THE EFFECT OF IN OVO FEEDING OF NICOTINAMIDE RIBOSIDE ON BROILER MYOGENESIS

by

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(Under the Direction of John Michael Gonzalez)

ABSTRACT

Broiler chicken weight gain is a result of genetics and nutrition, with increased muscle mass attributed to accelerated embryonic myogenesis and post-hatch muscle growth. During the avian incubation period, *in ovo* injection may be used as a strategy to deliver exogenous supplements into growing embryos for improving skeletal muscle development and growth. Nicotinamide riboside (**NR**), a vitamin B3 analog, is a human performance supplement used to stimulate mitochondria biogenesis and elevate tissue NAD^+ levels. Research showed injecting NR into the chick embryonic yolk sac increased breast muscle weight and muscle satellite cell numbers and proliferation rate. Therefore, the objective of this body of work was to determine: (1) effects of *in ovo* feeding dose of NR on commercial broiler muscle growth; (2) effects of low dose and location of *in ovo* feeding on high-yield broiler muscle growth; (3) different effects of *in ovo* feeding dose of NR on commercial and high-yield broiler muscle growth. Through the Cobb 500 broiler dose study, we confirmed that 250 mM is the best dose of NR to improve broiler breast muscle weight. Furthermore, low dose (2.5 mM NR) and location study indicated high-yield broiler egg yolk sac the optimum location for *in ovo* feeding. This led to increased hatched chick breast muscle morphometrics, which coincided with an increase in muscle fiber

density and tended to decrease fiber cross-sectional area (**CSA**). Increased Sirtuin1 and cyclin D mRNA expression of hatched chicks from eggs injected with 2.5 mM NR into yolk sac also indicated a potential NR regulated Sirtuin1/cyclin D molecular mechanism mediating chicken muscle early development. At last, the strain study suggested compared with Cobb 700 broilers, NR supplemented *in ovo* has more potential to enhance hypertrophy of the PM muscle of Cobb 500 chicks through increasing PM muscle fiber CSA and thickness.

INDEX WORDS: broiler, pectoralis major, muscle fiber, nicotinamide riboside, Cyclin D

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CHAPTER 1

INTRODUCTION

The Cobb 500 broiler is a modern strain commercially available throughout the world. Because of the commercial impact this strain has on poultry markets worldwide, and the characteristics in growth rate and yielding ability that separate it from other commercially available strains, investigation is warranted regarding *in ovo* feeding strategies. An increase in consumer demand for breast fillets and value-added products has resulted in an increased number of broilers being processed to heavier weights (Dozier et al., 2007). Due to its high carcass and breast meat yields at higher weights, the Cobb 700 broiler was developed for those markets where meat yield is a key performance indicator (Cobb-Vantress, 2008). Regarding the breast meat yield difference between these two strains, our study also focused on if *in ovo* feeding strategy could improve breast muscle growth of Cobb 500 broilers, making it catch up with the deboned meat yield of Cobb 700.

Over the past 6 decades, the poultry industry has made impressive strides to increase muscling of broiler chickens. Many of the gains are attributed to genetics and nutrition, but biologically, greater muscle mass reflects altered *in ovo* myogenesis and posthatch muscle growth. Similar to mammalian species, avian embryonic muscle development occurs in 2 phases: primary myogenesis from embryonic day (**E**) 3 to 7 and secondary myogenesis from E8 until hatching (Biressi et al., 2007). During embryonic myogenesis, progenitor cells undergo myogenic determination to form myoblasts capable

of proliferation and fusion to form multinucleated myotubes which mature to become myofibers (Abmayr and Pavlath, 2012). Myofiber expansion occurs during secondary myogenesis to create fixed muscle fiber number present at hatch or birth. These fibers constitute the foundation for future muscle growth through protein accretion.

In ovo feeding was first reported in the 1980s for vaccination against Marek's disease (Sharma and Burmester, 1982) and subsequently developed as an early nutritional technology to enhance poultry embryonic development to better prepare chicks for intensive development (Givisiez et al., 2020). During *in ovo* feeding, exogenous natural nutrients such as amino acids, carbohydrates, and vitamins can be injected into different locations of the egg based on stage of incubation. Gonzalez and Jackson (2020) reported injecting nicotinamide riboside **(NR)** into the chick embryonic yolk sac increased pectoralis major muscle (**PMM**) weight. Numerous studies reported NR, a vitamin B3 analog, increased nicotinamide adenine dinucleotide (**NAD^+**) availability (Bieganowski and Brenner, 2004; Trammell et al., 2016; Elhassan et al., 2017), which is linked to increased muscle satellite cell numbers and proliferation rate in mice (Zhang et al., 2016). Rathbone et al (2009) reported SIRT1 positively regulated muscle precursor cells' cell cycle proliferation. Further novel observations also found SIRT1 over-expression decreased p21Waf/Cip1 expression, an inhibitor of cyclin D–cdk4/6 activity, and increased phosho-Rbser780, a target of increased cyclin D–cdk4/6 activity on aged mice. Since muscle precursor cells are primary source of nuclei for postnatal skeletal muscle and are required for growth and repair to maintain structure and function of skeletal muscle through lifetime (Conboy et al., 2005), these data implied SIRT1 by increasing muscle precursor cell proliferation, and thus providing more muscle precursor cells,

could facilitate hypertrophy and regeneration of skeletal muscle in elderly animals. Given NR's positive effects on muscle form and function, the objective of the study was to determine NR effects commercial- and high-yield broiler chick muscle development through *cyclin D* and *SIRT1* mRNA expression.

CHAPTER 2

LITERATURE REVIEW

The development and growth of skeletal muscle and its molecular regulation

Myogenesis

Skeletal muscle is a highly complex and heterogeneous tissue serving a multitude of functions in the organism. The process of myogenesis which generates muscle can be divided into several distinct phases (Tajbakhsh 2009). During embryonic myogenesis, mesoderm-derived structures generate the first muscle fibers, and additional fibers are generated along these template fibers subsequently (Parker et al., 2003; Sambasivan and Tajbakhsh, 2007). Muscle resident myogenic progenitors initially proliferate extensively but later decrease as myonuclei number reaches a steady state and myofibrillar protein synthesis peaks (Schultz, 1996; Davis and Fiorotto, 2009). Once muscle has matured, these progenitors will enter quiescence and henceforth reside within in it as satellite cells.

Cell position and identity will form the three germ layers determined during early gestation (Arnold and Robertson, 2009). Somitogenesis precedes myogenesis. Somites are the first mammalian embryonic metameric structures. The prepatterned embryo subsequently develops the ectoderm, mesoderm, and endoderm. The mesoderm is anatomically separated into paraxial, intermediate, and lateral mesoderm. During

development, local oscillations in gene expression and signaling molecule gradients induce pairwise condensations of the paraxial mesoderm into somites (Aulehla and Pourquie, 2006). The somite's most dorsal portion remains epithelial and becomes the dermomyotome. Skeletal muscles of the body are derived from cells of this structure. The lips of the dermomyotome will mature into the myotome, a primitive muscle structure containing committed muscle cells expressing high levels of MyoD and Myf5 (Sassoon et al., 1989; Cinnamon et al., 2001; Kiefer and Hauschka, 2001; Ordahl et al., 2001). MyoD and Myf5 are both considered terminal specification markers to the muscle lineage (Pownall et al. 2002). When the embryo develops, the central part of the dermomyotome disintegrates, and muscle progenitors enter into the primary myotome (Ben-Yair and Kalcheim, 2005; Gros et al., 2005; Manceau et al., 2008). This progenitor population gives rise to a fraction of the satellite cells residing in postnatal skeletal muscle (KassarDuchossoy et al., 2005; Relaix et al., 2005; Schienda et al. 2006).

As for the chicken, myogenesis in the developing embryo proceeds through distinct stages in which multiple types of myoblasts and myofibers appear (Bonner and Hauschka, 1974; Seed and Hauschka, 1984; Stockdale and Miller, 1987; Stockdale et al., 2002; Hutcheson et al., 2009). The first differentiated muscle cells appear in the myotomal compartment of the rostral somites by Hamburger Hamilton (**HH**) stage 14 on embryonic day 2 (**E2**); and these somitic myocytes begin to co-express both fast and slow myosin heavy chain (**MyHC)** isoforms shortly after they form (Sacks et al., 2003). In chicken embryo limb buds, the first myofibers begin to form by E3-E4, and these primary myofibers are of at least two distinct types: a fast type that expresses only fast MyHC(s) and a fast/slow type that co-expresses both fast and slow MyHCs (Crow and Stockdale,

1986). This initial diversification of fast and fast/slow myofibers does not depend on functional innervation (Stockdale and Miller, 1987; Crow and Stockdale, 1986; Kelly and Rubinstein, 1986). Embryonic chicken limbs also contain distinct types of myoblasts that are committed to form either fast or fast/slow myotubes (DiMario et al., 1993; Van Swearingen and Lance-Jones, 1995). As embryonic development begins on E8, a distinct population of fetal myoblasts appears and secondary myofibers are formed alongside the primary fibers in the limbs (Kelly and Rubinstein, 1986).

To conclude, during skeletal muscle development, a remarkably finetuned extrinsic regulatory system directs distinct fates of self-renewal or differentiation to myogenic precursors.

Myogenic regulatory factors (MRFs)

Davis et al. (1987), using myoblast cDNA libraries, identified the basic helix-loop-helix factor MyoD by its ability to transform a selection of cell types, such as fibroblasts, into myoblast-like cells which were capable of fusing into myotubes. Subsequently, three more myogenic basic helix-loop-helix factors: Myf5, myogenin, and MRF4 which are also able to induce myoblast traits in various cell lines were discovered (Braun et al. 1989; Edmondson and Olson 1989; Rhodes and Konieczny 1989; Braun et al. 1990; Miner and Wold 1990). Eventually, Weintraub et al (1991) and Rudnicki-Jaenisch (1995) groups identified that the highly conserved MyoD, Myf5, myogenin, and MRF4 genes are collectively expressed in skeletal muscle lineage and are therefore referred to as myogenic regulatory factors (**MRF**).

Myf5 is the first MRF expressed during embryonic development, being transiently upregulated in the paraxial mesoderm and then concurrently with other MRF during the myotome formation (Ott et al. 1991; Buckingham 1992). Unexpectedly, Braun et al. (1992) found Myf5 knockout mice displayed delayed embryonic myogenesis that proceeded normally from the stage at which MyoD expression initiated and *vice versa*. MyoD knockouts were compensated by increasing and prolonging Myf5 expression (Rudnicki et al. 1992). These surprising results proved the redundancy of Myf5 and MyoD in myogenesis. Moreover, analysis of myogenin knockout mice revealed the expression of several differentiation markers, such as myosin heavy chain (**MHC**) and MRF4, was reduced, whereas MyoD levels were normal (Hasty et al., 1993).

Taken together, these studies suggest Myf5 and MyoD act upstream of myogenin and MRF4 to commit myoblasts to a myogenic lineage. Myogenin and MRF4 are more directly involved in terminal differentiation and trigger the expression of myotube-specific genes.

Besides, *Pax3* and *Pax7* also play indispensable roles during myogenesis. They are members of paired-type homeodomain transcription factors belonging to the large category of homeodomain transcription factors which control development and differentiation (Gehring, 1987). A study showed mouse embryos with *Pax3* loss-of-function mutation do not develop the hypaxial domain of the somite and consequently do not form limb or diaphragm muscles, but epaxial-derived muscles are less affected (Bober et al. 1994; Daston et al. 1996; Tremblay et al. 1998). Classifying *Pax3* as genetically upstream, no *MyoD* transcript can be detected in the limbs of *Pax3* mutants. Placing *Myf5* as the only MRF next to *Pax3* upstream of *MyoD*, *Pax3:Myf5:MRF4* triple-

mutant mice lose all body muscles, lack expression of *MyoD* and all the other downstream myogenic factors (Tajbakhsh et al. 1997); however, *Pax7* is likely to be dispensable for embryonic muscle development (Seale et al. 2000). Proving that *Pax3* and *Pax7* are able to compensate partially for each other during embryonic myogenesis, Hutcheson et al. (2009) showed loss of *Pax3* lineage was embryonically lethal and prevents emergence of *Pax7*-positive cells, whereas ablation of *Pax7*-expressing cells only leads to defects in later stages of development, causing smaller skeletal muscle size with fewer myofibers in the limbs at birth (Seale et al. 2000; Hutcheson et al. 2009).

Influencers of skeletal muscle development and growth: Sirtuin-1 and Cyclin D

A broad range of signaling molecules instructs myogenesis during embryonic development and in postnatal life (Kuang et al. 2008; Bentzinger et al. 2010). The molecular mechanisms that integrate various environmental and inherent factors to establish myogenic cell character and enhance skeletal muscle growth throughout animal's whole lifespan of are a matter of intense research. Sirtuin-1 (**SIRT1**) is a member of the sirtuin family. It constitutes a family of NAD^+-dependent enzymes. The mammalian sirtuin family consists of seven members, SIRT1-7 which differ in their sub-cellular localization. Sirtuin-1, SIRT6 and SIRT7 reside predominately in the nucleus. Inside, a large fraction of SIRT1 is associated with euchromatin, whereas SIRT6 associates with heterochromatin and SIRT7 is found in the nucleolus (Michishita et al., 2005). The sirtuin that resides most prominently in the cytoplasm is SIRT2 (Frye, 1999; North et al., 2003). Sirtuin-3, SIRT4 and SIRT5 were described as mitochondrial sirtuins. Besides, SIRT1 and SIRT5 exhibit robust and weak deacetylase activity, respectively

(North et al., 2003; Vaziri et al., 2001). Sirtuin-4 and SIRT6 are mono-ADP-ribosyl transferases (Haigis et al., 2006; Liszt et al., 2005; Shi et al., 2005), and both deacetylase and mono-ADP-ribosyl tranferase activities were detected for SIRT2 and SIRT3 (Frye, 1999; North et al., 2003; 39). The literature does not report robust activity for SIRT7 yet.

Sirtuin-1, the most studied member of this family, plays an important role in several processes ranging from cell cycle regulation to energy homeostasis (Nogueiras et al., 2012). It is a NAD^+ dependent deacetylase and removes acetyl groups from many histones and nonhistone proteins. Sirtuin-1 can deacetylate a variety of substrates and is, therefore, involved in a broad range of important physiological functions ranging from apoptosis, adipocyte and muscle cell proliferation and differentiation to energy expenditure (Li-Youcef et al., 2007). In humans, sirtuins exist throughout the body; for example, SIRT1 is expressed in the brain, liver, pancreas, adipose tissue, muscle, and heart. The second target described for mouse and human SIRT1 was the tumor suppressor p53. Sirtuin-1-dependent deacetylation of p53 inhibits its transactivation activity and suppresses apoptosis in response to oxidative stress and DNA damage (Vaziri et al., 2001; Luo et al., 2001).

The adult skeletal muscle stem cell [satellite or muscle precursor cells (**MPC**)] is almost solely responsible for the remarkable ability of adult skeletal muscle fibers to regenerate and enlarge in size (Adams et al., 2002; Barton-Davis et al., 1999). Fulco et al. (2003) demonstrated Sirtuin-1 inhibited MPC differentiation. A Sirtuin-1-induced decrease in MPC differentiation would suggest Sirtuin-1 might decrease skeletal muscle regeneration, which is contrary to the putative anti-aging function of Sirtuin-1; however, Fulco et al. (2003) did not study the effects of Sirtuin-1 on MPC proliferation. Rathbone

et al. (2009) found SIRT1 facilitated hypertrophy, re-growth, and skeletal muscle regeneration in elderly individuals by increasing MPC proliferation. In the Fulco et al. (2003) study, SIRT1 retarded muscle differentiation through its interaction with MyoD. Sirtuin-1 did not interact directly with MyoD but formed a complex with the acetyltransferase p300/CBP-associated factor (**PCAF**) and deacetylated both PCAF and MyoD. Also, myocyte enhancer factor-2 (**MEF2**) is a family of transcription factors with important functions in muscle cell differentiation and apoptosis (McKinsey et al., 2002). Zhao et al (2005) suggested SIRT1 potently induced MEF2 deacetylation during muscle cell differentiation, indicating their potential negative regulation on cardiac muscle cell differentiation and hypertrophy pathogenesis is worthy of further exploration as a therapeutic mechanism.

The proliferation of mammalian cells is governed by cyclins and their associated cyclin-dependent kinases (**CDK**). Cyclins represent regulatory subunits that bind, activate, and provide substrate specificity for their catalytic partners, CDK. These cyclin-CDK complexes phosphorylate critical cellular substrates, thereby allowing cell cycle progression. Among all cyclin classes, the family of D-type cyclins (cyclins D1, D2, and D3) stand out as very unique components of the cell cycle apparatus. D-cyclins are induced by the mitogenic stimulation, and their levels decline when mitogens are withdrawn (Sherr and Roberts, 1999). Once induced, D-cyclins associate with partner cyclin-dependent kinases CDK4 and CDK6. These cyclin D-CDK complexes phosphorylate the retinoblastoma tumor suppressor protein (**pRb**), which binds to and regulates E2F transcription factors during the early G1 phase of the cell cycle. The subsequent induction of cyclin E and its association with CDK2 is responsible for

extensive pRb phosphorylation, release of E2F factors, and thus transcription of genes required for the G1/S-phase transition in the cell cycle (Sherr, 1995; Dick et al., 2013; Narashima et al., 2014).

Lazaro et al (2002) concluded Cyclin D-CDK4 activity repressed skeletal muscle differentiation in proliferating cells by blocking MEF2 association and concomitantly disrupted the association of these factors with punctuate nuclear subdomains within the cell. Among D-cyclins, Cyclin D1 is an important regulator of G1 to S phase progression in many different cell types and can function as a key regulator of cell metabolism (Fajas, 2013). For example, cyclin D1 has been shown to inhibit mitochondrial function, lipogenesis and adipogenesis (Fu et al., 2005; Lee et al., 2014; Bhalla et al., 2014). Cyclin D2 critically regulates islet β-cell postnatal growth and glucose homeostasis (Georgia and Bhushan, 2004; Kushner et al., 2005). A study also showed Cyclin D3 played a cell-autonomous and nonredundant function in regulating the dynamic balance between myocyte proliferation, differentiation, and self-renewal that normally establishes an appropriate pool size of adult satellite cells (Luca et al., 2013). Moreover, Cyclin D3 performs a novel function, regulating muscle fiber type-specific gene expression. Mice lacking Cyclin D3 display an increased number of myofibers with greater oxidative capacity in fast-twitch muscle groups, primarily composed of myofibers that utilize glycolytic metabolism. The result suggests that Cyclin D3 regulates muscle fiber type phenotype, and consequently whole-body metabolism by antagonizing the activity of MEF2 (Giannattasio et al., 2018).

