The effects of *in ovo* nicotinamide riboside dose on broiler myogenesis

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ABSTRACT The objective of this study was to determine the effects of *in ovo* injection of nicotinamide riboside (**NR**) on broiler embryonic myogenesis. Fertilized Cobb 500 broiler eggs ($N = 240$) were sorted by weight and within each strata, 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 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 ($P > 0.06$). 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 adenine dinucleotide, nicotinamide riboside, pectoralis major

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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.,

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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 Kansas State University 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 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-cm^2 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 CO_2 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 E19 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

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

	Nicotinamide riboside dose					
Item	0 mmol	250 mmol	500 mmol	1 mol	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.

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 mm^2 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 r^2 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 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).

Table 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.

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). Treatment did not affect all whole-body measurements ($P > 0.08$) for E19 embryos with the exception of head circumference ($P = 0.04$; Table 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 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 ($P > 0.06$).

Table 3. Body and pectoralis major morphometrics of embryonic day 15 embryos injected *in ovo* at day10 of embryogenesis with increasing doses of nicotinamide riboside.

	Nicotinamide riboside dose					
Item	0 mmol	250 mmol	500 mmol	1 mol	SEM	*P*-value
n	18	15	15	16		
Body measurements						
Weight, g	13.6	14.5	14.2	13.9	0.86	0.90
Dimensions, mm						
Crown-rump length	59.4	61.4	61.1	61.0	1.46	0.75
Head width	13.6	13.7	13.7	14.0	0.28	0.73
Head length	17.1	17.6	17.8	17.4	0.86	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	17.6	18.3	17.8	18.5	0.71	0.77
Width	13.7	14.7	15.2	14.9	0.54	0.22
Depth	6.9	7.2	7.1	6.6	0.25	0.35

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

Table 4. Body and pectoralis major morphometrics of embryonic day 19 embryos injected *in ovo* at day 10 of embryogenesis with increasing doses of nicotinamide riboside.

	Nicotinamide riboside dose					
Item	0 mmol	250 mmol	500 mmol	1 mol	SEM	*P*-value
n	17	18	18	19		
Body measurements						
Weight, g	37.9	37.7	37.9	38.1	1.42	1.00
Dimensions, mm						
Crown-rump length	85.0	84.3	85.9	86.3	0.62	0.08
Head width	17.0	15.4	15.3	15.4	0.76	0.34
Head length	17.8	17.7	17.4	17.6	0.17	0.49
Head circumference	53.0^{a}	53.2^{a,c}	54.8^{b,c}	55.0^{b}	0.06	0.04
Chest circumference	57.0	59.5	60.1	61.5	0.12	0.08
Chest length	18.3	18.6	18.5	18.3	0.31	0.89
Chest width	15.6	15.5	16.3	15.4	0.32	0.14
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	15.1^{a}	17.2^{b}	17.7^{b}	17.6^{b}	0.38	<0.01
Width	5.1	5.5	5.7	5.6	0.17	0.12
Depth	2.7	2.9	2.7	2.9	0.10	0.29

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

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 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 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). 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 ($P > 0.69$).

Table 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					
Item	0 mmol	250 mmol	500 mmol	1 mol	SEM	*P*-value
N	17	11	15	18		
Body measurements						
Weight, g	45.3	46.9	46.1	46.6	0.61	0.16
Dimensions, mm						
Crown-rump length	93.2	95.4	95.0	93.0	1.04	0.16
Head width	15.6	15.5	15.7	15.5	0.16	0.83
Head length	17.8^{a}	18.7^{b}	18.2^{a,b}	17.8^{a}	0.29	0.05
Head circumference	54.7	55.7	55.6	53.9	0.07	0.17
Chest circumference	60.9	61.2	62.5	61.4	0.08	0.42
Chest length	20.5	21.9	21.2	21.1	0.47	0.14
Chest width	15.9^{a}	17.1^{b}	16.6^{a,b}	16.1^{a}	0.33	0.05
Heart weight, g	0.29	0.31	0.31	0.32	0.010	0.08
Liver weight, g	0.88	0.84	0.90	0.96	0.051	0.28
Pectoralis major measurements						
Weight, g	0.17^{a}	0.23^{b}	0.23^{b}	0.22^{b}	0.009	<0.01
Dimensions, mm						
Length	17.6^{a}	19.9^{b}	20.3^{b}	19.1^{a,b}	0.69	<0.01
Width	4.6^{a}	5.5^{b}	5.6^{b}	5.4^{b}	0.23	<0.01
Depth	2.8^{a}	3.1^{a,b}	3.3^{b}	3.0^{a}	0.15	0.02

