Characterization of Increased Muscle Growth in a Heavy Weight Line of Japanese Quail

THESIS

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By

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Abstract

The poultry industry is of great economic importance in the United States. Increasing the muscle yield of poultry is of great interest to the poultry industry, but the mechanisms that control muscle growth and development are not fully understood. Broiler-type chickens and turkey have been shown to have increased muscle yield, especially pectoral muscle (breast muscle), and this increased muscularity has been shown to be due to increased muscle fiber number (hyperplasia) and/or increased muscle fiber size (hypertrophy). Some lines of quail have been shown to have similarly increased pectoral muscle size, and to exhibit hypertrophy or hyperplasia. However, the heavy weight (HW) line of quail has not been characterized, and is of particular interest as previous studies indicate a possible increase in pectoral muscle size relative to body weight compared to a control line.

Embryonic and posthatch growth curves are reported here for the HW line as well as a control line (RBC). HW quail show an initially smaller size, but quickly surpass RBC line quail during embryonic growth. At hatch, HW quail are significantly larger than RBC, and continue to grow more quickly than RBC quail, to a 2.3 fold increase compared to RBC at 85 days posthatch. The HW line also shows an increased right pectoralis major (r. p. major) size and weight compared to the RBC line from posthatch day 4, and is a greater percentage of body weight by day 8 posthatch. By day 85 posthatch, the HW r. p. major is 2.7 times larger than that of the RBC.
The HW r. p. major has more DNA than the RBC from the day of hatch, indicating the presence of more nuclei, and probably a greater cell number. At day 15 posthatch, the HW r. p. major has 1.6 fold more fibers than the RBC r. p. major, and the mean fiber cross-sectional area is 3.6 fold larger. By day 85, the RBC fiber cross-sectional area is 471.45μm² and the HW is 839.22μm². No significant differences were seen in satellite cell proliferation rates at day 6 or 15 posthatch, although the HW line has a greater number of cells at day 6 posthatch. Further study would be required to determine whether satellite cell proliferation differences are a factor in the muscle growth differences seen in HW and RBC quail in earlier or later development. The increased size of HW muscle is due both to hypertrophy and hyperplasia.
Acknowledgments

I would like to thank Dr. Kichoon Lee for allowing me to work with him over the past year. Without his assistance and guidance, this work would not have been possible.

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Chapter 1: Introduction

The U.S. is the world's largest poultry producer, totaling over 43 billion pounds per year. Consumption has increased significantly over the last 50 years, more than doubling between 1970 and 2004 from 27.4 pounds per person to 59.2 pounds. The U.S. broiler industry alone was worth approximately $45 billion in 2010. (USDA) In the poultry industry, growth performance lines (broilers) have been intensively bred to increase growth weight, feed efficiency, and mature size, especially muscle size, in order to increase profit. It has been estimated that increasing breast muscle size by 1 percent would cause an increase in the poultry industry of $36 million per year. Despite the economic importance of increased muscle growth, the mechanisms that control differences in muscle growth are still only partly understood.

Embryonic muscle growth is characterized by proliferation of muscle precursor cells, myoblasts, differentiation and adhesion of myoblasts to form multi-nucleated myotubes, and further differentiation to form muscle fibers (Velleman, 2007). Increased muscle fiber number, hyperplasia, is accompanied by increased muscle size (Scheuermann et al., 2004). Posthatch muscle growth is primarily due to incorporation of nuclei from satellite cells, resulting in increased DNA and increased transcription and
translation, and subsequently increased protein synthesis. This results in a larger muscle fiber size. Muscle fiber size has been shown to be larger in broiler than leghorn chickens, and larger muscle fiber size is positively correlated with greater muscle mass and body weight. Broilers display both increased number (hyperplasia) and size (hypertrophy) of muscle fibers as compared to leghorns (Allen, 1979; Smith, 1963; Moss, 1968a; Velleman, 2007; Scheuermann et al. 2004, Burke and Henry 1997).

Previous studies of growth selected and control quail have shown a significant difference in body size and muscle weight (Fowler et al., 1980; Lilja and Olsson 1987; Ricklefs and Marks, 1985). Also, differences in somite development and myogenic regulators including myosin heavy chain have been found in growth selected quail (Coutinho et al., 1993). Dr. Nestor, an emeritus professor at OSU, has produced lines of Japanese quail selected over 40+ generations for four week body weight. The heavy weight (HW) line has been shown to have a roughly 2 fold larger body weight at sexual maturity, and a 2.4 fold greater breast muscle weight compared to the random-bred control (RBC) line. Interestingly, the HW line exhibited a slightly higher muscle size as a percentage of body weight (Reddish et al., 2003). However, the physiological characteristics of this difference and the mechanisms which control it have not been elucidated.

Although many studies have examined aspects of muscle development in broilers, and some in quail, the mechanisms behind the increased muscle growth of HW quail have not been examined. Furthermore, these studies examine hyperplasia and hypertrophy singly. A thorough investigation of embryonic and posthatch growth and
muscle development is necessary to determine whether the differences in HW muscle growth are due to hyperplasia, hypertrophy, or a combination of the two.
1.1 Aims

**Aim 1.** Establish embryonic and posthatch growth curves for RBC and HW lines and identify any important temporal differences in growth.