Influencers of skeletal muscle development and growth: PI3K/mTOR/Akt signaling pathway

The phosphatidylinositol -3-kinase (**PI3K**)/protein kinase B (**Akt**) and the mammalian target of rapamycin (**mTOR**) signaling pathway (**PI3K/Akt/mTOR**) is an intracellular signaling pathway important in regulating skeletal muscle hypertrophy. Therefore, it is directly related to cellular quiescence, proliferation, cancer, and longevity. PI3K activation phosphorylates and activates Akt, localizing it in the plasma membrane (King et al., 2015). Akt can have a number of downstream effects such as inhibiting p27, localizing FOXO in the cytoplasm, and activating mTOR which can affect transcription of p70 or 4EBP1 (Rafalski and Brunet, 2011). Both leptin and insulin recruit PI3K signaling for metabolic regulation (Garcia-Galiano et al., 2019).

Akt promoted muscle hypertrophy by activating downstream signaling pathways previously implicated in activating protein synthesis: the pathways downstream of mammalian target of rapamycin (mTOR) and the pathway activated by phosphorylating and thereby inhibiting glycogen synthase kinase 3 (**GSK3**). In contrast, in addition to demonstrating calcineurin does not mediate IGF-1-induced hypertrophy, a study showed that IGF-1 unexpectedly acts via Akt to antagonize calcineurin signaling during myotube hypertrophy (Rommel et al., 2001). In the skeletal muscle, increased protein synthesis is mediated by a time course initiated by mechanical stimuli, which can be modulated by anabolic inputs, such as mechanical and circulating factors. The anabolic stimuli can exert distinct contributions to signaling pathways, although both reflect on protein synthesis. For example, growth factors released by the skeletal muscle in response to mechanical stimulation are generally accepted to promote increased protein synthesis

through PI3K/Akt/mTOR pathway (Rommel et al. 2001; Bodine et al. 2001). Moreover, growth factors leading to PI3K/Akt/mTOR activation seem to be part of a late component of protein synthesis signaling, since mechano-growth factor (**MGF**) mRNA expression increases can be observed around 2.5 h following a resistance training session (Hameed et al., 2003; Haddad and Adams, 2002). These observations indicate that PI3K/Akt/mTOR could be a crucial regulatory target to improve postnatal skeletal muscle growth. A study also revealed that persistent overexpression of IGF-I in mice skeletal muscle results in hypertrophy, which is likely mediated via the mTOR/p70S6K pathway, potentially via an Akt-independent signaling pathway (Song et al., 2005). In addition, it was demonstrated that chronic stretching of skeletal muscle was capable of increasing its mass/length through activation of mTOR pathway (Aoki et al., 2006). Genetic activation of IGF1 (Coleman et al., 1995; Musaro et al., 2001) or Akt was shown to be sufficient in inducing muscle hypertrophy, as demonstrated by tissue specific transgenic mouse models. Lai et al. produced an inducible constitutively active Akt model, which allowed for the demonstration that even in an adult animal, relatively short-term activation of Akt resulted in doubling of skeletal muscle mass (Lai et al., 2004). A study found that Sonic hedgehog (**Shh**) signaling exerts a protective role through the AKT/mTOR/p70S6K signaling pathway during skeletal muscle ischemia/reperfusion (**I/R**) injury, indicating Shh-AKT signaling pathway may be a therapeutic target for protecting skeletal muscle from I/R injury (Zeng et al., 2017).

Nicotinamide riboside and its many uses

Nicotinamide adenine dinucleotide (**NAD^+**) is an essential coenzyme that catalyzes cellular redox reactions, becoming reduced to nicotinamide adenine dinucleotide hydrogen (**NADH**), in many fundamental metabolic processes such as glycolysis, fatty acid beta oxidation, or the tricarboxylic acid cycle (Haigis & Sinclair, 2010; Imai & Yoshino, 2013; Verdin, 2014). There are five major precursors and intermediates that synthesize NAD^+: tryptophan, nicotinamide, nicotinic acid, nicotinamide mononucleotide (**NMN**), and nicotinamide riboside (**NR**). Nicotinamide riboside, claimed to be a new pyridine-nucleoside form of vitamin B_3, was first identified in a conserved eukaryotic NAD^+ biosynthetic pathway and is a natural product found in cow's milk (Bieganowski & Brenner, 2004). Subsequently, Dartmouth College were granted patents for nutritional and therapeutic uses of NR in which the compound was promoted as an anti-aging supplement (Brenner, 2006). Since then, the number of studies investigating the therapeutic potential of NR has grown rapidly.

Nicotinamide riboside was shown to elicit beneficial effects on age-associated disorders. In prediabetic and diabetic mice under a high-fat diet, NR administration improved steatosis of the liver, glucose tolerance, and weight gain (Trammel et al., 2016a; Trammel et al., 2016b). With age, the regenerative capacity of muscle decreases as muscle stem cells enter senescence. This is concomitant with a decrease in NAD^+ and a reduction of the mitochondrial unfolded protein response (**mtUPR**) (Zhang et al., 2016). When NR (400 mg/kg/day for 6-8 weeks) is administered, the muscle stem cell self-renewal capacity is restored and mtUPR is activated, improving the mitochondrial

stress response (Zhang et al., 2016). Additionally, Zhang et al showed mice receiving 400 mg/kg of body weight of NR supplementation at two years of age showed a significant, moderate extension of life span. The NR-mediated NAD+ repletion in aging mice protected against muscle degeneration and delayed muscle stem cell senescence in models of muscular dystrophy through improved mitochondrial function, resulting in enhanced regenerative capacity and more favorable phenotypes. Moreover, Zhang et al. (2016) found that there were more satellite cells in both young and old mice after NR treatment. In metabolic diseases such as obesity, diabetes, and nonalcoholic fatty liver disease (**NAFLD**), protective effects of exogenous NR delivery include enhanced metabolic flexibility, mitochondrial function, improved glucose tolerance, reduced weight gain and prevention of hepatic steatosis and other pathological factors of NAFLD (Yoshino et al. 2011, Cantó et al. 2012, Trammell et al. 2016b, Zhou et al. 2016, Shi et al. 2017). In 2016, Khan et al. concluded that fine tuning of cellular signaling with vitamin cofactors, especially NAD^+ precursors like NR, is an intriguing and straightforward therapeutic strategy that should be explored on mitochondrial myopathy, the most common type of adult-onset mitochondrial disorders.

Regarding skeletal muscle and exercise performance, administration of NR ameliorated functional deficits and restored muscle mass in mice genetically modified to exhibit low NAD^+ levels (Frederick et al., 2016). In addition, NR administration enhanced oxidative metabolism and increased exercise performance in obese mice (Cantó et al., 2012). Dietary supplementation of NR in mice can also negate the metabolic consequences of high fat diet and increase oxidative performance (Canto et al., 2012), delay disease progression in mice with mitochondrial myopathy, inducing sirtuin

dependent mitochondrial biogenesis and the mitochondrial unfolded protein response (Khan et al., 2014). In addition, NR improves glycemic control and opposes development of diabetic neuropathy in mice and allows rats to resist development of chemotherapeutic neuropathy (Trammel et al., 2016b; Hamity et al., 2017). Moreover, NR prevents alcohol induced liver injuries. Activation of Sirt1 plays an important role in the protective effect of NR on livers, in which NR serves as an NAD+ precursor, regulates intracellular NAD homeostasis, activates SirT1-PGC-1α axle, refurbishes mitochondrial function and regulates lipid metabolism, reversing liver injuries induced by alcohol. This study eventually suggested NR is a promising food supplement to prevent the adverse effects induced by alcohol consumption (Wang et al., 2018). Similarly, another research group also founded that chronic administration of NR increased NADPH and glycogen levels in liver, which may regard NR as the best available nutritional supplement to increase NADPH levels and alleviate major liver-related diseases (Kourtzidis et al., 2018). In the previous report, this group also indicated that a 21-day NR supplementation protocol leads to dysregulation in redox and energy metabolism and impaired exercise performance in healthy young rats (Kourtzidis et al., 2016). In meat-producing agricultural species, NR supplementation could be a novel strategy to improve meat production and quality. Feeding NR at the end of the finishing period may enhance visual pork loin chop color during simulated retail display (Gonzalez et al., 2020). In poultry studies, injecting NR into the egg yolk of the developing Cobb 500 broiler embryo increased pectoralis major muscle (**PMM**) development (Davis et al., 2018a) and injecting up to 5.0 mM NR increased PMM measures, indicating NR influenced avian myogenesis. (Davis et al., 2018b). Additionally, at 10^{th} hatching day, 250 mM NR *in ovo*

fed into Cobb 500 broiler egg yolk sac drastically increased PMM fiber density, which caused increased PMM weight (Gonzalez and Jackson, 2020). Xu et al (2022) found that Cobb 700 broiler eggs *in ovo* fed by 2.5 mM NR into yolk sac also hatched chicks with increased PMM weight, length, and fiber density, in which increased expression of *SIRT-1* and the *Cyclin D* may be responsible.

Application of in ovo feeding technology in the poultry industry

In ovo feeding was first reported in the 1980s for vaccination against Marek's disease (Sharma and Burmester, 1982) and developed as an early nutritional technology to enhance poultry embryonic development and better prepare poultry chicks for intensive development. During *in ovo* feeding, exogenous natural nutrients such as amino acids, carbohydrates, and vitamins can be injected into different locations of the egg based on stage of incubation. According to Ebrahimi et al. (2012), when eggs are in the initial stages of embryonic development, compounds are administered to the egg protein at a depth of 12 mm, which deposits the compound as close to the germinal disc as possible. At a later stage of embryonic development, nutrients are usually administered to the yolk sac due to its surface area and ability to digest nutrients. After day 17 of incubation, the yolk sac is resorbed and the air chamber and amnion are the preferred sites of injection (Kucharska-Gaca et al., 2017).

***In ovo* feeding of amino acids**

In embryonic development of chicken, all amino acids are essential. Thus, the absence of any amino acid causes protein synthesis impairment and a disturbance of

homeostasis, which in turn results in impaired growth and development (Ohta et al., 2001). Gaafar (2009) reported injection of amino acids increased hatched chick weight, which resulted in an increased 2.4% of chick mass ratio. A number of studies were also conducted in which only individual amino acids were administered. Coşkun et al. (2014) demonstrated DL-methionine administered in the amniotic fluid caused greater chick to total egg weight. Yu et al. (2018) showed *in ovo* feeding of L-arginine (**Arg**) to broiler embryos improved breast muscle weight, hormones level, amino acid **(AA**) concentration, and mammalian target of rapamycin (**mTOR**) signal pathway messenger RNA expression of breast muscle until 21-day post-hatch.

In ovo feeding of carbohydrates

Rapid growth requires a high energy requirement, especially during late embryogenesis. Therefore, *in ovo* feeding of supplemental carbohydrates may be beneficial for broiler embryos. Chicken eggs are rich in protein and lipids but are poor in carbohydrates (Burley and Vadehra, 1989). Among several tested nutrients, *in ovo* feeding of carbohydrates were widely studied because their concentration inside the egg is <1% of the total nutrients, and only 0.3% is free glucose (Campos et al., 2011). The main argument for carbohydrate supplementation is that increasing available carbohydrates could reduce the use of amino acids in gluconeogenesis, thus favoring protein synthesis in the muscle (Kornasio et al., 2011). Therefore, naturally available carbohydrates in eggs may not be sufficient to meet the immediate metabolic demands of the embryo (Zhai et al., 2011). Several studies indicated administration of different types of carbohydrates into the amnion undoubtedly increased the level of available energy for

an embryo and reduced energy consumption from the metabolism of proteins and lipids. Zhai et al. (2011) showed injecting a mixture of glucose, sucrose, maltose and dextrin (0.25 g/ml) significantly increased the body weight of chicks in direct proportion to the volume of the administered solvent.

Chicks that received a mixture of dextrin and β-hydroxy-β-methylbutyrate-calcium salt increased muscle mass in the post-hatching period due to increased glycogen in the liver and muscles, as well as increased proliferation of satellite cells (Kornasio et al., 2011). Chen et al. (2009) found *in ovo* injection of glutamine and carbohydrates improved postnated small intestine development and pectoralis mass, which was probably due to sparing of breast muscle protein during embryo development.

In ovo feeding of vitamins

Due to the positive effects of vitamins on embryonic development, several research groups have begun to examine the efficacy as an *in ovo* technology. As shown in previously conducted studies, administration of exogenous vitamins contributes to the growth of birds and can modulate the immunity of broiler chickens (Kucharska-Gaca et al., 2017). The positive effect of vitamins on the final body weight was also found in the research by Selim et al. (2012), which were conducted on duck eggs. The study showed *in ovo* injection of vitamin E resulted in higher hatchability percentage compared to the uninjected control. The ducklings hatched from eggs were characterized by higher body weight after the administration of vitamin E (10 mg) and vitamin C (3 mg) on the12^{th} day of incubation at starting and growing period during post-hatch growth.

The use of *in ovo* feeding technology may lead to significant improvement in the efficiency and profitability of broiler production. *In ovo* injection improved not only hatching, but also the nutritional status of the chicks and resulted in a greater predisposition to growth (Liu et al., 2011; Selim et al., 2012; Ebrahimi et al., 2012). In addition, the use of Arg could regulate protein deposition while contributing to the meat yield of commercial poultry. Over the years, further studies were conducted on the experimental injection of small amounts of drugs, vaccines, and nutrients into the egg during incubation. These early developments led to increased research into *in ovo* techniques for poultry to achieve improved starting weights, better feed utilization, faster growth, and greater body weights, which is the reason why early *in ovo* feeding of poultry chicks has become increasingly appealing (for review see Kadam et al., 2013).

In summary, NR supplement improves mice skeletal muscle development and growth at both prenatal and postnatal stage through salvage pathway enhancing NAD^+ level. Moreover, *in ovo* feeding is a widely used avian exogenous supplement delivery method. Therefore, it is postulated *in ovo* feeding of NR could improve broiler skeletal muscle growth, which is potential to be utilized for enhancing chicken meat productivity.

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CHAPTER 3

THE EFFECT OF *IN OVO* NICOTINAMIDE RIBOSIDE DOSE ON COMMERCIAL AND HIGH YIELD BROILER MYOGENESIS: DOSE STUDIES

The effects of *in ovo* nicotinamide riboside dose on broiler myogenesis. Xu, X., A. R. Jackson, and J. M. Gonzalez. (2021) (Journal of Poultry Science) 100:100926. Reprinted here with permission of the publisher.

Abstract

The objective of this study was to determine the effects of *in ovo* feeding of nicotinamide riboside (NR) on broiler embryonic myogenesis. Fertilized Cobb 500 (N = 240) broiler eggs were sorted by weight and within each stratum, randomly assigned to 1 of 4 NR dose treatments (0 mmol, 250 mmol, 500 mmol, or 1 mol; final concentration in yolk of 0, 2.5, 5.0, or 10.0 mmol) of NR. At day 10 of incubation, 100 µL of the assigned NR dose was injected into the yolk sac of the developing embryo, and chicks were euthanized within 24 h of hatching. Pectoralis major muscle (**PMM**) and individual fiber morphometrics were collected. Chicks injected with NR had greater PMM weight and length ($P < 0.01$) but did not differ from each other ($P > 0.14$). Chicks from eggs injected with NR had greater PMM weight and width than control chicks ($P < 0.01$) but did not differ from each other ($P = 0.86$). Chicks from eggs injected with 500 mmol NR had greater PMM depth than control and 1mol chicks ($P < 0.04$), which did not differ ($P = 0.24$) from each other. Chicks from eggs injected with 250 mmol NR did not differ in PMM length compared with all other treatments ($P > 0.06$). There was no treatment effect ($P = 0.20$) for PMM fiber cross-sectional area; however, there was a treatment effect ($P < 0.01$) for muscle fiber density. Chicks from eggs injected with 1 mol NR had greater fiber density than all other treatments ($P < 0.01$). Chicks injected with 250 and 500 mmol NR had greater fiber density than control chicks ($P < 0.01$) but did not differ ($P < 0.06$) from each other. Injecting developing embryos at day 10 of incubation increased hatched chick PMM morphometrics, which were partly because of the NR catalyzed increase in muscle fiber density.

Key Words: Broiler; muscle fiber; nicotinamide riboside; pectoralis major muscle

Introduction

Over the past 6 decades, the poultry industry has made impressive strides to increase the muscling of broiler chickens. Many of the gains are attributed to genetics and nutrition, but biologically, the larger muscle mass reflects altered *in ovo* myogenesis and posthatch muscle growth. Similar to mammalian species, avian embryonic muscle development occurs in 2 phases: primary myogenesis from embryonic day (**E**) 3 to E7, and secondary myogenesis from E8+ (Biressi et al., 2007). During embryonic myogenesis, progenitor cells undergo myogenic determination to form myoblasts capable of proliferation and fusion to form multinucleated myotubes that mature to myofibers (Abmayr and Pavlath, 2012). Expansion of myofibers occurs during secondary myogenesis to create the fixed number of muscle fibers present at hatch or birth. These fibers constitute the foundation for future muscle growth through protein accretion.

In ovo injections were first used in the 1980s to administer vaccinations against Marek's disease (Sharma and Burmester, 1982). Building upon the technology, *in ovo* injection of amino acids, carbohydrates, and vitamins have been used to improve chick embryonic muscle and intestine development likely through enhanced nutritional status leading to a greater predisposition for growth (Uni et al., 2005; Liu et al., 2011; Selim et al., 2012; Zielinska et al., 2012). Recently, Gonzalez and Jackson (2020) reported injecting 250 mmol of nicotinamide riboside (**NR**) into the chick embryonic yolk sac increased pectoralis major muscle (**PMM**) size. The natural vitamin B3 analog increases nicotinamide adenine dinucleotide (**NAD^+**) availability (Bieganowski and Brenner, 2004; Trammell et al., 2016; Elhassan et al., 2017), which is linked to increased muscle satellite cell numbers and proliferation rate in mice (Rathbone et al., 2009; Zhang et al., 2016).

Given the positive effects of NR on muscle form and function, the objective of the study was to determine the effective dose of NR needed to improve broiler chick PMM development at hatch.

Materials and Methods

All experimental procedures were approved by the University of Georgia Institutional Animal Care and Use Committee.

