loss percentage with a target weight loss of 0.67% per day.

Solution Preparation and Injection Procedure

Solutions were prepared immediately prior to injection procedures. To achieve a 250m*M* solution, 6.38g of NR was added to 0.1L of 0.9% sterile saline and covered with foil to prevent exposure to light. To determine a final concentration in the egg, the average volume of the yolk from Cobb 500 eggs was determined to be 10mL. Injecting 100µl of the 250m*M* NR solution into the 10mL yolk led to a final concentration of 2.5mM NR. The albumin injection groups were given the same amount of 100µl of solution. The control group also received 100µl of the 0.9% sterile saline. The injection solutions were stored at room temperature before and during the injection process. Sets of 20 eggs representing equal treatment numbers were removed from the incubator at a time. Eggs were candled to determine location of the yolk and albumen and the injection site was cleaned with 70% ethanol. Eggs were turned at a 90°angle and a 2.54-cm, 20-guage hypodermic needle was used to create an opening in the shell. The needle was pressed approximately 1 cm into injection site and 100µl of the assigned treatment was expelled into the egg. A 1-cm^2 portion of medical tape (Nexcare; 3M, Maplewood, MN) was positioned over the injection location and eggs were returned to the incubator.

Hatching, Euthanasia, and Processing

On d 18 of incubation, the relative humidity of the incubator was increased to 60 ± 2% and eggs were placed into hatching boxes at the bottom of the incubator. As chicks began to hatch on d 21, they were removed from the incubator, marked for treatment, and relocated to a

box positioned underneath a heat lamp. Approximately 12 to 24 h after hatch, chicks were euthanized by prolonged exposure to CO_2 gas and decapitation. Chick weights were recorded and calipers (Traceable Digital Calipers; Fisher Scientific, Pittsburg, PA) were utilized to measure crown to rump length **(CR)**, head width, head length. Head and chest circumference were also collected by wrapping a string around the designated area and comparing the length of the measurement against a ruler. Chicks were sprayed with 70% ethanol, and the skin and feathers were pulled back to reveal the *Pectoralis major* muscles. Once exposed, the overall chest width and length were measured using digital calipers. Using a scalpel, the left and right PM muscles were removed, careful to not remove the *Pectoralis minor* muscles. The left PM was weighed and dimensions of this muscle were collected using digital calipers, including PM length, width, and depth. The left PM was positioned onto a tongue depressor and placed into a -80°C freezer, where it was stored until used for histology sectioning. The right PM was stored in a microcentrifuge tube at -80°C until further analyses. The heart and liver were also removed, weighed, and then discarded.

Histology and Immunohistochemistry

The left PM was removed from the tongue depressor and embedded in tissue embedding media (Fisher Scientific), cooled with liquid nitrogen, and stored at -80°C until further analysis. When sectioned, cuts were made parallel to the length of the PM muscle. Six cryosections were collected (10μM thick) on positively charged slides (Diamond White Glass; Globe Scientific Inc., Paramus, NJ) and the methods of Noel et al. (2016) were followed for immunohistochemistry with modifications. Briefly, cryosections were incubated in a blocking solution containing 5% horse serum and 0.2% TritonX-100 in Phosphate Buffered Saline (**PBS**) for 30 min. Slides were incubated for 1 h at room temperature in a primary antibody solution

consisting of blocking solution and 1:500 α-dystrophin (Thermo Scientific, Waltham, MA). Cryosections were washed 3 times for 5 min each in PBS and then incubated for an additional 30 min with blocking solution containing 1:1,000 Alexa-Flour 594 goat-anti-rabbit heavy and light chains (Life Technologies; Carlsbad, CA). Cryosections were washed again as stated above, 5μl of 9:1 glycerol in PBS was placed on each section, and coverslips were added for imaging. Cryosections were imaged at 200× magnification using a Nikon Elipse TI-U inverted microscope (Nikon Instruments Inc., Melville, NY). A Nikon DS-QiMC digital camera (Nikon Instruments Inc.) was used to photograph cryosections and an average of 1,000 fibers were analyzed using NIS-Elements Imaging software (Basic Research, 3.3; Nikon Instruments Inc.) to determine fiber cross-section area **(CSA)**.

NAD+ Quantification

Skeletal muscle NAD+ content was quantified using the NAD+/NADH Assay Kit (Abnova, Taipei, Taiwan). Briefly, about 25mg of tissue from the right PM of two samples within the same treatment were pooled together to reach a weight of 50mg of muscle tissue. The pooled samples were homogenized in 100μl of NAD extraction buffer. Samples were heated at 60°C for 5 min and 20 μl assay buffer and 100 μl of NADH extraction buffer were added. Samples were vortexed, centrifuged at 17,500 x g for 5 minutes, and the supernatants were extracted. Supernatants and the working reagent were aliquoted and absorbance was measured at 565 nm at time points 0 and 15 minutes using a spectrophotometer (Eon; BioTek, Winooski, VT, USA). The NAD+ concentration was calculated using the change in optical density (**OD**) by subtracting OD_0 from OD_{15}, deducting the OD value of the blank, and dividing that value by the slope of the NAD+ standard curve. (Data not presented).

Statistics

Data were analyzed as a completely randomized design with a 2 × 2 factorial arrangement and egg as the experimental unit. All models were analyzed using the MIXED procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC) with treatment (**TRT**) and injection location (**LOC**) as fixed effects. Statistical significance was set at $P \leq 0.05$.

RESULTS

Body Morphometrics

There were no TRT × LOC interactions for all measures ($P > 0.07$; Table 3.1), except PM weight and length ($P < 0.01$; Table 3.2). Pectoralis major weights of NR chicks injected in the yolk were greater than all other treatment groups ($P < 0.01$), which did not differ from each other ($P > 0.09$). Length of PM from NR chicks injected into the albumen was longer than control chicks injected into either the yolk or albumen ($P < 0.04$), but was shorter ($P < 0.01$) than NR chicks injected into the yolk. Treatment did not affect whole body or organ measures ($P > 0.12$; Table 3.1); however, NR treatment did increase length, width, and depth of PM ($P < 0.01$). The main effect of location showed no effects ($P > 0.06$), with the exception of an increased chest width and PM weight when injection took place in the yolk ($P \leq 0.01$).

Muscle Fiber Characteristics

There was no TRT × LOC interaction for muscle fiber CSA ($P = 0.73$; Figure 3.1). Neither treatment nor location affected muscle fiber CSA ($P \geq 0.06$). There was a TRT × LOC interaction for muscle fiber number per 1000 square microns ($P < 0.01$; Figure 3.2). There were no differences between treatments injected into the albumin or between NR injected into the yolk ($P \geq 0.16$); however, when NR was injected into the yolk of the egg, muscle fiber number was significantly larger than all other treatments ($P < 0.01$).

NAD+ Quantification

At the time of publication of this thesis, NAD+ data was proprietary. This data is expected to be published in a peer reviewed journal at a later date.

DISCUSSION

Body Morphometrics

Over the last 60 years, poultry consumption has increased by 229% (USDA, 2018). To keep up with demand, the industry has improved rate of growth in broilers with decreased market age and increased market weight (National Chicken Council, 2018). Poultry scientists are investigating ways to capitalize on poultry growth. In the past, *in ovo* injected growth hormone did increase poultry growth by increasing overall body weight (Hargis et al., 1989); however, in present day, consumers no longer are tolerable towards growth hormones. Due to these consumer preferences, several studies have examined the effects on injecting alternatives that consumers may find more favorable. Although, these same studies report no effects on overall body weight of chicks after *in ovo* injections with differing compounds including selenium, probiotics, and vitamin E (Macalintal et al., 2012; Bednarczyk et al., 2016; Araujo et al., 2018). Research studies have yet to examine the effects of nicotinamide riboside on whole body and skeletal muscle growth in meat producing animals, including in ovo injection of NR in broilers. However, several studies have looked at the effect of NR on these qualities in rodents. Under normal diet conditions, mice fed NR did not show changes in body weight (Canto et al., 2012). Addition of NR only affected overall body weight in mice fed high-fat diets, with mice supplemented with NR gaining less weight than mice that were not given NR (Canto et al., 2012). In the current study, *in ovo* injection of NR into either location did not affect overall body weight in hatched chicks. This is supported by the fact that NR injection also did not affect any

other whole body measurement for d 21 hatched chicks. Overall, the lack of significance on overall body weight of the current study are similar to literature regarding *in ovo* injection studies of other vitamins and minerals as well as similar to the addition of NR in the feed of rodents under normal conditions.