**Aim 2.** Determine the effects of hypertrophy and/or hyperplasia in HW and RBC lines.

  Objective 1. Determine total muscle fiber number of pectoralis major for RBC and HW lines, and confirm by total DNA comparison.

  Objective 2. Determine the average muscle fiber size of HW and RBC lines at several posthatch timepoints and identify differences in hypertrophy.

  Objective 3. Perform a proliferation assay to determine if there are differences in posthatch myogenic activity between RBC and HW lines.
Chapter 2: Review of Literature

2.1 Myogenesis

A. Embryonic Muscle Growth

In early embryonic growth, the paraxial mesoderm forms somites, which further differentiate into the sclerotome and the dermomyotome, characterized by Pax1/Pax9 and Pax3/Pax7/myf5 expression, respectively (Deutsch et al., 1988; Brand-Saberi et al., 1993; Christ and Brand-Saberi, 2002; Pourquié et al., 1993; Goulding et al., 1994; Kiefer and Hauschka, 2001). Cells in the dermomyotome compartmentalize into lateral and medial portions which will differentiate into muscle (Christ et al., 1992; Christ and Ordahl, 1995). The medial dermomyotome forms the epaxial myotome and begins myogenesis in response to signaling from Sonic hedgehog (Shh) (Fan et al., 1995). The lateral dermomyotome develops a hypaxial myotome, which will develop into thoracic and abdominal muscles, and releases migratory muscle precursor cells, expressing Lbx-1, N-cadherin, and Scatter factor/hepatocyte growth factor (SF/HGF) (Jagla et al., 1995; Dietrich et al., 1998; Brand-Saberi et al., 1996; Heymann et al., 1996; Bladt et al., 1995).
The muscle precursor cells aggregate and form premuscular masses (Christ et al., 1977). Some muscle precursor cells continue expressing Pax3 and myf5 and proliferate, while others begin expressing MyoD and form differentiating myoblasts. Continued embryonic muscle growth is due to both proliferation and differentiation, and the balance between these two processes can determine muscle size (Patel et al., 2002; Fuchtbacher, 2002). Myoblasts exit the cell cycle and fuse to form multi-nucleated myotubes, which will aggregate and form primary and secondary muscle fibers in specific muscle groups following interaction with the extracellular matrix and tendon structures (Mintz and Baker, 1967; Christ and Jacob, 1980; Grim and Wachtler, 1991; Kardon, 1999; Stockdale and Holtzer, 1961).

B. Posthatch Muscle Growth

Muscle fibers formed during embryonic development exhibit hypertrophy, or increase in size, in posthatch growth. This hypertrophy is due to incorporation of nuclei from satellite cells resulting in increased DNA content and concomitant increased protein synthesis (Smith, 1963; Moss, 1968 B; Goldberg et al., 1975; Barnett et al., 1980). In addition to hypertrophy of existing fibers, increased fiber number in young, adult, and aged animals has been shown in several species following injury or stretch-induced growth (Alway et al., 1989; 1990; Alway, 1991; Gonyea et al., 1986; Kennedy et al., 1988; McCormick and Schultz, 1992; Antonio and Gonyea, 1993; Carson et al., 1995). There is some disagreement as to whether the growth of these fibers involves satellite cells only, or some other cell type(s) (Christ and Brand-Saberi, 2002). Although Smith,
1963 stated that fiber number was fixed at hatch, increased fiber number has also been recorded in postnatal/posthatch vertebrate species without injury or exercise treatment (Fowler et al., 1980; Aberle and Stewart, 1983; Tamaki et al., 2002; Scheuermann et al., 2004).

Differentiated myoblast cells, in cell culture or once incorporated into multinucleated myotubes have been shown to be unable to synthesize DNA, even when stimulated by a point-mutagen (Stockdale and Holtzer, 1961; Cohen et al., 1977). However, 50-99% of the DNA in muscle has been shown to accumulate postnatally (Allen et al., 1979). The increase in DNA has been shown to be due to satellite cell proliferation and incorporation into muscle fibers (Mauro, 1969; Moss and Leblond, 1970; 1971). Hypertrophy is believed to be due to an increase in protein synthesis relative to degradation as the number of nuclei within the fiber is increased by satellite cell incorporation (Moss, 1968; Winick and Noble, 1966; Allen et al., 1979). DNA cannot be used as a direct measure of hypertrophy, as satellite cell proliferation is also involved in adult muscle fiber formation, but it can indicate key timepoints for muscle growth (Carson et al., 1995; Antonio and Gonyea, 1993).

2.2 Hypertrophy and Hyperplasia in Chickens, Turkey, and Quail

Differences in muscle growth between broiler and layer lines of chickens are well established. Smith (1963) showed a significant difference in m. sartorius muscle size and muscle cell size between Leghorn and White Gold (broiler) chickens. Scheuermann et al. (2004) found that two broiler lines of chicken had a higher body weight and breast muscle weight than Leghorn chickens of the same age. A previous study of the HW line
and an unselected randombred control line indicated a difference in body weight at sexual maturity, although no significant difference was shown in p. major as a percentage of BW (Reddish et al., 2003). Aberle and Stewart (1983) showed that broiler type chickens had a larger body weight and muscle weight throughout development, as well as a greater number and area of fibers in m. sartorius.