Egg Procurement, Incubation, and in ovo Injection

Fertilized Cobb 500 broiler eggs (N = 240; Cobb Vantress, Siloam Springs, AR) were transported to Kansas State University Muscle Biology Laboratory (Manhattan, KS), egg weights were recorded, and within each 4 egg strata, eggs were randomly assigned to an NR treatment (0 mmol, 250 mmol, 500 mmol, or 1 mol NR; Table 3-1). Eggs were incubated at 37°C with 40 ± 4% relative humidity (Sportsman 1502 incubator, GQF Manufacturing Company Inc., Savannah, GA) and hourly rotated to reposition eggs. Egg weights were recorded daily with daily weight loss ranging from 0.65% to 0.75% of total mass.

At day 10 of incubation, the methods of Gonzalez and Jackson (2020) were followed for treatment administration. Eggs were briefly removed from the incubator, and before injection, the injection site was cleaned with 70% ethanol. One-hundred microliters of 0.9% sterile saline containing the assigned concentration of NR was injected into the yolk sac using a 2.54-cm, 20-gauge injection needle inserted approximately 1-cm. Solutions of each treatment dose were made immediately before

injection of all eggs by premixing the NR into warm 0.9% saline. Once injected, the concentration of NR in the yolk sac was approximately 2.5, 5.0, and 10.0 mmol. The injection site was covered with a 1-cm2 portion of medical tape (Nexcare; 3M, Maplewood, MN) and placed back into the incubator under the conditions described above. At day 18 of incubation, eggs were removed from their trays, placed in hatching boxes, and the humidity of the incubator was increased to 60 ± 2%.

Harvest and Sample Collection

Embryos at day 15 (E15; n = 18, 15, 15, and 16 embryos for 0 mmol, 250 mmol, 500 mmol, and 1 mol, respectively) and 19 (E19; n = 17, 18, 18, and 19 embryos for 0 mmol, 250 mmol, 500 mmol, and 1 mol, respectively) of incubation and chicks at 24 h posthatch (n = 17, 11, 15, and 18 embryos for 0 mmol, 250 mmol, 500 mmol, and 1 mol, respectively) were euthanized by exposure to CO2 and decapitation. Crown to rump length, head width, and head length were measured by calipers (Traceable Digital Calipers; Fisher Scientific, Pittsburg, PA). Head and chest circumference were collected by curling a string around the target area and measuring the length of the curled string by a ruler. Breasts were sprayed with 70% ethanol, skinned to expose the PMM, and chest width and length were measured by calipers. Both sides of the PMM for E18 and hatched chicks were removed, and the left side was weighed, followed by length, width, and thickness measurement collection. The left side of the rib cage, including the pectoralis minor and major muscles, was removed, and the measurements above were collected.

The following methods were conducted for hatched chicks only. The left PMM was submerged in optimal cutting temperature tissue freezing medium compound, slowly

frozen at -20°C for 1 h, and stored at -80°C until cryosectioning. The right side of PMM was stored in a 1.5 ml microcentrifuge tube and stored at -80°C until NAD + analysis. The left biceps femoris muscle was collected and stored at -80°C for quantitative PCR analysis. The heart and liver of each chick was removed, weighed, and discarded.

Immunohistochemistry and Histology

The methods of Gonzalez and Jackson (2020) were followed for immunohistochemistry analysis. On 2 separate slides, 6 cryosections (10 μm thick) were collected on positively charged slides (Diamond White Glass; Globe Scientific Inc., Paramus, NJ). Cryosections were incubated in 5% horse serum and 0.2% TritonX-100 in phosphate buffered saline (PBS) for 30 min to block all nonspecific binding sites. Cryosections were incubated for 16 h at 4°C with a primary antibody solution consisting of blocking solution and 1:500 rabbit α-dystrophin (Thermo Scientific, Waltham, MA) and 1:2 chicken α-Pax7 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA). Cryosections were washed 3 times for 5 min with PBS and incubated for 30 min with 1:1,000 Alexa-Flour 594 goat-anti-rabbit H&L (Life Technologies, Carlsbad, CA), 1:1,000 Alexa-Flour 488 chicken-anti-mouse IgG1 (Life Technologies), and 1:1,000 Hoescht Dye 33,342 (Life Technologies) secondary antibodies in blocking solution. After washing in PBS 3 times for 5 min, 5 μL of 9:1 glycerol in PBS was placed on each cryosection, and slides were coverslipped for imaging.

The methods of Noel et al. (2016) were followed for succinate dehydrogenase (SDH) staining. Slides were incubated at 37°C for 1 h in a prewarmed incubation solution containing nitro blue tetrazolium solution, phosphate buffer, and sodium succinate

solution. After washing in Milli-Q water 3 times for 1 min each, 5 μL of 9:1 glycerol in PBS was placed on each cryosection, and they were coverslipped for imaging.

All cryosections were imaged at 200-fold magnification using a Nikon Eclipse TI-U inverted microscope (Nikon Instruments Inc., Melville, NY). Immunohistochemistry photomicrographs were collected with a DS-QiMC digital camera (Nikon Instruments Inc.), and SDH cryosection images were collected with a Nikon DS-Fil color digital camera (Nikon Instruments Inc.) White light intensity was kept constant for SDH photomicrographs. All photomicrograph collection and image analyses were conducted using NIS Elements Basic Software (Nikon Instruments Inc.). Cross-sectional area (CSA) of a minimum of 1,000 muscle fibers per chick was determined as the area within the dystrophin border. The number of satellite cell was determined as nuclei co-staining for Pax 7 and Hoechst dye located at the periphery of muscle fiber. Satellite cells were quantified as number of cells per mm2 and number of cells per fiber. A minimum of 50 muscle fiber bundles per chick were analyzed for SDH mean intensity using the same software. The scale for mean intensity ranged from 0 (black or the most intense staining) to 250 (white or the least intense staining). All immunohistochemistry and histology measurements were averaged for each experimental unit and used in the statistical analyses.

NAD^+ Quantification

Nicotinamide adenine dinucleotide content of the right PMM was quantified using a commercial NAD/NADH assay kit (Abnova, Taipei, Taiwan) following the methods of Van Bibber-Kruger et al. (2020). Twenty-milligram of PMM tissue was homogenized in a propriety NAD extraction buffer, heated at 60°C for 5 min, and propriety assay and

NADH buffers were added. Samples were centrifuged at 20,817 × g for 5 min, and the supernatant was used for analysis. Standards, samples, and working reagent [propriety assay buffer, enzymes, lactate, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were loaded onto 96-well plates, and absorbance was read at 565 nm at 0 and 15 min. The change in absorbance was used to calculate amount of NAD+. Extrapolation of unknowns to a standard curve with an average r2 of 0.97 was used to calculate concentration. All samples had coefficient of variations less than 10%.

Cyclin D mRNA Expression

The methods of Burnett et al. (2016) were followed with minor modifications. Briefly, nucleic acids were extracted and purified from 200 mg of the left biceps femoris muscle from hatched chicks using Trizol (Life Technologies), followed by affinity column isolation of total RNA (PureLink RNA Mini Kit, Life Technologies, Carlsbad, CA). Total RNA concentration and 260 nm/280 nm ratio quantified RNA isolates with a ratio greater than 1.9 were used for real-time PCR analysis. Trace genomic DNA decontamination and reverse transcription were conducted on 50 ng of total RNA using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Gene specific primers were designed, efficiencies determined, and validated for qPCR (Table 3-2). Complementary DNA was amplified in duplicate for each sample using PerfeCTa SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD) and the appropriate gene specific forward and reverse primers (20 pm) in an Eppendorff Mastercycler realplex2 S PCR System (Eppendorf North America, Hauppauge, NY). Thermal cycling parameters were initial heating at 50°C for 2 min, denaturing at 95°C for 10 min, 50 cycles of 15 s at

95.0°C, annealing at 60.5°C for 30 s, and extension for 20 s at 68.0°C. A final dissociation step was included at 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. Expression was normalized to 18S ribosomal RNA expression (ΔCt, where Ct refers to the threshold cycle) and calibrated to control chick (0 mmol) mRNA expression (ΔΔCt). Housekeeping gene expression was statistically analyzed and found not to be affected by treatment. Gene fold change expression levels were calculated as $2^{-\Delta\Delta Ct}$ as previously described by Livak and Schmittgen (2001).

Statistics

All data were analyzed as a completely randomized design with embryo/chick as the experimental unit. Treatment served as the fixed effect, and all models were analyzed using the Mixed procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC). Pairwise comparisons between the least squares means of the factor level comparisons were computed using the PDIFF option of the LSMEANS statement. Statistical significance was determined at $P \leq 0.05$.

Results

Body Morphometrics and Muscle Characteristics

There were no treatment effects for all measures collected on E15 embryos ($P > 0.22$; Table 3-3). Treatment did not affect all whole-body measurements ($P > 0.08$) for

E18 embryos with the exception of head circumference ($P = 0.04$; Table 3-4). Embryos injected with 500 mmol and 1 mol NR had larger head circumferences than 0 mmol embryos ($P < 0.05$) but did not differ ($P = 0.83$) from each other. Embryos injected with 250 mmol NR had smaller ($P = 0.04$) head circumferences than 1 mol embryos but did not differ from all other treatments ($P > 0.07$). Treatment did not affect PMM measures ($P > 0.12$), except PMM weight and length ($P < 0.01$). Embryos injected with NR had greater PMM weight and length compared with control embryos ($P < 0.01$) but did not differ from each other ($P > 0.25$).

There were no treatment effects on all hatched chick whole body measures ($P > 0.08$; Table 3-5), except head length and chest width ($P = 0.05$). Chicks from eggs injected with 250 mmol NR had greater head lengths and chest widths than control chicks and chicks from eggs injected with 1 mol NR ($P < 0.03$), which did not differ from each other ($P > 0.54$). Head length and chest width from hatched chicks injected with 500 mmol NR were not different from all other treatments ($P > 0.10$).

In hatched chicks, treatment affected all PMM measures ($P < 0.02$). Chicks from eggs injected with NR had greater PMM weight and width than control chicks ($P < 0.01$) but did not differ from each other ($P = 0.86$). Chicks from eggs injected with 250 and 500 mmol NR had longer PMM than control chicks ($P < 0.01$) but did not differ ($P = 0.63$) from each other. Chicks from eggs injected with 1 mol NR did not differ in PMM length compared with all other treatments ($P > 0.06$). Chicks from eggs injected with 500 mmol NR had greater PMM depth than control and 1M chicks ($P < 0.04$), which did not differ ($P = 0.24$) from each other. Chicks from eggs injected with 250 mmol NR did not differ in PMM length compared with all other treatments ($P > 0.06$).

Muscle Fiber Morphometrics and Satellite Cell Content

There was no treatment effect ($P = 0.20$) for muscle fiber CSA; however, there was a treatment effect ($P < 0.01$) for muscle fiber density (Figure 3-1). Chicks from eggs injected with 1 mol NR had greater muscle fiber density than all other treatments ($P < 0.01$). Chicks from eggs injected with 250 and 500 mmol NR had greater muscle fiber density than control chicks ($P < 0.01$) but did not differ ($P < 0.06$) from each other. There was a treatment effect ($P < 0.01$) for satellite cell density from hatched chicks, but there was no treatment effect ($P = 0.28$) for number of satellite cells per muscle fiber (Figure 3-2). Chicks from eggs injected with 500 mmol or 1 mol NR had a greater satellite cell density than chicks from the other 2 treatments ($P < 0.01$) but did not differ ($P = 0.69$) from each other. Chicks from eggs injected with 250 mmol NR had a greater ($P < 0.01$) satellite cell density than control chicks.

Succinate Dehydrogenase Staining Intensity and NAD^+ Content

There was no treatment effect ($P > 0.81$) for SDH staining intensity on hatched chicks, but there was a treatment effect ($P < 0.01$) for NAD+ content of PMM (Figure 3-3). Hatched chicks injected with 1 mol NR had more NAD+ than the other 3 treatment groups ($P < 0.01$), which did not differ from each other ($P > 0.69$).

Cyclin D mRNA Expression

There were no treatment effects for cyclin D1 and 2 mRNA expression ($P > 0.76$); however, treatment did affect ($P = 0.01$) cyclin D3 expression (Figure 3-4).

Control chicks had greater cyclin D3 expression than all NR treatments ($P < 0.04$), which did not differ from each other ($P > 0.26$).

Discussion

As reported by the National Chicken Council (2020), from 1925 through 2019, the poultry industry increased broiler market weight by 153%, while improving feed efficiency 61%. Equally impressive, birds now reach market weight in 58% less time. Advancements in genetics and nutrition that maximize muscle development and growth are the main factors for these advancements in production efficiency. Despite these production efficiency improvements, the poultry industry is constantly looking to improve growth and muscle deposition in its birds utilizing novel methods. *In ovo* feeding of nutrients constitutes one such method researchers have examined for over 2 decades.

In ovo feeding is defined as direct administration of a compound into eggs during incubation (Saeed et al., 2018). Studies demonstrated this technique supplied nutrients directly into chicken embryos which improved muscle development and growth, enhanced breast meat yield and feed utilization (Bhanja et al., 2008), and decreased embryo mortality and morbidity by strengthening immunity and protection status against important infectious pathogens (Madej and Bednarczyk, 2016). When used for growth purposes, *in ovo* injection of compounds appears to affect body weight in a compound-dependent manner. Compounds injected *in ovo* such as amino acids or creatine pyruvate increased chick body weight (Al-Murrani, 1982; Ohta et al., 2001), whereas L-carnatine, a compound that functions similar to NR, did not affect body weight (Zhao et al., 2017).

In the current Cobb 500 study, NR did not affect body weight during embryo development and at hatching; however, head circumference was affected by NR at E19, and head length and chest width were affected at hatching. These improvements contrast the previous study where injecting 250 mmol of NR into the albumen or yolk sac of the developing embryo did not affect all whole-body measures (Gonzalez and Jackson, 2020). The larger head measurements could signal advancements in brain growth; however, the fact other measures were not affected, the affected measurements occurred inconsistently by dose, and the magnitude of the improvements were small indicated these improvements may not be biologically significant. Because the chest width measurement was taken without the skin and feathers, improvement in the 250 mmol treatment may indicate there was increased muscling on the carcasses of those chicks.

Previously, Gonzalez and Jackson (2020) reported injecting 250 mmol of NR into the egg of the developing embryo increased PMM weight by 38% and length, width, and depth by 21, 9, and 10%, respectively. In the aforementioned study, measurements were only collected at hatch; however, the current study collected PMM data 2 times before hatching. At E15, NR had no effect on PMM measures, but by E19 NR increased weight and length by a minimum of 21 and 13%, respectively. At hatch, NR increased the weight advantage to 35% and maintained the 13% increase in PMM length. Therefore, the additional weight may have been because of the 17 and 18% increase in PMM width and depth, respectively. The 250 mmol NR response of the current study is similar in magnitude compared with the Gonzalez and Jackson (2020) response, and they also indicate injecting more than 250 mmol of NR does not provide any extra benefit for global PMM morphometrics.

With *in ovo* injections occurring on day 10 of incubation, NR affected the events associated with secondary muscle fiber development. Similar to Gonzalez and Jackson (2020), NR injections did not affect muscle fiber CSA, but did increase muscle fiber density. The absence of an effect on fiber CSA but increased density is biologically surprising; however, the difference in response is most likely because of the methods employed to calculate density. While not statistically significant, CSA appeared to decrease as dose increased. Therefore, this led to increased density when fiber number was calculated on a per mm^2 basis. Unlike the global PMM response, injecting 250 and 500 mmol NR increased muscle fiber density by an average of 34% compared with control chicks, whereas 1 mol injections increased density by 75%. The 1 mol maximum response is the greatest response of the two NR broiler studies and is also greater than other studies injecting various compounds into duck and chickens (Liu et al., 2012; Zielinska et al., 2012). These results imply the increase in PMM morphometrics may have been because of the development of more muscle fibers during secondary myogenesis.

Embryonic muscle development involves the proliferation and differentiation of somitic progenitor cells into myoblasts, which terminally differentiate and fuse into myotubes. Satellite cells, the resident muscle stem cell pool responsible for adult muscle growth and repair (Collins and Partridge, 2005), originate from Pax7 expressing myogenic progenitor cells in the dermomyotome central domain (Gros et al., 2005). The literature does not contain studies documenting the effects of NR supplementation on embryonic and fetal myogenesis. Because several studies reported NR supplementation increased muscle NAD^+ levels (Khan et al., 2014; Zhang et al., 2016), an important

substrate in siturin-1 pathway control of stem cell proliferation (Rathbone et al., 2009), this could be the mechanism responsible for NR-stimulated increased satellite cell number per gram of tissue in aged and young mice (Zhang et al., 2016). Okabe et al. (2019) showed NR increased NAD^+ level, and NAD^+ acts as a substrate for the sirtuin family from which sirtuin-1 regulates myogenesis during early development by down regulation of MyoD expression (Nogueiras et al., 2012). In the current study, the increase in muscle fiber density was also accompanied by an increase in satellite cell density. At hatching, chicks from eggs injected with 250 mmol NR had satellite cell density increase by 73% compared with control, and 500 mmol and 1 mol chicks had an average density increase of 116%. When put on a fiber basis, the NR treatment effect on satellite cell number was eliminated, indicating the increase in density was primarily because of more muscle fibers being formed. More interesting, NAD^+ levels were only greater in 1 mol chicks, which may be the reason why these chicks had a much greater muscle fiber density than the other treatments. Additionally, the lack of a NR effect on SDH staining intensity indicates there was not an increase in mitochondria biogenesis, and the increase in NAD^+ level was because of a rise in the efficiency of production.

The cyclins and their dependent kinases serve as regulatory subunits that regulate cell cycle progression. The type D cyclins (D1, D2, and D3) act as unique cell cycle components that sense mitogenic elements in the extracellular environment to increase proliferation (Sherr and Roberts, 1999). In developing skeletal muscle, myoblast cyclin D1 content increased to prevent terminal differentiation (Rao and Kohtz, 1995). Kiess et al. (1995) and Rao and Kohtz (1995) demonstrated cyclin D3 expression stimulated differentiation of myoblasts to myotubes. With chicks supplemented NR appearing to

possess a greater fiber density, the cyclin D pathway may be influenced by the compound. In the current study, control chicks had greater cyclin D3 mRNA expression by an average of 38%. With cyclin D1 and 2 mRNA expression unchanged, this may indicate control chicks were farther behind in myogenesis compared with NR chicks and were continuing to form myotubes.

