Effect of incubation temperature variation and genetic selection on broiler chicken growth performance, skeletal muscle growth characteristics, and meat yield

by

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Abstract

Throughout the last 5 decades, the broiler industry has focused its efforts toward higher meat yields and more efficient production, leading to the adoption of state-of-the-art technology for production and as a way of furthering our knowledge about physiological and nutritional factors that affect broiler production. Currently, topics such as incubation temperature and molecular aspects concerning muscle growth and their importance on growth performance are a target as a way to increase productivity. Therefore, one experiment was conducted to determine whether subtle changes in incubation temperature may impact growth performance and carcass yield when applied during crucial stages of myogenesis. Eggs from 3 different tray locations (Bottom, BOT; Middle, MID; Top, TOP) were exposed to the treatment temperature (± 0.3 ºC) from d 4 to 11 of incubation and were exposed to the same temperature (< ± 0.1 °C) from d 1 to 3 and 12 to 18 of incubation and before hatching. Subtle changes in incubation temperature during primary and secondary myogenesis did not affect broiler growth performance. However, broilers from BOT trays had heavier breast and tender weights than those from MID trays. These results indicate that variations in incubation temperature as little as 0.3 ºC among incubator tray locations during myogenesis, can significantly impact broiler meat yields. The second experiment attempted to elucidate the differences in myogenic stem cell populations (Myf-5+, MyoD+, Pax7+, Myf-5+:MyoD+, MyoD+:Pax7+, and Myf-5+:MyoD+:Pax7+) and muscle fiber characteristics between 2 muscle types and

sexes in 2 different genetic strains of chickens, Red Ranger (RR), and Ross 708 x Ross 708 (ROSS). Muscle cross sectional area was similar among the 2 strains and sexes. Myofiber distributions were different between muscle types and chicken strains. BF muscles had higher number of smaller myofibers compared to PM. Furthermore, RR chickens had higher number of smaller myofibers compared to fast-growing ROSS broilers. Differences in cross-sectional area, nuclear density, and populations of Myf-5+ and Pax7+ were mainly between muscle types. ROSS broilers exhibited a 27% larger population of Myf-5+:MyoD+:Pax7+ stem cells compared with RR. These results indicated that ROSS and RR chickens have similar myogenic stem cell populations on d 43 and that perhaps major differences in performance are due to changes in gene expression profile during early stages of post-hatch growth. Future research should evaluate changes in myogenic stem cell populations as a result of changes in incubation temperature during myogenesis and the subsequent effects on broiler growth performance. These data will allow for the development of practical management interventions during the incubation period that might positively impact broiler growth efficiency and meat yield.

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To the King of Kings and Lord of Lords, Jesus Christ, my Lord, for all things were made by Him; and without Him was not anything made that was made. For the Lord giveth wisdom: out of His mouth cometh knowledge and understanding. He goes first in everything, for all things are from Him and to Him. Therefore, all the praise and glory be to Him, for the strength and wisdom He gave me to overcome and finish this thesis and degree, and for what is coming next.

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I. Introduction

The broiler industry has undergone a myriad of structural and organizational changes in the last 8 decades. All of those changes have increased the production. Much of the success can be attributed to the improvement of production technologies and structural organization (National Chicken Council, 2018). Implementation of genetic improvements through traditional breeding has allowed the reduction of the rearing time needed to obtain the same weight as 8 decades ago and since the duration of the incubation period stays the same, more emphasis has been given to incubation parameters (Havenstein, et al., 2003). Incubation temperature is the most important physical factor during chicken embryogenesis and is often the most difficult to regulate (Gigli, et al., 2009). Current broiler industry has adopted multi-stage incubators due to their versatility, low energetic costs and ease handling. Multi-stage incubators provide the same air temperature to eggs from different ages using the principle of heat exchange between surfaces (French, 1997). However, it is well known that the heat production curve of a chicken embryo is not constant and, therefore, such changes in embryo heat production should be offset through artificial heat from the incubator (Lourens, et al., 2006). Even though numerous reports indicate that large variations in incubation temperature can impair embryonic development, little is known about the effect of subtle changes in incubation temperature during important windows of muscle development that have not yet been investigated. Therefore, the 3rd chapter of the thesis presented therein presents

the results of exposing chicken eggs to subtle changes of temperature during incubation on growth performance and carcass part weights and yields.

Besides the production technologies and management, one of the most recent fields of study in the broiler industry is related with myogenic stem cells populations, myogenic regulatory factors and their role in skeletal muscle development and growth (Xiao, et al., 2017). Implementation of genetic selection has allowed the improvement of feed efficiency and the general production parameters through selection of dominant genes that regulate such traits (Emmerson, 1997). Muscle is regulated by a myriad of myogenic regulatory factors that, together, play a critical role in the signaling and final myofibrillar protein synthesis response to environmental and nutritional factors during the grow-out period (Gerrard and Grant, 2006). There are many reports regarding the differences in growth performance between old and new strains, however, little is known about the differences between sexes and different broiler strains from a molecular and cellular standpoint. Understanding the mechanisms behind muscle growth and development may allow us to further understand and improve the current management practices that will in turn improve broiler performance and productivity in both short and long-term. Therefore, the 4th chapter of the thesis presented therein is dedicated to investigation of differences in muscle growth between 2 different strains of broiler chickens.

II. Literature Review

Introduction

In the United States, per capita broiler chicken consumption has increased above that of other meats since 1992 and currently it has tripled consumption since 1960 (National Chicken Council, 2013). Production of broiler meat accounts for about 70% of the total sales from the poultry industry and currently, chicken production continues to increase over time (USDA, 2017). This increase in global per capita consumption is associated with the low cost, high quality and health benefits attributed with chicken meat. To meet this demand, the poultry industry is always seeking new alternatives to increase its productivity. Currently, incubation is one of the major concerns when producing broilers since it is one of the first steps in raising a profitable flock (Decuypere et al., 2001). The reduction of the duration of the rearing period during broiler production has increased the percentage of time spent in the embryonic stage. Therefore, incubation parameters have gained more importance and currently, major efforts are directed towards improvement of such conditions (Hulet, 2007).

To meet the growing demand for chicken meat and to ensure the sustainability of food production systems, it is essential that we continue the increase of our understanding of how commercial management practices during key stages of incubation impact today's high-yielding broilers from a physiological standpoint.

Broiler Egg Incubation Parameters

Overview. Egg incubation is an integrated process by which adequate conditions of temperature, ventilation, humidity and rotational movements are provided to meet embryonic chick requirements and to optimize survival, chick quality, and growth performance (Boleli et al., 2016). The current incubation process has the same objective as 3,000 years ago, however, the effectiveness in handling incubation parameters has been improved because of the development of the broiler industry and improvement in physiological knowledge.

In the past 5 decades, substantial changes in the growth post-hatch and development of commercial broiler chickens have occurred. Havenstein et al. (2003) reported comparative production data using diets corresponding to each period, for each of the broiler production systems between 1957 and 2001. These data show that in 1957, a broiler carcass of 2 kg could be achieved at 57 d of age, whereas in 2001, broilers were able to achieve the same BW in about 43 days. The increases in BW gain were accomplished with no re-evaluations of the incubation parameters. Given that broiler chickens post-hatch period duration has been shortened, embryonic growth now accounts for an increasing percentage of the total development time of the bird; now it represents 35 to 45% of the total lifetime of a broiler chicken, whereas 4 decades ago, incubation period (21 days) represented only 20 to 30%. Therefore, incubation conditions have gained more importance in broiler production as a way to guarantee good pre and post hatch growth and development (Hulet, 2007). Although incubation parameters are discussed individually herein, it is important to underscore that they are strongly related and play an integral and critical role during embryonic growth and development.

Incubation Temperature. One of the most influential physical factors when incubating eggs is incubation temperature, which influences embryonic organ formation, metabolic regulation of the developing embryo, and general cellular activities (Molenaar et al., 2011; Maatjens et al., 2016). Within the incubator, heat transfer is the result of temperature differentials between surfaces. During incubation of eggs, exchanges of heat between eggs and the incubator environment are driven by conduction, convection, radiation, and evaporation mechanisms that are directly associated with egg characteristics. Embryo temperature is a medley of interactive physical factors regulated by incubator temperature, ability of air to flow among eggs, and internal heat production. All these factors can, as shown by French (1997), be summarized in the following equation,

$$Tei = Ti + \frac{(He - Hw)}{K}$$

Where the Tei is the internal egg temperature, Ti is the temperature within the incubator, He is the embryo internal heat production, Hw is the heat loss as vapored water, and K is the thermal conductance or the ability of the egg to allow thermal flow from its external surroundings within the incubator.

Convection is the main way by which eggs exchange heat with the surrounding air; this happens by generating convection currents that exchange warmer and cooler air. These are crucial to promote conductive heat loss, lowering the value of Hw . Heat exchanges between eggs and their environment are necessary for normal embryonic development and the level of these exchanges is concomitant to the embryo physical characteristics and embryonic metabolic rate (Baarendse et al., 2007).

An embryo metabolic rate increases thorough incubation (Masters and Vleck, 1980); newer eggs have low metabolic rate and, therefore, generate low internal heat (He). Conversely, as the embryo metabolic functions are increased, metabolic heat increases (He). Since the embryo lacks the ability to generate or dissipate heat (Hw), its requirements must be covered with external sources (Ti) and must be maintained within a limited range (Romijn and Lojhorst, 1960; Spotila et al., 1981).

In broiler eggs, most of the water is deposited in the yolk and albumen constituting about 70% of the egg's weight. Temperature plays a crucial role in the exchange of water vapor from different pressure gradients, this process is known as evaporative heat loss (French, 1997). Whilst most of this water is absorbed by the embryo, a low volume of the water is evaporated as water heat loss (Ar and Rahn, 1980), forming the air chamber which is imperative for gas exchange.

Relative Humidity. Even though the egg seems to be completely isolated, the egg shell allows exchanges mainly driven by diffusion mechanisms (Boleli et al., 2016). Egg water loss during incubation, which is controlled by relative humidity, is crucial to maintain the same relative water content in the egg thorough all the cellular processes (Ar and Rahn, 1980). It also allows the formation of the air chamber which occupies about 15% of egg volume and is the place for gas exchange (Rahn et al., 1977). Air cell allows lung ventilation after pipping and an auspicious hatching. Rates of egg water loss are intrinsically correlated with the vapor pressure gradients driven by relative humidity, where large variations to either direction, result in abnormal embryo internal water loss and impairing of metabolic functions, leading to embryonic mortality (Davis et al., 1987; van der Pol et al., 2013).

Within the incubator, relative humidity gradients are controlled by regulation of air flow and the addition of water vapor from a reservoir. Therefore, seasonal external environmental relative humidity heavily impacts the internal incubator environment. Such humidity gradients change during incubation period and must be met to achieve optimal embryonic development and growth.

Ventilation. As any other living organism, an egg requires gas exchange during incubation to carry out cellular metabolic functions and important developmental cellular activities that can be stimulated or impaired based on oxygen: carbon dioxide concentrations (Metcalfe et al., 1981). During incubation, dwindling air movement among eggs results in lower heat loss by conduction and convection, impairing embryo growth and development. Ventilation is a crucial process that allows enough oxygen to come in and remove the carbon dioxide and vapored water, influencing oxygen concentrations and embryonic metabolism (Tona et al., 2005). Molenaar et al. (2011) showed that reduced concentrations of oxygen can reduce embryonic internal activity, leading to malnutrition and mortality. Adequate oxygen concentrations provided through ventilation, guarantees utilization of glucose precursors as a main source of embryonic energy to carry out developmental pathways appropriately and assure an adequate embryonic growth. Furthermore, entry of fresh air facilitates the cooling process since most of the incubators do not have enough cooling capacity to be completely independent of air ventilation.

Turning. Rotational movements are required to guarantee an adequate development and absorption of extraembryonic membranes (Eycleshymer, 1907). Turning should be provided during the whole incubation process to achieve the maximum

hatchability (New, 1957). Tazawa (1980) showed that rotational movements provided to fertile eggs during incubation have a very critical role in oxygen-carbon dioxide metabolism, and the lack of egg turning leads to lower oxygen pressure impairing embryonic cellular activity which leads to poor embryonic growth. Egg turning during incubation assures adequate embryonic metabolism and is crucial for achieving a higher utilization of albumen, increasing chick weight at hatch by preventing adhesion of embryonic yolk and allantois that would result in malnutrition and early or late embryonic mortality (Eycleshymer, 1907; Tona et al., 2005). Elibol and Brake (2006) showed that an increase in daily rotational movements (4 hourly; 96 daily) thorough incubation and exposure to acute angle of turning (45 degrees) had the lowest incidence of mispositioned embryos compared with eggs exposed to 35 degrees and therefore, resulting in less incidence of possible malformations.

Practices Regarding Incubation

History of multi-stage and single-stage incubators. Incubation practices have been developed and improved for over 3,000 years, starting with the early Egyptians, who established the principles for what nowadays we call single-stage incubators. Ancient single-stage incubators were made up of mud bricks, with the ability to retain heat; they had a central passage that connected little entrances for each incubation room. The burning of straw at the top of the incubator was the source of heat for growing embryos, ventilation was provided by small vents in the roof of the chambers and as expected, egg turning was provided manually twice a day and relative humidity was controlled by temperature and ventilation. Strength of the fire was used to regulate the temperature, and size of vents helped to regulate ventilation. In these ancient incubators, all eggs were in and out at the same time. Chinese egg producers started a novel

incubation technology 200 years after the introduction of the Egyptians primitive incubators by utilizing the concept of heat transfer. They realized that older eggs were able to produce heat, whereas, newer eggs needed transfusion of heat, therefore, they created a new incubation method by mixing eggs from different ages, where older eggs helped to heat up newer eggs. This was the beginning of multi-stage incubators (Paniago, 2005).