As seen with overall body weight, only *in ovo* injection with growth hormone successfully increased the breast muscle weight (Hargis et al., 1989). While NR has not previously been injected into broiler eggs, NR feed supplementation in rodents has resulted in varied effects across many studies. Canto et al. (2014) demonstrated that healthy mice fed NR had similar lean muscle mass compared to mice not supplemented with NR. Only under unusual circumstances did NR increase skeletal muscle mass in rodents. NAMPT knockout mice with characteristically lower muscle mass compared to normal mice, showed increases in skeletal muscle similar to control mice when fed NR (Frederick et al., 2016). Similar results in the muscular dystrophy model were observed in a study by Ryu et al. (2016). Feeding NR to healthy mice did not lead to an increase in muscle mass; however, NR led to an increase in muscle mass of mice with muscular dystrophy by an average of 43% (Ryu et al., 2016). In the current study, only injection of NR into the yolk of the egg led to a 35% increase in PM weight. Additionally, injection of NR into both the yolk and the albumin increased PM length by an average of 14%. These differing results among studies may be due to the prenatal method at which NR was delivered to broiler eggs in the current study, whereas in past studies NR was given as a daily feed additive in mice postnatally.

Muscle Fiber Characteristics

In previous literature regarding use of NR, muscle fiber CSA has been examined only in the NAMPT knockout and muscular dystrophy mice models which characteristic of smaller muscle fibers. In both cases, increases in muscle fiber CSA were reported with the addition of NR in the diet (Frederick et al., 2016; Ryu et al., 2016). In the current study, the results reported were contrasting to previous literature. Fiber CSA was not altered due to an injection of NR into either location and fiber size was similar across all treatments.

These findings would indicate that the overall growth of the PM observed was not due to changes in size of the muscle fibers. For growth to have occurred without an increase in fiber size, fiber number was altered. In the current study, fiber number per 1000μm was increased by 37.9% when an injection with NR took place in the yolk of the developing egg. No other studies concerning either NR or *in ovo* injections evaluated muscle fiber number. Due to the fact that in past literature, NR was given postnatally, fiber number could not be altered in previous rodent studies, whereas in the current study, NR was injected prenatally. While fiber size was not altered by NR injection into either the yolk or albumin, fiber number was significantly increased in the YO+ treatment. Overall, the current study's findings were not comparable to past literature, though this may largely be due to the differences in species and method at which NR was administered.

CONCLUSION

Injection of 2.5 m*M* nicotinamide riboside at d 10 in incubation into both the yolk and albumin did not affect overall body growth or organ weight in hatched broiler chicks; however, skeletal muscle from the *pectoralis major* was altered. Increased PM weight was observed from

chicks given NR into the yolk of the egg. Length of the PM was also increased by injection of NR into both locations, though the longest muscles came from chicks that were supplemented with NR in the yolk. While muscle fiber size in the PM was unchanged by treatment and injection location, muscle fiber number was increased by the YO+ treatment. These findings indicate that while body measurements were unchanged, positive transformations were made to PM muscle size and fiber number with a 2.5mM injection into the yolk. Overall, the new injection technology may be a solution to improving growth rate in broilers and could be utilized to meet the consumer demand for chicken.

Table 3.1 Whole body muscle measurements from hatched broiler chicks injected with 0mM Nicotinamide Riboside (NR) in 0.9%sterile saline or 2.5mM NR in 0.9% sterile saline into either the yolk (Y) or albumin (A) at day 10 incubation

	Treatment							
	0 m*M* NR		2.5 m*M* NR			*P*-values		
Item	A	Y	A	Y	SEM	TRT[1]	LOC[2]	TRT×LOC
Chick weight, g	42.69	42.99	43.19	44.02	6.4	0.17	0.31	0.63
Body measurements, mm								
Crown-rump length	95.60	96.93	96.14	89.36	3.70	0.12	0.23	0.07
Head width	15.40	15.74	15.56	15.45	0.40	0.67	0.48	0.21
Head length	21.79	21.86	21.29	21.73	0.19	0.42	0.51	0.63
Head circumference	32.38	32.08	32.14	31.49	1.60	0.70	0.66	0.87
Chest length	22.01	21.96	21.98	22.68	0.55	0.36	0.39	0.29
Chest width	16.90	17.35	16.57	17.69	0.33	0.98	0.01	0.28
Heart weight, g	0.36	0.36	0.35	0.36	0.02	0.75	0.55	0.24
Liver weight, g	1.09	1.08	1.05	1.11	0.06	0.64	0.36	0.19

[abc] of treatment within row differs ($P < 0.05$).

[1]TRT = treatment of 0 m*M* or 2.5m*M* NR in 0.9% sterile saline.

[2] LOC = location of injection.

Table 3.2 Pectoralis major measurements from hatched broiler chicks injected with 0mM Nicotinamide Riboside (NR) in 0.9% sterile or 2.5mM NR in 0.9% sterile saline into either the yolk (Y) or Albumin (A) at day 10 of incubation

	Treatment							
	0 m*M* NR		2.5 m*M* NR			*P*-values		
Item	A	Y	A	Y	SEM	TRT[1]	LOC[2]	TRT×LOC
Pectoralis major weight, g	0.13[a]	0.13[a]	0.14[a]	0.18[b]	0.01	<0.01	<0.01	<0.01
Pectoralis major dimensions, mm								
Length	17.55[a]	17.04[a]	18.68[b]	20.74[c]	0.43	<0.01	0.06	<0.01
Width	4.59	4.62	4.81	5.21	0.22	<0.01	0.13	0.20
Depth	2.27	2.32	2.43	2.65	0.09	<0.01	0.09	0.30

[abc] of treatment within row differs ($P < 0.05$).
[1]TRT = treatment of 0 m*M* or 2.5m*M* NR in 0.9% sterile saline.
[2] LOC = location of injection.