Comparisons of broiler- and layer-type chickens have shown that broilers tend to exhibit greater muscle hypertrophy than layers at similar ages or body weights. Broilers also show an earlier hypertrophy and earlier switch in dominant fiber type than layers under similar conditions (Smith, 1963; Rémignon et al., 1993; Scheuermann et al., 2004; Aberle and Stewart 1983; Aberle et al., 1979). Similar differences have been seen in broiler type turkey (Velleman et al., 2003) and geese (Klowsowska et al., 1993). In addition, growth-selected, 'P' line quail exhibit greater β muscle fiber hypertrophy than non-selected 'C' line quail (Fowler et al., 1980). Some broiler-type chickens have been shown to have a greater fiber number than layer-type chickens (Scheuermann et al., 2004; Aberle and Stewart, 1983). Similarly, P line quail have been shown to exhibit greater fiber number than C line quail (Fowler, 1980).

2.3 Characterization of Growth-Selected Quail

Growth selected quail grow at different rates than non-selected quail during both embryonic and posthatch development. During embryonic growth, P line quail have been shown to have fewer somites at 42 and 47 hrs of incubation than C line quail, although the initiation of growth is not delayed (Lilja and Olsson, 1987; Coutinho et al., 1993). P line quail are smaller than control quail up to 4 days of incubation, have no significant
difference in size at 5 days of incubation, and are significantly larger than control quail from day 6 of incubation through hatch (Lilja and Olsson, 1987). Following hatch, P line quail grow more quickly than control quail during the first two weeks of hatch, and have greater body weight and greater pectoral muscle weight and percent of body weight (Ricklefs and Marks, 1985). P line quail have been shown to have greater fiber number than control line quail, as has been seen in broiler chickens (Fowler, 1980).

A heavy weight (HW) line of quail has been developed by selection for four-week body weight from a random bred control line (R1; RBC) for over 40 generations (Nestor et al., 1982; 2002). The HW line has a BW at sexual maturity twice that of the RBC line, as well as a greater egg-size, although it is not known what mechanisms are responsible for this difference. The HW line has been shown to have a larger pectoral muscle weight than the RBC line, both in actual size and as a percentage of body weight, although the significance of this difference has not been reported (Reddish et al., 2003).
Chapter 3: Increased Muscle Growth in Heavy Weight Quail is Due to Hypertrophy and Hyperplasia

3.1 Abstract

Growth and development of muscle in a heavy weight (HW) line of quail selected for four-week body weight was compared to that of its parent non-selected random bred control line (RBC). Body weight (BW) was found to be significantly different in embryonic growth from day 8 of incubation (p<0.05), and remained significantly different throughout embryonic and posthatch growth such that HW weighed 2.3 fold more than RBC at 85 days posthatch. The pectoralis major (p. major) muscle of the HW line was significantly larger than the RBC by day 4 posthatch, and by day 8 when expressed as a percentage of BW. The HW line showed both a greater fiber number and greater fiber cross-sectional area than RBC, with hypertrophy occurring earlier in the HW line. The HW line has a greater amount of DNA in the right p. major than the RBC line from the day of hatch (p<0.001) and a greater number of nuclei in the right p. major at day 6 posthatch (p<0.001). No difference was seen in satellite cell proliferation percentages between lines at day 6 or 15 posthatch. Our data indicated that the greater weight of HW birds is partially due to greater muscularity, and that the HW line exhibits
both hyperplasia and hypertrophy greater than that of the RBC line, with differences occurring both in early embryonic growth and posthatch muscle development.

3.2 Introduction

The increasing economic importance of white meat to the poultry industry in the United States has led to an interest in increased breast yield. Genetic selection has led to an increase in meat yield of broiler chickens and an interest in further understanding of the physiological mechanisms involved in increased muscle yield.

Muscle growth occurs in two distinct phases, embryonic and posthatch. During embryonic muscle growth, cells from the dermomyotome are designated as muscle precursor cells, and migrate to the myotome or (for limb muscle) myogenic zones of growing limb buds. Here, the muscle precursor cells proliferate, and some exit the cell cycle and further differentiate into myoblasts. These myoblasts fuse to form primary and then secondary muscle fibers and increase in number and length during embryonic growth and in the first week posthatch. Posthatch muscle growth is due to hypertrophy of existing muscle fibers through incorporation of satellite cells, formed from the proliferation of muscle precursor cells during embryonic growth, and subsequent increase in protein expression within the fibers (Allen et al., 1979; Christ and Brand Saberi, 2002; Steinbacher et al., 2006; Velleman, 2007).

Studies of genetically selected lines of Japanese quail have shown an increase in four-week body weight compared to non-selected controls, and this body weight increase has been shown to be mostly due to increased muscle mass, comparable to broiler lines of chicken (Ricklefs and Marks, 1984; Reddish et al., 2003). However, it is not known if
this increased muscle mass is due to hypertrophy, hyperplasia, or a combination of both factors.