Conclusion

In ovo feeding of NR increased PMM morphometrics of E19 of embryos and hatched chicks. Similar to the previous NR *in ovo* feeding study, increased PMM measurements coincided with an increase in muscle fiber density and no effect on fiber CSA in hatched chicks. While increasing the dose of injected NR did not affect hatched chick PMM morphometrics, dose increased muscle fiber and satellite cell density. The lack of increased PMM weight, width, length, and depth because of NR administration indicated there is no advantage when injecting more than 250 mmol of NR; however, the drastic increase is muscle fiber density because of elevating the dose may have implications for future growth or meat quality characteristics.

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Table 3-1. Summary statistics of beginning egg weights separated by assigned harvest group.[1]

	Nicotinamide riboside dose, mM					
Items	0	250	500	1000	SEM	*P*-value
Egg weight, g						
Embryonic d-15[2]						
n	20	20	20	20		
Average	66.7	66.8	66.9	67.3	0.77	0.98
Minimum	63.1	63.1	63.2	63.2		
Maximum	72.6	72.7	72.7	77.6		
Embryonic d-19[2]						
n	20	20	20	20		
Average	60.7	60.8	60.9	61.0	0.40	0.99
Minimum	55.2	55.8	56.7	57.5		
Maximum	63.0	63.0	63.0	63.1		
Hatch						
n	20	20	20	20		
Average	65.5	65.5	65.6	65.6	0.17	1.00
Minimum	64.2	64.3	64.3	64.4		
Maximum	66.8	66.8	66.8	67.0		

[1]Eggs were Cobb 500 (Cobb-Vantress, Siloam Springs, AR).
[2]Day of incubation embryos were harvested for data collection.

Table 3-2. Gene specific primers utilized for real-time PCR analysis.

Gene	Forward primer (5' to 3')	Reverse primer (5′ to 3′)	T_m[1], °C	Amplicon length, bp	Efficiency	GeneBank accession
Cyclin						
D1	GCTACCTGCATGTTTGTGGC	GGGTCTGATGGAGTTGTCGG	64	92	93	NM_205381
D2	TGAGAACTGCCCTGCTCTTG	CAGAGGACCTAGCAGCCAAC	64	84	92	XM_015292118
D3	CAGAACTTGCTGAGCCAGGA	TCCGCATGTAGGGCTTGATC	64	87	97	NM_001008453.1
18S rRNA[2]	GAACGAGACTCTGGCATGCT	TCAATCTCGGGTGGCTGAAC	64	96	90	XR_003078044

[1]Melting temperature.
[2]Normalizing gene. Expression was not affected by treatment.

Table 3-3. Body and pectoralis major morphometrics of E15 chick embryos injected *in ovo* at day 10 of embryogenesis with increasing doses of nicotinamide riboside.

	Nicotinamide riboside dose, mM					
Items	0	250	500	1000	SEM	*P*-value
n	18	15	15	16		
Body morphometrics						
Weight, g	13.6	14.5	14.2	13.9	0.86	0.90
Dimensions, mm						
Crown to rump length	59.4	61.4	61.1	61.0	1.50	0.75
Head width	13.62	13.70	13.72	14.02	0.279	0.73
Head length	17.06	17.63	17.78	17.41	0.858	0.93
Heart weight, g	0.13	0.14	0.13	0.14	0.007	0.23
Liver weight, g	0.27	0.27	0.25	0.28	0.018	0.68
Pectoralis major measurements						
Weight[1], g	0.73	0.81	0.82	0.84	0.063	0.60
Dimensions, mm						
Length	17.60	18.25	17.79	18.50	0.713	0.77
Width	13.73	14.65	15.18	14.86	0.543	0.22
Depth	6.93	7.20	7.07	6.61	0.249	0.35

[1]Weight includes rib cage and pectoralis minor and major muscles.

Table 3-4. Body and pectoralis major morphometrics of E19 chick embryos injected *in ovo* at day 10 of embryogenesis with increasing doses of nicotinamide riboside.

	Nicotinamide riboside dose, mM					
Items	0	250	500	1000	SEM	*P*-value
n	17	18	18	19		
Body morphometrics						
Weight, g	37.9	37.7	37.9	38.1	1.42	1.00
Dimensions, mm						
Crown to rump length	85.0	84.3	85.9	86.3	0.62	0.08
Head width	16.95	15.38	15.31	15.40	0.757	0.34
Head length	17.78	17.67	17.43	17.58	0.167	0.49
Head circumference	53.0[a]	53.2[a,c]	54.8[b,c]	55.0[b]	0.064	0.04
Chest circumference	57.0	59.5	60.1	61.5	0.127	0.08
Chest width	15.56	15.49	16.32	15.44	0.317	0.14
Chest length	18.34	18.60	18.45	18.30	0.309	0.89
Heart weight, g	0.22	0.22	0.22	0.23	0.006	0.56
Liver weight, g	0.63	0.64	0.64	0.63	0.024	0.98
Pectoralis major measurements						
Weight, g	0.14[a]	0.17[b]	0.18[b]	0.17[b]	0.007	<0.01
Dimensions, mm						
Length	15.12[a]	17.23[b]	17.72[b]	17.65[b]	0.380	<0.01
Width	5.11	5.48	5.66	5.57	0.173	0.12
Depth	2.68	2.91	2.74	2.87	0.099	0.29

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

Table 3-5. Body and pectoralis major morphometrics of hatched chicks injected *in ovo* at day 10 of embryogenesis with increasing doses of nicotinamide riboside.

	Nicotinamide riboside dose, mM					
Items	0	250	500	1000	SEM	*P*-value
n	17	11	15	18		
Body morphometrics						
Weight, g	45.3	46.9	46.1	46.6	0.61	0.16
Dimensions, mm						
Crown to rump length	93.2	95.4	95.0	93.0	1.04	0.16
Head width	15.56	15.53	15.65	15.48	0.16	0.83
Head length	17.76[a]	18.66[b]	18.22[a,b]	17.76[a]	0.29	0.05
Head circumference	5.47	5.57	5.56	5.39	0.07	0.17
Chest circumference	6.09	6.12	6.25	6.14	0.08	0.42
Chest width	15.91[a]	17.07[b]	16.56[a,b]	16.14[a]	0.33	0.05
Chest length	20.47	21.90	21.18	21.11	0.47	0.14
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
Pectoralis major measurements						
Weight, g	0.17[a]	0.23[b]	0.23[b]	0.22[b]	0.01	<0.01
Dimensions, mm						
Length	17.61[a]	19.89[b]	20.33[b]	19.13[a,b]	0.69	<0.01
Width	4.63[a]	5.50[b]	5.56[b]	5.39[b]	0.23	<0.01
Depth	2.78[a]	3.14[a,b]	3.33[b]	2.97[a]	0.15	0.02

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

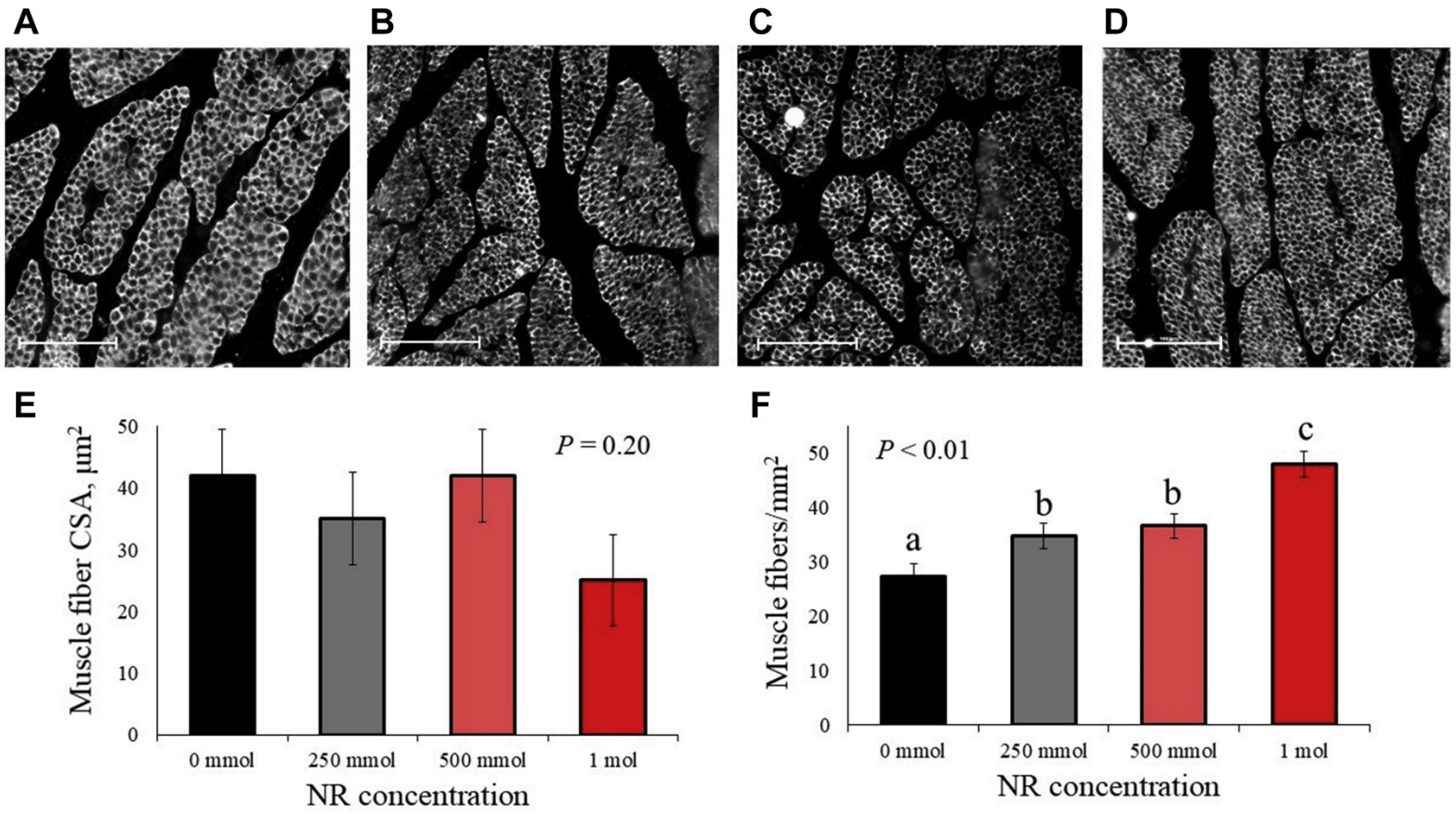

Figure 3-1. Representative 200-fold photomicrographs of pectoralis major cross-sections from (A) 0 mmol, (B) 250 mmol, (C) 500 mmol, and (D) 1,000 mmol chicks immunostained for dystrophin. (E) Muscle fiber cross-sectional area (**CSA**) and (F) muscle fiber density from hatched chicks administered 1 of 4 nicotinamide riboside (**NR**) doses during embryonic development. Fertilized Cobb 500 eggs were injected with the appropriate NR dose in 100 µL of 0.9% sterile saline at day 10 of incubation. Scale bars are 100 µm. [a,b,c]Treatments with different superscripts within a panel differ ($P < 0.05$).

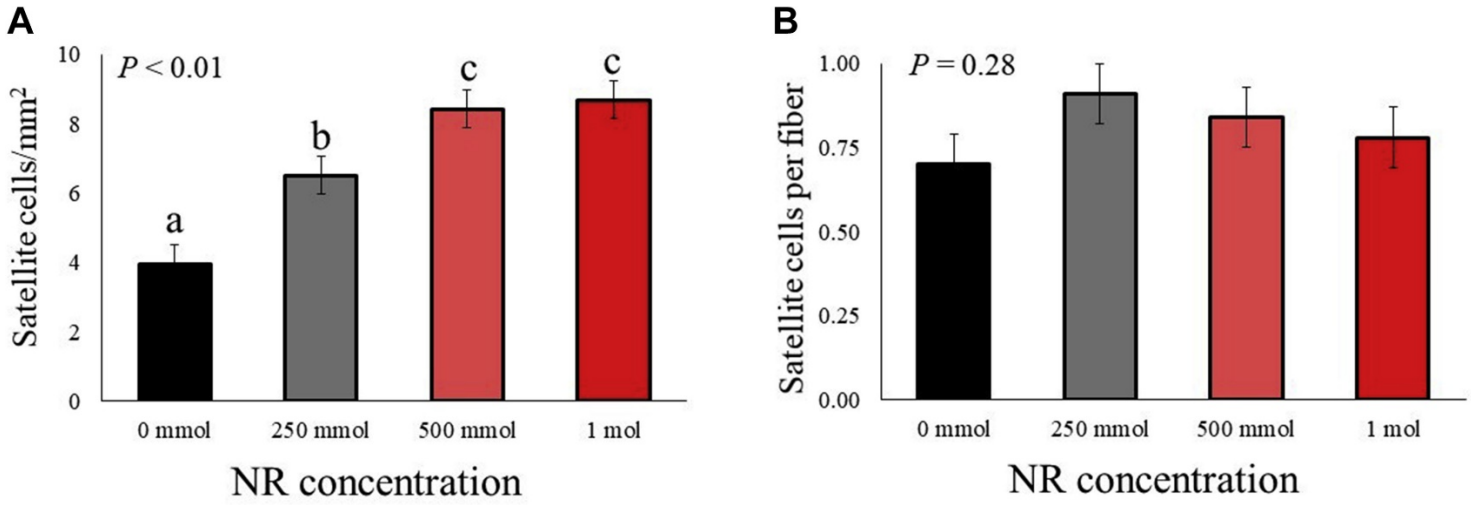

Figure 3-2. Pectoralis major (A) satellite cell density and (B) satellite cell number per fiber from hatched chicks administered 1 of 4 nicotinamide riboside (**NR**) doses during embryonic development. Fertilized Cobb 500 eggs were injected with the appropriate NR dose in 100 µL of 0.9% sterile saline at day 10 of incubation. [a,b,c]Treatments with different superscripts within a panel differ ($P < 0.05$).

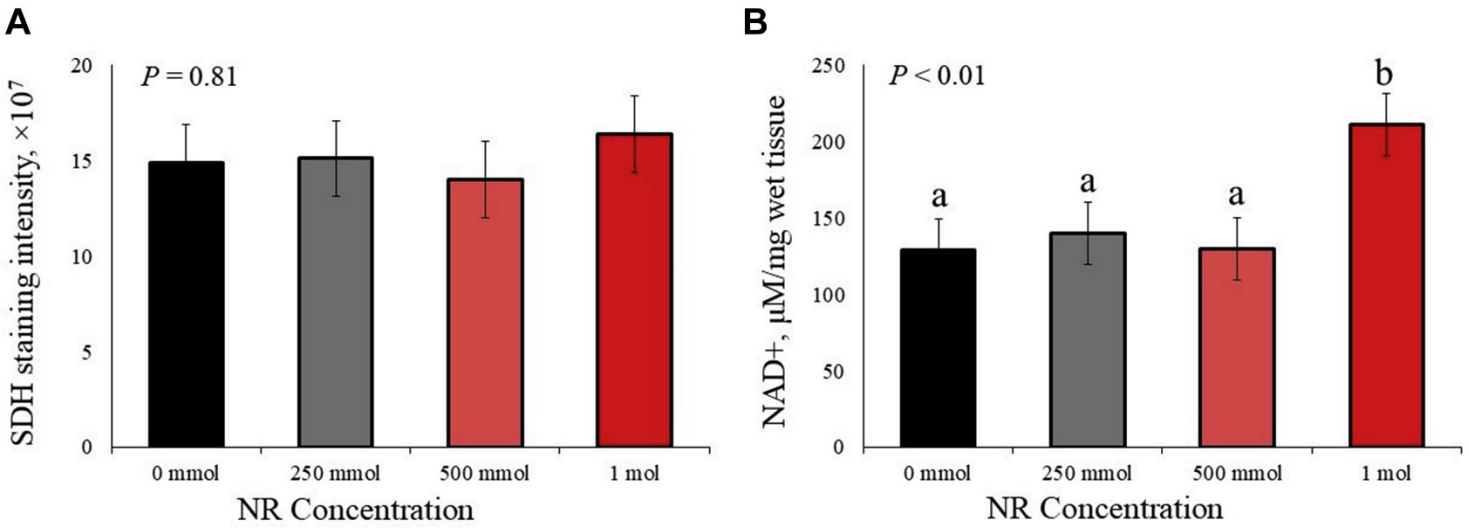

Figure 3-3. Pectoralis major (A) succinate dehydrogenase (**SDH**) staining intensity and (B) NAD^+ content from hatched chicks administered 1 of 4 nicotinamide riboside (**NR**) doses during embryonic development. Fertilized Cobb 500 eggs were injected with the appropriate NR dose in 100 µL of 0.9% sterile saline at day 10 of incubation. [a,b,c]Treatments with different superscripts within a panel differ ($P < 0.05$).

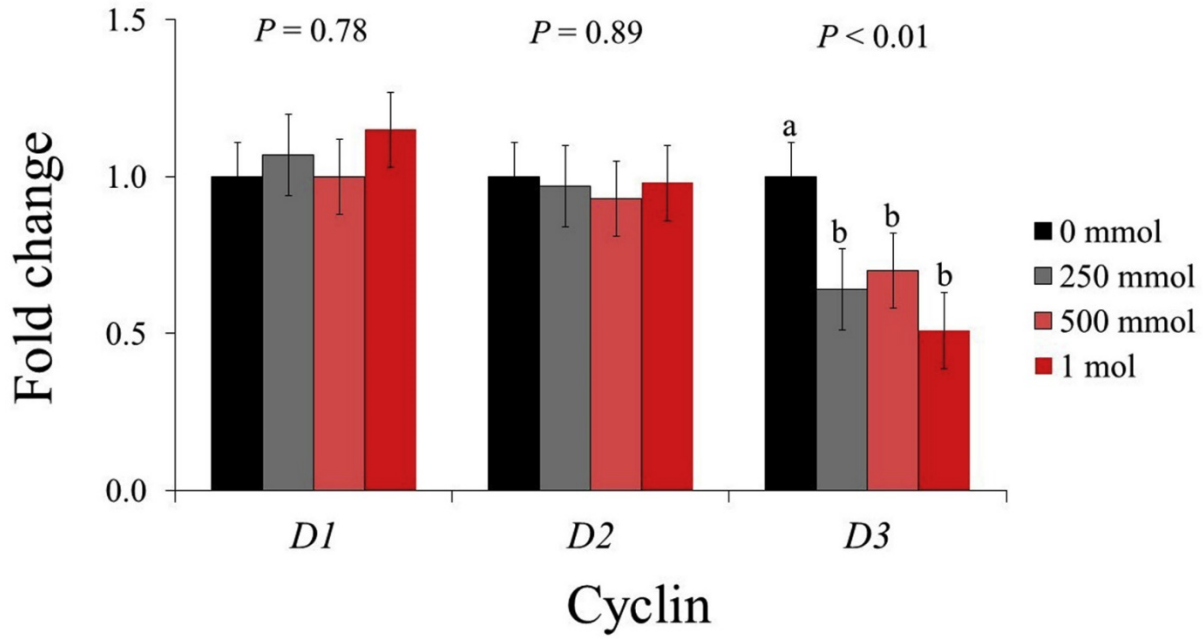

Figure 3-4. Biceps femoris cyclin D mRNA expression from hatched chicks administered 1 of 4 nicotinamide riboside (**NR**) doses during embryonic development. Fertilized Cobb 500 eggs were injected with the appropriate NR dose in 100 µL of 0.9% sterile saline at day 10 of incubation. [a,b,c]Treatments with different superscripts within a gene differ ($P < 0.05$).