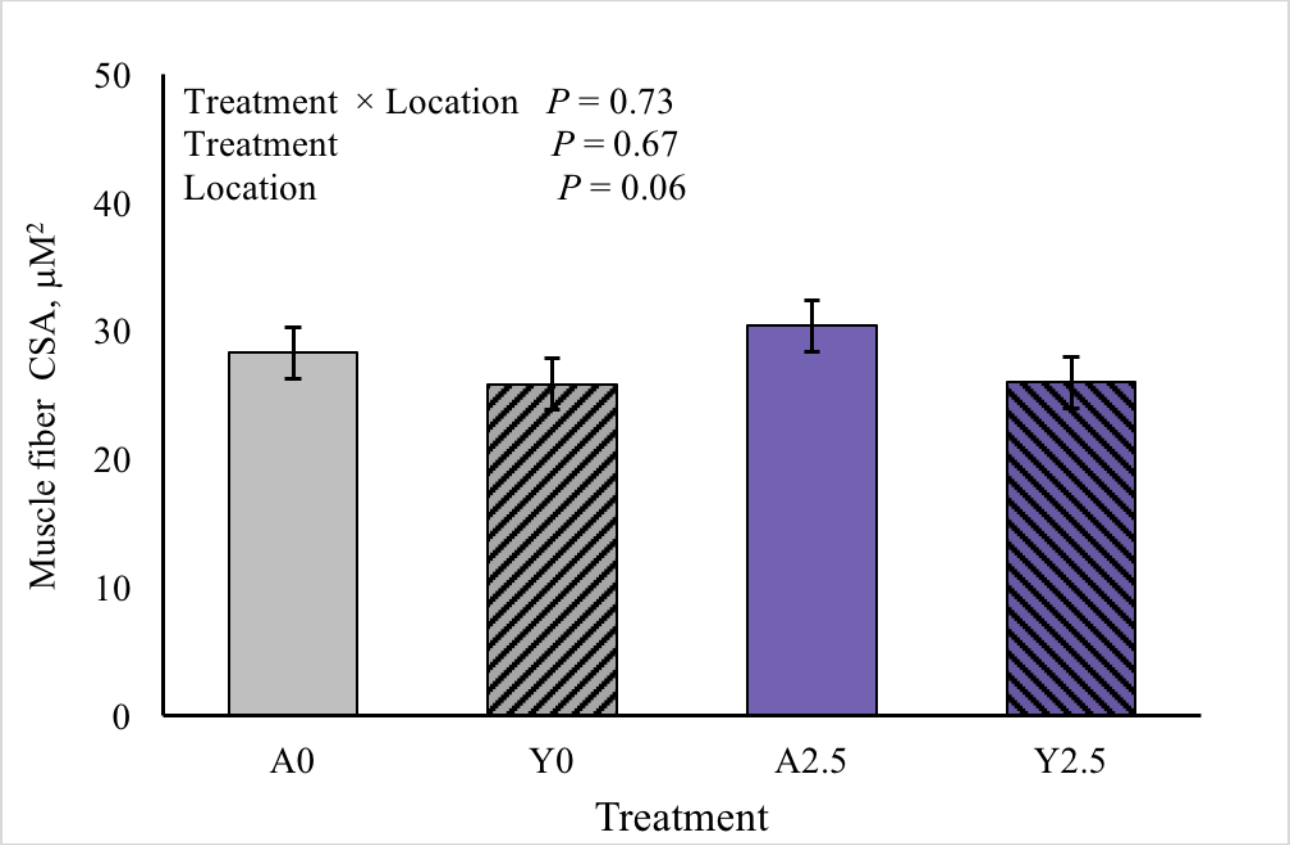
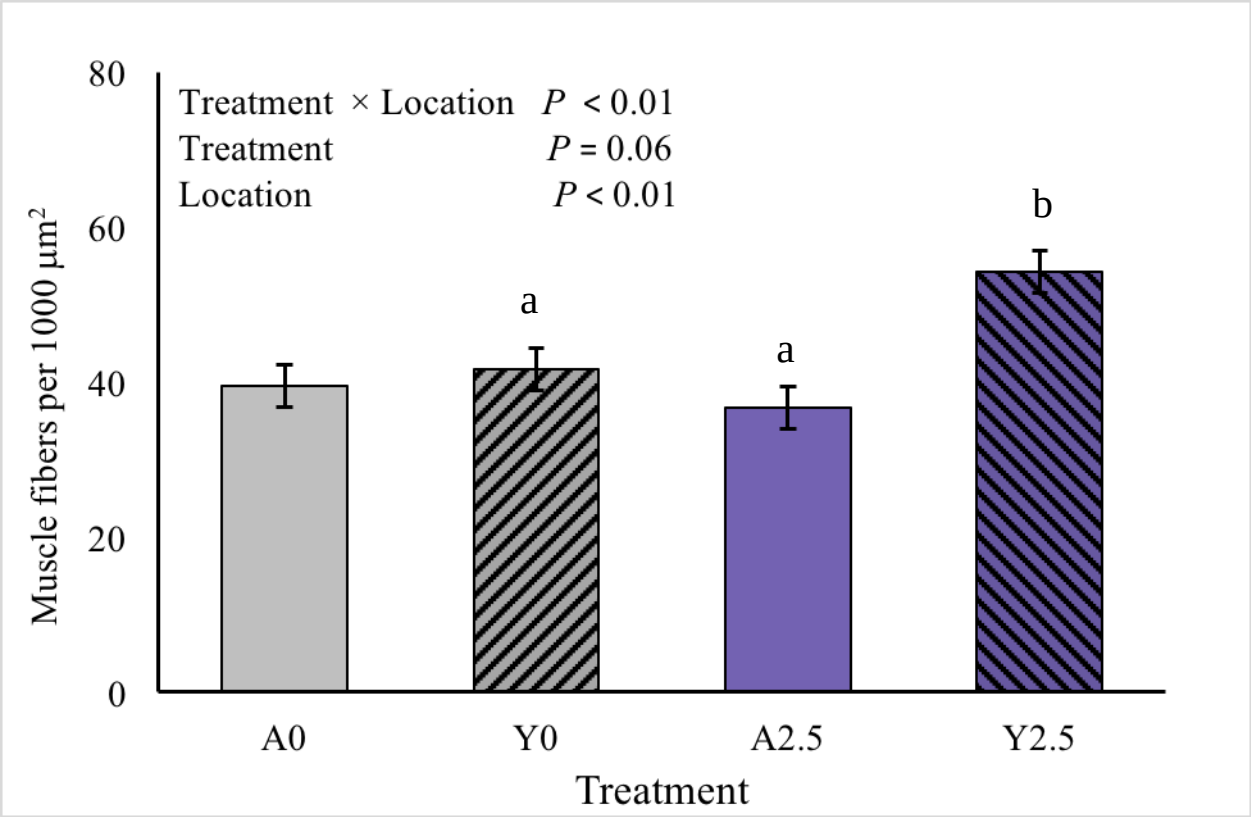


Figure 3.1 Muscle fiber cross-sectional area (CSA) from the *pectoralis major* (PM) of hatched broiler chicks injected with 0 m*M* nicotinamide riboside (NR) in 0.9% sterile saline or 2.5m*M* NR in 0.9% sterile saline into either the yolk (Y) or albumin (A) at day 10 of incubation. The PM muscle was embedded in a sectioning compound, sectioned onto a slide, and stained with Dystrophin antibody (Thermo Scientific). For each sample, the area of 1000 fibers were analyzed on the NIS-Elements Imaging software (Basic Research, 3.3; Nikon Instruments Inc.).


Treatment × Location P < 0.01
Treatment P = 0.06
Location P < 0.01
Muscle fibers per 1000 µm²
80
60
40
20
0
a
a
b
A0
Y0
A2.5
Y2.5
Treatment

Chapter 4 - Increasing the concentration of in ovo injected Nicotinamide Riboside has a positive influence on avian myogenesis

ABSTRACT

The objective of this study was to determine the effect of nicotinamide riboside concentration on avian embryonic myogenesis. Fertilized broiler eggs (*n*=420; Cobb 500) were randomly assigned to 1 of 4 treatments: 0.0, 2.5, 5.0, or 10.0 m*M* NR made in 0.9% sterile saline. Eggs were assigned to day of euthanasia on d15 of embryonic incubation, d19 of embryonic incubation, or d21 after hatch. At d10 of incubation, 100µl of allotted treatment was injected into the egg yolk. Eggs were incubated at 37±3°C and 40±2% relative humidity for 18 days and raised to 60±2% for the final 3 days. At all time points, embryos and chicks were euthanized. Measurements collected include: body weight; crown-rump length; chest and head measures; organ weights; and either chest plate weight and dimensions at d15, or left *Pectoralis major* (**PM**) weight and dimensions at d19 and d21. Leg area and muscle fiber characteristics were analyzed. There was no treatment effect on body measures for d15 embryo weight ($P \geq 0.23$). For d19 embryos and d21 chicks, there were no treatment differences for body measures ($P \geq 0.08$), excluding head circumference in d19 embryos and head length and chest width in d21 chicks. Head circumference was increased ($P=0.04$) in the 5 and 10mM d19 embryos, while d21 chick head length and chest width were larger ($P \geq 0.05$) from 2.5m*M* NR. There was no treatment effect ($P \geq 0.22$) for d15 chest plate measures; however, d19 PM weight and length were increased ($P<0.01$) at all NR levels. The weight and width of chick PM were increased ($P<0.01$) at every NR level. Chicks given 2.5 and 5mM NR had longer PM measurements ($P \leq 0.01$) than saline chicks. Injection of 5m*M* NR increased PM depth ($P \leq 0.04$) from d21 chicks. Treatment did not

affect leg muscle area or muscle fiber CSA ($P \geq 0.20$). All NR injections increased muscle fiber number ($P < 0.01$); though 10mM injection led to the largest increase in fibers ($P < 0.01$). Injecting up to 5.0 mM NR in the yolk of the developing embryo did not affect body weight but increased PM measures, indicating NR influenced avian myogenesis.

Key Words: broiler, *in ovo* injection, nicotinamide riboside, muscle fiber, *pectoralis major*

INTRODUCTION

Demand for poultry meat has continued to rise as chicken consumption has more than doubled over the past 60 years (National Chicken Council, 2019). A major goal of the agricultural industry is to be able provide for these changing consumer preferences. To maximize the availability of this protein, researchers are investigating ways to create a more efficient broiler. Several methods may be used to meet these demands including increasing production of broilers, increasing the growth rate of broilers, or increasing the amount of muscle found on each broiler. One approach to achieving increased growth rate or muscle accumulation is to modify myogenesis as the broiler embryo is developing.

Nicotinamide riboside (**NR**), a vitamin B3 analogue and NAD+ precursor, is one compound that has been shown to positively impact skeletal muscle qualities. When fed NR, NAD+ concentration in the skeletal muscle is significantly increased, which is important for energy production in the muscle (Canto et al., 2012; Frederick et al., 2016; Agerholm et al., 2017; Fletcher et al., 2017). Mitochondria biogenesis, is also increased with the addition of NR to the diet in mice (Khan et al., 2014). Due to the increase in mitochondria production, ATP levels are also amplified in the skeletal muscle of mice given NR (Khan et al., 2014; Ryu et al., 2016; Frederick et al., 2016; Zhang et al., 2016). The changes observed in the muscle physiology may explain increases in muscle performance in studies performed by Canto et al. (2012), Ryu et al. (2016), and Zhang et al. (2016).