The 16th and 17th centuries were chock-full of new ideas to offset the vast range of incubation physical conditions and guarantee good embryonic development, seeking constant incubator conditions even under extreme external fluctuating conditions. Different types of heat sources were tested such as, charcoal, hot water, etc.; however, it was not until the 19th century that technological advancements such as modern thermometers and usage of electricity as a source of heat gave rise to the modern incubator. In the 20th century, implementation of forced-draught incubators gave the opportunity for massive chick production, whereas, in the 21st century the increase of the poultry industry has led to the construction of the most state-of-the-art incubators with automatic sensors to detect fluctuations in incubation parameters. Throughout all this time, the broiler industry has been alternating the usage of single- and multi-stage incubators basing their decisions on their objectives (Hubbard 2017).

In the last century, single-stage incubators had the hegemony in the industry as a retrofit technology, where old-fashioned concepts about incubation were melded with actual technology and better knowledge of physiology to generate such incubators. However, single-stage incubators were costly since they had to provide heat during the first days of incubation and dissipate embryonic heat at the end of incubation. At this

point, the broiler industry decided to adopt multi-stage incubators where older eggs helped to provide heat to newer eggs (Hubbard, 2017). Over the last decade, the commercial broiler industry has used both incubation systems, basing their decisions on incubator management versatility (Boleli, 2016).

Current Commercial Broiler Chicken Industry Incubation Temperature Practices

Current commercial incubation practices strive to provide a homogeneous temperature along the different incubation stages since too high or too low temperatures impair embryonic metabolism, development, growth, hatchability and further post-hatch characteristics (Decuypere and Michels, 1992; Willemsen et al., 2010). The modern broiler industry continually seeks for improvements in their productivity, and as expected, the idea of better chick quality, hatchability rates and livability, obtained through good temperature management, which is concomitant with efficiency, has been one of the targets in modern incubator complexes (Boleli, 2016). In the last 50 years, the number of hatcheries has decreased in United States. Broiler industry has shifted from large number of small hatcheries to a few larger-sized. Moreover, incubator egg capacity has been increased due to adoption of multi-loading systems (NASS, 2005) which are primarily known by their versatility and lower operation (energetic) expenses. In these systems, eggs from different days of age, are placed in the same incubator where heat transfers between exothermic eggs (older) and endothermic eggs (newer eggs) take place (Hubbard, 2017). This heat exchange is responsible for increases in temperature variation depending on the profile and management provided. At this point, utilization of a common temperature is a potentially good idea, however, most of the time, when a good uniformity of temperature thorough incubation cannot be accomplished, obtaining and

adequate average does not seem to guarantee the maximization of embryonic growth potential (Meijerhof, 2009). Even though incubation machinery is set to provide adequate conditions, in one of their research studies, Gigli et al. (2009) showed that temperature and relative humidity values, within commercial multi-stage incubators, were lower than those recommended for satisfactory embryogenesis, concluding that multi-stage setter environmental conditions fluctuate from small, until considerable changes based on management; such changes impact embryonic growth and development. Boerjan (2006), claims that even though multi-stage incubators are known by their simplicity, incubation parameters (temperature, humidity, ventilation) are set at a fixed point and cannot create optimum conditions for every egg set, explaining that hatchability and chick quality will, therefore, be impaired.

In single-stage incubation, in contrast to multi-stage, broiler chicken eggs from the same stage of embryonic development are placed and taken out at the same time in the commonly known as “all in, all out”. Constant concerns about effect of temperature on physiological parameters during incubation are leading to a re-adoption of retrofit models of the single-stage incubators which have shown to provide constant egg shell temperatures thorough incubation (Hubbard, 2017). Single-stage incubation also favors disinfection procedures at the end of the incubation process and evades opening of the incubator until egg transfer to the hatchery, maintaining a consistent temperature, which allows provision of adequate environmental conditions that bolster embryonic growth and development (Fernandes et al., 2017).

Skeletal Muscle Development and Growth

Embryonic primary and secondary myogenesis. During embryogenesis, paraxial mesoderm matures and arranges in blocks of somatic cells in both sides of the

embryonic notochord. These somatic cells evolve from an epithelial sphere surrounding the mesenchymal chord and compartmentalize into a dorsal and a ventral section (Pourquie et al., 1995). The ventral section forms the multipotent mesenchymal cells while the dorsal section forms the myotome. At this point of myogenesis, uncommitted cells from both sites require the induction of myogenic regulatory factors (**MRF**) to change their fate and enter the muscle lineage.

The major myogenic regulatory factors are Myogenic regulatory factor 5 (**Myf-5**), Myogenic regulatory factor 1 (**MyoD**), Myogenin, and Myogenic regulatory factor 4 (**MRF4**). In myogenic cells, Pax3 and Pax7 function as important transcription factors, inducing the upregulation of MyoD and Myf-5. MyoD binds to sites on the DNA of other muscle-specific genes, and in conjunction with Myf-5, can regulate myoblast proliferation in tissues (Gilbert, 2000). Undifferentiated cells are primarily induced by paracrine and environmental factors to synthesize MyoD and Myf-5 and enter the muscle lineage. Once in the muscle lineage, these cells migrate from the paraxial somatic mesoderm to sites of muscle formation to form the myoblasts (Qin et al., 2013).

Myoblasts are mononucleated, immature muscle cells that are replication competent. After some replications, they reach a threshold from which they leave the cell cycle, align together and fuse to form post-mitotic multinucleated primary myotubes (Boudjelida and Muntz, 1987). The process of developing primary myofibers seems to be autonomous, where external input does not seem to have an important impact on development of primary myotubes. Primary myotubes are the first muscle fiber to develop and functions as a scaffold around which other myoblasts align and begin to fuse, forming the secondary myotubes (Gerrard and Grant, 2006). Primary myotubes are

formed by the fusion of embryonic myoblasts whereas, formation of secondary myotubes is achieved by the fusion of fetal myoblasts. Secondary muscle fiber number is dependent on the number of primary myotubes and is more susceptible to environmental changes. Most of the myoblasts will fuse to form primary and secondary myotubes however, there is a small population that does not fuse. These cells are called muscle satellite cells (SC) or muscle stem cells, which retain mitotic ability, can proliferate, fuse and are mainly regulated by stress and injury (Buckingham et al., 2003).

Myogenin and MRF4 regulate cellular fates by changing the gene expression profile of myogenic cells and allowing cell maturation (Fujisawa, 1992). During maturation of myoblasts, cell membranes fuse together forming immature muscle fibers in a nuclear desultory organization. At this point of myogenesis, MRF up-regulate muscle-specific genes coding for myosin, actin, titin and other important proteins required for muscle fiber maturation. Insulin-like growth factor (**IGF**) play an important role by promoting hyperplasia of newly-committed muscle cells and induction of early (MyoD and Myf-5) and late MRF (Myogenin and MRF4) expression, required for growth and development. All these events eventually, allow the formation of mature multinucleated muscle cells containing contractile units and external proteins, critical for mature myofiber activities (Fiorotto, 2012).

Post-hatch Skeletal Muscle Growth. During postnatal growth, the increment of skeletal muscle mass is mainly by increase in muscle fiber size. In the muscle, multinucleated muscle fibers are the cellular unit. The expansion in fiber requires additional nuclei for transcription of DNA and synthesis of proteins. Each nucleus has a nuclear domain or effective zone to which it provides DNA for protein synthesis

however, mature muscle cells are post-mitotic cells unable to replicate (Gerrard and Grant, 2006). Satellite cells are mononucleated promoter cells lying between the muscle fiber sarcolemma and the basement membrane characterized by their expression of Pax7 (Lepper et al., 2011). These cells function as myogenic stem cells that fuse with adjacent cells and donate nuclei. Allen et al. (1979) reported that most of the nuclei in mature muscle cells originate from SC. Fusion of SC increases the total DNA template within the muscle fiber, that is available for transcription of myofibrillar genes such as myosin, actin, troponin, titin and desmin. Thus, SC population increase the potential for myofibrillar protein synthesis (Allen et al., 1979; Yablonka-Reuveni, 2011). These cells are characterized by their little proportion of cytoplasm compared to nucleus (Mauro, 1961). These cells are quiescent in the basal lamina and are very sensitive to environmental cues that activate them to start cell cycle and differentiate into nuclei donors (Gerrard and Grant, 2006).

Temperature during incubation can impact satellite cell activity via hormonal changes that may impair or improve muscle development depending on the direction and intensity of the change (Morita et al., 2016; Xiao et al., 2017), thus, adequate temperature management should be provided to guarantee maximum potential of muscle growth post-hatch.

Effect of Temperature Variations During Different Stages of Embryonic Development

Incubation temperature variation is a multifactorial parameter that can be explained in terms of frequency, duration and amplitude of variations. These variations determine the magnitude, direction of the change and the possible impact on embryonic development (Willemsen et al., 2011). Within the incubator, provision of optimal

incubation temperature is a challenge that when not handled well, can impact productivity by impairing pre and post-hatch characteristics (Lourens et al., 2006). Barott (1937) demonstrated that the highest hatchability and chick quality are obtained with incubation temperature of 37.8 ºC, where deviations of ± 0.3 ºC from that point can impair normal embryonic metabolism.

High Incubation Temperature. Incubation temperature above the required, has different levels of impact during various stages of embryogenesis. It is well known to impair chick embryo developmental trajectory, at any stage, by increasing metabolic rate (Maatjens et al., 2016). When these changes are substantial, can result in problems on embryonic nutrition, hatchability, chick quality and changes in metabolic regulation (Willemsen et al., 2010; Molenaar et al., 2011). Von Blumroder and Tonhardt (2002), showed that high temperature increases the hypothalamus-adrenal axis by increasing the *in-ovo* production of catecholamines and corticosterone. High temperatures during late embryogenesis diminished yolk-free weight, and nutrient utilization by shortening the incubation period until more than 10 hours, causing premature hatching and less time to carry out important metabolic activities (Molenaar et al., 2010). Willemsen et al. (2011) showed that metabolism of respiration, carbohydrates and lipids is shifted by intermittent changes in temperature during the last week of incubation, that somehow, are buffered by the embryo once exposed to normal conditions. Yet, when those changes are constant during incubation, they can undermine the normal embryonic metabolism (Molenaar et al., 2010).

Low Incubation Temperature. While eggs exposed to high temperatures show an accelerated embryonic growth, the opposite is true for eggs exposed to low

temperatures. Willemsen et al. (2010) showed that eggs incubated at 3 °C below the required during the late embryogenesis, had a lower metabolism leading to retardation of the hatching process. However, their development and growth were similar to those of the control group. Incubation temperature manipulations impact hormonal and myogenic activity and can be used as a tool to induce epigenetic adaptation. Morita et al. (2016) showed that eggs exposed to low temperatures (36 °C) had lower plasma T3 and growth hormone, where eggs exposed to high temperatures (39 °C) had lower plasma T3 compared to the control treatment, claiming that these changes have long-lasting effects post-hatch that can impact development. However, Willemsen et al. (2010) showed that even though the hatching process of eggs exposed to lower incubation temperature is delayed, their final efficiency in the grow out period seems to be strikingly similar to those exposed to recommended temperatures.

Impact of Genetic Selection on Modern Commercial Birds, Heritage and Current Breeds

Overview. The current broiler industry started in the early 1800s with the presence of 4 main breeds: White and Brown Leghorn, Rhode Island Red, White Rock, and Cornish (Derry, 1945). The initial commercial poultry industry was focused on egg production, whereas meat was seen as a byproduct of this activity. Since identification between sexes was impossible before 7 weeks after hatching, males were reared for meat production as a byproduct. In 1940, the introduction of vent sexing of chicks allowed the ramification of dual-purpose broiler industry into broilers and laying hens with their own merits (Griffin and Goddard, 1994). Broilers, for meat production mainly, were derived from White Rock and Cornish whereas, hens for egg production were derived from

Leghorn. The modern broiler genetic selection is done using pure lines, where the major objective is to increase growth rate and meat yield (Muir and Aggrey, 2004).

Growth Performance and Carcass Characteristics. From the beginning of genetic selection in 1950, until the current newest lines, broiler growth has increased by over 400% with a 50% improvements in feed efficiency (Zuidhof et al., 2014). At the beginning, genetic selection was focused on rate of growth only, resulting in heavier carcasses with little effect on the developmental trajectory. Selected strains had an increase of all carcass parts including fat, resulting in poor feed conversion efficiency (Griffin and Goddard, 1994). However, current genetic selection has branched towards feed efficiency and leaner carcasses. Current broilers present leaner and heavier carcasses, resulting in more feed-efficient strains (Havenstein et al. 1994). In the last 3 decades, more emphasis towards improving carcass proportions and weights, specifically in *Pectoralis Major* (breast), has been applied on breeding programs. Current broilers devote approximately 20% of their body weight to breast muscles, which is approximately 10% greater than old strains from 1955 (Collins et al., 2014). Heritability of carcass part composition is independent, which facilitates genetic improvement across different traits in a balanced breeding program (Bailey et al., 2015).