CHAPTER 4

THE EFFECT OF *IN OVO* FEEDING OF NICOTINAMIDE RIBOSIDE ON HIGH YIELD BROILER MYOGENESIS: A DOSE×LOCATION STUDY

Effects of *in ovo* injection of nicotinamide riboside on high-yield broiler myogenesis. Xu, X., H. M. Alcocer, M. E. Gravely, A. R. Jackson, and J. M. Gonzalez. (2022) (Journal of Animal Science) 100:1-10. Reprinted here with permission of the publisher.

Abstract

The objective of this study was to determine the effects of *in ovo* injection of high-yield broiler embryos with nicotinamide riboside (**NR**) on pectoralis major muscle (**PMM**) development, growth, and gene expression. Fertilized Cobb 700 broiler eggs were randomly assigned to one of four treatments within a 2 × 2 factorial design. Factor 1 consisted of NR dose (**DOS**) with eggs receiving 0- or 2.5-mM NR. Factor 2 consisted of injection location (**LOC**), with treatments injected into either the yolk sac or albumen. At day 10 of incubation, 100 µL of the assigned NR dose was injected into the yolk sac of the developing embryo and chicks were euthanized within 24 h of hatching. Chick PMM and individual fiber morphometrics, and expression of genes associated with cell cycle progression were analyzed. There were DOS × LOC interactions for hatched chick PM weight and length ($P < 0.04$). When NR was injected into the albumen, PMM weight decreased ($P < 0.05$); when NR was injected into the yolk, PMM weight increased ($P < 0.05$). Pectoralis major length was not affected ($P > 0.05$) when NR was injected into the albumen but was increased ($P < 0.05$) when NR was injected into the yolk. There was a DOS × LOC interaction ($P = 0.04$) for muscle fiber density and tended to be a DOS × LOC interaction ($P = 0.07$) for muscle fiber CSA. Pectoralis major muscle fiber density was not affected when NR was injected into the albumen ($P > 0.05$), but density increased when NR was injected into the yolk ($P < 0.05$). There were DOS × LOC interactions for hatched chick COXII, cyclin D, and SIRT1 expression ($P \leqslant 0.04$), which may indicate NR improves skeletal muscle development and growth by enhancing myoblast proliferation during embryonic development.

Lay Summary

Broiler chicken weight gain is a result of genetics and nutrition, with increased muscle mass attributed to accelerated embryonic myogenesis and posthatch muscle growth. During the avian incubation period, *in ovo* injection may be used as a strategy to deliver exogenous supplements into growing embryos for improving skeletal muscle development and growth. Nicotinamide riboside (**NR**), a vitamin B3 analog, is a human performance supplement used to stimulate mitochondria biogenesis and elevate tissue NAD^+ levels. Research showed injecting NR into the chick embryonic yolk sac increased breast muscle weight and muscle satellite cell numbers and proliferation rate. Therefore, our objective was to determine the effects of *in ovo* injection of high-yield broilers with NR on broiler breast muscle development and growth. Our study showed *in ovo* injection of NR into the yolk sac increased hatched chick breast muscle morphometrics, which coincided with an increase in muscle fiber density and tended to decrease fiber cross-sectional area. Increased Sirtuin1 and cyclin D mRNA expression of hatched chicks from eggs injected with 2.5 mM NR into yolk sac indicate a potential NR regulated Sirtuin1/cyclin D molecular mechanism mediating chicken muscle early development.
Key words: cyclin D, mitochondria, muscle fiber, nicotinamide riboside, pectoralis major

Introduction

Over the past six decades, the poultry industry has made impressive strides to increase muscling of broiler chickens. Many of the gains are attributed to genetics and nutrition, but biologically, greater muscle mass reflects altered *in ovo* myogenesis and posthatch muscle growth. Similar to mammalian species, avian embryonic muscle development occurs in two phases: primary myogenesis from embryonic day (**E**) 3 to 7 and secondary myogenesis from E8

until hatching (Biressi et al., 2007). During embryonic myogenesis, progenitor cells undergo myogenic determination to form myoblasts capable of proliferation and fusion to form multinucleated myotubes which mature to become myofibers (Abmayr and Pavlath, 2012). Expansion of myofibers occurs during secondary myogenesis to create a fixed number of muscle fibers present at hatch or birth. These fibers constitute the foundation for future muscle growth through protein accretion. *In ovo* feeding was first reported in the 1980s for vaccination against Marek's disease (Sharma and Burmester, 1982) and later developed as a nutritional technology to enhance poultry embryonic development and better prepare chicks for intensive development (Givisiez et al., 2020). During *in ovo* feeding, exogenous natural nutrients such as amino acids, carbohydrates, and vitamins can be injected into different locations of the egg based on stage of incubation. Gonzalez and Jackson (2020) reported injecting NR into a commercial-yield chick embryonic yolk sac increased PMM weight through increased muscle fiber density. In a subsequent study, Xu et al. (2021) found no advantage on PMM weight when levels of NR were fed above 250 mM; however, muscle fiber density was increased when levels were fed above 250 mM. The biological mechanisms responsible for increased PMM weight and fiber density remain unexplored.

Numerous studies reported NR, a vitamin B3 analog, increased NAD^+ availability (Bieganowski and Brenner, 2004; Trammell et al., 2016; Elhassan et al., 2017), which was linked to increased muscle satellite cell numbers and proliferation rate in mice (Zhang et al., 2016) through sirtuin-1 (**SIRT1**) activity (Rathbone et al., 2009; Zhang et al., 2016). Further novel observations found SIRT1 over-expression decreased p21Waf/Cip1 expression, an inhibitor of cyclin D–cdk4/6 activity, and increased phosho-Rbser780, a target of increased cyclin D–cdk4/6 activity. Cyclin-D serves as one of the master regulators of cell cycle progression from G1 to S

phase of the cell cycle (Sherr and Roberts, 1999). Therefore, these data imply SIRT1, by increasing muscle precursor cell proliferation, could facilitate hypertrophy and generation of skeletal muscle. Given the positive effects of NR dose on commercial-yield broiler embryo myogenesis, the objective of the study was to determine low dose × location effects of NR *in ovo* feeding on high-yield broiler embryo muscle development through regulation of *cyclin D* and *SIRT1* mRNA expression.

Materials and Methods

All experimental procedures were approved by the University of Georgia Institutional Animal Care and Use Committee.

Egg Procurement, Incubation, and in ovo Injection

Fertilized Cobb 700 broiler eggs ($N = 600$; Cobb-Vantress Inc., Siloam Springs, AR) were transported to the University of Georgia Muscle Biology Laboratory (Athens, GA), egg weights were recorded, and within each four-egg strata, eggs were randomly assigned to an NR treatment within a 2 × 2 factorial design: Factor 1 consisted on NR dose; 0 or 2.5 mM. Factor 2 consisted of *in ovo* injection location: albumen or yolk sac. Each four-egg strata was randomly assigned a harvest day (E15, E18, or hatch). Eggs were incubated in one of three Sportsman 1502 incubators (GQF Manufacturing Company Inc., Savannah, GA) at 37 °C, 40 ± 4% relative humidity, and rotated hourly. Egg weights of four random eggs per incubator were recorded daily with daily weight loss ranging from 0.65% to 0.75% of total mass.

At incubation day 10, the methods of Gonzalez and Jackson (2020) were followed for treatment administration. Eggs were removed from the incubator and the injection site was cleaned with 70% ethanol. One-hundred microliters of 0.9% sterile saline containing the assigned concentration of NR was injected into the yolk sac using a 2.54-cm, 20-gauge injection needle inserted approximately 1 cm. Solutions of each treatment dose were made immediately before injection by premixing NR into warm 0.9% saline. Once injected, the final concentration of NR in the albumen or yolk sac was approximately 0.025 mmol. The injection site was covered with a 5 mm2 portion of medical tape (Nexcare; 3M, Maplewood, MN) and placed back into the incubator under the conditions described above. At incubation day 18, eggs were removed from their trays, placed in hatching boxes, and the humidity of the incubator was increased to 60 ± 2%.

Harvest and Sample Collection

At E15 (n = 24, 22, 22 and 24 embryos for albumen-0 mM NR, albumen-2.5 mM NR, yolk-0 mM NR, and yolk-2.5 mM NR, respectively), E18 (n = 21, 20, 19, and 19 embryos for albumen-0 mM NR, albumen-2.5 mM NR, yolk-0 mM NR, and yolk-2.5 mM NR, respectively) of incubation, and 24-h posthatch (n = 22, 23, 15, and 18 embryos for albumen-0, albumen-2.5 mM NR, yolk-0 mM NR, and yolk-2.5 mM NR, respectively), embryos and chicks were euthanized by exposure to CO2 and decapitation. Crown to rump length, head width, and head length were measured by calipers (Traceable Digital Calipers; Fisher Scientific, Pittsburg, PA). Head and chest circumference were collected by curling a string around the target area and measuring the length of the curled string by a ruler. Breasts were sprayed with 70% ethanol, skinned to expose the PMM, and chest width and length were measured by calipers. Both sides

of the PMM were removed, and the left side was weighed, followed by length, width, and thickness measurement collection. The right-side PMM was stored in a 1.5-mL microcentrifuge tube and stored at −80 °C for PCR analysis. The left PMM was submerged in optimal cutting temperature tissue freezing medium compound, slowly frozen at −20 °C for 1 h, and stored at −80 °C until cryosectioning. The heart and liver of each chick were removed, weighed, and discarded.

Immunohistochemistry and Histology

The methods of Gonzalez and Jackson (2020) were followed for immunohistochemistry analysis. On each slide, two cryosections (10 μm) were collected on a positively charged slide (Diamond White Glass; Globe Scientific Inc., Paramus, NJ). Cryosections were incubated in 5% horse serum and 0.2% TritonX-100 in phosphate-buffered saline (PBS) for 30 min to block all nonspecific binding sites. Cryosections were incubated for 45 min at room temperature with a solution consisting of blocking solution and 1:1,000 wheat germ agglutinin-Alexafluor 594 (cat no. W11262; ThermoFisher Scientific, Waltham, MA). After washing in PBS three times for 5 min, 5 mL of 9:1 glycerol in PBS was placed on each cryosection, and slides were coverslipped for imaging.

Cryosections were imaged at 200-fold magnification using an ECHO Revolve microscope (ECHO, San Diego, CA). Photomicrographs were collected, stored, and analyzed using ECHO Pro software (ECHO) operating on a iPad Pro (Apple, Cupertino, CA). Cross-sectional area (CSA) was determined for 1,000 fibers (minimum) as the area within the wheat germ agglutinin border. To determine muscle fiber density, the area of the entire field of vision was divided by the average CSA of the fibers in the photomicrograph.

Semiquantitative Real-Time PCR for DNA Copy Number and mRNA Expression

The PMM tissue was minced using a scalpel blade at room temperature and total DNA was extracted using the Qiagen DNeasy tissue 'on column' system (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA was eluted in RNase- and DNase-free water and spectrophotometry used to quantify abundance and purity. Semiquantitative PCR was run using PerfeCTa SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD) and the appropriate gene-specific forward and reverse primers (10 μm; Table 4-1) in Bio-Rad CFX 96 iCycler PCR System (Bio-Rad Laboratories, Hercules, CA). Thermal cycling parameters were initial denaturing at 95 °C for 3 min, 40 cycles of 15 s at 95.0 °C, and extension at 60 °C for 45 s. Gene fold-change expression levels were calculated as $2-\Delta\Delta Ct$ as previously described by Livak and Schmittgen (2001). Expression was normalized to 18S ribosomal RNA expression (ΔCt, where Ct refers to the threshold cycle) and calibrated to control chick (0 mmol) mtDNA replication (ΔΔCt).

The methods of Burnett et al. (2016) were followed with minor modifications for mRNA expression. Briefly, nucleic acids were extracted and purified from 200 mg of the right PM muscle using the Trizol:chloroform (Life Technologies) extraction method, followed by affinity column isolation of total RNA (PureLink RNA Mini Kit, Life Technologies). Total RNA concentration and 260/280 nm ratio were quantified, and RNA isolates with a ratio greater than 1.9 were used for real-time PCR analysis. After total RNA trace genomic DNA decontamination and reverse transcription were conducted on 50 ng of total RNA using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Gene-specific primers were designed, efficiencies determined, and validated for real-time PCR. Complementary DNA was amplified in duplicate for each sample using PerfeCTa SYBR Green FastMix (Quanta Biosciences,

Gaithersburg, MD) and the appropriate gene-specific forward and reverse primers (10 μm) in Bio-Rad CFX 96 iCycler PCR System (Bio-Rad Laboratories, Hercules, CA). Thermal cycling parameters were initial heating at 50 °C for 2 min, denaturing at 95 °C for 10 min, 50 cycles of 15 s at 95.0 °C, annealing at 60.5 °C for 30 s, and extension for 20 s at 68.0 °C. A final dissociation step was included at 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. Gene fold-change expression levels were calculated as $2^{-\Delta\Delta Ct}$ as previously described by Livak and Schmittgen (2001). Expression was normalized to 18S ribosomal RNA expression (ΔCt, where Ct refers to the threshold cycle) and calibrated to control chick (0 mmol) mRNA expression (ΔΔCt). Housekeeping gene expression was statistically analyzed and found not to be affected by treatment.

Statistics

Data from each harvest day were analyzed separately in a completely randomized design with a 2 × 2 factorial arrangement and utilizing embryo/chick as the experimental unit. Nicotinamide dose (**DOS**), injection location (**LOC**), and their interaction served as fixed effects. All models were analyzed using the Mixed procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC). Pairwise comparisons between the least squares means of the factor level comparisons were computed using the PDIFF option of the LSMEANS statement. Statistical significance was determined at $P \leqslant 0.05$ and tendencies were between $0.05 < P < 0.10$.

Results

Body Morphometrics and Muscle Characteristics

There were no DOS × LOC interactions for all measures collected on E15 ($P > 0.18$; Table 4-2) and E18 embryos ($P > 0.11$; Table 4-3). There were no DOS or LOC main effects for E15 and 18 measures ($P > 0.10$), except for E15 LOC and DOS effects for head length and heart weight, respectively ($P < 0.05$). Day-15 embryos receiving NR in the yolk sac had longer ($P = 0.05$) head length than albumen injected embryos. Day-15 embryos injected with NR had smaller ($P < 0.01$) heart weight than embryos injected with saline.

There were no DOS ×LOC interactions for body morphometric measures ($P > 0.25$), except for head circumference and chest length ($P < 0.03$; Table 4-4). Chicks from embryos injected with NR in the albumen had larger head circumference and smaller chests length than chicks from albumen-saline injected embryos ($P < 0.01$), whereas there was no difference for both measures when NR was injected into the yolk sac ($P > 0.67$). Chicks from embryos injected in the yolk sac has smaller crown to rump length, head width, and chest circumference than chicks from embryos injected in the albumen ($P < 0.03$). There were no DOS effects for all body morphometrics not associated with interactions ($P > 0.24$).

There were DOS × LOC interactions for all PM muscle measures ($P \leqslant 0.04$), except for PMM thickness ($P = 0.14$). Chicks from embryos injected in the yolk sac with NR had greater ($P = 0.03$) PMM weight than chicks from embryos injected with saline. Chicks from embryos receiving albumen NR had smaller ($P < 0.05$) PMM weight than chicks from embryos injected with saline. Chicks from embryos injected with NR in the yolk had greater ($P = 0.05$) PMM length than chicks from embryos injected with saline in the yolk, whereas chicks from embryos

injected with NR and saline in the albumen did not differ ($P = 0.33$). Chicks from embryos injected with NR in the albumen tended to have smaller ($P = 0.07$) PMM width than chicks from embryos injected with saline in the albumen ($P < 0.01$), whereas chicks injected with NR in the yolk sac tended to have greater ($P = 0.10$) width than chicks from embryos injected with saline. Chicks from embryos injected with saline had greater ($P = 0.02$) PMM thickness than chicks injected with NR.

There was no DOS × LOC interaction ($P = 0.45$) for heart weight, but chicks from embryos injected in the albumen or with saline had greater heart weights than chicks from embryos injected in the yolk or with NR ($P < 0.01$). There was a DOS × LOC interaction ($P = 0.04$) for liver weight. Compared to chicks from embryos injected with saline in the albumen, liver weight was smaller ($P = 0.04$) from chicks injected with NR in the albumen, but there was no difference ($P = 0.33$) in weight when NR was injected in the yolk sac.

Chicks from eggs injected with 2.5 mmol NR into yolk sac had greater PM weight than the other groups ($P < 0.01$). Chicks from eggs injected with 2.5 mmol NR into yolk sac had longer PM length than chicks from eggs injected with 2.5 mmol NR into albumen ($P = 0.01$) but did not differ ($P > 0.06$) from the other treatments. Chicks from eggs injected with 2.5 mmol NR into yolk sac had greater PM width compared with chicks from eggs injected with 2.5 mmol NR into albumen ($P < 0.01$) but did not differ ($P > 0.09$) from the other groups of chicks.

Muscle Fiber Morphometrics

There tended to be a DOS × LOC interaction for muscle fiber CSA ($P = 0.07$) and DOS × LOC interaction ($P = 0.04$; Figure 4-1) for muscle fiber density. When NR was injected into the yolk sac, chicks had smaller muscle fiber CSA and greater muscle fiber density than chicks from

embryos injected with saline in the yolk sac ($P < 0.05$). Chicks injected with saline and NR in the albumen did not differ in muscle fiber CSA and density ($P > 0.63$).