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

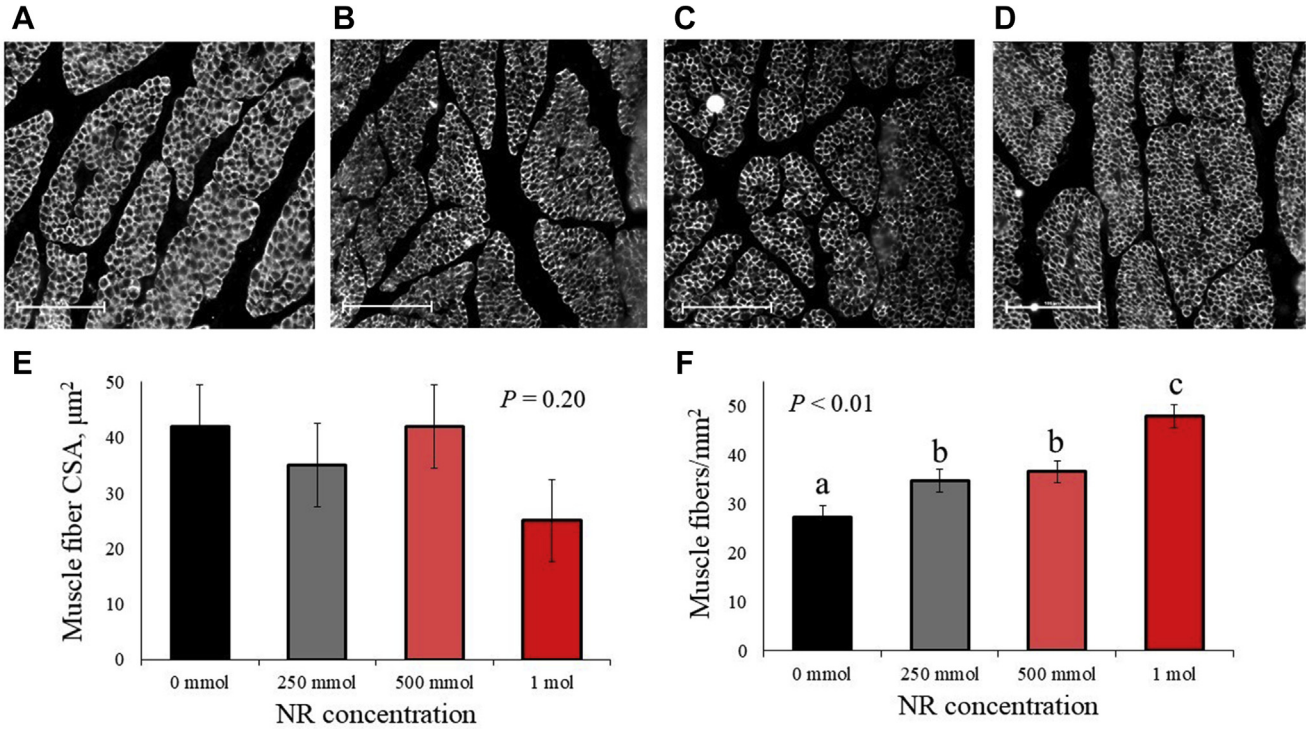

Figure 1. Representative 200-fold photomicrographs of pectoralis major cross-sections from (A) 0 mmol, (B) 250 mmol, (C) 500 mmol, and (D) 1,000 mmol chicks immunostained for dystrophin. (E) Muscle fiber cross-sectional area (CSA) and (F) muscle fiber density from hatched chicks administered 1 of 4 nicotinamide 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 ($P < 0.05$).

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 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