Physiological Changes. Much attention has been given to genetic selection and endocrine control of growth in the last decades. However, changes in myogenic stem cells derived from modern broiler chickens indicates that selection for rapid growth may be strongly altered by mechanisms intrinsic to the cell instead of a simple effect of circulating hormone concentrations (Griffin and Goddard, 1994). Intrinsic cellular protein metabolism has shown improvement, leading to greater muscle development and lower

protein turnover when compared based on fractional rates (Maruyama, et al., 1978). Modern broiler myogenic stem cell populations and muscular cellular activity have increased *in ovo,* bolstering subsequent post-hatch skeletal muscle growth when compared with layer lines (Al-Musawi et al., 2011). Indeed, broiler chickens have developed muscle fibers with greater size and their breast muscle can growth 8 times as fast as breast muscle in layers (Hassanpour et al., 2010). Xiao et al. (2017) demonstrated that newer strains have improved their paracrine and endocrine regulation of growth and myogenic regulatory factors, allowing changes in gene expression profile that increase efficiency in synthesis of proteins in the muscle. It has been said that muscle growth in current broiler chicken strains has been achieved without changes in muscle cell composition, however, the ratio between fast and slow-twitch muscle fibers is different in PM muscles from the modern strains compared with layers (Zheng et al., 2009). These changes at molecular and cellular level drive the overall genetic potential that has allowed the development of the modern broiler industry.

The research reported herein is focused in determining the effect of temperature variations during key stages of embryogenesis on meat yield, and genetic selection on muscle development from a molecular perspective by assessing differences in satellite cell activity, muscle morphometrics and growth efficiency among broiler chickens. Data generated by this work should provide the basis for practical decisions during incubation and breeding as well as better understanding of the molecular mechanisms behind skeletal muscle hypertrophic growth. This may provide a pathway by which we can further enhance the efficiency of broiler production from initial stages, as well as help to elucidate the cellular mechanisms behind embryonic and post-hatch development.

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III. Effects of Early Incubation Temperature Variations on Broiler Chicken Growth Performance and Carcass Part Yields

Abstract

Large variations in hatching egg incubation temperatures have been previously shown to negatively impact embryonic and post-hatch growth in broiler chickens. Therefore, our objective was to determine whether small incubation temperature variations (± 0.3 °C) due to tray location effect, from embryonic day 4 to 11, which encompasses both primary and secondary myogenesis, could significantly impact post-hatch broiler growth performance and carcass yields. Broiler hatching eggs were obtained from a 40-wk-old commercial broiler breeder flock and incubated in a single-stage incubator at a commercial hatchery running a temperature profile designed to generate a 0.3 °C temperature differential among trays placed in the top (TOP), middle (MID), and bottom (BOT) of the racks (n = 4 racks per location). Eggs were exposed to the temperature treatment (± 0.3 °C) from d 4 to 12 and incubated at similar temperatures from d 1 to 3 and 12 to 18. From d 4 to 11, average internal egg temperatures were 37.81, 37.75, and 37.57 °C in the TOP, MID, and BOT trays, respectively ($P > 0.05$). Chicks hatched from the 3 incubator tray locations (LOC; n = 720 per LOC) were vent sexed, vaccinated, and separate-sex reared with 12 birds per pen for 41 d on a common corn and soybean-meal based diet in a floor-pen facility. At d 41, all birds (n = 720) were processed to determine carcass and parts yields. No significant differences were observed

in the growth performance of broilers incubated in different incubator tray locations ($P > 0.05$). However, broilers from BOT trays had heavier tender and breast weights than broilers from MID trays ($P < 0.05$). In addition, broilers from the BOT trays had significantly higher breast meat yield as a proportion of carcass weight (25.00%) than MID (24.54%) broilers ($P < 0.05$). LOC also had a significant impact on proportion of carcass yield. Interestingly, broilers from MID trays had greater carcass yield than those from TOP trays ($P < 0.05$). As expected, male broilers had significantly heavier carcass, breast, tender, wings, drumsticks and thighs weights than females ($P < 0.05$). Overall, these data suggest that changes in incubation temperature as little as 0.3 °C because of incubator tray location, during myogenesis, can significantly impact broiler carcass and breast meat yields.

Introduction

Incubation temperature is the most influential physical factor during chicken embryogenesis since it determines embryonic and post-hatch growth, metabolism, and developmental characteristics (Sozcu and Ipek, 2015). However, achieving consistent internal egg temperature to cover the embryonic requirements thorough incubation, is a challenge in the current broiler industry and even when machinery is set to provide adequate conditions, most of the time, those conditions are not met (Gigli et al., 2009). Significant changes in incubation temperature during embryonic phases, generally lead to cumulative negative impacts on the post-hatch growth (Tong et al., 2013). During embryogenesis, striated skeletal muscle development is achieved mainly by hyperplasia. Once the egg is fertilized, myogenesis begins around the 48^{th} hour of incubation with the formation of the somatic cells (Bellairs and Osmond, 1998). Cells from the somites will,

posteriorly, be committed into the muscle lineage to form primary and secondary myofibers. In chickens, the first wave of myogenesis takes place from Embryonic Day (**ED**) 4 to 7 of incubation by the fusion of embryonic myoblasts and results in the formation of primary myofibers. Posteriorly, the second wave of myogenesis happens from ED 8 to 12 of incubation by the fusion of fetal myoblasts and results in the formation of secondary myofibers (Crow and Stockdale, 1986; Al-Musawi et al., 2011). The primary myofibers serve as a scaffold for the formation of the secondary myofibers (Gerrard and Grant, 2006). Therefore, the number and size of primary myotubes heavily determines the total number of myofibers at hatch (Zhang and McLennan, 1999). The secondary myofibers are known to be more susceptible to environmental changes compared with primary myofibers. In chickens, it is known that primary and secondary myogenesis are completed by embryonic day 12 (Yablonka-Reuveni, 1995). Primary and secondary myogenesis are the most important myogenic developmental windows and can be altered by incubation temperature. Hammond et al. (2007) demonstrated that stimulation of embryonic movement through egg incubation temperature results in muscle fiber hyperplasia and muscle growth *in ovo* by induction of myogenic factors. In addition, during the period of incubation, the chicken's embryo undergoes a series of physiological and thermoregulatory transitions from a poikilothermic to a homeothermic status, which must be considered when adjusting incubator settings (Lourens et al., 2006). Indeed, it is known that significant metabolic activity and heat production begins around d 4 of incubation (Black and Burggren, 2004; Hulet et al., 2007). Therefore, recent attention has focused on understanding chick embryonic development and the adequate temperature that must accompany those changes to ensure maximum growth potential.

Recent studies have shown that extremely low temperatures during different stages of the incubation period result in lower metabolism and higher mortality, whereas, high incubation temperatures result in shortening of the incubation period, malnutrition, and embryonic mortality (Suarez et al., 1996; Leksrisompong et al., 2007; Willemsem et al., 2010). Although numerous reports indicate that large variations in incubation temperature, to either direction, can heavily impact embryonic developmental trajectory and, therefore, growth post-hatch, limited formal research has been conducted to determine the effect of subtle changes of incubation temperature during key stages of myogenesis. Therefore, an experiment was conducted to determine the effect of subtle variations in incubation temperature during primary and secondary myogenesis.

Materials and Methods

The Institutional Animal Care and Use Committee at Auburn University approved the use of live birds in this experimental protocol (PRN 2015-2770).

Experimental Design

Eggs from a 40-week-old commercial broiler breeder flock were incubated in a single-stage incubator in a commercial hatchery. Racks within the incubator were spatially divided in 3 sections, bottom (BOT), middle (MID), and top (TOP). There was a total of 12 trays per rack (n = 4 trays per location; 150 eggs per tray). The incubator temperature profile was divided into 3 periods. From embryonic day 1 (ED1) to embryonic day 3 (ED3), incubator temperature profile was designed to generate homogeneous temperature among tray locations (BOT, MID, and TOP). Air temperature from ED1 to ED3 was 38 ± 0.1 °C for all locations. From ED4 to ED11, eggs were

exposed to the temperature treatment. During this period, incubator temperature profile was designed to generate a minor (0.3 ºC) temperature difference among tray locations within the racks. Internal egg temperatures were measured during the temperature treatment period by randomly selecting 4 eggs per location in a daily basis, and measurements from internal embryo temperature were collected using invasive methodologies. Internal egg temperatures were 37.8, 37.7, and 37.5 °C in the TOP, MID, and BOT trays, respectively (Figure 3.1). Finally, from ED12 to ED18 and before hatching, the eggs were exposed to a homogeneous air temperature of 37.7 ± 0.2 °C in all locations. No measurements of O_2 or CO_2 were recorded during the experiment. Thermometers had a higher limit error of ± 0.1 ºC.

Bird Husbandry

Chicks hatched from 3 different incubator tray locations (LOC; n = 720 per LOC) were randomly selected, vent sexed-separated, separated by tray location, and randomly placed into 60 floor pens (0.21 m^2 per bird; n = 12 birds per pen) for 41 days. Chicks were vaccinated at the hatchery for Marek's Disease, Newcastle Disease, and Infectious Bronchitis. All pens were equipped with a hanging feeder and a nipple drinker line. Birds consumed feed and water *ad libitum* basis. All birds were fed a common corn and soybean-meal based diet divided into 3 phases, starter from d 1 to d 13, grower from d 14 to d 33, and finisher from d 34 to d 41. The starter was offered in crumble form and the grower and finisher were offered in pellet form. All diets were formulated to meet or exceed the nutrient recommendations (Table 3.1). Ambient temperature set points consisted of 33 °C at placement with lowering in temperature based on bird comfort to reach a final temperature of 20 °C. Birds were exposed to 23 hours of light (23L) and 1

hour of darkness (1D) from placement to 7 d of age, followed by an 18L:6D photoperiod for the remainder of the experiment. Light intensity was set at 30 lux from 1 to 7 d of age, 10 lux from 8 to 14 d of age, and 5 lux from 14 to 41 d of age. At d 41, all birds (n = 720) were processed to determine carcass and carcass part yields.

Measurements

Pen feed intake and individual bird body weights were recorded on days 13, 33, and 41 for the determination of mortality-corrected body weight gain **(MC BWG)** and mortality-corrected feed intake **(MC FI)**. To calculate MC BWG, initial group-pen weight was recorded on day 1 (12 birds per pen) and an individual bird ID was given on day 6 to track individual growth. Mortality was recorded on a daily basis and feed conversion ratio **(MC FCR)** was corrected for mortality (Table 3.2). On day 41, during the night, feeders were raised 6 hours before processing. The next day, birds were transported in coops to the Auburn University Pilot Processing Plant, electrically stunned, exsanguinated, scalded, and manually eviscerated. Carcasses were chilled on ice for 3 hours before measuring carcass and fat pad weights. The following day, the weights of *Pectoralis major* (boneless and skinless breast), *Pectoralis minor* (boneless tender), both wings (bone-in weight), drums (bone-in weight), and thighs (boneless and skinless thighs) were recorded after the separation carried out by professional deboners. Carcass proportion was calculated relative to live body weight on day 41. The remainder of carcass part yields were calculated as proportions of chilled carcass weights. The breast muscles of each bird were visually evaluated and scored on a 3-point scale for Wooden Breast (**WB**) and White striping (**WS**) myopathies, being 0 the absence of the myopathy and 3 the most severely affected birds. All fillets were scored by the same evaluator.

Statistical Analyses

The experiment was analyzed as 3 × 2 factorial with 3 incubator locations (BOT, MID, TOP) and 2 sexes. Each treatment was represented by 10 replicate pens arranged in a randomized complete block design, with pen as the experimental unit and pen location as the blocking factor. Incubator tray location (**LOC**) and sex were used as fixed effects. Growth performance and proportions of carcass parts were subjected to analysis of variance using PROC GLIMMIX of SAS 9.4 (SAS Institute, 2013) by the following glimmix-effects model:

$$Y_{ij} = \mu + \pi_i + \alpha_j + \pi\alpha_{ij} + \varepsilon_{ij}$$

Where μ is the grand mean; π_i are independently normally distributed random block effects with mean 0 and variance σ^2_a; α_j are the mean factor levels analogous to the jth treatment such that $\sum \alpha_j = 0$; $\pi\alpha_{ij}$ are the interactions between the fixed factors levels and the treatments with mean 0 and variance σ^2_b; and finally, ε_{ij} are independently and identically distributed random errors with mean 0 and variance σ. Satterthwaite adjustment was used to correct the degrees of freedom.

Proportions of carcass parts were analyzed using PROC GLIMMIX of SAS 9.4 (SAS Institute, 2013) using the events/experiments syntax with a R-side covariance structure. For all hypothesis tests, treatment means were separated using the PDIFF option and declared different when $P < 0.05$.

Results and Discussion

Hatchability and Mortality

The percentage of hatchability was 92%, 88.67%, and 88.67% at the bottom, middle, and top trays. The mortality from d 0-7 after hatch was 2.3%, 0%, and 1.6% ($P > 0.05$); (Not

shown). Incubator tray location did not impact significantly the percentage of mortality during the grow-out period (P >0.05); (Table 3.2).