A recent study of a specific selected quail line, heavy weight (HW), indicated a possible difference in breast muscle weight as a proportion of body weight between the HW line and a random bred control, non-selected line (RBC) at sexual maturity (Reddish et al., 2003). In addition to the overall difference in body weight, this indicates an important selection difference between the two lines which merits further investigation.

The purpose of this study was to characterize the growth and muscle development of the HW line of quail in order to better understand the mechanisms leading to increased muscle yield.

3.3 Materials and Methods

Growth Study

Fertile eggs from the HW and RBC lines were obtained from The Ohio State University Ohio Agricultural Research and Development Center poultry facility in Wooster, OH, weighed, and incubated. At 6, 7, 8, 10, 11, 12, 14, and 16 days of incubation, eggs were removed from the incubator and opened, and embryos were extracted with forceps, killed, blotted dry and weighed. Embryos were fixed in 10% neutral buffered formalin (NBF) for sectioning.

Fertile eggs from the HW and RBC lines were also incubated to hatch, and length of incubation recorded. Twelve hours after hatching, chicks were weighed and moved to battery cages. Birds were maintained in battery cages grouped by line and hatch. At one
week intervals, a sample of 10 birds from each line was weighed, and their weights used to construct a growth curve. Measurements were repeated with multiple hatches.

*Tissue Samples*

At posthatch day 0, 2, 4, 6, 8, 15, 30, and 85, a sample of five birds from each line was killed by CO$_2$ inhalation, the breast muscle exposed, and the entire right pectoralis major (r. p. major) removed, weighed, and flash frozen on dry ice. The entire left pectoralis major was removed, weighed, and a cross-section taken and fixed in 10% NBF for sectioning.

*Tissue Sectioning-Fiber Area Determination*

Cross-sectional samples were taken from the r. p. major muscle belly at a region of unidirectional muscle fiber arrangement. Samples were taken from 5 individuals of each line at posthatch day 8, 15, 30, and 85, fixed in 10% NBF for 24-72h and sent to the Goss Histology Lab (Histology and Immunohistochemistry Core, Ohio State University College of Veterinary Medicine, Columbus, OH) for sectioning. Samples were dehydrated by ethanol, embedded in paraffin and 8-10μm sections cut perpendicular to the direction of fiber growth. The sections were fixed on slides and stained with hematoxylin and eosin for determination of mean fiber area and number. For day 8 and 15 samples, whole muscle cross-sections were used. For day 30, a whole muscle cross-section was cut in half after initial fixation and split between two tissue blocks. For day 85, a 3cm long portion of the muscle cross-section was used.
**Muscle Fiber Area Determination**

Muscle cross-section slides from the Goss Histology lab were viewed at 40x magnification with a light microscope and a minimum of 3 images per individual taken with QCapture software (QImaging). Images were analyzed with Image J (NIH) software and 100 fibers per individual were traced and cross-sectional area determined. Muscle fiber density (MFD) was determined by counting the number of fibers present in a 4.0mm$^2$ area. Whole muscle area was similarly determined from images taken with a bifocal dissecting microscope. Muscle fiber number was estimated by the equation: $(MFD/4)*$Whole muscle cross-sectional area in mm$^2$.

**DNA extraction**

The whole r. p. major was ground in a volume of cell lysis buffer scaled to the weight of the muscle using a Tissuemiser homogenizer (Fisher Scientific, Waltham, MA). The homogenate was kept at -20°C. DNA was extracted using a phenol-chloroform isopropyl alcohol procedure, and DNA concentration measured using a Nano-drop spectrophotometer (Thermo Scientific, Waltham, MA). This was used to calculate the total DNA of the tissue.

**BrdU Injection**

At 6 and 15 days posthatch five each RBC and HW birds were weighed and given an intraperitoneal injection of BrdU 10mg/100g BW. Two hours following the injection, birds were killed by CO$_2$ inhalation, and left and right pectoral major removed and weighed. The left p. major was flash frozen on dry ice and stored at -80°C. The r. p. major was fixed in 10% NBF for 48 hrs prior to sectioning.
Tissue Sectioning-Immunohistochemistry

A cross section of r. p. major for each bird was sent to the Goss Histology Lab (Histology and Immunohistochemistry Core, Ohio State University College of Veterinary Medicine, Columbus, OH) for sectioning. Samples were dehydrated by ethanol, embedded in paraffin and 5µm sections cut perpendicular to the direction of fiber growth. The slides were stored in the dark at room temperature (RT) prior to immunohistochemistry.