Mitochondria DNA Replication

There were no DOS, LOC, or DOS × LOC effects for E15 and E18 embryo mitochondria DNA (**mtDNA**) COXII expression ($P > 0.26$; Figure 4-2), but there was a DOS × LOC interaction for hatched chicks ($P = 0.03$). When NR was injected into the yolk sac, chicks had greater mtDNA COXII expression than chicks from embryos injected with saline in the yolk sac ($P < 0.03$). Chicks injected with saline and NR in the albumen did not differ in mtDNA COXII expression ($P > 0.41$).

Sirtuin-1 and cyclin D mRNA Expression

At E15, there were no DOS × LOC interactions for all four genes of interest ($P > 0.27$; Figure 4-3). There were no LOC effects for SIRT1 or Cyclin D2 mRNA expression ($P > 0.33$), but embryos injected in the yolk sac had greater Cyclin D1 and D3 mRNA expression than albumen injected embryos ($P < 0.08$). Embryos injected with NR had greater expression of all four genes compared to embryos injected with saline ($P < 0.01$).

At E18, there were no DOS × LOC interactions for all four genes ($P > 0.23$; Figure 4-4). There were no LOC effects for all genes ($P > 0.18$), except yolk sac injected embryos had greater ($P = 0.01$) Cyclin D1 mRNA expression than albumen injected embryos. Embryos injected with NR had greater SIRT1, Cyclin D1, and Cyclin D3 mRNA expression than saline injected embryos ($P < 0.03$). Embryos injected with NR tended to have greater Cyclin D2 mRNA expression than saline injected embryos ($P = 0.08$).

There were DOS × LOC interactions for hatched chick mRNA expression of all genes ($P < 0.04$; Figure 4-5). Chicks from embryos injected with NR in the yolk and albumen had greater SIRT1 expression than chicks from embryos injected with saline in the same locations ($P < 0.001$), but these embryos did not differ ($P = 0.28$) in expression. Chicks from embryos injected with NR in the yolk sac had greater ($P < 0.001$) SIRT1 expression than chicks from embryos injected with NR in the albumen. Chicks from yolk sac NR injected embryos had greater expression of all Cyclin D forms than chicks from embryos injected with saline in the yolk sac ($P \leqslant 0.01$). Chicks injected with saline and NR in the albumen did not differ in Cyclin D1 and 2 expression ($P \geqslant 0.58$); however, chicks injected with NR in the albumen tended to have greater ($P = 0.08$) Cyclin D3 expression than chicks from embryos injected with saline.

Discussion

The poultry industry is considered one of the most successful sectors of agriculture worldwide. Advancements in genetics and nutrition that maximize muscle development and growth are the main factors for improved production efficiency. Despite these production efficiency gains, the poultry industry is constantly looking to improve growth and muscle deposition utilizing novel methods. *In ovo* injection of nutrients constitutes one such method researchers have examined for over two decades.

In mammals, prenatal nutrition is important for embryonic development, healthy fetal growth, and even has persistent consequences to adult offspring due to the 80utria-epigenetics (Li et al., 2018, 2019). Unlike mammals, broiler embryos are separated from the mother during embryonic development; therefore, egg contents are the only source of nutrition. Fortunately, *in*

ovo injection enables producers to supplement nutrients during embryonic development. Effects of the *in ovo* application of various nutrients including proteins (Foye et al., 2006), peptides (Dalloul et al., 2005), nucleotides (Dalloul et al., 2005), and vitamins (El-Senousey et al., 2018) have been tested. Zhang et al. (2018) used hand injection to administer 0.5, 1.5, 4.5, or 13.5 mg of vitamin C (L-ascorbic acid) to individual Ross 708 broiler eggs at 17 d of incubation. It was observed that none of the vitamin C treatment levels reduced the adverse effects of the holding period on chick BW or the rate of yolk sac absorption. With NR being a fairly new compound, only three studies document effects of the product on broiler embryonic development.

Injecting 250 mM NR into the albumen or yolk sac of Cobb 500 (commercial-yield line) developing affected only chick chest width (Gonzalez and Jackson, 2020). Utilizing the same broiler strain, Xu et al. reported increasing NR doses up to 1 M did not affect whole body morphometrics of E15 and E19 embryos and increased chest width by 8% of chicks from embryos injected with 250 mM NR. Using the same broiler strain as the current study, Alcocer et al. (2021) reported injecting increasing levels of NR from 85 to 340 mM did not affect E18 and hatched chick body weights. In the current study, NR did not affect body weight of late-stage embryos or at hatch; however, head circumference and chest length were affected by NR at hatch day. These improvements contrast the previous study where larger head circumference may indicate better brain development and growth, and increased chest length may imply improved skeletal muscle mass on carcasses of these chicks.

Similar to the current study but in the commercial-yield broiler, Gonzalez and Jackson (2020) reported injecting 250 mM of NR into the yolk sac of the developing embryo increased PMM weight by 38% and its length, width, and thickness by 21%, 9% and 10%, respectively. In the dose titration study, injecting NR at increasing levels affected E19 embryo and hatched chick

PPM weight by 24% and 33%, respectively. Pectoralis major weight increased at E19 due to an increase in length, whereas hatched chick PMM weight increased due to increases in all three measures (Xu et al., 2021). The Alcocer et al. (2021) study found all high-yield broiler PMM measures were unaffected at E18, but weight and depth increased up to 15% and 17%, respectively, for hatched chicks. During the current study, NR injection had no effect on E15 or 18 PMM measures, but PMM weight and length increased by 12%, and 8%, respectively, at hatch. Similar to Gonzalez and Jackson (2020), results of the current study indicated high-yield broiler embryos also have increases in PMM development and growth when NR is injected into the yolk sac. Additionally, the tempered response in PMM growth and development in comparison with Gonzalez and Jackson (2020) was most likely due to embryos being injected with 100 times less NR. When comparing with Alcocer et al. (2021), the similar PPM response to NR may indicate high-yield embryos' response to NR *in ovo* injection plateaus at reduced levels.

In ovo NR injections at day 10 of incubation affected events associated with chicken secondary muscle fiber development similar to past studies. No changes in CSA in response to 250 mM NR were noted but the supplement increased muscle fiber density by 45% (Gonzalez and Jackson 2020). When up to 1M NR was injected in commercial-yield broiler embryo yolk sac, muscle fiber CSA also was not affected but fiber density was increased by nearly 75% (Xu et al., 2021). In the current study, injecting NR at the reduced dose compared to the other studies, tended to decrease fiber CSA by 21%, which increased myofiber density by 35%. Although these high-yield broiler embryos were injected with a reduced NR dose, their increased PMM weight, length, width combined with increased fiber density may indicate NR stimulated pathways associated with increased muscle fiber density during secondary myogenesis.

Khan et al. (2014) demonstrated NR increased NAD^+ levels and mitochondria biogenesis in mice with a mitochondrial myopathy. Cytochrome c oxidase subunit II (COXII) is the second subunit of cytochrome c oxidase and also one of the three mtDNA-encoded subunits (MT-COX1, MT-CO2, MT-CO3) of respiratory complex IV. mtDNA-encoded subunit-CO2 is one of the three subunits which are responsible for the formation of the functional core of the cytochrome c oxidase. mtDNA-encoded subunit-CO2 plays an essential role in the transfer of electrons from cytochrome c to the bimetallic center of the catalytic subunit 1 (The UniProt Consortium, 2017). Therefore, expression of COXII can be utilized to estimate muscle mitochondria amount. Expression analysis of the current study indicated NR injection in the yolk sac did not affect COXII expression at E15 and 18, but increased expression in hatched chicks by 74%. Although muscle NAD^+ content was not measured, this result could indicate embryos injected with NR in the yolk sac may have had increased levels of NAD^+. The role of mitochondria and mitochondrial metabolic efficiency in maximal muscle development were demonstrated by intrauterine restricted sheep muscle having reduced oxygen consumption and complex 1 activity (Pendleton et al., 2020). Therefore, stimulating mitochondria biogenesis may help muscle development in high-yield broilers.

Sirtuin-1 possess NAD^+ dependent enzymatic catalytic activity that is regulated by dynamic changes in NAD^+ level and NAD^+/NADH ratio (for review see Ghinis-Hozumi et al., 2012). Such requirement for NAD^+ as a co-substrate suggests sirtuin-1 might be a sensor of energy status in the cell (Michan and Sinclair, 2007). Injecting NR into the albumen and yolk sac increased E15 and 18 embryo SIRT1 expression. Consistent with COXII expression, injecting NR only in the yolk sac increased hatched chick SIRT1 expression level by 56%. Because SIRT1

can affect cyclin D, NR may be regulating chicken muscle fiber hyperplasia during embryonic stage through the NAD+-sirtuin 1-cyclin D molecular pathway.

Type D cyclins (cyclin D1, D2, and D3) are essential for cell cycle progression from G1 to the S phase of mitosis, where they bind to and activate cyclin-dependent kinases (CDK4 and CDK6) to trigger phosphorylation of retinoblastoma protein and initiation of DNA synthesis. There is a principal mechanism that augment cyclin D abundance thus activation of CDK4/6, which is through elevating cyclin D protein translation, mediated via PI3K-Akt-mTOR (mammalian target of rapamycin)-S6K1 (S6 kinase 1) signaling (Muise-Helmericks et al., 1998; Koziczak and Hynes, 2004). In developing skeletal muscle, myoblast cyclin D1 content increased to prevent terminal differentiation (Rao and Kohtz, 1995). Kiess et al. (1995) demonstrated cyclin D3 expression stimulated differentiation of myoblasts to myotubes in vitro. Similar to COXII and SIRT1 expression levels, injecting NR into the albumen and yolk sac increased E15 and 18 embryo cyclin D1, 2, and 3 mRNA expression levels. At hatch, chicks supplemented with NR into yolk sac had greater cyclin D1, D2, and D3 expression by an average of 49%, which may be the reason that the NR yolk group had greater fiber density. With chicks supplemented NR possessing greater fiber density, the cyclin D pathway may be influenced by the compound to catalyze this response.

Conclusion

In ovo injection of NR into yolk sac increased PMM morphometrics of hatched chicks. Similar to previous NR *in ovo* injection studies, increased PMM measurements coincided with an increase in muscle fiber density and decreased fiber CSA. The patterns of COXII, SIRT1, and

Cyclin D expression across all embryos and hatched chicks indicate NR catalyzed changes in expression between d 18 of incubation and hatch may be responsible for increased PPM fiber density and weight. Despite injecting high-yield broiler embryos with 100 times less NR, the drastic increase in muscle fiber density may have implications for future growth or meat quality characteristics.

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Conflict of interest statement

Mrs. Ria Jackson is a Pedigree Processing Plant Manager with Cobb-Vantress, Inc. She assisted with egg procurement and incubation assistance but did not assist in data interpretation.

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Table 4-1. Gene-specific primers utilized for real-time PCR analysis.

Gene	Forward primer (5' to 3')	Reverse primer (5′ to 3′)	T_m[1], °C	Amplicon length, bp	Efficiency	GeneBank accession
SIRT1	AAGACCTGCTCCCAGAAACG	ACAGCAAGGCGTGCATAGAT	61.4	247	93	NM_001004767.1
Cyclin D1	GCTACCTGCATGTTTGTGGC	GGGTCTGATGGAGTTGTCGG	63.4	92	93	NM_205381
D2	TGAGAACTGCCCTGCTCTTG	CAGAGGACCTAGCAGCCAAC	63.4	84	92	XM_015292118
D3	CAGAACTTGCTGAGCCAGGA	TCCGCATGTAGGGCTTGATC	62.4	87	97	NM_001008453.1
18S rRNA[2]	GAACGAGACTCTGGCATGCT	TCAATCTCGGGTGGCTGAAC	54.0	96	90	XR_003078044
COXII	GGCGCATCAGTAGACCTAGC	CAGCTGCTAGGACGGGTAAG	60.0	193	91	NC_053523.1

[1]Melting temperature.
[2]Normalizing gene. Expression was not affected by treatment.

Table 4-2. Body and pectoralis major morphometrics of embryonic day-15 embryos injected *in ovo* at day 10 of embryogenesis with 2.5 mM nicotinamide riboside into albumin or yolk sac.

Items	Treatment[1]				SEM	*P*-value		
	A0	A2.5	Y0	Y2.5		Dose	Location	Dose × Location
Body morphometrics								
Body weight, g	15.2	14.4	14.9	14.9	0.32	0.20	0.66	0.25
Crown to rump length, mm	62.7	61.4	62.8	63.0	0.57	0.30	0.14	0.18
Head width, mm	15.3	15.4	15.3	15.6	0.15	0.16	0.63	0.51
Head length, mm	19.8	19.9	20.4	20.1	0.20	0.63	0.05	0.32
Head circumference, mm	44.3	43.5	44.5	43.4	0.60	0.10	0.97	0.80
Chest circumference, mm	44.1	43.5	45.3	44.0	0.65	0.13	0.18	0.54
Chest width, mm	16.7	16.6	16.7	16.7	0.20	0.76	0.64	0.84
Chest length, mm	16.5	16.5	16.7	16.5	0.22	0.63	0.78	0.49
Pectoralis major morphometrics								
Weight, g	0.42	0.42	0.44	0.42	0.02	0.51	0.63	0.62
Length, mm	17.1	16.9	16.7	17.1	0.41	0.79	0.89	0.48
Width, mm	8.8	8.7	9.0	8.9	0.20	0.71	0.38	0.72
Thickness, mm	5.6	5.7	5.9	5.6	0.21	0.75	0.62	0.40
Heart weight, g	0.12	0.12	0.13	0.11	0.004	<0.01	0.66	0.55
Liver weight, g	0.24	0.24	0.23	0.23	0.01	0.79	0.64	0.98

[1]A0, 0 mM NR *in ovo* injected (100 µL 0.9% saline) into albumin; A2.5, 2.5 mM NR *in ovo* injected into albumin; Y0, 0 mM NR *in ovo* injected (100 µL 0.9% saline) into yolk sac; Y2.5, 2.5 mM NR *in ovo* injected into yolk sac.

Table 4-3. Body and pectoralis major morphometrics of embryonic day-18 embryos injected *in ovo* at day 10 of embryogenesis with 2.5 mM nicotinamide riboside into albumin or yolk sac.

Items	Treatment[1]				SEM	*P*-value		
	A0	A2.5	Y0	Y2.5		Dose	Location	Dose × Location
Body morphometrics								
Body weight, g	26.1	26.1	26.3	26.9	0.55	0.56	0.32	0.64
Crown to rump length, mm	75.5	77.8	76.5	78.8	1.56	0.13	0.50	0.98
Head width, mm	15.7	15.4	16.0	16.1	0.26	0.64	0.06	0.35
Head length, mm	20.7	21.3	20.7	21.2	0.33	0.10	0.95	0.99
Head circumference, mm	53.3	53.2	53.3	53.8	0.98	0.88	0.75	0.74
Chest circumference, mm	54.9	53.4	53.3	55.3	1.26	0.86	0.91	0.17
Chest width, mm	15.5	15.2	15.9	15.2	0.53	0.33	0.63	0.68
Chest length, mm	16.2	16.8	17.1	16.5	0.38	0.91	0.42	0.11
Pectoralis major morphometrics								
Weight, g	0.40	0.40	0.44	0.42	0.02	0.66	0.19	0.74
Length, mm	18.7	18.6	19.3	18.5	0.45	0.35	0.55	0.39
Width, mm	9.9	10.0	10.1	9.6	0.32	0.55	0.69	0.42
Thickness, mm	5.6	6.0	6.0	5.9	0.21	0.43	0.46	0.23
Heart weight, g	0.17	0.19	0.18	0.18	0.007	0.21	0.57	0.27
Liver weight, g	0.41	0.47	0.41	0.42	0.02	0.08	0.15	0.20

[1]A0, 0 mM NR *in ovo* injected (100 µL 0.9% saline) into albumin; A2.5, 2.5 mM NR *in ovo* injected into albumin; Y0, 0 mM NR *in ovo* injected (100 µL 0.9% saline) into yolk sac; Y2.5, 2.5 mM NR *in ovo* injected into yolk sac.

Table 4-4. Body and pectoralis major morphometrics of hatched chicks injected *in ovo* at day 10 of embryogenesis with 2.5 mM nicotinamide riboside into albumin or yolk sac.

Items	Treatment[1]				SEM	*P*-value		
	A0	A2.5	Y0	Y2.5		Dose	Location	Dose × Location
Body morphometrics								
Body weight, g	43.0	43.3	43.0	42.9	0.59	0.87	0.70	0.80
Crown to rump length, mm	90.3	88.5	87.5	87.5	0.88	0.24	0.02	0.25
Head width, mm	16.2	16.4	15.9	16.0	0.16	0.38	0.03	0.62
Head length, mm	23.3	23.1	23.6	23.7	0.35	0.84	0.20	0.58
Head circumference, mm	53.5^{a}	56.5^{b}	54.0^{a}	54.4^{a}	0.68	0.01	0.21	0.03
Chest circumference, mm	56.4	56.8	54.7	54.9	0.82	0.66	0.02	0.89
Chest width, mm	16.4	16.7	16.0	16.14	0.27	0.31	0.06	0.66
Chest length, mm	19.9^{a}	18.7^{b}	19.2^{a,b}	20.0^{a}	0.38	0.61	0.37	<0.01
Pectoralis major morphometrics								
Weight, g	0.26^{a}	0.22^{b}	0.26^{a}	0.29^{c}	0.01	0.94	<0.01	<0.01
Length, mm	18.4^{a,b,x}	17.9^{a,b}	18.2^{a}	19.4^{b,y}	0.47	0.42	0.16	0.04
Width, mm	8.3^{a,b,x}	7.7^{a,y}	8.2^{a,b,x,y}	8.8^{b,z}	0.28	0.95	0.03	0.02
Thickness, mm	3.7	3.3	3.6	3.5	0.12	0.02	0.36	0.14
Heart weight, g	0.35	0.32	0.29	0.28	0.01	<0.01	<0.01	0.45
Liver weight, g	0.81^{a}	0.72^{b}	0.73^{a,b}	0.78^{a,b}	0.04	0.53	0.77	0.04

[1]A0, 0 mM NR *in ovo* injected (100 µL 0.9% saline) into albumin; A2.5, 2.5 mM NR *in ovo* injected into albumin; Y0, 0 mM NR *in ovo* injected (100 µL 0.9% saline) into yolk sac; Y2.5, 2.5 mM NR *in ovo* injected into yolk sac.