Growth Performance

No significant interactions between LOC and SEX were observed for growth performance. Therefore, only the main effects, LOC and SEX, are reported. Incubator tray location did not have any effect on feed intake, MC FCR, MC BWG, or mortality percentage thorough the rearing period until d 41 (P > 0.05); (Table 3.2). This is in contrast with Leksrisomp et al. (2007), and Molenaar et al. (2011) who reported that broilers from eggs exposed to higher eggshell temperatures (39.5 and 38.9 ºC, respectively) during the last third of the incubation period, had lower BW during the grow-out period, because high temperatures reduced the yolk-free body mass and organ weights at hatch, causing subsequent impairments in the rearing period. In addition, it is known that chicks that survive semi-lethal high incubation temperatures consume less feed, grow slower and have a higher chance to die when compared with normal treatments (Ernst et al., 1984). Other studies have shown that eggs exposed to lower air temperatures (34.6 ºC), can grow slower than those exposed to higher temperatures, however, their embryonic growth and development is the similar to those eggs exposed to standard temperatures (Willemsen et al., 2010). This is also supported by Maatjens et al. (2017) who showed that even though the embryos with an eggshell temperature of 36.7 ºC during the last third of incubation have a delayed hatching process, their development and growth are the similar or even improved when compared with eggshell temperatures 37.8 and 38.9 ºC, resulting in a better chick quality and higher body weight during the first week of the grow-out period. This improvement was not caused by an increase in the

feed intake, therefore, the feed efficiency of such broilers was increased. Our results suggest that subtle changes such as 0.3 ºC in incubation temperature among tray locations from embryonic day 3 to embryonic day 11, may not be as influential as to have a substantial effect in the overall growth performance during post-hatch period. This might be the result of the embryonic resilience. It is known that intermittent changes in temperature have differential effects on embryonic metabolism without affecting embryonic growth or hatchability (Willemsen et al., 2011). Therefore, embryos might be capable to endure small changes in incubation temperature and maintain a steady-state throughout incubation not allowing major physiological changes that can impair the subsequent post-hatch growth. However, it is important to underscore that larger variations in incubation temperature have direct effects on embryonic growth and metabolism as seen in other studies (Molenaar et al., 2011; Lourens et al., 2006; Maatjens et al., 2016). Finally, it is known that in large-scale chicken egg incubation, most of the time, it is likely to find differences up to 5 ºC in incubation temperature due to structural and physical factors intrinsic to the incubator that last for the entire period of incubation and can really have a harmful effect on embryonic development and subsequently that might affect growth performance (Gigli et al., 2009).

Differences in growth performance were primarily due to the effect of sex. Male broilers had higher feed intake ($P < 0.004$) and heavier body weight gain ($P < 0.0001$) than females thorough the rearing period. Finally, male broilers had a better MC FCR ($P < 0.006$) than females thorough the rearing period (Table 3.3). It has been reported that males can consumed up to 13% more feed, gain 22% more weight and improve 7.3%

feed efficiency (Howlider and Rose, 1992) when exposed to the same environmental conditions and fed the same diet compared to females.

Carcass Characteristics

No significant interactions between LOC and SEX were observed for carcass parts weights. Therefore, only the main effects, LOC and SEX, are reported. Incubator tray location did not affect the carcass, abdominal fat pad, wing, drum and thigh weights ($P > 0.05$). However, broilers from BOT trays had significantly heavier breast ($P < 0.05$) and tender muscle weights ($P < 0.05$) than those from MID trays (Table 3.4). This is in contrast with previous studies that have shown that increases in incubation temperature during late embryogenesis, when satellite cells are being formed, can stimulate satellite cell proliferation, leading to higher potential for post-hatch skeletal muscle hypertrophic growth (Piestun et al., 2009). However, it is known that warmer temperatures during early or late incubation can impact carcass parts weights and yields negatively by affecting the embryonic developmental trajectory. The continuous exposure of eggs to high temperature generally results in impairments in embryonic development and significantly inadequate carbohydrate and lipid metabolism. The inability of the embryo to utilize all the nutrients from the yolk causes the retardation and disability of the embryonic growth, resulting in poor performance during the grow-out period (Willemsen et al., 2010). Clark et al. (2017) reported that broilers from eggs exposed to 12 hours of higher air temperature (39.5 °C) daily from ED14 to ED18, had lower PM weights compared to those from the eggs exposed to the same higher temperatures for just 3 hours daily. Even though, different studies seem to contrast each other, it is important to underscore that eggs were exposed to the treatment temperature in different phases

throughout embryogenesis. Therefore, since the duration of the temperature change and the period when it was applied were different, the variations on embryonic resilience, muscle development and growth post-hatch would be expected to have been different.

In the present study, incubator tray location did not have any effect on abdominal fat pads, tender, wing, drum and thigh proportions ($P > 0.05$). Even though broilers from MID trays had a lower breast and tender weight, they had a greater carcass yield as a proportion of d 41 BW than those from the TOP trays ($P < 0.05$). However, broilers from the MID trays had significantly lower breast meat yield as a proportion of carcass weight (24.54%) than BOT (25.00%) broilers ($P < 0.05$). In contrast with these results, Molenaar et al., (2011) reported that broilers exposed to a higher eggshell temperature (38.9 °C) from ED7 onwards, had 1% higher PM meat yield compared to those exposed to normal eggshell temperature (37.8 °C). These results indicate that even subtle variations in incubation temperature can affect carcass composition when they are applied during important embryonic developmental periods. Similar to those results, Janisch et al. (2015) reported that broilers from eggs incubated at higher air temperatures (38.8 °C) from ED7 to ED10 had heavier carcass and carcass parts than those from eggs exposed to lower air temperatures (36.8 °C). However, their carcass and carcass part yields were lower than those from lower temperatures. Also, previous studies demonstrated that post-hatch performance of broilers from eggs exposed to high temperatures is impaired due to inadequate embryonic growth resulting in lower weights and higher mortality (Molenaar et al., 2011). This is because high temperatures have slightly negative impacts on the development of cellular components that lead to cumulative negative effects on adults (Watcharapong et al., 2016). These variation in the results might be attributed to the

differences in the way the temperature measurements are recorded. Therefore, during broiler egg incubation, temperature intensity, direction, magnitude, and its interaction with other important physical factors such as humidity and ventilation, must be considered to draw reliable and accurate results given that the chicken embryo is undergoing constant physiological changes.

Males had heavier carcass and carcass parts except for abdominal fat pad when compared to females ($P < 0.001$). It is known that male and female broilers have distinctively different developmental trajectories that are led by changes in molecular and gene expression profile. Males had greater chilled carcass as a proportion of body weight on day 42, and also drums and thighs as proportions of chilled carcass when compared to females ($P < 0.01$). This is in accordance with other studies and commercial broiler performance guides that predict that male broilers have heavier carcass and greater proportions of carcass and carcass parts when compared to females (Bogosavljevic-Boskovic et al., 2006; Ross 2014; Cobb 2015). On the other hand, females had greater proportion of fat pad and tenders as proportions of chilled carcass than males (Table 3.5). No differences were observed in PM yield between sexes ($P < 0.05$).

The presence of WB was merely due to the effect of sex (Table 3.7). Females had higher incidence of WB score 1 and WB score 2 ($P < 0.02$), whereas the male broilers were more severely affected, showing a higher incidence of WB score 3 than females ($P < 0.001$). In contrast to these results, Clark et al. (2017), reported that male broilers from eggs exposed to 12 hours of high air temperature (39.5 °C) daily from ED 14 to ED18, had higher incidence of myopathies score 1 and lower score 2 compared to the male broilers from eggs exposed to 0 and 3 hours of high temperature daily. Incidence of WS

myopathy was neither affected by LOC nor by sex and there were not interactions among main effects (Table 3.6).

Changes as little as 0.3 °C in incubation temperature, due to incubator tray location during primary and secondary myogenesis, can impact carcass composition and yields. Broilers from MID trays had a 0.46% lower breast yield compared to those from BOT trays. This difference in breast yield equals to 17 g per broiler (Table 3.4). In USA, about 9 billion broilers are harvested yearly, therefore, changes as little as 0.3 °C could impair the production of broilers by at least 150 ton of breast meat per year resulting in considerable losses per complex per year. This underscores the importance of careful incubator temperature management, whether a multi-stage or a single-stage incubator is used.

In conclusion, primary and secondary myogenesis are the most important myogenic developmental windows during embryogenesis that require the synchronization of a myriad of physiological changes that need to be offset throughout incubation. Any alteration in those physiological events can divert the normal course of muscle tissue formation, shifting the normal developmental trajectory curve to either direction based on the direction, duration, and magnitude of the change in incubation temperature. Some studies are in accordance and others contrast in the results obtained about the effects of changes in incubation temperature on embryonic and post-hatch growth. Obtaining more accurate results it is important to reduce the error-introducing factors by using a same chicken strain, magnitude, direction, duration and pattern of the temperature change (Tona et al., 2010; Willemsen et al., 2011). Another important factor to be considered is the way the temperature measurements are taken. Even though the incubator's air

temperature, eggshell and internal embryo temperature are correlated, it is not adequate to draw conclusions considering these measurements at the same level of reliability since internal embryo temperature is a multifactorial parameter (French, 1997).

The present study has shown that incubation temperature variations as small as 0.3 ºC can impact growth carcass composition and yields when it is applied constantly during primary and secondary myogenesis. In commercial hatcheries, the dimensions of the setters make the adequate management of incubation parameters harder, specifically constant temperature, and even though when the setters are set to meet embryonic requirements, most of the time those requirements are not met, leading to higher embryonic mortality, poor chick quality, and reduced growth efficiency. The mechanism behind all these changes in carcass composition and characteristics is still unknown and merits further investigation. Therefore, further studies are needed to determine the effect of subtle changes in incubation temperature during primary and secondary myogenesis, on muscle fiber number, myogenic stem cell populations, and skeletal muscle growth characteristics. These data will provide a better understanding about effect of incubation temperature on embryonic development, which can be used to improve the current management practices that will in turn improve broiler performance and productivity in both short and long-term.

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Table 3.1 Ingredient and calculated nutrient composition of diets fed to broilers reared to 41 d of age

	Starter d 1 to 13	Grower d 14 to 33	Finisher d 34 to 41
Ingredient (%)			
Corn	57.65	61.09	68.16
Soybean meal (48% CP)	35.15	31.66	25.61
Poultry oil[1]	3.06	3.00	2.98
Salt	0.45	0.45	0.46
Dicalcium phosphate	1.72	1.48	1.38
Limestone	1.28	1.18	1.00
DL-Methionine	0.31	0.89	0.16
L-Lysine, 98%	0.13	---	---
Vitamin premix[2]	0.10	0.05	0.05
Mineral premix[3]	0.10	0.10	0.10
Choline chloride[4] (60%)	0.05	0.05	0.05
Total	100.00	100.00	100.00
Calculated values			
AME_n (kcal/kg)	3,064	3,090	3,182
CP, %	21.50	20.30	17.50
Methionine + Cysteine, %	1.00	1.50	0.75
Lysine, %	1.30	1.10	0.93
Calcium, %	0.95	0.85	0.75
Available phosphorus, %	0.45	0.40	0.38

[1]Poultry oil was added with 1% placed in the mixer and 2% spray-applied post-pelleting.
[2]Vitamin premix provided the following per kilogram of diet: Vitamin A (Vitamin A acetate), 9,370 IU; Vitamin D (cholecalciferol), 3,300 IU; Vitamin E (DL-alpha tocopheryl acetate), 33 IU; menadione (menadione sodium bisulfate complex), 2 mg; Vitamin B12 (cyanocobalamin), 0.02 mg; folacin (folic acid), 1.3 mg: D-pantothenic acid (calcium pantothenate), 15 mg; riboflavin (riboflavin), 11 mg; niacin (niacinamide), 44 mg; thiamin (thiamin mononitrate), 2.7 mg; D-biotin (biotin), 0.09 mg; and pyridoxine (pyridoxine hydrochloride), 3.8 mg.
[3]Mineral premix includes per kg of diet: Mn (manganese sulfate), 120 mg; Zn (zinc sulfate), 100 mg; Fe (iron sulfate monohydrate), 30 mg; Cu (tri-basic copper chloride), 8 mg; I (stabilized ethylenediamine dihydriodide), 1.4 mg; Se (sodium selenite), 0.3 mg.
[4]Choline chloride-60 (Balchem Corporation, New Hampton, NY).

Table 3.2 Effect of incubator tray location on growth performance of broilers reared to 41 d of age[1]

	Incubator tray location[2]				
Item[3]	Bottom	Middle	Top	SEM[4]	*P*-value
d 0 to 13 MC FI, g	461	457	460	3	0.777
d 0 to 13 MC BWG, g	391	384	388	3	0.203
d 0 to 13 MC FCR	1.177	1.192	1.185	0.006	0.264
d 0 to 41 MC FI, g	4,937	4,939	4,927	68	0.991
d 0 to 41 MC BWG, g	3,269	3,290	3,300	51	0.906
d 0 to 41 MC FCR	1.513	1.515	1.511	0.026	0.993
d 0 to 13 mortality, %	3.7	2.0	2.0	0.012	0.458
d 0 to 41 mortality, %	5.0	3.7	3.3	0.014	0.668

[1]Broilers from eggs incubated in different incubator tray locations were vent-sexed, sex-separated, and randomly assigned to group pens upon arrival. All birds received a common corn and soybean-meal based diet. Diets were provided in 3 phases: starter (d 1 to 13), grower (d 14 to 33), and finisher (d 34 to 41). Values represent least-square means of 10 replicate pens per treatment (12 birds per pen).
[2]There were 4 trays per location. Internal embryo temperatures were 37.5, 37.7, and 37.8 °C in the bottom, middle, and top trays, respectively.
[3]MC FI = mortality corrected feed intake, MC BWG = mortality corrected body weight gain, MC FCR = mortality corrected feed conversion ratio.
[4]SEM = largest pooled standard error of the pairwise mean comparisons.
[a-b]Means within a row with different superscript differ ($P < 0.05$).