Along with the molecular changes observed after NR supplementation, muscle accumulation and conformation has been altered in some studies. Although NR did not increase overall muscle mass in healthy mice (Canto et al., 2012), increased in muscle growth as well as

an increase muscle fiber cross sectional area were demonstrated in mice with muscular dystrophy (Ryu et al., 2016). Zhang et al. (2016) detected an increase in number of muscle satellite cells after supplementation of NR, which could explain improvements to muscle repair seen in the muscular dystrophy model.

While supplementation of NR has been primarily through feed, one way to administer NR to developing broilers would be through *in ovo* injection. *In ovo* injection has previously been used to add compounds such as probiotics and vitamins (Bednarczyk et al., 2016; Araujo et al., 2018), but no studies have examined the effect of *in ovo* injection of NR. Therefore, the objective of this study was to observe the effects of *in ovo* injection of nicotinamide riboside on whole-body growth measurements, NAD+ content, mitochondrial quantity, satellite cell number, and muscle characteristics in broilers.

METHODS

Egg Collection, Treatment Assignment, and Incubation

Fertilized broiler eggs (n=420) with an average weight of 65.6g (Cobb 500; Cobb Vantress, Siloam Springs, AR) were transported to Kansas State University (Manhattan, KS). Immediately following arrival, egg weights were recorded and eggs were ordered by weight. Within each 4 egg strata, eggs were randomly assigned to 1 of 4 treatments to be injected into the yolk of the egg at d 10 of incubation. Treatment consisted of 0, 2.5, 5, and 10m*M* NR made up in 0.9% sterile saline that was to be injected into the yolk of the egg. Eggs were equally delegated to both NR treatment and day of processing, which included d15 of incubation, d19 of incubation, and day 21 or day of hatch. After designation of treatments, eggs were positioned with equal treatment representation onto trays and placed in an incubator (Sportsman 1502; GQF Manufacturing Company Inc., Savannah, A) with a setting of 37°C and a relative humidity of 40

± 2%. The incubator shelves rotated hourly to turn eggs and the trays were relocated daily throughout the incubator to account for variation. Tray weights were recorded daily to determine percent weight loss with a goal of 0.67% loss per day.

Solution Preparation and Injection Procedure

The methods of the previous study were followed with some modifications. Briefly, solutions were prepared immediately prior to injection. To achieve a 1000m*M*, 500m*M*, and 250m*M* solutions, 25.52g, 12.76g, and 6.38g of NR were added to 0.1L of autoclaved 0.9% sterile saline, respectively. To figure a final concentration in the egg, the average volume of the yolk from Cobb 500 eggs was determined to be 10mL. Injection with 100µl of the 1000, 500, or 250m*M* NR solution into the 10mL yolk created the final concentration 2.5, 5, and 10m*M* NR. The control solution solely consisted of 0.9% sterile saline. All solutions were stored at room temperature before and during injection process. Candling verified the location of the yolk for injections and the injection site was cleansed with 70% ethanol. A 2.54-cm, 20-guage injection needle was used to create an opening in the shell and the needle was inserted approximately 1-cm into injection site. Expulsion of 100µl of assigned treatment took place in the yolk. A 1-cm^2 portion of medical tape (Nexcare; 3M, Maplewood, MN) was pressed over the opening. Treated broiler eggs were returned to the incubator under normal conditions until d 18 of incubation.

The procedures of the previous study were followed with modifications. Briefly, at day 15 of incubation, the assigned 120 eggs were removed from the incubator and the shells were penetrated at allow euthanasia with exposure to CO_2 gas. After 25 minutes, embryos were removed from the eggs and were processed. Embryo weight, CR, head width, and head length were recorded. The ribs, *pectoralis major*, and *pectoralis minor* muscles were removed and kept

intact. This chest section was weighed, and the dimensions of length, width, and depth were also documented. Both the heart and liver were removed and weighed.

After 18 d of incubation, relative humidity was increased to 60 ± 2% and eggs were moved to hatching boxes. The processing procedures of d19 embryos and d21 hatched chicks were similar. The delegated 180 eggs for d19 embryo processing were removed from the incubator and exposed to prolonged CO_2 gas and decapitation. As the remaining 120 eggs began to hatch on d21, chicks were marked by treatment and were placed into a heated environment under a lamp. All chicks were euthanized by prolonged exposure to CO_2 gas and decapitation within 24h after hatch. For both the d19 embryos and d21 chicks, overall weight, CR, head width, head length, head circumference, and chest circumference were documented. The embryos and chicks were sanitized with 70% ethanol and the skin was removed to expose the breast muscles and the overall chest width and length were measured using digital calipers (Traceable Digital Calipers; Fisher Scientific) Both the left and right PM muscles were removed with a scalpel. The left PM weight and the dimensions of this muscle, including PM length, width, and depth, were recorded. The left PM was placed onto a tongue depressor and moved to a -80°C freezer for storage until use for histological sectioning. The right PM was also stored for further analyses. The left leg was removed caudal to the hip joint and the *rectus femoris*, *vastus lateralis*, *vastus intermedius*, and *vastus medialis* still attached to the femur, were blotted on paper to measure the approximate area of the leg. The heart and liver were removed for organ weights.

Histology and Immunohistochemistry

The methods of the previous study were followed with modifications. The left PM was embedded into tissue embedding media (Fisher Scientific, Pittsburg, PA), cooled with liquid

nitrogen, and stored in a -80°C until further histological analysis. When sectioned, cuts were made perpendicular to the length of the PM muscle. To analyze muscle fiber size and satellite cell number via immunohistochemistry, six cryosections were cut (10μM thick) and positioned onto positively charged slides (Diamond White Glass; Globe Scientific Inc., Paramus, NJ). One additional slide per sample with six cryosections (20μM thick) were obtained for SDH staining. The methods of Noel et al. (2016) were followed for fiber and satellite cell immunohistochemistry with modifications. Briefly, cryosections were incubated in a 5% horse serum and 0.2% TritonX-100 in PBS blocking solution for 30 minutes and then for 1h at room temperature in a primary antibody solution. The primary antibody solution consisted of 1:2 blocking solution, 1:2 Pax7 antibody (Developmental Studies Hybridoma Bank), and 1:500 α-dystrophin (Thermo Scientific, Waltham, MA). Cryosections were washed with PBS 3 times for 5 minutes and then incubated again 30 minutes with blocking solution containing 1:1,000 Alexa-Flour 594 goat-anti-rabbit heavy and light chains (Life Technologies), 1:1,000 Hoescht Dye 33342 (Life technologies), and 1:1000 Alexa-Flour 488 goat-anti-mouse IgG1 (Life Technologies). Cryosections were washed again as stated previously. Every two sections received 5μl of 9:1 glycerol in PBS and coverslips were added and affixed with clear fingernail polish.

Succinate dehydrogenase staining methods from Noel et al. (2016) were followed. Slides containing six cryosections were incubated in a pre-warmed solution containing a nitro-blue tetrazolium solution, phosphate buffer, and a sodium succinate solution for 1h at 37°C. After incubation, slides were washed in Milli-Q (**MQ**) water 3 times each for 5 minutes. A 9:1 glycerol in PBS solution was placed on every two cryosections and slides were cover-slipped for imaging.

All cryosections were imaged at 20X magnification using a Nikon Elipse TI-U inverted microscope (Nikon Instruments Inc., Melville, NY). A Nikon DS-QiMC digital camera (Nikon Instruments Inc.) was used to photograph sections for muscle fiber CSA and satellite cell number. Photomicrographs for SDH were taken using a Nikon DS-Fil color digital camera (Nikon Instruments Inc.). An average of 1000 fibers were analyzed using NIS-Elements Imaging software (Basic Research, 3.3; Nikon Instruments Inc.) to determine fiber CSA for each sample and the corresponding area was analyzed for satellite cell number. An average of 50 skeletal muscle bundles was analyzed for SDH mean intensity on the same software. The scale for mean intensity starts with a value of 0, which indicates black or the most intense staining, to a value of 250, which signifies white or the least intense staining. (Satellite cell number and succinate dehydrogenase staining data not presented).