Immunohistochemistry

Slides were deparaffinized with xylene, then rehydrated following usual accepted procedures. Slides were then treated with 0.3% Triton-X 100 in PBS for 15 minutes at RT, followed by 2M HCl for 30 minutes at 37°C. HCl was neutralized with 0.1M Borate Buffer, pH8.5, and slides washed in PBS. The slides were then blocked with 5% chicken serum in PBS for 20 minutes, washed, and covered with 300µL mouse monoclonal anti-BrdU antibody (G3 G4, Developmental Sutides Hybridoma Bank, Iowa City, IA) 1:100 in PBS overnight at room temperature. Slides were washed, and covered with 300µL donkey anti-mouse FITC-conjugated Antibody (715-095-150, Lot 95147, Jackson Immunoresearch, West Grove, PA) 1:100 for 30 minutes at RT. Slides were then washed, and covered with 300µL DAPI (D1306, Lot 24194W, Invitrogen, San Diego, CA) for 10 minutes at RT, washed, air-dried, and covered. Five images per slide were taken immediately with a fluorescent microscope with filters at excitation 330-385nm (UV-DAPI staining) and 460-490nm (FITC staining). Slides were stored in the dark at 4C.
following immunohistochemistry. Percent of proliferating nuclei was calculated by
(number of BrdU + nuclei/ number of total nuclei)*100 for each image.

3.4 Results

*Growth curve*

A sample of birds weighed at one week intervals from each line was used to
close a growth curve for each line (Figure 1). From the day of hatch, birds of the HW
growth-selected line have a significantly greater weight than birds of the RBC line.
(p<0.001) As the birds grow, the difference between the HW line and the RBC line
increases, from a 1.3 fold difference at day 0 to a (mature) difference of 2.3 fold by day
85 posthatch. As the difference in weight was present at the time of hatch, a further study
was conducted to determine at what point during embryonic growth the difference in
weight occurs.

Embryos were collected at regular intervals during incubation, weighed, and a
growth curve constructed. (Figure 2) At day 6 of incubation, the HW embryos appear to
be smaller than the RBC (p=0.07). At day 7, embryos of both lines appear to have the
same weight, however, by day 8 of incubation, the HW line embryos are larger than the
RBC line embryos (p<0.05). This difference in weight continues to increase to day 14 of
incubation, when the HW line is roughly 1.5 times the weight of the RBC line. In
addition to the difference in size, the HW line has a longer average hatching time than the
RBC. While the RBC line generally hatches at day 17 of incubation, the HW were
observed to hatch at about day 18.
It was suggested that the increase in HW size could be due to the larger size of HW eggs, and thus a greater availability of space and nutrients for the growing embryo. To examine this, egg size and corresponding embryo weight were tracked for both HW and RBC for several timepoints throughout embryonic growth. Comparisons of RBC and HW lines indicated that, although the HW line eggs were an average of 2.5g larger than the RBC eggs, there was no consistent correlation between individual egg size and embryo weight. When the two lines are compared by timepoint, there is an initial negative correlation at day 6, a near-zero correlation at day 8, and an increasingly positive correlation for days 10-14, as might be expected with the increasingly significant difference in embryo weight that follows that same pattern. However, when compared within a line, by timepoint, there is no clear pattern.

_Muscle Growth_

To examine a possible difference in muscle growth characteristics, whole right and left p. major weight was compared for RBC and HW quail at each posthatch timepoint (Figure 3). Average muscle weights for RBC and HW are listed in Table 1. There is a difference in muscle size from posthatch day 4 (p<0.001). The difference in muscle weight increases with age, and the mature HW p. major is 2.7 times larger than the RBC. The greatest difference in muscle weight occurs between posthatch days 4 and 8, when the RBC muscle weight increases 6.08 fold while the HW muscle weight increases 9.43 fold, compared to a growth between days 8 and 15 of 2.18 and 3.01 fold, respectively.
As there is a significant difference in body weight for HW and RBC birds throughout the posthatch period, we examined the difference in p. major weight as a percent of body weight (Figure 4). This data, shown in table 3, shows a significant difference between RBC and HW from posthatch day 8 (p<0.02), with the difference increasing to day 15. Again, the period from posthatch day 4 to 8 shows the greatest proportional growth for both lines, with the HW line showing a greater growth than the RBC.

Table 1 RBC and HW R.P. Major Weights

<table>
<thead>
<tr>
<th></th>
<th>d0</th>
<th>d4</th>
<th>d8</th>
<th>d15</th>
<th>d30</th>
<th>d85</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB</td>
<td>0.0471±0.00</td>
<td>0.0848±0.01</td>
<td>0.5162±0.04</td>
<td>1.1274±0.18</td>
<td>3.4418±0.13</td>
<td>5.9380±0.34</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>8</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>HW</td>
<td>0.0522±0.01</td>
<td>0.1471±0.04</td>
<td>1.3867±0.17</td>
<td>4.1675±0.89</td>
<td>13.5034±0.31</td>
<td>16.0125±0.72</td>
</tr>
<tr>
<td></td>
<td>0.22</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
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Values are mean ± standard error. P-values are from a Student’s t-test, comparison between lines at each timepoint.

_Hypertrophy and Hyperplasia_

To examine the difference in hypertrophy of RBC and HW line muscle fibers, muscle fiber cross-sectional area was determined and the average and distribution plotted for various timepoints. (Figure 5, Table 2). There was no significant difference in average fiber area at posthatch day 8, with average areas of 130.92μm² and 142.09μm² for RBC and HW. At day 15 posthatch, the difference in average fiber area was significant at
p<0.001, with RBC and HW areas of 144.58μm² and 512.63μm² respectively. There was no significant growth in either line between day 15 and day 30, although the difference between lines was significant at p<0.0001. However, at day 85 posthatch, both lines showed hypertrophy compared to day 30, with an average of 471.45μm² for RBC and 839.22μm² for HW. This indicates a definite difference in growth pattern between the two lines, with change in HW mean fiber area occurring much earlier than in RBC.