Table 3.3 Effect of broiler sex on growth performance from d 1 to 41[1]

	Sex			
Item[2]	Female	Male	SEM[3]	*P*-value
d 0 to 13 MC FI, g	452	467	2	<0.001
d 0 to 13 MC BWG, g	376	400	2	<0.001
d 0 to 13 MC FCR	1.202	1.169	0.005	<0.001
d 0 to 41 MC FI, g	4,707	5,162	56	<0.001
d 0 to 41 MC BWG, g	2,975	3,597	42	<0.001
d 0 to 41 MC FCR	1.583	1.440	0.021	<0.001
d 0 to 13 mortality, %	2.5	2.4	0.008	0.986
d 0 to 41 mortality, %	3.1	4.9	0.012	0.252

[1]Broilers from eggs incubated in different incubator tray locations were vent-sexed, sex-separated, and randomly assigned to group pens upon arrival. All birds received a common corn and soybean-meal based diet. Diets were provided in 3 phases: starter (d 0 to 13), grower (d 14 to 33), and finisher (d 34 to 41). Values represent least-square means of 10 replicate pens per treatment (12 birds per pen).
[2]MC FI = mortality corrected feed intake, MC BWG = mortality corrected body weight gain, MC FCR = mortality corrected feed conversion ratio.
[3]SEM = largest pooled standard error of the pairwise mean comparisons.

Table 3.4 Effect of incubator tray location on carcass parts weights and yields of broilers reared to 41 d of age[1]

	Incubator tray location[2]				
Item	Bottom	Middle	Top	SEM[3]	*P*-value
Carcass part weights, g	2,361	2,337	2,328	13.7	0.158
Abdominal fad pad, g	45.35	44.82	43.65	0.8	0.297
Breasts (boneless, skinless), g	590[a]	573[b]	578[ab]	4.8	0.035
Tenders, g	130[a]	127[b]	128[ab]	1.1	0.046
Wings (bone-in), g	264	261	260	1.6	0.155
Drums (bone-in), g	298	298	296	2.1	0.737
Thighs (boneless, skinless), g	323	319	322	3.1	0.549
Proportion of carcass parts[4]					
Chilled carcass, %	71.62[ab]	72.16[a]	71.43[b]	0.21	0.041
Abdominal fat pad, %	1.91	1.91	1.86	0.03	0.502
Breasts, %	25.00[a]	24.54[b]	24.88[ab]	0.13	0.029
Tenders, %	5.55	5.46	5.50	0.04	0.276
Wings, %	11.18	11.17	11.16	0.05	0.978
Drums, %	12.60	12.76	12.71	0.06	0.269
Thighs, %	13.67	13.65	13.86	0.10	0.269

[1]On d 41, during the night, feeders were raised 6 hours before processing. The next day, all birds (n = 720) were processed and carcasses were chilled for 3 hours before measuring carcass and fat pads weights. The carcasses were stored in ice for 16 hours and then carcass parts were measured and recorded.
[2]There were 4 trays per location. Internal embryo temperatures were 37.5, 37.7, and 37.8 °C in the bottom, middle, and top trays, respectively.
[3]SEM = largest pooled standard error of the pairwise mean comparisons.
[4]Chilled carcass is shown as proportion of live body weight on d 41 and carcass parts are shown as proportions of chilled carcass weight.
[a-b]Means within a row with different superscript differ ($P < 0.05$).

Table 3.5 Effect of broiler sex on carcass parts weights and yields of broilers reared to 41 d of age[1]

	Sex			
Item	Female	Male	SEM[2]	*P*-value
Carcass part weights, g	2,138	2,546	10.4	<0.001
Abdominal fad pad, g	49.38	39.84	6.5	<0.001
Breasts (boneless, skinless), g	532.8	628.3	3.9	<0.001
Tenders, g	122.5	134.7	0.9	<0.001
Wings (bone-in), g	239	284	1.3	<0.001
Drums (bone-in), g	264	331	1.7	<0.001
Thighs (boneless, skinless), g	290	353	2.5	<0.001
Proportions of carcass parts[3]				
Chilled carcass, %	71.42	72.04	0.17	0.011
Abdominal fat pad, %	2.3	1.56	0.02	<0.001
Breasts, %	24.92	24.69	0.11	0.113
Tenders, %	5.72	5.28	0.03	<0.001
Wings, %	11.18	11.16	0.04	0.740
Drums, %	12.36	13.02	0.06	<0.001
Thighs, %	13.58	13.88	0.08	0.011

[1]On d 41, during the night, feeders were raised 6 hours before processing. The next day, all birds (n = 720) were processed and carcasses were chilled for 3 hours before measuring carcass and fat pads weights. The carcasses were stored in ice for 16 hours and then carcass parts were measured and recorded.
[2]SEM = largest pooled standard error of the pairwise mean comparisons.
[3]Chilled carcass is shown as proportion of live body weight on d 41 and carcass parts are shown as proportions of chilled carcass weight.

Table 3.6 Effect of incubator tray location on incidence and severity of wooden breast and white striping of breast fillets of broilers reared to 41 d of age[1]

	Incubator tray location[2]				
Item[3]	Bottom	Middle	Top	SEM[4]	*P*-value
Wooden breast score 0, %	1.84	2.79	1.78	0.91	0.740
Wooden breast score 1, %	18.89	21.41	24.56	3.57	0.500
Wooden breast score 2, %	47.04	42.54	44.95	3.58	0.675
Wooden breast score 3, %	29.40	28.85	24.91	3.80	0.651
White striping score 0, %	32.72	34.14	39.23	4.02	0.479
White striping score 1, %	52.02	50.85	50.89	3.46	0.963
White striping score 2, %	13.99	14.36	9.37	2.82	0.339
White striping score 3, %	-	-	-	-	-

[1]The breast muscles of all birds (n = 720) were visually evaluated and scored on a 3-point scale for Wooden Breast and White Striping myopathies. All fillets were scored by the same evaluator.
[2]There were 4 trays per location. Internal embryo temperatures were 37.5, 37.7, and 37.8 °C in the bottom, middle, and top trays, respectively.
[3]Percentage of the total count for myopathy incidence per treatment.
[4]SEM = largest pooled standard error of the pairwise mean comparisons.
[a-b] Means within a row with different superscript differ ($P < 0.05$).

Table 3.7 Effect of sex on incidence and severity of wooden breast and white striping of breast fillets of broilers reared to 41 d of age[1]

	Sex			
Item[2]	Female	Male	SEM[3]	*P*-value
Wooden breast score 0, %	2.01	2.18	0.86	0.882
Wooden breast score 1, %	32.45	13.55	2.98	<0.001
Wooden breast score 2, %	49.54	40.23	2.9	0.027
Wooden breast score 3, %	15.77	43.88	3.23	<0.001
White striping score 0, %	37.88	32.83	3.27	0.278
White striping score 1, %	48.21	54.29	2.84	0.130
White striping score 2, %	13.07	11.72	2.19	0.663
White striping score 3, %	-	-	-	-

[1]The breast muscles of all birds (n = 720) were visually evaluated and scored on a 3-point scale for Wooden Breast and White Striping myopathies. All fillets were scored by the same evaluator.
[2]Percentage of the total count for myopathy incidence per treatment.
[3]SEM = largest pooled standard error of the pairwise mean comparisons.

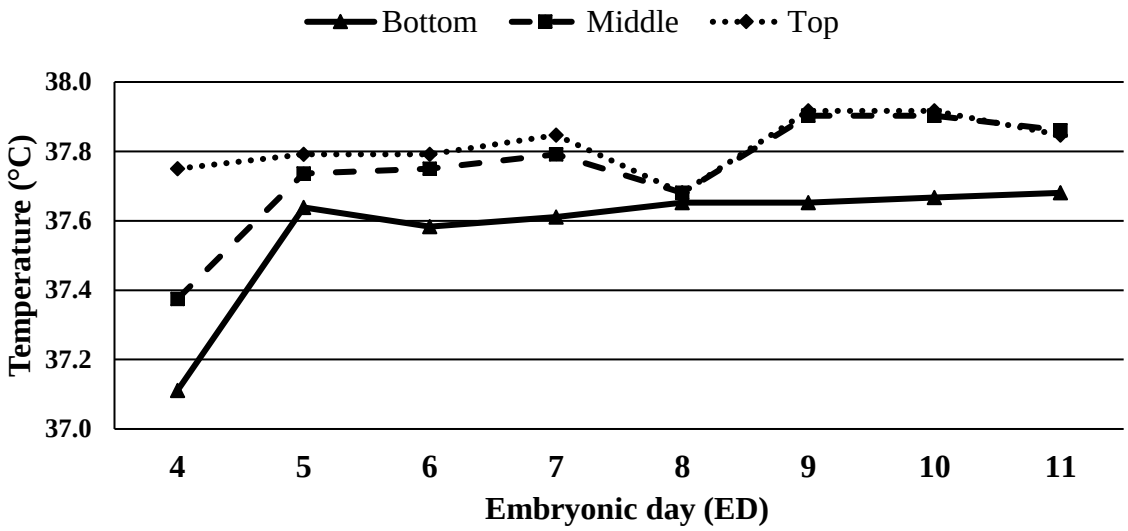

Figure 3. 1 Internal embryo temperature from the bottom (BOT), middle (MID), and top (TOP) trays from embryonic day (ED) 4 to 11 were measured by randomly selecting 4 eggs per location in a daily basis. Internal embryo temperature was collected using invasive methodologies. Internal embryo temperatures were 37.5, 37.7, and 37.8 ºC in the BOT, MID, and TOP trays respectively.

IV. Evaluation of Broiler Chicken Myogenic Stem Cell Population Heterogeneity and Skeletal Muscle Fiber Morphometrics

Abstract

Myogenic stem cells play a key role in mediating post-hatch skeletal muscle growth in broiler chickens through their donation of nuclei to existing muscle fibers which ultimately contributes to the myofibrillar protein synthesis potential of those fibers. Information regarding myogenic stem cell populations and muscle fiber size distributions in modern broiler strains of both sexes is scarce. Therefore, we evaluated myogenic stem cell populations and skeletal muscle fiber size from both sexes in 2 functionally different muscles (*Pectoralis major*, PM and *Biceps femoris*, BF) from 2 chicken strains, Red Ranger (RR; S&G Poultry, Clanton, AL) and Ross 708 x Ross 708 (ROSS; Aviagen Group, Huntsville, AL). Male and female broilers from both strains (n = 80 birds per strain) were penned separately (20 birds per pen) and fed a common corn and soybean meal-based diet. At 43 d of age, 8 birds per sex per strain (n = 32 total) were euthanized and samples from the PM and BF muscles of each bird were collected and stored for subsequent analysis by cryohistology, immunofluorescence staining, and fluorescence microscopy. Cryosections from each muscle from each bird were immunofluorescence stained to detect Myf-5, MyoD, and Pax7-expressing myogenic stem cells. Myogenic stem cell populations (Myf-5+, MyoD+, Pax7+, Myf-5+:MyoD+, MyoD+:Pax7+, and Myf-5+:MyoD+:Pax7+) were enumerated and the cross-sectional area (CSA) of each

fiber in the representative digital image was measured. Mean fiber CSA was greater in the PM compared to the BF muscle ($P = 0.006$). Fiber CSA was similar among the 2 strains and sexes ($P > 0.05$). However, nuclear density and total myogenic cell populations were greater in the BF compared to the PM muscle ($P < 0.05$). The Myf-5+ and Pax7+ population sizes were similar among muscles ($P > 0.05$). ROSS broilers exhibited a 27% larger population of Myf-5+:MyoD+:Pax7+ stem cells compared with RR ($P = 0.04$). No differences were observed due to sex effect. Overall, we observed that the major differences in broiler myogenic stem cell populations were among the 2 functionally different muscles and not among sexes or broiler strains.

Introduction

During embryogenesis, striated skeletal muscle arises from paraxial mesoderm, which matures and organizes into blocks of somatic cells in the dorsal and ventral section of the embryonic notochord (Palacios and Puri Pier, 2006). The newly formed somatic cells rapidly form the multipotent mesenchymal cells and the myotome. These uncommitted cells are induced by myogenic regulatory factors (**MRF**) to enter the muscle lineage and to form myoblasts (Bismuth and Relaix, 2010). Myoblasts are mononucleated and immature muscle cells that will fuse to form primary and secondary myotubes. The paired-homeobox family of transcription factors Pax3 and Pax7 are important upstream regulators of MyoD, triggering proliferation of myoblasts and embryonic myogenesis (Jin, et al., 2016). MyoD, Myf-5, Myogenin, and MRF4 are crucial MRF which play an important role by activating the myogenic programming and myoblast proliferation. Myogenin is a key gene in the activation of the muscle

differentiation program and the formation of myotubes (Parker, et al., 2003). Myotubes will fuse to form the primary and secondary myofibers prior to muscle maturation. After the formation of myotubes, the arrangement of myofibrillar components into a cross-striated pattern within myofibers bestows the contractile ability which is characteristic of the mature muscle tissue (Engel and Horvath, 1960). Mature skeletal muscle fibers are classified in 3 types based on their speed of contraction and metabolism, slow oxidative (type I), and fast-twitch (type II), which are further divided into oxidative (type IIa) and glycolytic (type IIb). *Pectoralis major* (**PM**) muscle is mainly composed of type IIb muscle fibers while the *Biceps femoris* (**BF**) is predominantly composed by type I muscle fibers. Different types have different anatomy, function and molecular composition (Zierath and Hawley, 2004).