Mitochondrial Quantification

Mitochondrial extraction and western blotting was used to quantify mitochondrial cytochrome C. The extraction procedure of Smith et al. (1967) was as follows with modifications. Briefly, leg muscle tissue was powdered and a maintenance buffer containing sucrose, mannitol, HEPES, and K_2PO_4 was added before homogenization. Homogenized sample was filtered with double layer cheesecloth and spun at 650 × *g* for 10 minutes at 4°C. Supernatant was strained with the cheesecloth. The centrifugation and straining steps were completed one additional time. The collection of supernatant was spun at 7750 × *g* for 25 minutes at 4°C. The resulting supernatant was decanted and the pellet was washed three times in maintenance buffer. The cleaned pellet was suspended in maintenance buffer and stored at -80°C

for subsequent western blot analysis. Protein concentrations were determined using a Pierce BCA Assay Kit (Thermo Scientific Rockford, IL).

The western blot method of Phelps et al. (2016) was followed with modifications. Proteins were separated via electrophoresis on 7.5% polyacrylamide gels and transferred to nitrocellulose membranes (88018; Thermo Scientific) using a TE77X Semi-dry Transfer Unit (Hoefer Inc., Holliston, MA). Blots were blocked with 5% nonfat dry milk in TBS-T for 30 minutes and then incubated overnight with a 1:1000 Cytochrome C mouse monoclonal antibody (Santa Cruz Biotechnology Inc., Dallas, TX). Blots were washed with TBS-T 3 times for 5 minutes each and then incubated in, IgG HRP-linked secondary an,tibody (7076; Cell Signaling Technology, Danvers, MA) for 1 h. Blots were washed as mentioned above and enhanced with chemiluminescence. A ChemiDoc-It 415 Imaging System (UVP LLC; Upland, CA) was used to image blots and band intensities were analyzed using VisionWorksLS Image Acquisition and Analysis Software (UVP, LLC). (Mitochondrial Cytochrome C data not presented).

NAD+ Quantification

The methods of the previous study were followed to quantify levels of NAD+ with a NAD+/NADH Assay Kit (Abnova, Taiwan). Two samples were equally pooled from the same treatment to reach a weight of 50mg of muscle tissue. The combined samples were homogenized in 100µl of NAD extraction buffer and heated at 60°C for 5 minutes. Assay buffer and the opposing NADH extraction buffer were added, samples were vortexed and centrifuged at 17,500 x g for 5 minutes. The supernatants were extracted and aliquoted along with the working reagent, and the absorbance was measured at 565nm at time points 0 and 15 minutes using a spectrophotometer (BioTek, Winooski, VT, USA). To calculate the NAD+ concentration, using

the change in optical density (OD) from 0 minutes to 15 minutes and dividing by the slope of the NAD+ standard curve. (NAD+ data not presented).

Cyclin Quantification

Cyclin D1, D2, and D3 were quantified utilizing the methods of Barnett et al. (2016) using Real Time (**RT**) polymerase chain reaction (**PCR**). Briefly, 50mg of the quadriceps muscle was homogenized in 3ml of Trizol. Samples were incubated at room temperature for 20 minutes, 600µl of chloroform was added, and was centrifuged at 3220 x g for 1h. The remaining top, aqueous layer was moved to a new conical tube and an equal amount of Isopropanol was added, then incubated at room temperature for 30 minutes. The sample was centrifuged again as specified previously and the supernatant was discarded. The pellet was washed with 75% ethanol. For RNA extraction, a PureLink RNA Mini Kit was used (12183018A; Thermo Scientific). The previous pellet was resuspended in 600µl of the kit lysis buffer, 600µl of 70% ethanol was added, and then the sample was vortexed. The mixture was aliquoted to a spin cartridge and centrifuged at 12,000 x g for 30 seconds. The flow though was discarded, 650µl of Was Buffer I was added to the cartridge, and centrifuged at the same settings as the previous spin. The flow through was discarded and 80µl of PureLink DNase was added to the cartridge. After an incubation for 15 minutes at room temperature, 650µl of Wash Buffer I was added and centrifuged as before. The flow through was discarded and 500µl of Wash Buffer II was aliquoted to the cartridge placed in a recovery tube. Centrifugation was at 12,000 x g for 1 minute. After the addition of 30µl of RNase Free Water and an incubation of 1 minute at room temperature, samples were centrifuged at 12,000 x g for 2 minutes. The liquid in the collection

tube was kept and heated at 65ºC and the RNA concentration was determined on the NanoDrop (Thermo Scientific).

A RT reaction was used to synthesize cDNA. Each sample was calculated to run a 50ng reaction. Each sample was brought up to 8µl with sterile filtered water. One microliter of both DNase enzyme and DNase Buffer was added for a total volume of 10µl. The sample was ran for 30 minutes at 37ºC. A volume of 1µl of DNase Stop Solution was added and the stop step was run at 65ºC for 10 minutes. A master mix was created containing 10X RT Buffer, 25X dNTP mix, 10X primer, Multiscribe Reverse Transcriptase, RNase inhibitor, and sterile filtered water. Nine microliters of the master mix were added to each sample. Each sample was ran for the RT reaction utilized the flowing cycle: 25ºC for 10 minutes, 37ºC for 120 minutes, 85ºC for 5 seconds, and held at 4ºC.

The RT PCR reaction utilized a standard curve with 8 standards. This was created by pooling 30µl of each cDNA sample to create standard one, and was diluted 4-fold until there were 8 total standards. To run each sample, 20µl of the RT reaction was diluted 1:16 for all three cyclins. A SYBR Green master mix was created with SYBR green, forward primer, reverse primer, and water, and 10µl of this mix was added to each sample. The PCR used the following thermocycle: 50ºC for 2 minutes, 50ºC for 10 minutes, 95ºC for 10 minutes, and 95ºC for 15 seconds. The cyclins were measured by the amount of DNA fluoresced. (Cyclin D quantification data not presented).

Statistics

Data were analyzed as a completely randomized design with egg as the experimental unit. All models were analyzed using the MIXED procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC)

with treatment (**TRT**) and injection location (**LOC**) as fixed effects. Statistical significance was set at $P \leq 0.05$.

RESULTS

Body Morphometrics

There was no treatment effect on d15 embryos for all body measures ($P \geq 0.23$; Table 4.1). Treatment did not affect all whole body measures ($P \geq 0.08$; Table 4.2) for d19 embryos with the exception of head circumference, where the 5.0 and 10.0m*M* NR concentrations were larger ($P < 0.05$) compared to the saline control. Embryos injected with 2.5m*M* NR had smaller head circumferences ($P < 0.04$) than the 10.0m*M* embryos, but were similar to all other treatments ($P \geq 0.07$). There was no treatment effect on all d21 chick body measurements ($P \geq 0.08$; Table 4.2); though injection of 2.5m*M* NR increased both head length and chest width ($P \leq 0.03$) compared to the saline and 10.0mM chicks. Head length and chest width from hatched chicks injected with 5.0mM NR was similar ($P \geq 0.10$) to all other treatments. Treatment did not affect leg blot area of hatched chicks (P = 0.84; Figure 4.3).

There was no treatment effect on all chest plate measurements ($P > 0.22$; Table 4.3). For d19 embryos, treatment with NR at every level increased both PM weight and length ($P < 0.01$), which were not different from each other ($P \geq 0.25$). Nicotinamide riboside treatment did not affect PM width or depth ($P \geq 0.12$). Nicotinamide riboside treatment did affect all PM measures taken from hatched chicks ($P \leq 0.02$). Both PM weight and width were increased by injection of NR at all three concentrations ($P < 0.01$), which did not differ from each other ($P \geq 0.44$). Hatched chicks injected with 2.5 and 5.0mM NR had longer PM measurements ($P \leq 0.01$) than the saline treatment, while the 10.0m*M* concentration group was similar to all other treatments ($P \geq 0.06$). Injection of 5.0m*M* NR increased the depth of the PM ($P \leq 0.04$) from d21 chicks

compared to both the saline and 10m*M* treatments, although the 2.5m*M* NR group had similar PM depth ($P \geq 0.06$) to all other injection treatments.

Muscle Fiber Characteristics

There was no treatment effect on muscle fiber cross sectional area ($P = 0.20$; Figure 4.1). Treatment with NR did affect fiber number per 1000µm ($P < 0.01$: Figure 4.2). The addition of 2.5 and 5m*M* NR increased fiber number ($P \leq 0.01$) compared to the control, while injection of 10m*M* NR showed to largest increase in fiber number compared to all other treatments ($P < 0.01$).