<table>
<thead>
<tr>
<th></th>
<th>d8</th>
<th>d15</th>
<th>d30</th>
<th>d85</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RBC</strong></td>
<td>130.92±8.99</td>
<td>144.58±7.42</td>
<td>158.58±3.68</td>
<td>471.45±81.59</td>
</tr>
<tr>
<td><strong>HW</strong></td>
<td>142.09±8.05</td>
<td>512.63±98.43</td>
<td>538.12±86.83</td>
<td>839.22±82.21</td>
</tr>
</tbody>
</table>

Comparison of Average Muscle Fiber Area in μm. Significance is from a Student’s t-test between lines at each timepoint.

The differences in average fiber area are reflected by the distribution of fiber areas, those of HW showing a wider spread, increasing in size by day 15, and continuing to increase through day 85. The RBC distribution changes more slowly, with the most appreciable difference occurring between day 30 and day 85 (Figure 5).

To determine whether the greater size of HW quail muscle was due to hypertrophy or a combination of hypertrophy and hyperplasia, we also conducted counts of muscle fiber number for both lines. Muscle fiber density was determined, then the total number of fibers was estimated. A comparison of the number of muscle fibers at day 15 posthatch shows that, while the muscle fiber density is greater for RBC (as expected, due
to their smaller fiber size), the total fiber number is greater for HW than RBC. (p<0.005) HW have 1.6 fold more fibers than RBC at day 15 and 1.3 fold more at day 30 posthatch. At day 15 posthatch, RBC have an average of ~204,000 fibers, and HW an average of ~337,000 fibers. By day 30 posthatch, RBC have an average of ~467,000 fibers, and HW ~618,000.

_R. P. Major DNA Comparison_

HW line quail have a greater amount of total DNA in the r. p. major than RBC from day 0 (p<0.001) and the difference remains throughout posthatch muscle growth. (Figure 6) However, when expressed as micrograms of DNA per gram of tissue, there is no significant difference between the lines, although there is a significant difference between timepoints. DNA concentration decreases with increasing age. (Figure 7)

_Satellite Cell Proliferation (BrdU Staining)_

The percentage of proliferating nuclei at day 6 posthatch was 7.203% for HW and 7.512% for RBC. At day 15 posthatch, it was 7.814% for HW and 7.453% for RBC. There were no significant differences in percent proliferating nuclei between lines at either timepoint or between timepoints for either line. At day 6 posthatch, the HW line had a greater number of nuclei per slide than the RBC line. (p=0.0001) Data summarized in Table 2.
### Table 3 Comparison of RBC and HW Satellite Cell Proliferation

<table>
<thead>
<tr>
<th></th>
<th>Nuclei Number</th>
<th>BrdU+ Nuclei</th>
<th>Percent BrdU+</th>
<th>Significance Between Timepoints</th>
<th>Significance Between Lines</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RBC (P6)</strong></td>
<td>358.48±10.44*</td>
<td>26.90±1.52</td>
<td>7.51±0.377</td>
<td>p=0.461</td>
<td>p=0.305</td>
</tr>
<tr>
<td><strong>(P15)</strong></td>
<td>136.97±5.12</td>
<td>10.66±0.885</td>
<td>7.45±0.462</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HW (P6)</strong></td>
<td>424.11±13.00*</td>
<td>30.65±3.25</td>
<td>7.20±0.666</td>
<td>p=0.239</td>
<td></td>
</tr>
<tr>
<td><strong>(P15)</strong></td>
<td>126.15±3.64</td>
<td>9.88±0.755</td>
<td>7.81±0.536</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± standard error. P value is from a Student’s t-test between timepoints, within line and between lines, within timepoint for percentage of proliferating nuclei. *p=0.0001,  Student’s t-test between lines at day 6 posthatch for number of nuclei per image.

### 3.5 Discussion

HW quail have a pattern of growth distinct from that of RBC. During embryonic growth, the two lines are of similar size through day 6 of incubation; however, HW quail are significantly larger than their RBC counterparts by 8 days of incubation. The initial difference in size increases as embryonic growth continues, however, despite their faster increase in size, HW quail mature more slowly than RBC, requiring an average of one day longer of incubation prior to hatch, which has not been noted in other growth-selected quail lines. It has been shown that some lines of quail selected for body weight exhibit muscle hyperplasia accompanied by a slower early embryonic development than non-selected quail, with delayed expression of early myogenic developmental markers (Fowler et al., 1980; Coutinho et al., 1993). This delay might be related to slower differentiation of muscle precursor cells that allows for a longer period of cell proliferation, leading to greater muscle precursor cell number, and thus greater muscle size without increased fiber size.
The difference in embryo growth has been suggested to be due to differences in egg size, and corresponding nutrient availability. Lilja and Olsson (1987) examined embryonic growth of P line, growth selected, and C line, control, Japanese quail. They showed that the P line quail were delayed in early growth, with fewer somites at 42 hours of incubation, and a decreased embryo weight at days 3 and 4 of incubation, followed by more rapid growth, such that the P line embryos were larger from day 6 onwards. They concluded that this difference could be related to the earlier maturation of the yolk and allantois in the P line, and improved nutrient utilization in embryonic and posthatch growth. The HW line shows a similar pattern of growth. They tend to be smaller than RBC during early development (p=0.07), and surpass the RBC in later embryonic growth. Embryo size and egg weight correlation by line and timepoint indicates a possible effect of egg size, however, when compared within a line by timepoint, there is no consistent correlation. Effect of egg size cannot be ruled out; however, it does not sufficiently explain the observed differences in posthatch muscle growth.