During myogenesis, there is a small population of cells that do not fuse to form myotubes, these are called satellite cells (**SC**). These cells are mononucleated myogenic stem cells that retain their ability to fuse after hatch, recapitulating the embryonic development of multinucleated cells (Mauro, 1961). These mononucleated cells express Myf-5 and Pax7 and are mononucleated myogenic stem cells located in the basal lamina, within mature muscle fibers, whose function is to donate nuclei, which results in increase in the protein synthesis potential (Zammit, et al., 2006). Even though different lines of broilers present genetic closeness, there have been identified divergent muscle growth rates due to the differential expression of genes among them (Zheng, et al., 2009).

Genetic selection has allowed the maintenance of positive traits such as feed efficiency and higher proportions of breast and carcass yields. Indeed, about 40% of the phenotypic standard deviation in muscle fiber number and size of the new strains, is due

to genetic origin (Rehfeldt, et al., 2000). Newer strains are capable of reaching the same body weight in half the time it took 5 decades ago, and all these changes are mainly the consequence of selection programs based on growth and meat yields (Havenstein, et al., 2003). Furthermore, differences are not limited to genetic lines, great differences in growth can be seen between sexes. It is clear that male and female broiler chickens have divergent developmental trajectories, perhaps, attributed to different gene expression profiles (Scheuermann, et al., 2003). Genetic selection has improved meat production by increasing the potential for muscle growth. The metabolism of the modern broiler has evolved, resulting in birds that are highly efficient at converting feed to body mass along with achieving muscle yield (Griffin and Goddard, 1994). As shown by Tona, et al. (2010), modern lines of commercial crossbred broilers, have different developmental trajectories, which also impact the organs and other tissues development. In addition, it is well known that genotype can greatly impact hatchability and growth during embryonic and post-hatch development (Siegel, et al., 1968). Xiao, et al. (2017) demonstrated that modern strains have improved their paracrine and endocrine regulation of growth and MRF, allowing changes in gene expression profile that increase efficiency in synthesis of proteins in the muscle.

Although numerous reports claim that this improvement in growth are due to increased myogenic stem cell activity and number, there is little published research available where different myogenic population-mediated changes between sexes and different genetic lines have been investigated. Therefore, an experiment was conducted to determine the differences in myogenic stem cell populations and skeletal muscle fiber

morphometric characteristics in PM and BF muscles from 2 different strains of broiler chickens and their respective sexes.

Materials and Methods

The Institutional Animal Care and Use Committee at Auburn University approved the use of live birds in this experimental protocol (2016-2938).

Bird Husbandry

A total of 80 fast-growing ROSS 708 × ROSS 708 broilers (ROSS, Aviagen Inc., Huntsville, AL) were feather-sexed, separated by sex, and placed into 4 pens (0.12 m^2/bird; n = 20 per birds per pen); 80 Red Ranger (RR; S&G Poultry, Clanton, AL) dual purpose slow-growing chickens were placed as-hatched (**SR**) into 4 pens (0.12 m^2/bird; n = 20 per birds per pen). Chicks were vaccinated at the hatchery for Marek's disease, Newcastle disease, and infectious bronchitis. All pens were equipped with a hanging feeder and a nipple drinker line. Birds consumed feed and water *ad libitum* basis. All birds were fed a common corn and soybean-meal based diet divided into 3 phases, starter from d 1 to d 13, grower from d 14 to d 27, and finisher from d 28 to d 43. Started was offered as a crumble, while grower and finisher were offered as a pellet form. All diets were formulated to meet or exceed the nutrient recommendations of the breeder guidelines (Table 4.1). Ambient temperature set points consisted of 33 °C at placement with lowering in temperature based on bird comfort to reach a final temperature of 20 °C. Birds were exposed to a 23 hours of light (23L) and 1 hour of darkness (1D) from placement to 7 d of age, followed by an 18L:6D photoperiod for the remainder of the experiment. Light intensity was set at 30 lux from 1 to 7 d of age, 10 lux from 8 to 14 d

of age, and 5 lux from 14 to 43 d of age. At d 43, a total of 32 chickens (n = 16 per strain; n = 8 from each sex; Aviagen, 2014) were harvested for muscle sample collection.

Growth Performance

Birds and feed were weighed on d 1, and 43 of age for the calculation of body weight gain (**BW**), mortality corrected feed intake (**MC FI**), and mortality corrected feed conversion ratio (**MC FCR**).

Muscle Sample Collection and Cryohistological Analysis

Birds were euthanized using CO2 asphyxiation, followed immediately by cervical dislocation and the PM and BF muscles were collected from each bird. For PM, muscle samples were collected from the cranial portion of the muscle. For BF, muscle samples were collected from the dorso-cranial portion of the BF. Muscle samples collected from PM and BF of each bird were snap frozen in liquid nitrogen, and stored in cryotubes at −80 °C. Before cutting, samples were moved and held at −20 °C for 24 hours. Samples were cryosectioned at a thickness of 5-µm using a Leica CM 1950 cryomicrotome. After cutting, muscle samples (n = 5 per slide) were mounted on positively charged glass slides (VWR International, PA) and stored at 4 ºC prior to immunofluorescence staining.

Immunofluorescence Staining. Cryosections were fixed and stained according to the methods of Day, et al. (2009) and Meloche, et al. (2018), with minor modifications as described below. All procedures were conducted at room temperature unless otherwise noted. Slides were rehydrated in PBS (pH 7.0; Invitrogen, Carlsbad, CA) for 10 min, and then fixed in paraformaldehyde (4% in PBS; VWR International, Cleveland, OH) for 10 min, followed by 2 PBS rinses. Cryosections were then exposed to 0.5% Triton X-100 (VWR International, NJ) to make the cell membrane permeable. Tissue sections were

exposed to a blocking solution (300 µL) of 10% horse serum (Sigma-Aldrich), 2% bovine serum albumin (VWR International), and 0.2% Triton X-100 in PBS for 30 minutes to block nonspecific antigen binding. Antibody reactions were conducted in a dark, humidified box. Primary antibodies (diluted with blocking solution) were applied to the tissue and allowed to react 1 hour, followed by rinsing 3 times in PBS, 5 minutes per rinse. Secondary antibodies (diluted with blocking solution at 1:1000) were applied and allowed react with muscle tissue for 30 minutes followed by rinsing 3 times in PBS, for 5 minutes each rinse. After application of secondary antibodies, all slides were briefly exposed to 4',6-diamidino-phenylindole (DAPI; 1 ug/mL; VWR International) and immediately rinsed twice in PBS. Slides were mounted using Fluoro-gel Mounting Media (VWR International, Hatfield, PA), covered with thin glass coverslips (VWR International, Radnor, PA), and allowed to dry at 4 °C overnight. All cryosections were imaged within 48 h of immunofluorescence staining.

Primary and Secondary Antibodies. Primary antibodies were as follows: MyoD, Mouse IgG2b (1:50 dilution); Myf-5 Rabbit IgG (1:50 dilution); Pax7, Mouse IgG1 (1:10 dilution). Secondary antibodies were used at a 1:1000 dilution. Secondary antibodies were used for detection of primary antibodies. Secondary antibodies were: Alexa-Fluor 488 Goat anti-mouse IgG2b; Alexa-Fluor 546 goat anti-rabbit IgG Heavy and Light chain; and Alexa-Fluor 633 goat anti-mouse IgG1.

Image Analysis. Immunofluorescence-stained cryohistological slides were imaged at 10X and 20X magnification using an inverted fluorescence microscope (Nikon Eclipse, Ti-U; Nikon Instruments, Inc. Mellville, NY). Images were captured and analyzed using Elements Imaging software (Nikon Instruments, Inc.). A representative

image was captured from each slide. Cross-sectional area was measured on a μm^2-basis for every myofiber within each image. Total numbers of Myf-5+, MyoD+, Pax7+, Myf-5+:MyoD+, MyoD+:Pax7+, and Myf-5+:MyoD+:Pax7+ cells were enumerated for each image. The total number of DAPI-stained nuclei were counted in each image as a measure of nuclear density and detection of false positives. All cell and nuclear densities are presented on a per square micron basis.

Statistical Analysis. Strain and sex were the main effects for growth performance. Strain, sex and muscle type were the main effects for muscle cross-sectional area (**CSA**), myogenic stem cell populations and muscle-related characteristics. For growth performance, each treatment was represented by 2 replicate pens arranged in a randomized complete block design, with pen as the experimental unit and pen location as the blocking factor. For CSA and immunofluorescence, bird served as experimental unit thus, there were a total of 16 replicates per treatment. Data for growth performance, taxonomy, and CSA were subjected to analysis of variance using PROC GLIMMIX of SAS 9.4 (SAS Institute, 2013), using the individual mean GLIMMIX-effects described above for experiment 1. Data for proportions of myogenic stem cell population and taxonomy were subjected to analysis of variance using the events/experiments syntax with a R-side covariance structure. Satterthwaite adjustment was used to correct the degrees of freedom. For all hypothesis tests, treatment means were separated using the PDIFF option and declare different with a level of significance of $P < 0.05$.

Results and Discussion

Growth Performance

When compared by strain, ROSS broilers had heavier body weight (2.99 kg versus 1.32 kg); ($P < 0.001$), higher feed intake (4.83 kg versus 3.17 kg); ($P < 0.05$), and better FCR (1.68 versus 2.74); ($P < 0.05$) than Red Ranger chickens thorough the rearing period (Table 4.2). There is a clear difference in growth performance between strains in the present study. It is noticeable that genetic selection has increased growth rate and improved FCR. Zuidhof, et al. (2014) demonstrated that 48 years of genetic selection (from 1957 to 2005), have increased broiler growth over 400%, with a current reduction of 50% in FCR. All these improvements in the current broiler strains have been achieved through shifts in the developmental trajectory toward leaner and heavier carcasses, resulting in more feed-efficient chicken strains (Havenstein et al., 1994). In addition, straight-run dual-purpose chickens had the lowest body weight gain when compared with the ROSS male and females ($P < 0.001$) (Table 4.3). Both, males and females from the genetic line ROSS have been genetically improved to fast-growing strains. However, due to sexual dimorphism, males are more efficient than females.

CSA and Muscle Fiber Characteristics

No significant interactions among main effects strain, sex or muscle were observed for CSA. Therefore, only results for main effects are discussed. Surprisingly, maximum, minimum, and average fiber CSA were similar in both strains ($P > 0.05$); (Table 4.4) and and sexes ($P > 0.05$); (Table 4.5) when fibers from the same muscle were compared. This is in contrast with Geiger, et al. (2018) who found that quantitatively, the CSA and muscle fiber size of muscles from high weight selected strains, was greater than

slow-growing strains. They attributed this differences to the increase in the pool of activated satellite cells between strains which were able to proliferate and differentiate, increasing the total CSA within the muscle, stating that muscles of chickens selected for larger body size possess larger myofibers. This contrast might be due to the 2 lines used in their experiment came from the same ancestor White Plymouth Rock. However, one of them was selected for high weight and the other for low weight, whereas in our experiment we used a dual-purpose strain (RR) versus a fast-growing meat-type strain (ROSS) which are genetically from different origin. In accordance with our results, MacRae, et al. (2007) compared PM muscle characteristics among 5 different grand-parent lines and found that even though there were differences in BW between lines, no differences were observed in CSA. However, when CSA was analyzed in terms of myofiber distribution, dual purpose chickens (RR) had a higher number of smaller fibers ranging from 1500 to 2000 μm^2 (17% versus 12%); ($P = 0.035$) whereas the fast-growing chickens (ROSS) had higher number of bigger fibers ranging from 2500 to 3000 μm^2 than RR (12% versus 8%); ($P = 0.037$); (Figure 4.1). This suggests noticeable differences in myofiber size when comparing slow- and fast-growing chicken strains. Finally, sex did not have any effect in the distribution of myofiber CSA ($P < 0.05$); (Figure 4.2).

When we compare the mean fiber CSA between muscle types, it is noticeable that PM muscle has greater mean CSA than BF ($P = 0.006$); (Table 4.6). These results are consistent with Jaturasitha, et al. (2008) who showed that type-IIb muscle fibers from the PM of 5 different genetic lines of broiler chickens had greater CSA when compared to the type-I muscle fibers from the BF. Furthermore, when CSA is analyzed in terms of myofiber distribution, BF muscles had higher number of small myofibers ranging from

1000 to 1500 μm^2 compared to PM muscles (15% versus 10%); (P = 0.026); (Figure 4.3). This results were expected since type-IIb and type-I muscle fibers which commonly make up the white and red muscle fibers, respectively, have physiological and physical differences. White muscle fibers (type-IIb) contain greater amounts of the enzyme lactate dehydrogenase which represents glycolytic metabolism. Red muscle fibers (type-I) have abundance of the enzymes succinate dehydrogenase, isocitrate dehydrogenase, isocitrate dehydrogenase and nicotinamide which represent oxidative metabolism. Glycolytic metabolism can occur in the presence or absence of oxygen. Therefore, white fibers, such as PM, have a lower capillary density and greater diameter, whereas, type-I muscle fibers, such as BF, are oxidative or oxygen-dependent muscle fibers which, therefore, need to reduce the diffusion distances by having a smaller cross-sectional area, so the metabolic wastages and nutrients can be transported easily (Gerrard and Grant, 2006).