Additional Results

At the time of publication for this thesis, satellite cell number, succinate dehydrogenase stain fiber oxidation, mitochondrial quantity, NAD+ quantity, and Cyclin D expression were proprietary. These data sets are expected to be published in a peer reviewed journal at a later date.

DISCUSSION

Body Morphometrics

Over the last 60 years, demand for chicken has increased dramatically with yearly per capita consumption rising by 70 lbs. (USDA, 2018). Due to these changing consumer preferences, scientists are investigating ways to capitalize on poultry growth rates. In the past, *in ovo* injected growth hormone increased overall body weight of broilers (Hargis et al., 1989). In recent times, consumers do not look favorably at the use of growth hormones, making meat animal producers move away from these practices. Due to these consumer demands, some studies have observed the effects of injecting compounds that are more widely accepted. *In ovo*

injection with selenium, probiotics, and vitamin E did not affect body weight unlike injected growth hormone (Macalintal et al., 2012; Bednarczyk et al., 2016; Araujo et al., 2018).
No previous studies have tested the effects of nicotinamide riboside on improving the rate of growth in meat producing animals; however, several studies have looked at the effect of NR on whole body measurements and skeletal muscle in rodents. Studies by Canto et al. (2012) and Ryu et al. (2016) fed NR to mice and reported no change in overall weight after supplementation. In contrast, one study did demonstrate a decrease in overall weight gain, but only when mice were fed a high-fat diet with the addition of NR (Canto et al., 2012). In experiment one, neither injection of NR into the yolk or the albumin led to an increase in overall weight and other body morphometrics. The current study, experiment two, focused on broiler growth, in which *in ovo* injection of NR at several concentrations did not affect overall body weight in d 15 embryos, d 19 embryos, or hatched chicks. The unaffected body weight between treatments was supported by no changes in all other body measurements for d 15 embryos. Similarly, most body measurements were unaffected by treatment with the exception of a slight increase in head circumference for d 19 embryos as injection concentration increased, as well as increased head lengths and chest widths with the injection on 2.5mM NR in hatched chicks. Overall, the lack of significance on overall body weight of the current study are similar to literature regarding *in ovo* injection studies of other vitamins, minerals, and probiotics. Similarly, supplementation studies utilizing NR are in agreement with the current study; under normal conditions, NR does not lead to a change in body weight.

As seen with overall body weight, only *in ovo* injection with growth hormone successfully increased the breast muscle weight (Hargis et al., 1989), compared to other more consumer accepted compounds such as a vitamin E injection, which did not affect skeletal

muscle growth (Araujo et al., 2018). While NR has only previously been injected into broiler eggs, with the exception of experiment one, NR feed supplementation in rodents has resulted in varied effects across many studies. One study reported that healthy mice fed NR had similar lean muscle mass compared to mice not supplemented with NR (Canto et al., 2012). In contrast, NR demonstrated an increase in skeletal muscle mass in mice, but only under unusual circumstances. In two separate studies, NAMPT knockout mice and muscular dystrophy mice had lower muscle mass compared to normal mice, but showed improvements to skeletal muscle resulting in muscle mass similar to control mice with the addition to NR (Frederick et al., 2016; Ryu et al., 2016). In experiment one, injection of NR into the yolk of the egg led to a 35% increase in PM weight and injection of NR into both the yolk and the albumin increased PM length by an average of 14%. In the current study, injection of NR into the yolk of the egg had no effect on PM weight or dimensions from d 15 broiler embryos. However, at d 19 and day of hatch, injection of NR has positive effects on PM muscle development. *In ovo* injection of NR increased PM weight by an average of 24% and lengthened the PM by an average of 16% for d 19 embryos compared to the saline control. Even larger changes to the PM were observed from NR chicks at hatch, including an increase in weight by 33%, length by 16%, width by 18%, and depth by 20%. These differing results among past studies may be due to the prenatal method at which NR was delivered to broiler eggs, whereas in past studies NR was given as a feed supplementation in mice postnatally. However, the results observed between experiment one and two are similar, whereas NR injection increased breast muscle growth after d 15 of incubation.

Muscle Fiber Characteristics

In past studies, muscle fiber CSA after supplementation with NR has only been inspected in mice models in the situation of NAMPT knockout and muscular dystrophy. In both cases,

increases in muscle fiber CSA were reported with the addition of NR in the diet (Frederick et al., 2016; Ryu et al., 2016). The current study demonstrated conflicting results with past literature, with no changes in fiber size observed after an injection of NR into either the yolk of albumin of the egg at d 10 of incubation. In contrast to past literature, but in similarity to experiment one, no differences in PM muscle fiber CSA were detected between treatments. These findings would indicate that the overall growth of the PM observed was not due to decrease in size of muscle fibers. For growth to occur without the increase in fiber size, muscle fiber number must be altered. In experiment one, an increase in fiber number was observed by injection of 2.5m*M* NR into the yolk of the egg by 37.9%. Similarly, for the current study, muscle fiber number per $1000\mu m^2$ was significantly larger than the control chicks for all NR concentration treatments. Fiber number was increased by an average of 30% for the 2.5 and 5m*M* treatments and by 70% for the 10m*M* injection. No past studies concerning either *in ovo* injections of NR evaluated muscle fiber number. In past literature, NR was given postnatally so fiber number could not be altered in previous rodent studies. In the current study, NR was injected prenatally so that embryonic myogenesis may have been affected. While fiber size was not altered by NR injection of any concentration, fiber number was significantly increased by all three levels of NR, with 10mM leading to the largest number of skeletal muscle fibers. Overall, the current study's findings were not comparable to past literature, however were similar to the results of experiment one. This may largely be due to the differences in species and the method and time at which NR was given.

CONCLUSSION

Injection of nicotinamide riboside at d 10 in incubation into both the yolk of developing broiler eggs did not affect most measures of body growth; however, injection did positively

affect many skeletal muscle aspects. The three differing levels of NR did not affect any overall body measures for d 15 embryos, and similarly had no effects on most body measures for d 19 embryos and d 21 hatched chicks. Conversely, many skeletal muscle properties from the *pectoralis major* were altered. Injection of NR did positively affect some PM measures for d 19 embryos, but by d 21, all PM dimensions were increased. *Pectoralis major* development peaked with the 5m*M* NR concentration. In the PM of hatched chicks, muscle fiber size remained the same, while muscle fiber number increased with increasing NR concentration treatments.

These findings indicate that while body measurements were mostly unchanged for all time points, positive transformations were made to PM characteristics with an NR injection into the yolk. For most qualities, maximum effects were seen at the 5m*M* NR injection level, with the exception of the largest muscle fiber number per 1000 square microns from the 10m*M*. Overall, the new injection technology may be a solution to improving growth rate in broilers and could be utilized to meet the ever growing consumer demand for chicken.

Table 4.1 Whole body measurements from d15 broiler embryos injected with 0, 2.5, 5, or 10mM Nicotinamide Riboside (NR) at day 10 of incubation

	Nicotinamide Riboside dose, m*M*					
Item	0.0	2.5	5.0	10.0	SEM	TRT[1]
Chick weight, g	13.61	14.46	14.18	13.94	0.86	0.90
Body measurements, mm						
Crown-rump length	59.40	61.40	61.07	61.05	1.50	0.75
Head width	13.62	13.70	13.72	14.02	0.28	0.73
Head length	17.06	17.63	17.78	17.41	0.86	0.93
Heart weight, g	0.13	0.14	0.13	0.15	0.01	0.23
Liver weight, g	0.27	0.27	0.25	0.28	0.02	0.68

[abc] of treatment within row differs ($P < 0.05$).
[1]TRT = treatment of 0.0, 2.5, 5.0, and 10.0m*M* NR in 0.9% sterile saline

Table 4.2 Whole body measurements from d19 embryos and d21 hatched chicks injected with 0, 2.5, 5, and 10m*M* Nicotinamide Riboside (NR) at d10 of incubation

	Nicotinamide Riboside dose, m*M*					
Item	0.0	2.5	5.0	10.0	SEM	TRT[1]
D19 embryo						
Weight, g	37.93	37.74	37.89	38.06	1.42	0.99
Body measurements, mm						
Crown-rump length	84.95	84.34	85.87	86.32	0.62	0.08
Head width	16.95	15.38	15.31	15.40	0.76	0.34
Head length	17.78	17.67	17.43	17.58	0.17	0.49
Head circumference	52.96 [a]	53.17 [a,b]	54.79 [b,c]	54.97 [c]	0.06	0.04
Chest width	15.56	15.49	16.32	15.44	0.29	0.14
Chest length	18.34	18.60	18.45	18.29	0.31	0.89
Chest circumference	57.04	59.47	60.07	61.52	0.13	0.08
Heart weight, g	0.22	0.22	0.22	0.23	0.01	0.56
Liver weight, g	0.63	0.64	0.64	0.63	0.02	0.98
D21 chick						
Weight, g	45.30	46.91	46.13	46.60	0.81	0.16
Body measurements, mm						
Crown-rump length	93.24	95.44	95.08	93.01	1.04	0.16
Head width	15.56	15.53	15.64	15.48	0.21	0.83
Head Length	17.76 [a]	18.66 [b]	18.22 [a,b]	17.76 [a]	0.29	0.05
Head circumference	54.71	55.73	55.57	53.94	0.1	0.17
Chest width	15.90 [a]	17.07 [b]	16.56 [a,b]	16.14 [a]	0.29	<0.05
Chest length	20.47	21.90	21.18	21.11	0.62	0.14
Chest circumference	60.88	61.18	62.47	61.39	0.11	0.42
Heart weight, g	0.29	0.31	0.31	0.32	0.01	0.08
Liver weight, g	0.88	0.84	0.90	0.96	0.05	0.28

[abc] of treatment within row differs ($P < 0.05$).