^{a,b}Treatments with different superscripts within a row differ ($P < 0.05$).

^{x,y}Treatments with different superscripts within a row tend to differ ($P < 0.10$).

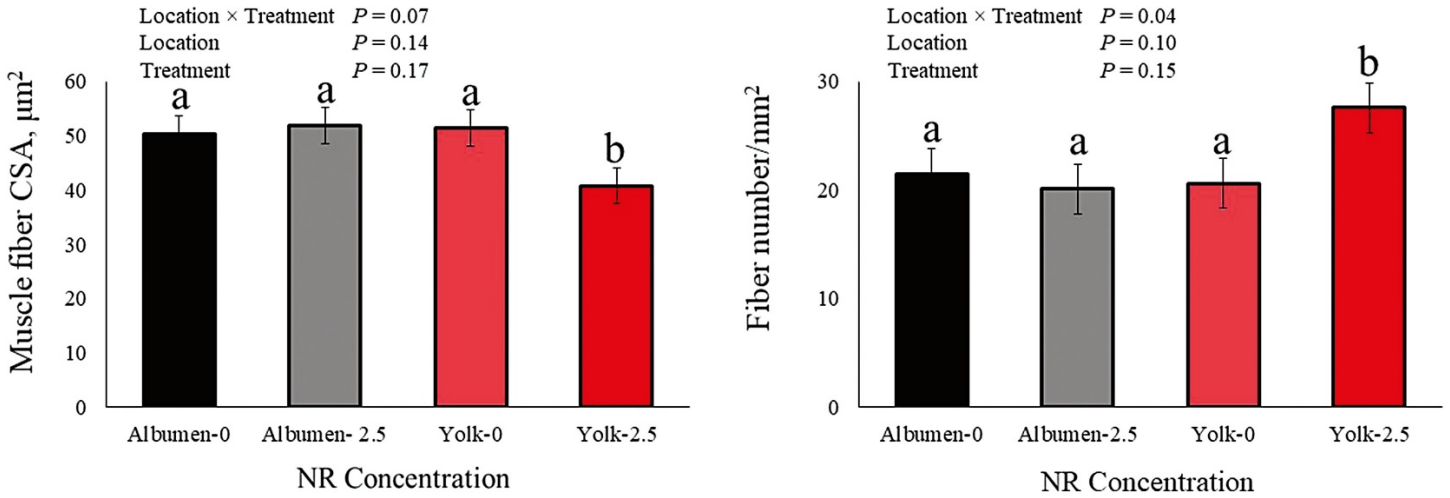

Figure 4-1. Muscle fiber cross-sectional area (**CSA**) and muscle fiber density from hatched chicks administered 0 or 2.5 mM nicotinamide riboside in the albumen or yolk sac during embryonic development. [a,b]Treatments with different superscripts within a panel differ ($P < 0.05$).

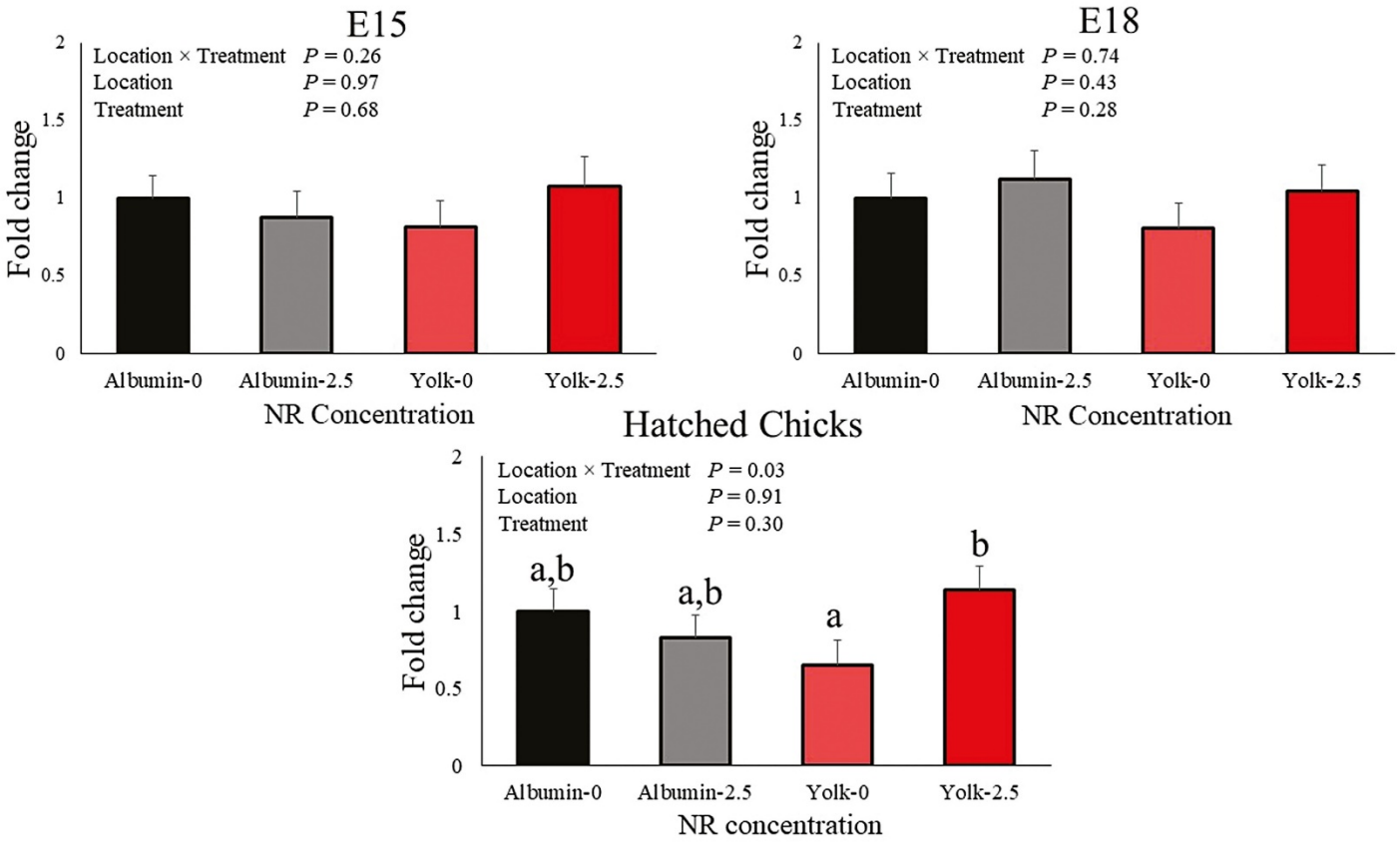

Figure 4-2. Pectoralis major mitochondrial COXII replication copy number from embryonic day-15 embryos, embryonic day-18 embryos and hatched chicks administered 0 or 2.5 mM nicotinamide riboside in the albumen or yolk sac during embryonic development. [a,b]Treatments with different superscripts within a gene differ ($P < 0.05$).

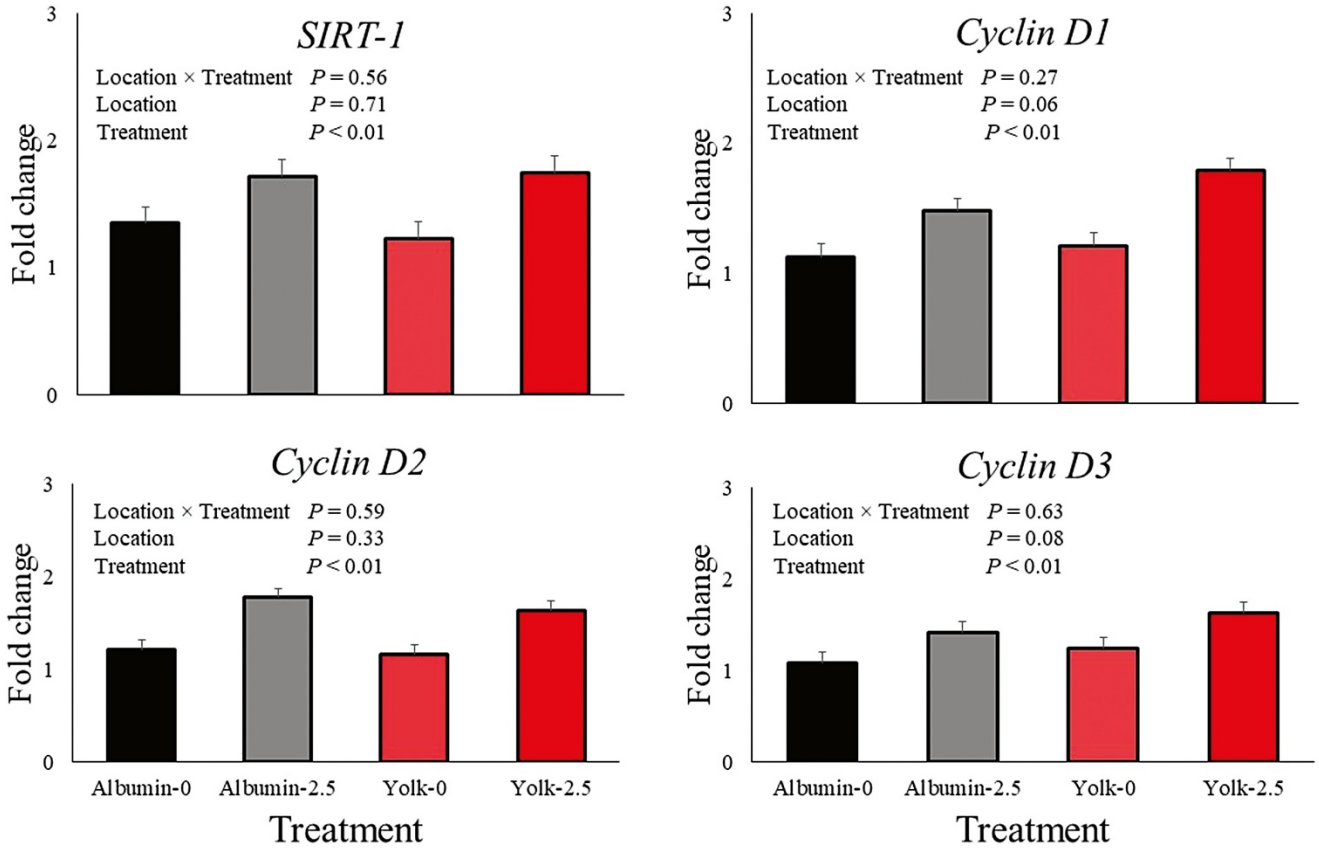

Figure 4-3. Pectoralis major Sirtuin-1 and Cyclin D mRNA expression from day-15 embryos administered 0 or 2.5 mM nicotinamide riboside in the albumen or yolk sac during embryonic development.

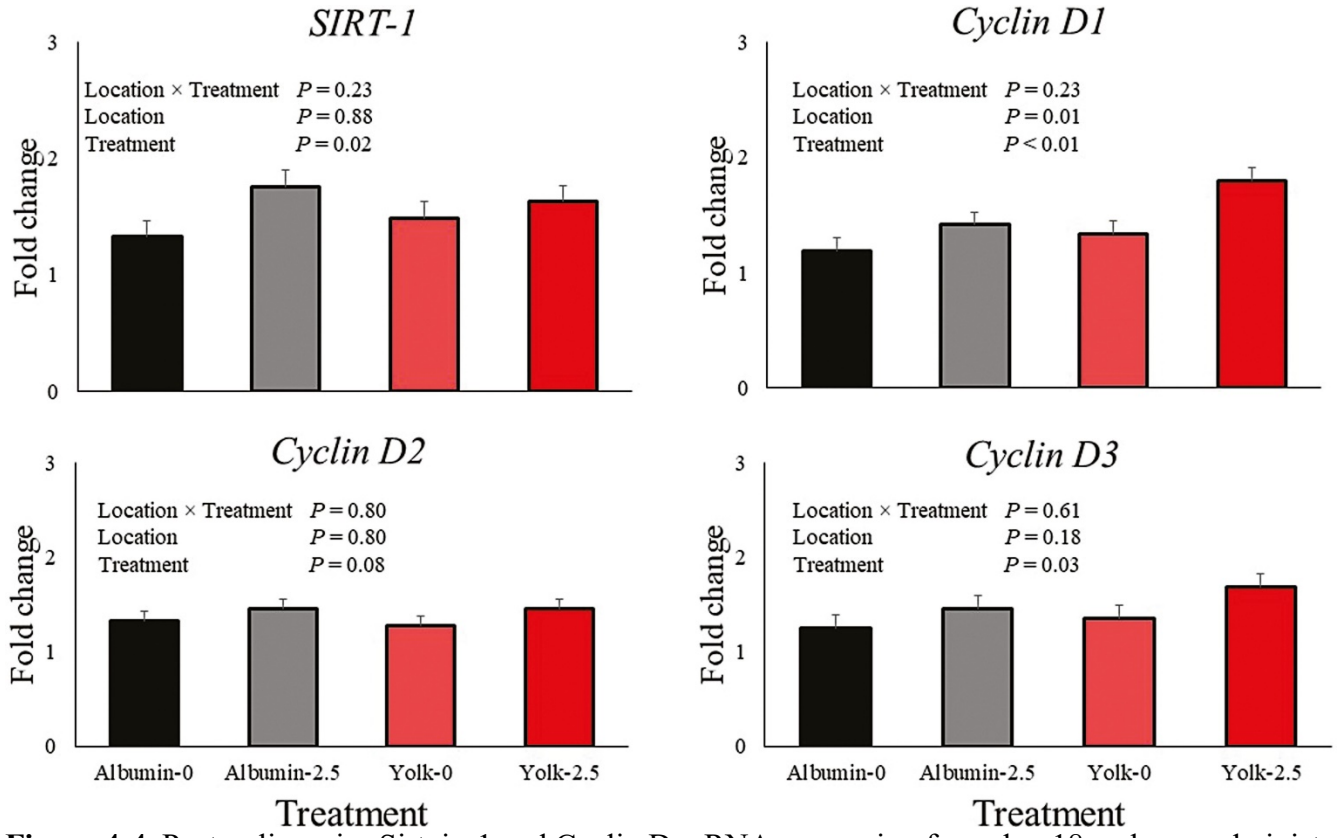

Figure 4-4. Pectoralis major Sirtuin-1 and Cyclin D mRNA expression from day-18 embryos administered 0 or 2.5 mM nicotinamide riboside in the albumen or yolk sac during embryonic development.

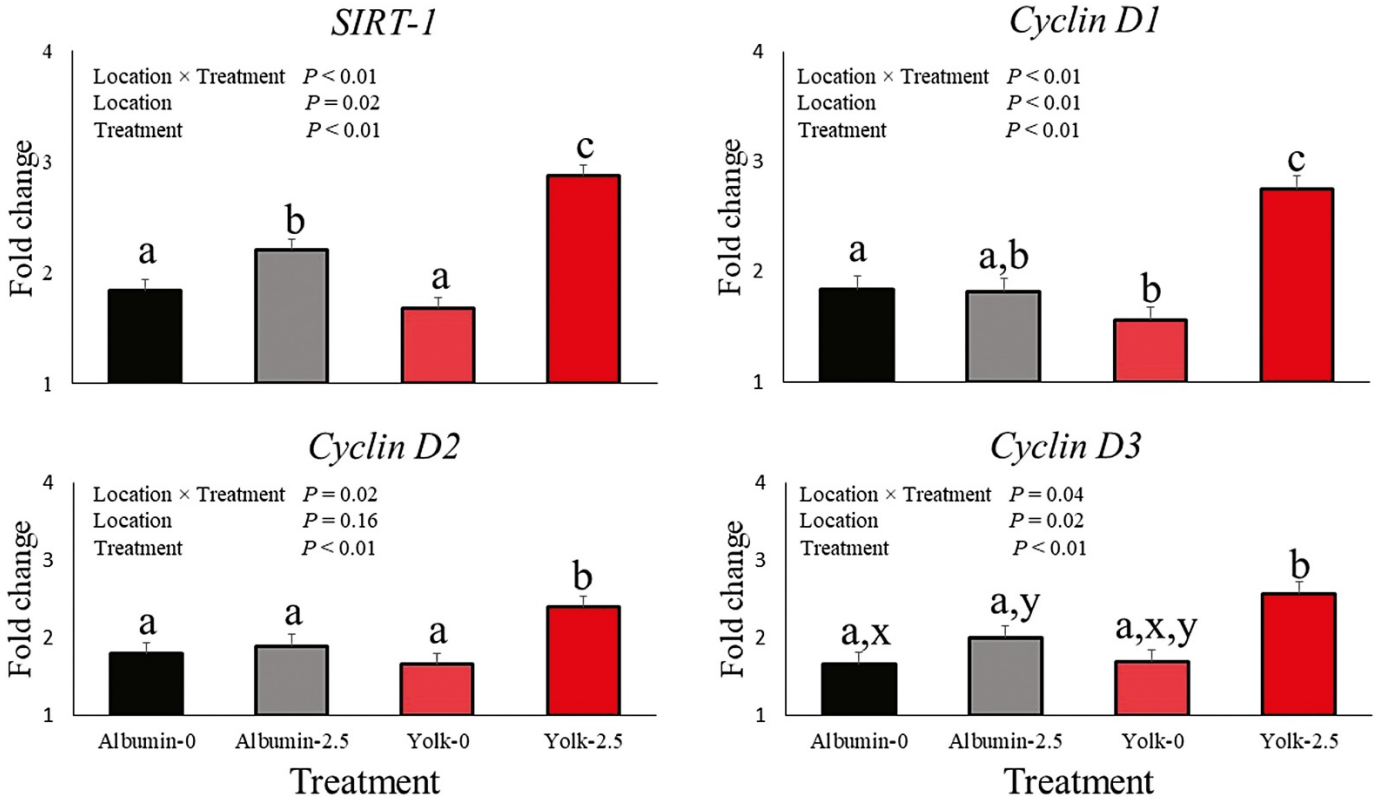

Figure 4-5. Pectoralis major Sirtuin-1 and Cyclin D mRNA expression from hatched chick embryos administered 0 or 2.5 mM nicotinamide riboside in the albumen or yolk sac during embryonic development. [a,b]Treatments with different superscripts within a row differ ($P < 0.05$). [x,y]Treatments with different superscripts within a row tend to differ ($P < 0.10$).