Overall, even though data in this topic is limited, some of the data collected suggest that skeletal muscle fiber-type composition is dependent on species, anatomical location, and sex. There is also evidence of the presence of sexual dimorphism with respect to muscle fiber-type composition (Haizlip et al., 2015), which was not the case in our experiment. Most of the differences found were among muscle type and not among strain or sexes for muscle characteristics.

Muscle myogenic stem cell populations and nuclear density

No significant interactions among the main effects strain, sex, or muscle were observed for myogenic stem cell populations. Therefore, only results of main effects are presented. ROSS broilers exhibited a 27% larger population of proliferative cells (Myf-5+:MyoD+:Pax7+) compared with RR (P = 0.04); (Table 4.7). This also indicates that the

myogenic stem cell activity remains active during older post-hatch stages in modern broiler strains selected for faster growth and increased muscle yield, even though the maximum peak of cellular activity is during the first 3 weeks after hatch before reaching a plateau and then slowly decrease their activity (Moss, et al., 1964). This indicates the higher activity of SC in muscle from fast-growing broiler chickens when compared with slow-growing strains. The SC pool from fast-growing strains contains a higher percentage of activated SC and with higher ability to proliferate bestowing a greater genetic potential for muscle hypertrophic growth (Geiger et al., 2018). Finally, no differences were observed in myogenic stem cell populations between sexes ($P > 0.05$); (Table 4.8) which indicates the similarity on muscle cells composition on chickens on day 43. However, we know that males and females present divergent developmental growth trajectories that might be driven by differences in the initial number of myogenic stem cell populations whose activity is reduced over time.

Nuclear density was greater ($P = 0.003$) in the BF compared to the PM muscle (Table 4.9). Morphological differences between muscles are correlated with differences in muscle-intrinsic characteristics. Individual muscle fibers differ substantially in many characteristics. The BF muscles have a higher metabolic activity requiring a more complex organization and presence of organelles within the muscle fibers (Gerrard and Grant, 2006). The myogenic populations sizes that were positive for either Myf-5+ and Pax7+ were similar among muscles ($P > 0.05$), which indicates that the level of determination activity of SC among the muscles is the same at 43 d of age. There were differences in the total number of myogenic cells between muscles (Table 4.9). BF muscles had higher number of total myogenic cells when compared with the PM ($P <$

0.05). This is in accordance with Harding, et al. (2016) who found that the proliferation of cell-cultured-myogenic-cells from the PM was lower than BF muscles at different temperatures. In addition, they found that the cell differentiation was greater in PM, indicating a more hypertrophic instead of proliferative activity in such muscle. However, it is crucial to point out that type I muscle fibers such as those found in the BF, have a greater protein turnover rate compared with type-IIb fibers meaning that the internal muscle activity in the type-I muscle fibers should be higher than type-IIb fibers.

In conclusion, there are more similarities than differences in the myogenic stem cell activity between the fast-growing (ROSS) and dual-purpose (RR) strains evaluated in this project. Such similarities might be due to the age at which the muscle cell activity was measured. It is known that myogenic stem cell activity does not stay constant over time, but it decreases as the SC populations reach a threshold from which they are no longer able to undergo further mitosis. However, myogenic stem cell activity can vary greatly depending on the strain and sex. It is well known that considerable differences in molecular and cellular characteristics can be seen when comparing meat-type versus dual purpose strains. In addition, PM muscles which are majorly composed of type IIb, and the Biceps femoris (BF), which is composed mainly by type I muscle fibers, vary greatly in structure and function, therefore, clear differences were observed between muscle types in this experiment. Finally, sex did not have any effect in the muscle characteristics or myogenic stem cell population activity on d 43, indicating that muscle cell activity is reduced over time. Therefore, further studies are needed to elucidate the presence and activity of myogenic stem cell populations during different stages throughout the grow-

out period and how these changes in cell populations affect the growth trajectory and performance.

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Table 4.1 Ingredient and calculated nutrient composition of diets fed to broilers reared to 43 d of age

	Starter d 1 to 13	Grower d 14 to 27	Finisher d 28 to 43
Ingredient (%)			
Corn	57.65	61.09	68.16
Soybean meal (48% CP)	35.15	31.66	25.61
Poultry oil[1]	3.06	3.00	2.98
Salt	0.45	0.45	0.46
Dicalcium phosphate	1.72	1.48	1.38
Limestone	1.28	1.18	1.00
DL-Methionine	0.31	0.89	0.16
L-Lysine, 98%	0.13	---	---
Vitamin premix[2]	0.10	0.05	0.05
Mineral premix[3]	0.10	0.10	0.10
Choline chloride[4] (60%)	0.05	0.05	0.05
Total	100.00	100.00	100.00
Calculated values			
AME_n (kcal/kg)	3,064	3,090	3,182
CP, %	21.50	20.30	17.50
Methionine + Cysteine, %	1.00	1.50	0.75
Lysine, %	1.30	1.10	0.93
Calcium, %	0.95	0.85	0.75
Available phosphorus, %	0.45	0.40	0.38

[1]Poultry oil was added with 1% placed in the mixer and 2% spray-applied post-pelleting.
[2]Vitamin premix provided the following per kilogram of diet: Vitamin A (Vitamin A acetate), 9,370 IU; Vitamin D (cholecalciferol), 3,300 IU; Vitamin E (DL-alpha tocopheryl acetate), 33 IU; menadione (menadione sodium bisulfate complex), 2 mg; Vitamin B12 (cyanocobalamin), 0.02 mg; folacin (folic acid), 1.3 mg: D-pantothenic acid (calcium pantothenate), 15 mg; riboflavin (riboflavin), 11 mg; niacin (niacinamide), 44 mg; thiamin (thiamin mononitrate), 2.7 mg; D-biotin (biotin), 0.09 mg; and pyridoxine (pyridoxine hydrochloride), 3.8 mg.
[3]Mineral premix includes per kg of diet: Mn (manganese sulfate), 120 mg; Zn (zinc sulfate), 100 mg; Fe (iron sulfate monohydrate), 30 mg; Cu (tri-basic copper chloride), 8 mg; I (stabilized ethylenediamine dihydriodide), 1.4 mg; Se (sodium selenite), 0.3 mg.
[4]Choline chloride-60 (Balchem Corporation, New Hampton, NY)

Table 4.2 Effect of broiler strain on growth performance from d 0 to 43[1]

	Strain			
Item[2]	ROSS	RR	SEM[3]	*P*-value
d 0 BW, g	52	45	0.006	0.425
d 43 BW, g	2,995	1,327	0.063	<0.001
d 0 to 43 MC BWG, g	2,940	1,280	0.068	<0.001
d 0 to 43 MC FI, g	4,830	3,177	0.384	0.022
d 43 MC FCR	1,687	2,742	0.301	0.048

[1]Ross 708 x Ross 708 (ROSS; Aviagen Group, Huntsville, AL) and Red Ranger (RR; S&G Poultry, Clanton, AL), were placed into 8 pens and were reared during 43 days. All birds received common corn and soybean-meal based diets. Diets were provided in 3 phases: starter (d 1 to 13), grower (d 14 to 27), and finisher (d 28 to 43). Values represent least-square means of total of 2 replicate pens per treatment (20 birds per pen).
[2]BW = body weight, MC BWG = mortality-corrected body weight gain, MC FI = mortality-corrected feed intake, MC FCR = mortality-corrected feed conversion ratio.
[3]SEM = largest pooled standard error of the pairwise mean comparisons.

Table 4.3 Effect of broiler sex on growth performance from d 0 to 43[1]

	Sex[2]				
Item[3]	Female	Male	As-hatched	SEM[4]	*P*-value
d 0 BW, g	58	47	45	0.009	0.546
d 43 BW, g	2,870[b]	3,120[a]	1,327[c]	0.059	<0.001
d 0 to 43 MC BWG, g	2,805[b]	3,075[a]	1,280[c]	0.064	<0.001
d 0 to 43 MC FI, g	4,610[ab]	5,050[a]	3,177[b]	0.578	0.084
d 43 MC FCR	1,690	1,685	2,742	0.466	0.172

[1]All birds received a common corn and soybean-meal based diet. Diets were provided in 3 phases: starter (d 1 to 13), grower (d 14 to 27), and finisher (d 28 to 43). Values represent least-square means of total of 2 replicate pens per treatment (20 birds per pen).
[2]ROSS broilers were feather sexed-separated right after hatch and randomly placed into 4 pens. Red Ranger chickens were placed as-hatched (SR) into 4 pens and were separated by sex on sampling day using secondary sexual characteristics.
[3]BW = body weight, MC BWG = mortality-corrected body weight gain, MC FI = mortality-corrected feed intake, MC FCR = mortality-corrected feed conversion ratio.
[4]SEM = largest pooled standard error of the pairwise mean comparisons.
[a-c]Means within a row with different superscript differ ($P < 0.05$).

Table 4.4 Effect of broiler strain on muscle fiber cross-sectional area (CSA)[1]

	Strain			
Item[2]	ROSS	RR	SEM[3]	*P*-value
Mean fiber CSA, μm^2	3,015	2,946	271	0.846
Minimum CSA, μm^2	265	338	87	0.523
Maximum CSA, μm^2	6,277	5,566	507	0.287
CSA standard dev., μm^2	1,642	1,440	141	0.279

[1]Both chicken strains, Ross 708 x Ross 708 (ROSS; Aviagen Group, Huntsville, AL) and Red Ranger (RR; S&G Poultry, Clanton, AL) were evaluated. Values are least-square means of 16 broiler replicates.
[2]Images were captured and analyzed using an inverted fluorescence microscope (Nikon Eclipse, Ti-U) and Elements Imaging software. A representative image was captured from each slide. Cross-sectional area was measured on a μm^2-basis for every myofiber within each image.
[3]SEM = largest pooled standard error of the pairwise mean comparisons.

Table 4.5 Effect of broiler sex on muscle fiber cross-sectional area (CSA)[1]

	Sex			
Item[2]	Female	Male	SEM[3]	*P*-value
Mean fiber CSA, μm^2	2692	3269	254	0.108
Minimum CSA, μm^2	213	390	82	0.127
Maximum CSA, μm^2	5559	6284	475	0.278
CSA standard dev., μm^2	1482	1600	132	0.525

[1]ROSS broilers were feather sexed-separated right after hatch and randomly placed into 4 pens. Red Ranger chickens were placed as-hatched into 4 pens and were separated by sex on sampling day using secondary sexual characteristics. Values are least-square means of 16 broiler replicates.
[2]Images were captured and analyzed using an inverted fluorescence microscope (Nikon Eclipse, Ti-U) and Elements Imaging software. A representative image was captured from each slide. Cross-sectional area was measured on a μm^2-basis for every myofiber within each image.
[3]SEM = largest pooled standard error of the pairwise mean comparisons.

Table 4.6 Effect of muscle type on cross-sectional area (CSA)[1]

	Muscle			
Item[2]	BF	PM	SEM[3]	*P*-value
Mean fiber CSA, μm^2	2,484	3,477	251	0.006
Minimum CSA, μm^2	181	422	81	0.039
Maximum CSA, μm^2	5,072	6,771	470	0.013
CSA standard dev., μm^2	1,291	1,792	131	0.008

[1]Muscle samples were collected on d 43 from the *Biceps femoris* (BF) and *Pectoralis major* (PM) and were coated in magnesium silicate, snap frozen in liquid nitrogen, and independently stored at -80 ºC for subsequent cryohistological analysis. Values are least-square means of 16 broiler replicates.

[2]Images were captured and analyzed using an inverted fluorescence microscope (Nikon Eclipse, Ti-U) and Elements Imaging software. A representative image was captured from each slide. Cross-sectional area was measured on a μm^2-basis for every myofiber within each image.

[3]SEM = largest pooled standard error of the pairwise mean comparisons.

Table 4.7 Effect of broiler strain on myogenic stem cell populations[1]

	Strain			
Cell type densities[2]	ROSS	RR	SEM[3]	*P*-Value
DAPI+ only	148.4	137.6	6.52	0.206
Myf-5+	10.2	6.7	1.89	0.155
MyoD+	11.2	11.4	2.52	0.967
Pax7+	1.7	1.4	0.44	0.597
Myf-5+:Pax7+	1.5	0.9	0.38	0.191
MyoD+:Pax7+	0.8	0.3	0.30	0.187
Myf-5+:MyoD+:Pax7+	15.5	11.2	1.58	0.043
Total myogenic cells	127.6	119.4	6.09	0.309

[1]Values are least-square means of 16 broiler replicates from 2 strains Ross 708 x Ross 708 (ROSS; Aviagen Group, Huntsville, AL) and Red Ranger (RR; S&G Poultry, Clanton, AL), and from both sexes.

[2]Images were captured and analyzed using an inverted fluorescence microscope (Nikon Eclipse, Ti-U) and Elements Imaging software. A representative image was captured from each slide to analyze taxonomy. Analysis of treatment effects on the absolute densities of specific cell types were conducted in PROC GLIMMIX on a per mm^2 basis.

[3]SEM = largest pooled standard error of the pairwise mean comparisons.