During posthatch growth, the difference in body weight between RBC and HW increases, and from an initial 1.3 fold difference, the 85 day posthatch HW is 2.3 times the size of RBC birds the same age. This difference in body weight is similar to that shown by broiler and layer type chickens (Smith, 1963; Scheuermann et al., 2004; Aberle and Stewart, 1983; Reddish et al., 2003). A previous study of HW and RBC line quail showed a significant difference in body weight, but no significant difference in pectoral muscle as a percentage of BW in the two lines at sexual maturity, however, sexual maturity occurs at different ages in the two lines (Reddish et al., 2003).
When the muscle size is compared on a percent body weight basis, the HW breast muscle is a greater percentage of body weight than RBC from posthatch day 8 onwards. It is interesting to note that the significant difference occurs at the end of the first week posthatch. In general, rapid muscle growth coincides with rapid DNA accretion and high satellite cell number (Allen et al., 1979). The large increase in muscle weight during a relatively short period of time between days 4-8 posthatch indicates that the first week posthatch is of importance for quail posthatch muscle growth, possibly due to satellite cell proliferation.

Ricklefs and Marks (1984) showed that growth-selected and randombred quail growth rates were different only up to 2 weeks posthatch, and that the pectoral muscles of the growth-selected line were a larger proportion of the body than non-selected quail. The increase in breast muscle relative to body weight has been shown to be greater than that of other muscle groups, such as gastrocnemius or supracoracoideus possibly due to difference in fiber type, but there are no apparent differences in breast muscle fiber type composition between lines (Moss, 1968 B; Fowler et al., 1980). When comparing the growth of the two lines, the majority of p. major muscle growth occurs prior to day 30 posthatch. However, as a percentage of body weight, the majority of growth occurs prior to day 15. The period of most rapid growth in both body weight and muscle size occurs in the first two-three weeks posthatch, similar to the previously reported growth weights for other growth-selected quail.

The temporal pattern of changes in muscle size and muscle fiber size are different in RBC and HW quail. HW muscle fiber cross-sectional area increases greatly between
day 8 and day 15 posthatch, while RBC fiber area changes little. Broiler type chickens showed an earlier increase in muscle weight and alteration in fiber type than layer-type chickens (Aberle and Stewart, 1983). Similarly, the HW line shows an earlier increase in fiber size than RBC. This early increase in fiber size continues during later muscle growth, and by day 85 posthatch, the average HW muscle fiber area is nearly twice that of the RBC.

Smith (1963) showed a difference in average muscle fiber area between leghorn and broiler type chickens of about 200μm² by 10 weeks of age. By day 85, HW quail show an average muscle fiber area more than 360μm² larger than RBC. Smith concluded that the difference in muscle size at 10 weeks for age could be accounted for solely by the difference in hypertrophy of muscle fibers. However, Scheuermann et al. (2004) showed that two broiler lines had a lower myofiber density (and presumed larger myofiber area) but a higher myofiber number than a leghorn line of chickens. Furthermore, they showed that the high breast yield selected broiler line had a higher myofiber number than the normal breast yield line, and indicated that difference in fiber number could account for difference in muscle size between the two broiler lines. Increased fiber number has also been reported in several other studies of vertebrate species during normal growth (Fowler et al., 1980; Aberle and Stewart, 1983; Tamaki et al., 2002; Scheuermann et al., 2004) and following injury or external stretch (Alway et al.; 1989;1990; Alway, 1991; Gonyea et al., 1986; Kennedy et al., 1988; McCormick and Schultz, 1992; Antonio and Gonyea, 1993; Carson et al., 1995).
Using methods similar to Scheuermann et al. (2004), we compared the apparent fiber number of HW and RBC and days 15 and 30 posthatch. At both day 15 and day 30 posthatch, HW have a greater number of fibers than RBC (p<0.005). This indicates that the HW line, similar to the P line previously characterized, does show hyperplasia greater than that of the non-selected quail.

The difference in posthatch muscle growth of RBC and HW quail includes differences in both hypertrophy and hyperplasia. Differences in amount and time course of hypertrophy posthatch indicate either a difference in satellite cell proliferation and incorporation, resulting in increased nuclei per muscle fiber and correlated increased protein synthesis, or if satellite cell proliferation and incorporation is the same, a difference in protein expression or degradation irrespective of the number of nuclei. To determine whether differences in proliferation could account for the observed developmental differences, we examined the DNA content and concentration of the r. p. major of RBC and HW quail at several important timepoints established by the previous study.