[1]TRT = treatment of 0.0, 2.5, 5.0, and 10.0m*M* NR in 0.9% sterile saline.

Table 4.3 Overall chest and Pectoralis major muscle measurements from d15 embryos, d19 embryos, and d21 hatched broiler chicks injected with 0, 2.5, 5, or 10mM Nicotinamide Riboside (NR) at day 10 of incubation

	Nicotinamide Riboside dose, m*M*					
Item	0.0	2.5	5.0	10.0	SEM	TRT[1]
[2]D15 Chest plate measurements						
Weight, g	0.73	0.81	0.82	0.84	0.06	0.60
Length, mm	17.60	18.25	17.79	18.50	0.71	0.77
Width, mm	13.73	14.65	15.18	14.86	0.54	0.22
Depth, mm	6.93	7.20	7.07	6.61	0.25	0.34
D19 *Pectoralis major* measurements						
Weight, g	0.14[a]	0.17[b]	0.18[b]	0.17[b]	0.01	<0.01
Length, mm	15.12[a]	17.23[b]	17.72[b]	17.65[b]	0.38	<0.01
Width, mm	5.11	5.48	5.66	5.57	0.17	0.12
Depth, mm	2.68	2.91	2.73	2.87	0.10	0.29
D21 *Pectoralis major* measurements						
Weight, g	0.17[a]	0.23[b]	0.23[b]	0.22[b]	0.02	<0.01
Length, mm	17.61[a]	19.89[b]	20.33[b]	19.13[a,b]	0.70	<0.01
Width, mm	4.63[a]	5.50 [b]	5.56 [b]	5.39[b]	0.30	<0.01
Depth, mm	2.78[a]	3.14[a,b]	3.33 [b]	2.97[a]	0.15	0.02

[abc] of treatment within row differs ($P < 0.05$).
[1]TRT = treatment of 0.0, 2.5, 5.0, and 10.0m*M* NR in 0.9% sterile saline.
[2] Chest plate includes the ribs, *pectoralis major*, and *pectoralis minor* muscles.

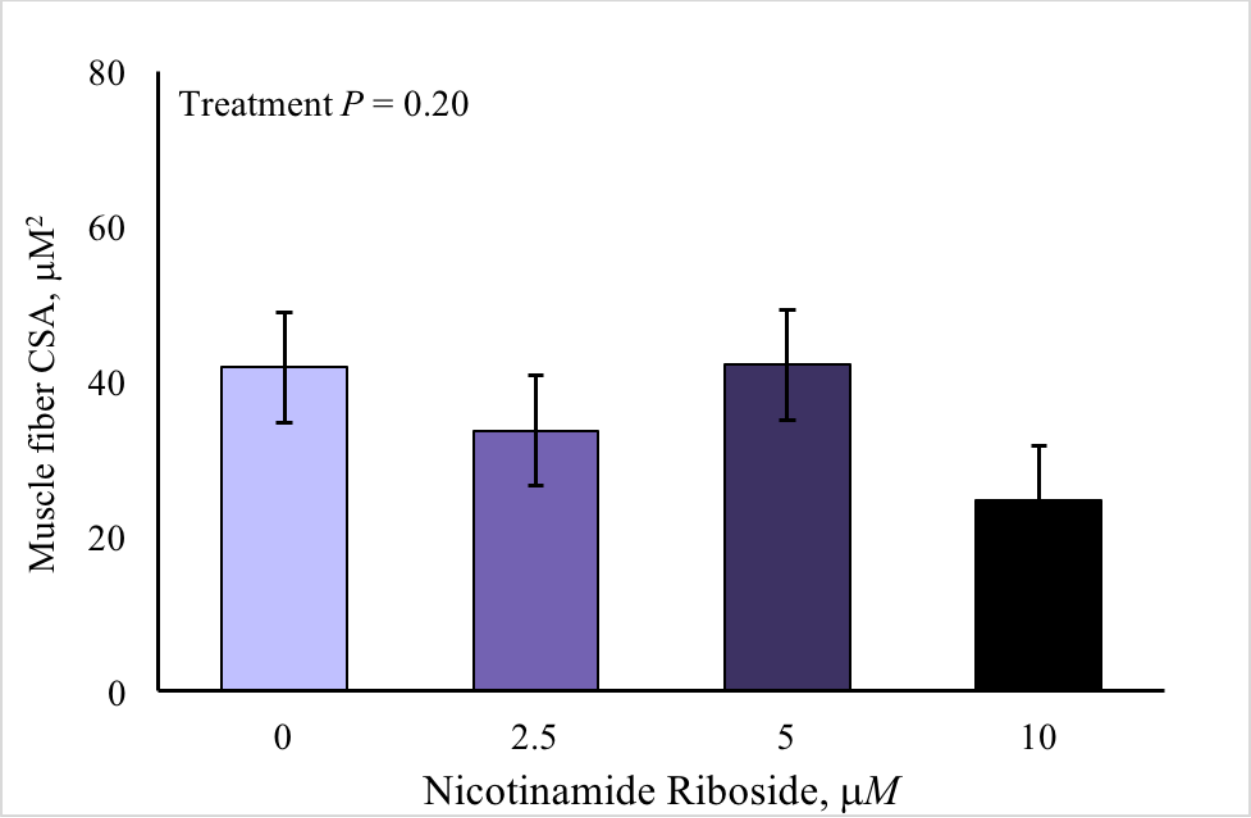


Figure 4.1 Muscle fiber cross-sectional area (CSA) from the *pectoralis major* of hatched broiler chicks injected with 0, 2.5, 5, or 10 m*M* Nicotinamide Riboside (NR) in 0.9% sterile saline into the yolk of fertilized broiler eggs at day 10 of incubation. The PM muscle was embedded in a sectioning compound, sectioned onto a slide, and stained with Dystrophin antibody (Thermo Scientific). For each sample, the area of 1000 fibers were analyzed on the NIS-Elements Imaging software (Basic Research, 3.3; Nikon Instruments Inc.).