Table 4.8 Effect of broiler sex on myogenic stem cell populations[1]

	Sex			
Cell type densities[2]	F	M	SEM[3]	*P*-Value
DAPI+ only	143.5	142.5	6.12	0.914
Myf-5+	6.7	10.2	1.77	0.153
MyoD+	11.2	11.5	2.36	0.926
Pax7+	1.5	1.6	0.41	0.922
Myf-5+:Pax7+	1.0	1.4	0.36	0.514
MyoD+:Pax7+	0.5	0.6	0.31	0.886
Myf-5+:MyoD+:Pax7+	14.8	12.0	1.48	0.185
Total myogenic cells	125.5	121.4	5.71	0.608

[1]ROSS broilers were feather-sexed, separated by sex, and randomly placed into 4 pens. Red Ranger chickens were placed as-hatched into 4 pens and were separated by sex on sampling day using secondary sexual characteristics. Values are least-square means of 16 broiler replicates from 2 strains (ROSS and Red Ranger), and from both sexes.

[2]Images were captured and analyzed using an inverted fluorescence microscope (Nikon Eclipse, Ti-U) and Elements Imaging software. A representative image was captured from each slide to conduct cell count. Analysis of treatment effects on the densities of specific cell types were conducted in PROC GLIMMIX on a per mm^2 basis.

[3]SEM = largest pooled standard error of the pairwise mean comparisons.

Table 4.9 Effect of muscle type on myogenic stem cell populations[1]

	Muscle			
Cell type densities[2]	BF	PM	SEM[3]	*P*-Value
DAPI+ only	156.0	129.0	6.05	0.003
Myf-5+	9.4	7.5	1.75	0.432
MyoD+	13.4	9.2	2.34	0.199
Pax7+	1.8	1.3	0.40	0.430
Myf-5+:Pax7+	1.2	1.2	0.35	0.897
MyoD+:Pax7+	0.6	0.5	0.30	0.823
Myf-5+:MyoD+:Pax7+	12.0	14.8	1.47	0.189
Total myogenic cells	132.7	114.3	5.65	0.024

[1]Samples from *Biceps femoris* (BF) and *Pectoralis major* (PM) were collected on d 43 to analyze myogenic stem cell populations. Values are least-square means of 16 broiler replicates.

[2]Images were captured and analyzed using an inverted fluorescence microscope (Nikon Eclipse, Ti-U) and Elements Imaging software. A representative image was captured from each slide to analyze cell taxonomy. Analysis of treatment effects on the absolute densities of specific cell types were conducted in PROC GLIMMIX on a per mm^2 basis.

[3]SEM = largest pooled standard error of the pairwise mean comparisons.

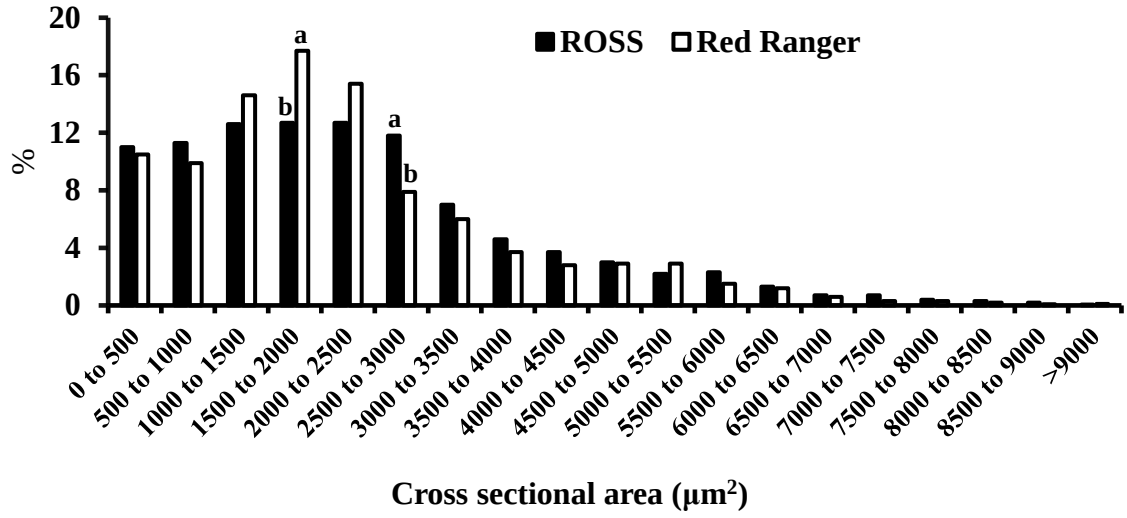

Figure 4.1 Distribution of myofiber cross-sectional areas of ROSS and Red Ranger chickens from 2 muscle types (*Pectoralis major* and *Biceps femoris*) and both sexes. Myofibers are clustered in bin intervals of 500 µm^2. Treatment effects were evaluated using PROC GLIMMIX on the proportions of total fibers ($P < 0.05$). $^{a\text{-}b}$Means within the same bin with different superscript, differ significantly ($P < 0.05$).

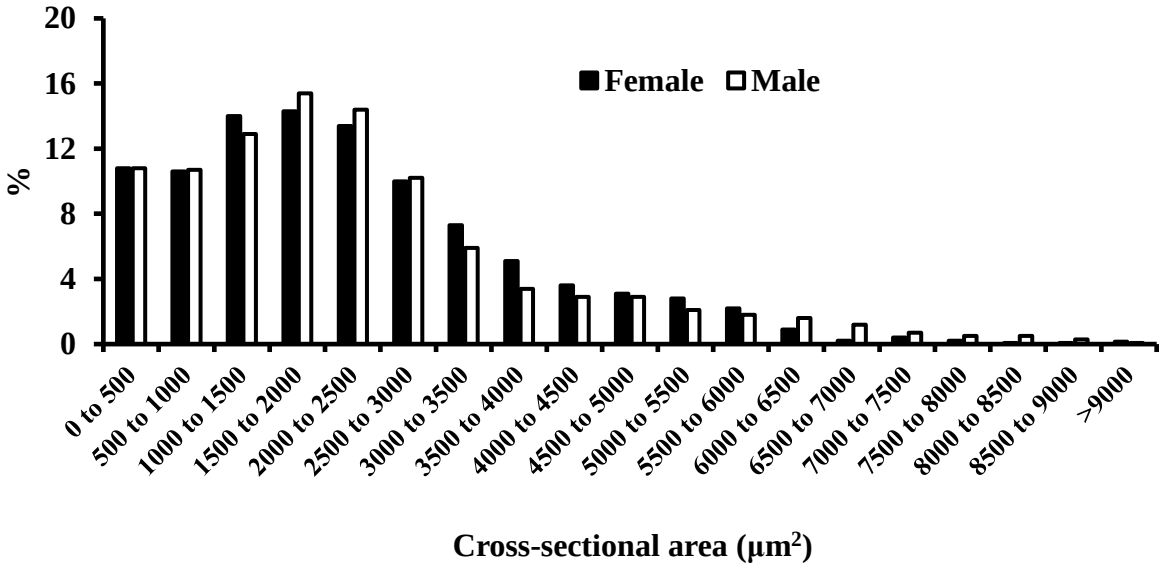

Figure 4.2 Distribution of myofiber cross-sectional areas of males and females from 2 chicken strains (ROSS and Red Ranger) and 2 muscle types (*Pectoralis major* and *Biceps femoris*). Myofibers are clustered in bin intervals of 500 µm^2. Treatment effects were evaluated using PROC GLIMMIX on the proportions of total fibers ($P < 0.05$). $^{a\text{-}b}$Means within the same bin with different superscript, differ significantly ($P < 0.05$).

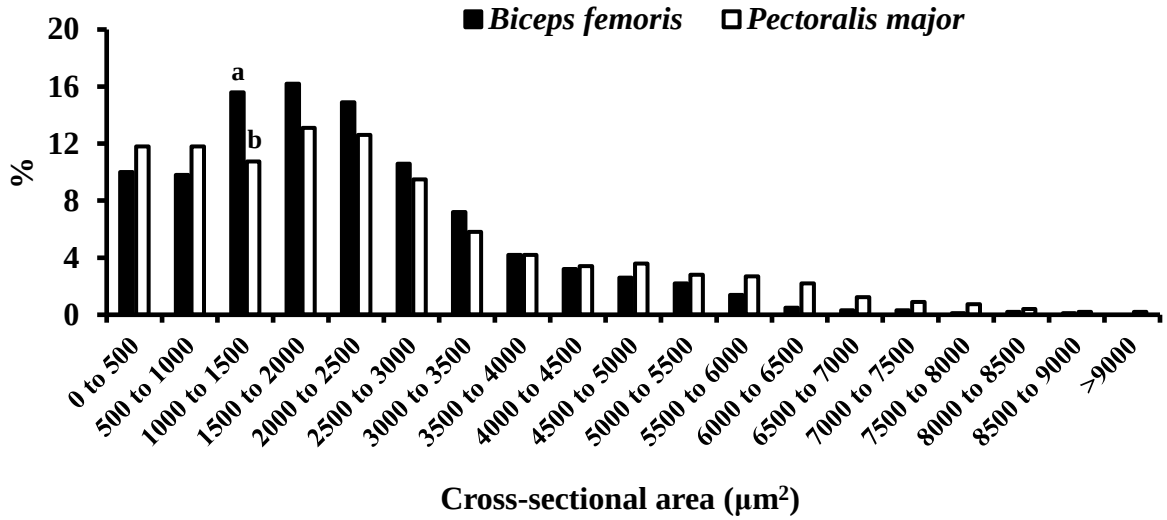

Figure 4.3 Distribution of myofiber cross-sectional areas of *Biceps femoris* and *Pectoralis major* muscles from 2 chicken strains (ROSS and Red Ranger) and both sexes. Myofibers are clustered in bin intervals of 500 µm^2. Treatment effects were evaluated using PROC GLIMMIX on the proportions of total fibers ($P < 0.05$). $^{a\text{-}b}$Means within the same bin with different superscript, differ significantly ($P < 0.05$).

V. Conclusions

The rapid growth of the broiler industry in the last 8 decades, has been the engine for substantial improvements to take place in all areas within the production chain. In the last 3 decades, most of the attention was focus on the growth post-hatch since this accounted for about 75% of the overall length of the production cycle. However, genetic selection has improved the overall efficiency shortening the production cycle. Now, the incubation process accounts for about 45% of the total lifespan of a broiler and therefore, it has gained more importance as a regulation point to improve production. Adoption of state-of-the-art incubators has improved hatchability rates and chick survival, however, large-scale incubators, even though they are programmed to meet embryonic requirements, often fail to offset the embryonic physiological changes, leading to loses and lower efficiency.

The first experiment evaluated the effect of changes in incubation temperature during primary and secondary myogenesis from embryonic day 4 to embryonic day 11 on growth performance and carcass yield, using commercial installations. Several studies had previously shown that large changes in incubation temperature either during the first or the second half of incubation can result in impairment of embryonic growth and development which would in turn, result in impaired growth post hatch. However, it was unknown what was the minimum change in incubation temperature needed in order to cause substantial changes in production that can result in loses. The results from this

experiment demonstrated that changes as little as 0.3 ºC due to incubator tray location, can cause differences in breast meat yield of about 0.46%, and differences in breast and tender weights, where broilers from eggs incubated in higher temperatures had the lower breast meat yield, and breast and tender weights. These results indicate the importance of a correct setting of the incubators whether it is a single-stage or a multi-stage. Therefore, it is critical to account for limitations that single- or multi-stage incubators may present based on their structure as a way to reduce those variations during incubation. Finally, it would be adequate to assess the effect of incubation temperature variation during important stages of myogenesis from a molecular and cellular level as a way to enhance our knowledge and to improve the current incubation practices and technology.

The second experiment was designed to evaluate and compare the differences in muscle fiber characteristics and myogenic stem cell populations size from both sexes in 2 functionally different muscles (*Pectoralis major*, PM and *Biceps femoris*, BF) from 2 chicken strains. There were clear differences in growth performance between the 2 strains with the commercial cross achieving a much greater weight more efficiently than the dual purpose strain. Current broiler strains such as ROSS, have been genetically improved to provide more output by unit of input. In this experiment, ROSS broilers had twice the weight and 40% better feed conversion ratio when compared with the dual-purpose strain (RR). Surprisingly, no significant differences were observed in myofiber CSA between strains. However, distributions of myofiber CSA suggest that dual purpose chicken strains have higher number of smaller myofibers than fast-growing chicken. Furthermore, fast-growing chickens had higher number of greater myofiber CSA than the dual purpose slow-growing chicken strain. Clear differences were observed in CSA between muscle

types. PM muscles had a greater CSA when compared with the BF. Also, BF had higher number of smaller myofibers than PM muscles. Most of the differences were between muscle type indicating the similarity in myogenic stem cell activity at day 43 between fast-growing and dual-purpose strains. However, there was a difference in Myf-5+:MyoD+:Pax7+ stem cells, where ROSS exhibited a 27% larger population of triple positives than RR. This indicates that the myogenic stem cell activity of fast-growing strains may be greater during the whole growth period, resulting in greater and more efficient growth post-hatch. However, these results don't provide adequate information to determine differences in myogenic stem cell activity during early post-hatch periods. Therefore, it is important to continue pursuing evaluations of different strains and incubation conditions to increase our understanding about genetic and molecular aspects behind skeletal muscle growth that may allow us to improve the productivity and current production practices.