The HW line has more DNA than the RBC line from the day of hatch, indicating that they already have a greater number of nuclei. This would be consistent with a greater number of fibers (greater hyperplasia) and of satellite cells, and thus greater hypertrophic potential. If one line exhibited greater proliferation of satellite cells than the other during a specific time, one would expect to see an increase in the amount of DNA for that line relative to the other between timepoints.
This is true for the HW between 0 and 4 days, 4 and 15 days, and 15 and 30 days, as the HW DNA increases more rapidly than the RBC. Between 30 and 75 days, the ratio of HW to RBC DNA decreases, from 4.35 fold to 2.97 fold. This is consistent with the data previously reported as between day 30 and day 85 posthatch the RBC line undergoes the greatest amount of hypertrophy of any time during posthatch development, while the HW line already showed hypertrophy between day 8 and day 15 posthatch. It is interesting to note that between days 15 and 30, when no significant hypertrophy was seen in either line, the amount of DNA increases in both lines (approximately 1.5 fold for RBC and 1.9 fold for HW.) This increase in DNA without an increase in muscle size is consistent with the significant increase in fiber number observed for both lines during this time period, as satellite cell proliferation is required for new fiber growth.

It was noted that the day 4 to day 8 posthatch time period showed the greatest relative growth for the two lines, and we hypothesized that this might be due to either an increase in fiber number or fiber size. To determine whether there was a difference in proliferation between the lines during this time, we compared the proliferation of the two lines using BrdU staining at day 6 posthatch. No difference in percentage of proliferating nuclei was seen between lines, although the percent of proliferating nuclei (~7%) was much higher than that reported in previous studies of similarly aged turkey (<1%), possibly due to the differences in metabolism and speed of growth between the two species (Mozdziak et al., 2002; Moore et al., 2005). However, it is important to note that although the percentage of proliferating nuclei was the same in both lines, the actual number of proliferating nuclei was greater in the HW, which supports the idea that
hyperplasia had already occurred in the HW line greater than in the RBC line, possibly during embryonic growth.

To determine whether the proliferation during this period was greater than during other periods of posthatch muscle growth, we also compared the proliferation of the two lines at day 15 posthatch. Again, no difference in percentage of proliferating nuclei was seen between lines, and there was no difference in percentage of proliferating nuclei between timepoints. It is possible that both periods studied have a high proliferation rate, as the day 6 proliferation is during a time of large increase in DNA and muscle size, and the day 15 proliferation rate may be tied to hypertrophy in the HW and increased muscle fiber number in both RBC and HW (as shown by the increase in fiber number between days 15 and 30 posthatch). Further studies are required to determine whether the proliferation rates seen at these two timepoints are normal for posthatch quail, or if there may be differences in proliferation between the lines during earlier or later posthatch growth, but it is clear that at both day 6 and 15 posthatch, satellite cell proliferation is an important factor in quail muscle growth.

Our data indicates that the difference in muscle growth between RBC and HW lines is due both to hyperplasia, which may be related to expression events in embryonic growth, and to differences in temporal action and extent of hypertrophy, which would be related to expression events in posthatch period. Further study of the possible muscle regulatory factor expression differences and temporal differences in proliferation would be of benefit in understanding the complex mechanisms regulating muscle growth.
Figures

Figure 1 Posthatch Growth Curve Comparison of RBC and HW Quail
Comparison of mean body weight. X axis: Days relative to hatch, Y axis: Mean body weight, g. Error bars are standard error of mean. * Indicates significance between lines of p<0.001.

Figure 2 Embryonic Growth Curve Comparison of RBC and HW Quail
Comparison of mean embryo weight. X axis: Days of Incubation, Y axis: Mean Embryo weight, g. Error bars are standard error of the mean. Significance between lines: #p=0.07, **p<0.05, ***p<0.001
Figure 3 Comparison of RBC and HW R. P. Major Weight
Comparison of mean r. p. major weight (g). X axis: timepoint, Y axis: weight (g). Error bars are standard error of mean. Significance between lines ***p<0.001.

Figure 4 Comparison of RBC and HW R.P. Major as a Percentage of Body Weight
Comparison of mean r. p. major weight as a percentage of body weight. X axis: timepoint, Y axis: %. Error bars are standard error of mean. Significance between lines *p<0.05, **p<0.01, ***p<0.001.
Figure 5 Comparison of RBC and HW Muscle Fiber Size

Images: Representative muscle cross-sections, scale bar=0.1mm, Graphs: Histograms of RBC and HW Muscle Fiber Area. X axis: Fiber Area ($\mu m^2$), Y axis: Percent of Measured Fibers
Figure 6 Comparison of RBC and HW DNA in Whole R. P. Major

Comparison of RBC and HW R. P. Major DNA. X axis: timepoint, Y axis: DNA (µg). Values are mean ± standard error. Significance between lines #p=0.066, ***p<0.001.

Figure 7 Comparison of RBC and HW R.P. Major DNA per Gram of Tissue

Comparison of RBC and HW DNA. X axis: timepoint, Y axis: DNA (µg per gram of tissue). No significance between lines.
References