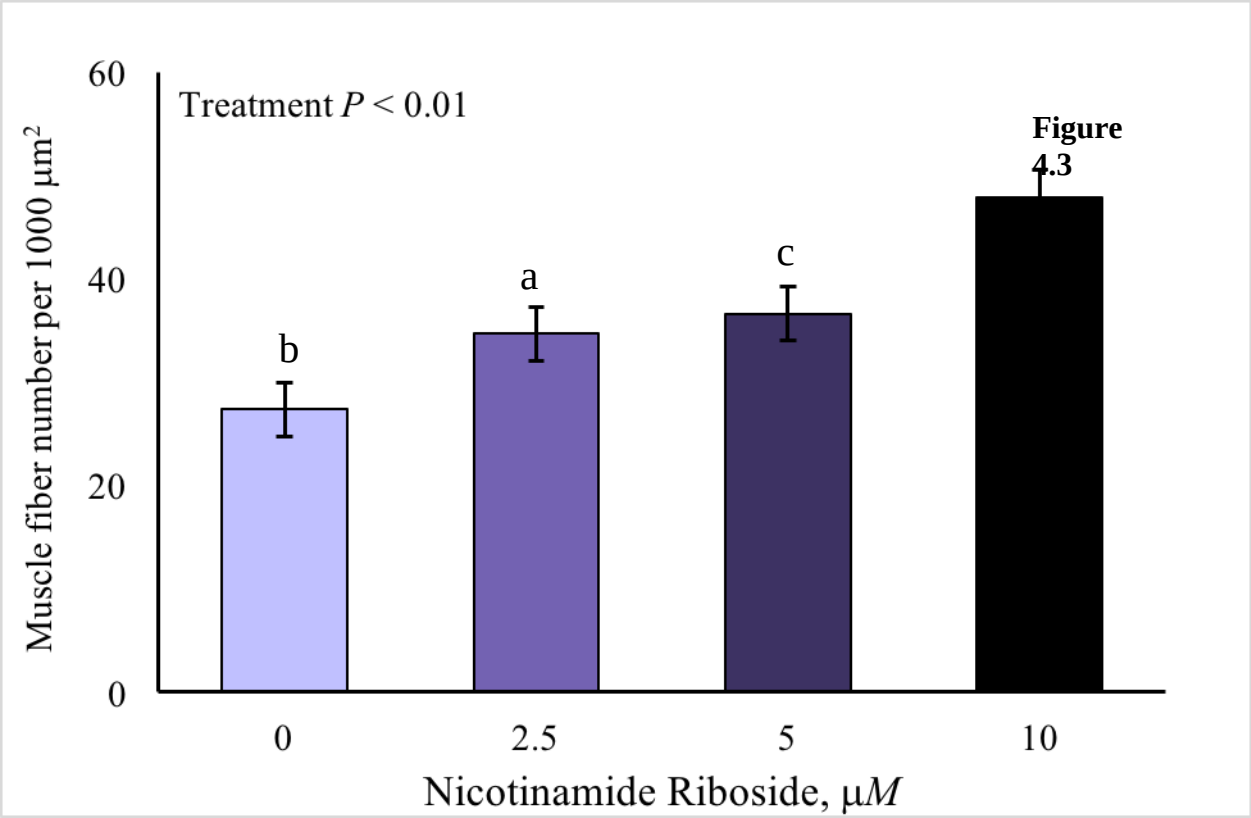


Figure 4.2 Muscle fiber number per 1000 square microns from the *pectoralis major* of hatched broiler chicks injected with 0, 2.5, 5, or 10 m*M* Nicotinamide Riboside (NR) in 0.9% sterile saline into the yolk of fertilized broiler eggs at day 10 of incubation. The PM muscle was embedded in a sectioning compound, sectioned onto a slide, and stained with Dystrophin antibody (Thermo Scientific). For each sample, fiber number was analyzed on the NIS-Elements Imaging software (Basic Research, 3.3; Nikon Instruments Inc.).

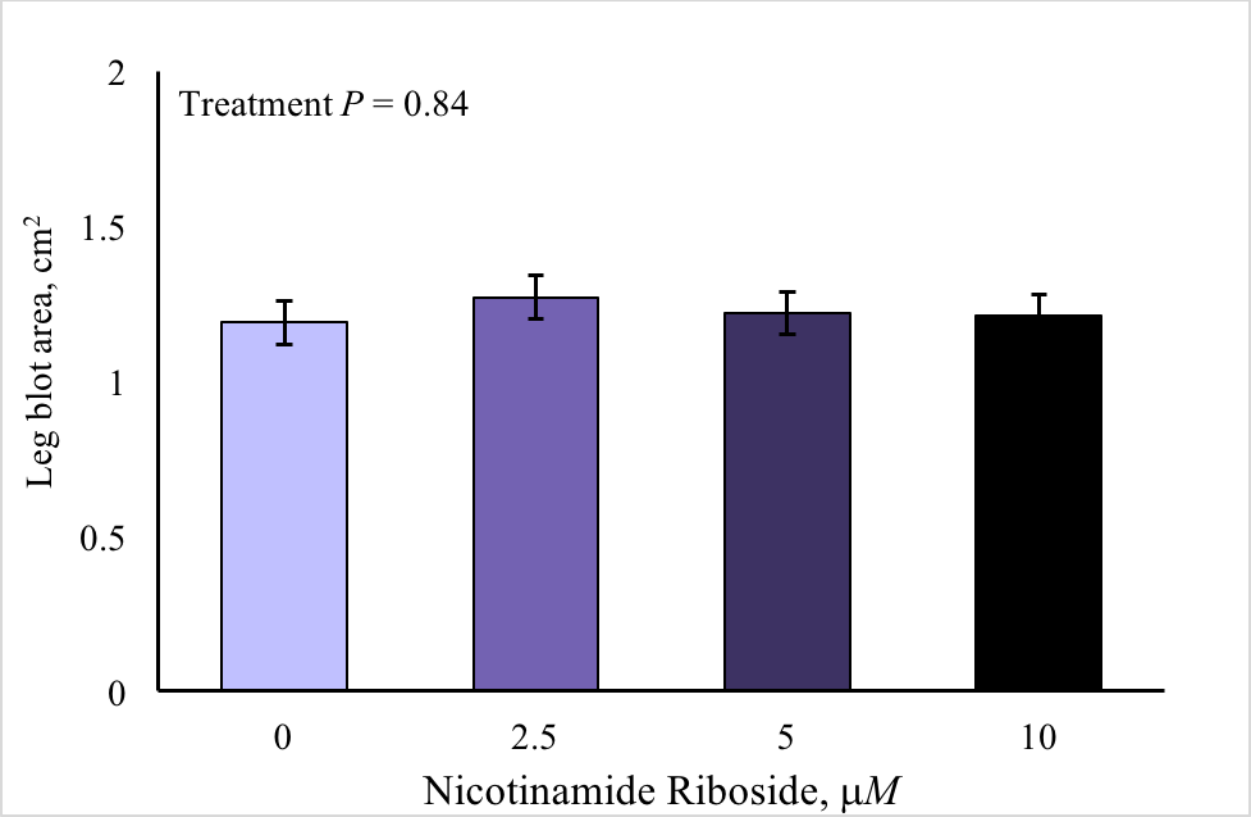


Figure 4.4 Effect of Nicotinamide Riboside (NR) concentration injected into fertilized broiler eggs at d 10 of incubation on leg blot area. The left leg was removed caudal to the hip joint and the *rectus femoris*, *vastus lateralis*, *vastus intermedius*, and *vastus medialis* still attached to the femur, were blotted on paper to measure the approximate area of the leg. The resulting space was traced, and the area was analyzed with NIS-Elements Imaging software (Basic Research, 3.3; Nikon Instruments Inc.).

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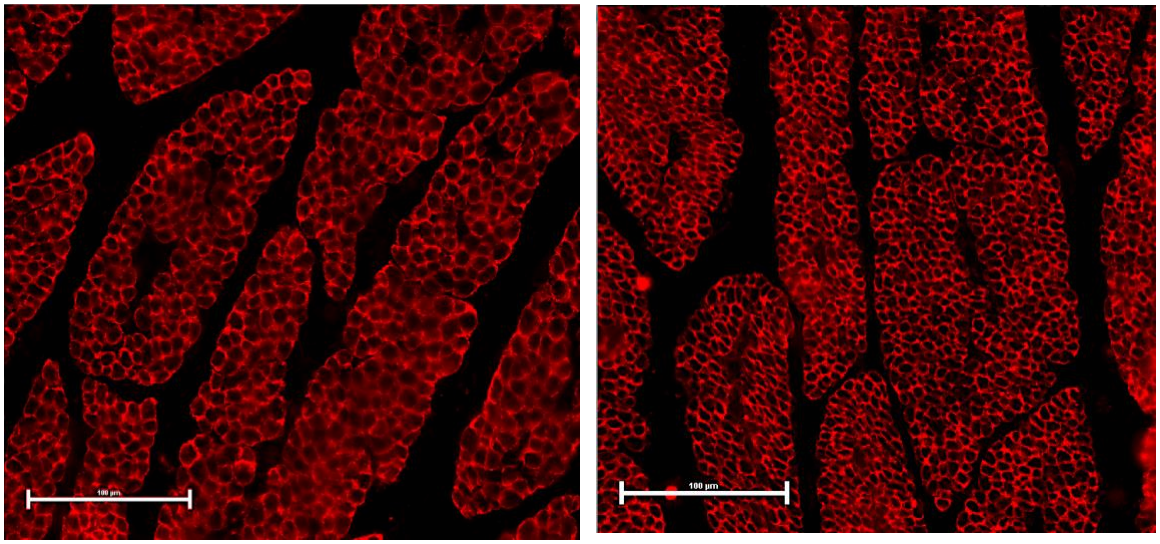
a b

Appendix A - Representative pictomicrograph of broiler chick skeletal muscle fibers from the *Pectoralis major*



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