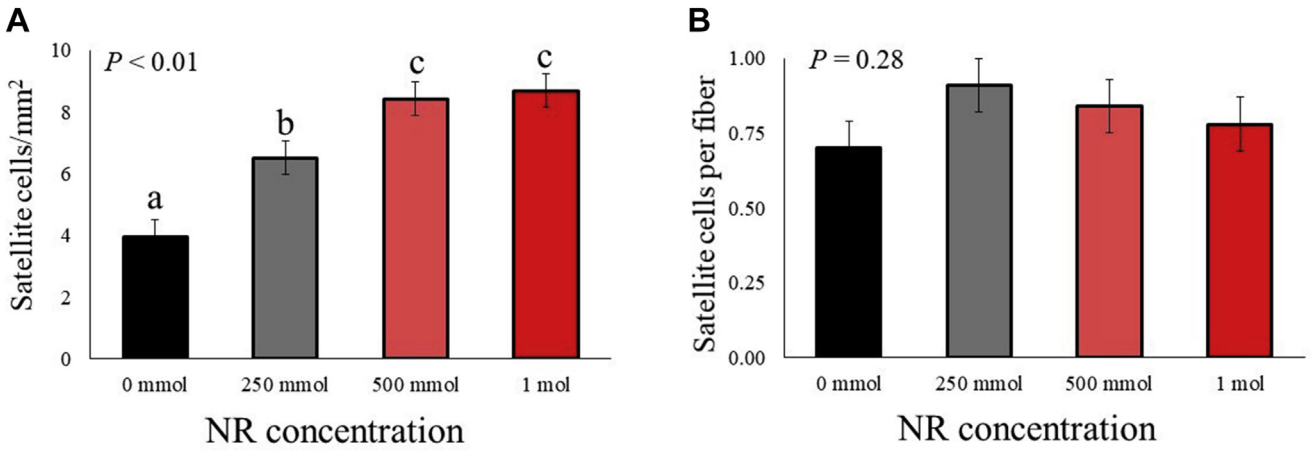

Figure 2. Pectoralis major (A) satellite cell density and (B) satellite cell number per fiber from hatched chicks administered 1 of 4 nicotinamide riboside (NR) doses during embryonic development. Fertilized Cobb 500 eggs were injected with the appropriate NR dose in 100 μL of 0.9% sterile saline at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

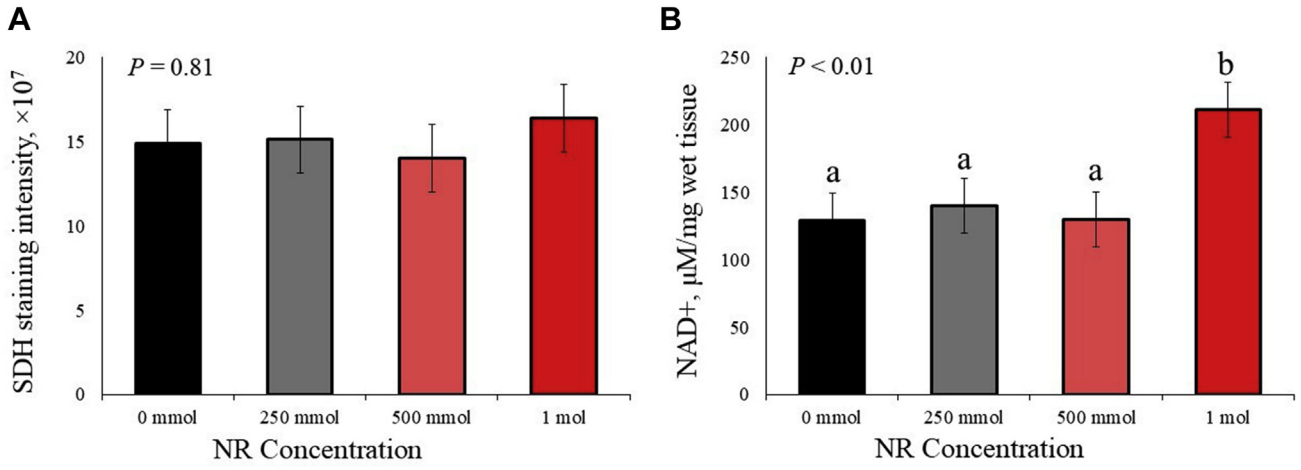

Figure 3. Pectoralis major (A) succinate dehydrogenase (SDH) staining intensity and (B) NAD + content from hatched chicks administered 1 of 4 nicotinamide riboside (NR) doses during embryonic development. Fertilized Cobb 500 eggs were injected with the appropriate NR dose in 100 µL of 0.9% sterile saline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

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 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

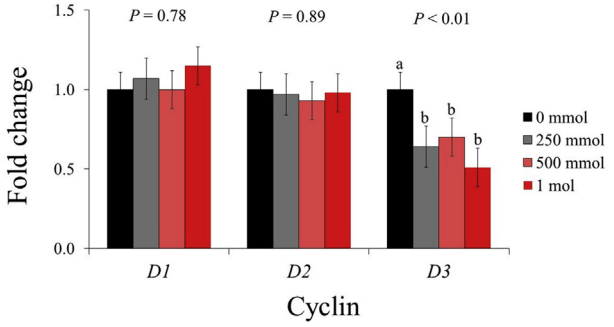

Figure 4. Biceps femoris *cyclin D* mRNA expression from hatched chicks administered 1 of 4 nicotinamide riboside (NR) doses during embryonic development. Fertilized Cobb 500 eggs were injected with the appropriate NR dose in 100 µL of 0.9% sterile saline at day 10 of incubation. [a,b,c]Treatments with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

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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DISCLOSURES

The authors have no conflict of interests to declare.

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