CHAPTER 5

THE EFFECT OF *IN OVO* FEEDING OF NICOTINAMIDE RIBOSIDE ON BROILER MYOGENESIS: A STRAIN STUDY

Xu, X., L. Motsinger, T. Haginouchi, C. Alena, A. N. Reyes, D. A. Gonzalez, S. S. Zedonek, J. M. Gonzalez. To be submitted to (Journal of Animal Science).

Abstract

Recent research showed that nicotinamide riboside (**NR**) *in ovo* injection into the yolk sac increased pectoralis major muscle weight. As such, the objective of this study was to determine the effects of NR injection of two different strains of Cobb broilers on body weight, *pectoralis major* muscle (**PMM**) morphometrics, and muscle fiber density. Fertilized Cobb 500 (N = 180) and 700 (N =180) broiler eggs were randomly allocated to one of two NR treatments (0- or 2.5-mM NR) within a 2 × 2 factorial design. Eggs were incubated for a total of 21 d. On d 10 of incubation, NR was injected into the egg yolk sac. On d 21, chicks hatched, and body weights were recorded. *Pectoralis major* muscle was then pulled out, and its morphometrics were measured. Cross-sectional area (**CSA**) and muscle fiber density were quantified. There was a main effect of dose, such that chicks harvested from Cobb 500 eggs injected with 2.5 mm NR had increased (P = 0.02) PM thickness. There were main effects of treatment for muscle fiber CSA and muscle fiber number/mm2 such that PM harvested from chicks from Cobb 500 eggs injected with 2.5 mm NR had increased (P < 0.01) muscle fiber CSA and decreased (P < 0.01) muscle fiber number/mm^2. These data indicate that 2.5 mM of NR injected into Cobb 500 eggs has the potential to enhance hypertrophy of the PMM through increasing the thickness and muscle fiber CSA of PMM, while decreasing muscle fiber number/mm^2.

Introduction

As the population continues to grow at an exponential rate, it is necessary to maximize efficiency of meat animal production. Over the past decades, the poultry industry has made immense progress to increase skeletal muscle growth of broiler chickens. Moreover, *in ovo* feeding was first reported in the 1980s for vaccination against Marek's disease (Sharma and

Burmester, 1982) and later developed as a nutritional technology to enhance poultry embryonic development and better prepare chicks for intensive development (Givisiez et al., 2020).

Recent research has found that injection of NR into the yolk sac increases the PMM weight and fiber density on commercial yield line broilers (Gonzalez and Jackson, 2020). This was later confirmed by Xu et al. (2021), who found that increasing nicotinamide riboside dose did not increase muscle weight, but increased muscle fiber density. These first two studies were conducted in a commercial yield broiler. Because high yield broilers possess a more significant genetic potential for ultimate muscle mass size, Alcocer et al. (2021) found chicks injected with 500 and 1,000 mM NR had greater PMM weights and depth on high yield Cobb 700 broilers. Moreover, Xu et al. (2022) further investigated effects of *in ovo* injection of high yield broilers with low dose nicotinamide riboside (**NR**) on broiler pectoralis major muscle (**PMM**) development, growth, and mRNA expression, proving *in ovo* injection of NR into egg yolk sac increased chick PMM weight and fiber density.

To the best of the authors' knowledge, no other studies have examined the effects of NR injection on two different broiler strains that differ in growth rate and PMM yield, and NR has been shown to increase NAD^+ availability (Bieganowski and Brenner, 2004) and improve proliferation of skeletal muscle cells in mice (Zhang et al., 2016). As such, we investigated the effects of *in ovo* NR injection into two different broiler strains (Cobb 500 or 700) on chick body weight, PMM morphometrics, and muscle fiber density.

Materials and Methods

The experimental procedures were reviewed and approved by the University of Georgia Institutional Animal Care and Use Committee.

Fertilized Cobb 500 (N = 180) and 700 (N = 180) broiler eggs (Cobb-Vantress Inc., Siloam Springs, AR) were randomly assigned to one of two NR treatments (0 or 2.5 mM NR) within a 2x2 factorial design. Eggs were incubated in one of three Sportsman 1502 incubators (GQF Manufacturing Company Inc., Savannah, GA) at 37 °C, 40 ± 4% relative humidity, and rotated hourly. Egg weights of four random eggs per incubator were recorded daily with daily weight loss ranging from 0.65% to 0.75% of total mass.

At incubation day 10, the methods of Gonzalez and Jackson (2020) were followed for treatment administration. Eggs were removed from the incubator and the injection site was cleaned with 70% ethanol. One-hundred microliters of 0.9% sterile saline containing the assigned concentration of NR was injected into the yolk sac using a 2.54-cm, 20-gauge injection needle inserted approximately 1 cm. Solutions of each treatment dose were made immediately before injection by premixing NR into warm 0.9% saline. Once injected, the final concentration of NR in the albumen or yolk sac was approximately 0.025 mmol. The injection site was covered with a 5 mm^2 portion of medical tape (Nexcare; 3M, Maplewood, MN) and placed back into the incubator under the conditions described above. At incubation day 18, eggs were removed from their trays, placed in hatching boxes, and the humidity of the incubator was increased to 60 ± 2%.

Harvest and Sample Collection

On day 21, chicks were euthanized by exposure to CO_2 and decapitation. Body weights were recorded. Both sides of the PMM were removed, and the left side was weighed, followed by length, width, and thickness measurement collection by calipers (Traceable Digital Calipers; Fisher Scientific, Pittsburg, PA). The left PMM was submerged in optimal cutting temperature tissue freezing medium compound, slowly frozen at −20 °C for 1 h, and stored at −80 °C until cryosectioning.

Immunohistochemistry and Histology

The methods of Gonzalez and Jackson (2020) were followed for immunohistochemistry analysis. On each slide, two cryosections (10 μm) were collected on a positively charged slide (Diamond White Glass; Globe Scientific Inc., Paramus, NJ). Cryosections were incubated in 5% horse serum and 0.2% TritonX-100 in phosphate-buffered saline (**PBS**) for 30 min to block all nonspecific binding sites. Cryosections were incubated for 45 min at room temperature with a solution consisting of blocking solution and 1:1,000 wheat germ agglutinin-Alexafluor 594 (cat no. W11262; ThermoFisher Scientific, Waltham, MA). After washing in PBS three times for 5 min, 5 mL of 9:1 glycerol in PBS was placed on each cryosection, and slides were coverslipped for imaging.

Cryosections were imaged at 200-fold magnification using an ECHO Revolve microscope (ECHO, San Diego, CA). Photomicrographs were collected, stored, and analyzed using ECHO Pro software (ECHO) operating on a iPad Pro (Apple, Cupertino, CA). Cross-sectional area (CSA) was determined for 1,000 fibers (minimum) as the area within the wheat

germ agglutinin border. To determine muscle fiber density, the area of the entire field of vision was divided by the average CSA of the fibers in the photomicrograph.

Statistics

Data were analyzed in a completely randomized design with a 2 × 2 factorial arrangement and utilizing embryo/chick as the experimental unit. Nicotinamide riboside dose, broiler strain, and their interaction served as fixed effects. All models were analyzed using the Mixed procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC). Pairwise comparisons between the least squares means of the factor level comparisons were computed using the PDIFF option of the LSMEANS statement. Statistical significance was determined at $P \leq 0.05$ and tendencies were between $0.05 < P < 0.10$.

Results

Whole Muscle and Fiber Characteristics

There were no Dose × Strain interactions for body weight and all PMM measures ($P > 0.08$). There were no Dose or Strain effects for all measures ($P > 0.08$), except NR injected eggs produced thicker ($P = 0.02$) PMM.

There was a Dose × Strain interaction ($P = 0.03$; Figure 5-1) for PMM fiber CSA, but not for muscle fiber density ($P = 0.16$; Figure 5-2). When NR was injected into Cobb 500 broiler eggs, chicks had larger muscle fiber CSA ($P < 0.01$); however, there was no effect ($P = 0.97$) when NR was injected into Cobb 700 eggs. Chicks from the 700 strain and chicks not injected with NR, had greater fiber densities ($P < 0.02$).

Discussion

As the population continues to grow at an exponential rate, it is necessary to maximize efficiency of meat animal production. Over the past few decades, the poultry industry has made immense progress to increase skeletal muscle growth of broiler chickens and is constantly exploring to improve skeletal muscle growth utilizing novel strategies. *In ovo* feeding of exogenous nutrients is one of such methods which researchers have examined for over 20 years.

Unlike mammalian animals, without maternal nutrition, broiler embryos are separated from the mother during embryonic development. Therefore, egg contents become the only nutrient resource. But *in ovo* feeding is capable of delivering nutrients into eggs during embryonic development. Effects of *in ovo* feeding of various exogenous nutrients including vitamins (Selim et al., 2012), carbohydrates (Zhai et al., 2011), amino acids (Coskun et al., 2014), and have been proved. Selim et al. (2012) *in ovo* injected vitamin E, resulting in duck egg higher hatchability percentage. The ducks hatched from eggs were characterized by higher body weight after the administration of vitamin E (10 mg) and vitamin C (3 mg) on the12th day of incubation at starting and growing period during post-hatch growth. since NR is a new type of supplement compound, only four studies tested effects of the product on broiler embryonic muscle development.

In the Cobb 500 broiler NR dose study, no changes in CSA in response to 250 mM NR were noted but the supplement increased muscle fiber density by 45% (Gonzalez and Jackson 2020). When NR was injected in Cobb 500 broiler egg yolk sac, muscle fiber CSA also was not affected but fiber density increased by nearly 75% compared with the control group (Xu et al., 2021). In the high yield broiler NR dosage study, Alcocer et al. (2021) found injecting increasing levels of NR did not affect E18 or hatched chick body weights but increased hatched chicks

PMM weight and depth. Moreover, most recent research found *in ovo* feeding of NR, into the yolk sac increased PMM weight of Cobb 700 high-yield broilers by (Xu et al., 2022). In this study, injecting NR at the reduced dose compared to the other studies, decreased fiber CSA by 21% and increased myofiber density by 35%. Although these high-yield broiler embryos were injected with a reduced NR dose, their increased density may indicate NR stimulated pathways associated with increased muscle fiber density during secondary myogenesis.

Therefore, based on current research literature, no other studies have examined the effects of NR injection on two different broiler strains that differ in growth rate and PMM yield. As such, we investigated the effects of *in ovo* NR injection into two broiler strains (Cobb 500 or 700) on chick body weight, PM morphometrics, and muscle fiber density. After *in ovo* feeding of 2.5 mM NR, the results showed no dose × strain interaction effect on chick body weight and PMM morphometrics. Different with previous studies, there were interaction on muscle fiber CSA. When NR was injected into Cobb 500 eggs, chicks had larger CSA by 26% compared with NR Cobb 700 chicks. Although there were no interaction on muscle fiber density, NR Cobb 700 chicks numerically had more than 23% of muscle fiber density than Cobb 500 boilers, which was consistent with the previous high yield broiler NR study. This result gives a chance to further investigate if NR stimulated specific signaling pathway during secondary myogenesis that led to improved muscle hypertrophy on Cobb 500 broilers but improved hyperplasia on Cobb 700 broilers.

In terms of the inconsistent results on all measures, issues with egg quality and increased rotten egg incidence may have influenced responses. Over 60% of eggs were pimpled, misshaped, or dirty and were not eligible for incubation. Additionally, rotten egg rate increased from 31% to 74% after day 10 NR injection, indicating early bacterial contamination was an

issue which was only worsened by the injection process. The presence of rotten eggs is often regarded as a major risk for the potential of contaminating nearby eggs and the whole incubator/hatching room when broken (Franzo et al., 2020). Tona (2005) stated elevated quality chicks at hatch is related to good quality eggs set in the incubator. Brand et al. (2016) reported floor eggs, which are often more contaminated, produced more rotten eggs, had greater mortality during incubation, which reduced hatchability by 18.2%. These eggs also produced chicks with reduced body weight at hatch and day 11 of the grow out period. Because so many issues were observed with egg quality and rotten egg presence, these results should not be taken as a typical NR response and the experiment should be conducted again.

Conclusions

In ovo feeding of NR increased Cobb 500 broiler muscle fiber CSA compared with Cobb 700 broilers, but there were no effect on muscle fiber density. Although Cobb 700 broiler CSA did not significantly decrease like previous studies, there is still the potential to improve Cobb 700 chick muscle fiber density, which is worth for further research to determine dose × strain interaction effects and validate the underlying mechanisms.

Acknowledgements

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Table 5-1. Average chick body weight and pectoralis major morphometrics of hatched Cobb 500 or 700 strain broiler chicks injected *in ovo* at day 10 of embryogenesis with 2.5 mM nicotinamide riboside.

Items	Treatment				SEM	*P* - value		
	500-0	500-2.5	700-0	700-2.5		Dose	Strain	Dose × Strain
N	23	22	23	15				
Body weight, g	43.12	45.60	43.75	43.12	1.03	0.72	0.31	0.08
Pectoralis major morphometrics								
Weight, g	0.17	0.18	0.17	0.17	0.01	0.64	0.43	0.23
Length, mm	16.52	16.46	17.03	17.13	0.39	0.96	0.08	0.82
Width, mm	6.39	6.70	6.23	6.22	0.28	0.55	0.19	0.51
Thickness, mm	3.54	3.82	3.77	4.00	0.12	0.02	0.08	0.74

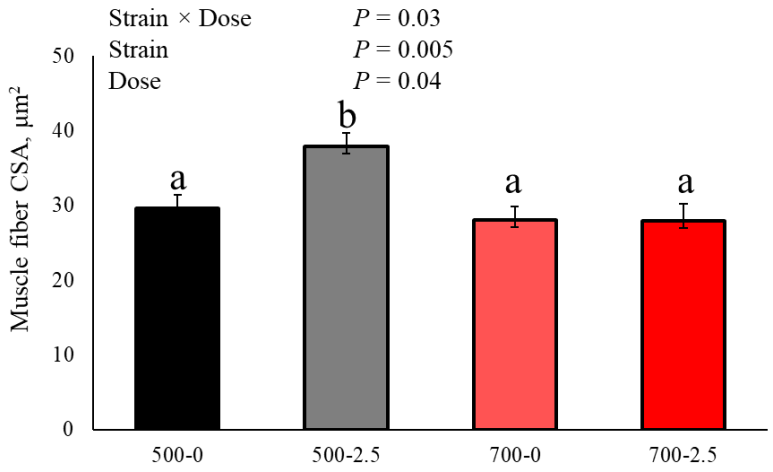

Figure 5-1. Average pectoralis major muscle fiber cross-sectional area (**CSA**) from hatched Cobb 500 or 700 strain broiler chicks injected *in ovo* at day 21 of embryogenesis with 2.5 mM nicotinamide riboside. Treatments with different superscripts within a row differ ($P < 0.05$).

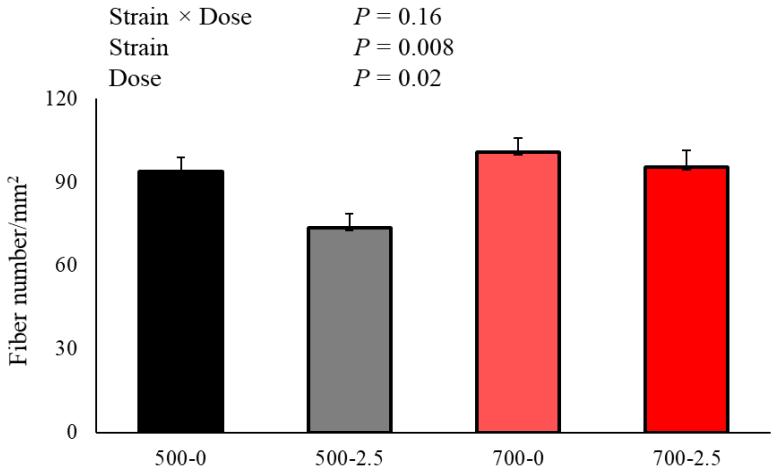

Figure 5-2. Average pectoralis major muscle fiber density from hatched Cobb 500 or 700 strain broiler chicks injected *in ovo* at day 21 of embryogenesis with 2.5 mM nicotinamide riboside.

CHAPTER 6

CONCLUSIONS

In ovo feeding of NR increased E19 embryos and hatched chicks PMM morphometrics. Similar to a previous NR *in ovo* feeding study, increased PMM measurements coincided with an increase in muscle fiber density and no effect on fiber CSA in hatched chicks. While increasing the dose of injected NR did not affect hatched chick PMM morphometrics, dose increased muscle fiber and satellite cell density. The lack of increased PMM weight, width, length, and depth because of NR administration indicated there is no advantage when injecting more than 250 mmol of NR; however, the drastic increase is muscle fiber density because of elevating the dose may have implications for future growth or meat quality characteristics. *In ovo* injection of NR into yolk sac increased PMM morphometrics of hatched chicks. Similar to previous NR *in ovo* injection studies, increased PMM measurements coincided with an increase in muscle fiber density and decreased fiber CSA. The patterns of COXII, SIRT1, and Cyclin D expression across all embryos and hatched chicks indicate NR catalyzed changes in expression between d 18 of incubation and hatch may be responsible for increased PPM fiber density and weight. Despite injecting high-yield broiler embryos with 100 times less NR, the drastic increase in muscle fiber density may have implications for future growth or meat quality characteristics. Overall, although Cobb 700 broiler CSA did not significantly decrease like previous studies, there is still the potential to improve Cobb 700 chick body weight and increase muscle fiber density, which is

worth for further research to determine dose × strain interaction effects and validate the underlying mechanisms.

To determine which Cobb broiler strain has more potential on improving skeletal muscle growth by *in ovo* feeding of NR, a mechanism and post-hatch broiler study can be conducted for further investigation. Through series of molecular biology experiments such as real-time PCR, western blot and immunoprecipitation, the NR-sirtuin-1 triggered regulatory pathway can be validated to figure out the if the mechanism leads to improved body and PMM growth on specific Cobb broiler strain. We also expect analyses to uncover if effects of *in ovo* feeding of NR can be extended to broiler postnatal muscle growth stage, showing higher body weight gain, lower feed convert ratio and greater breast muscle yield, during which NR triggered skeletal muscle growth associated signaling pathway will be validated. Taken together with the NR-sirtuin-1/cyclin D myogenic associated pathway, a whole muscle development and growth regulatory network will be built up. To accomplish this objective, massive animal harvest trials and in vitro cellular level tests needs to be conducted. If the positive effect on Cobb broilers can be testified, *in ovo* feeding of NR can be a potent and efficient strategy to enhance chicken meat productivity in poultry industry.

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