Proteomic analysis of 1-D Sarcoplasmic Protein Profiles of Pekin Duck Embryos’ Pectoralis Muscle as Influenced by Incubation Temperature

THESIS

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By

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Abstract

The objective of this study was to identify sarcoplasmic proteins responsive to incubation temperature in Pekin duck embryos. Previous studies reported that a 1-degree Celsius increase in incubation temperature during the first 10 days can accelerate embryonic development and this study was designed to identify the effects of early incubation temperature on embryonic myogenesis. Pekin duck eggs were incubated at 37.5°C or 38.5°C for the first ten days and subsequently transferred to 37.5°C for the rest of incubation (ED 11-28). The embryonic pectoralis muscle (PM) was collected at ED12, 18, 25 and hatch and sarcoplasmic proteins were subjected to 10% SDS-PAGE. Gels were digitized into TotalLab™ to acquire the mean band percentage (MBP) of bands. The body weight (BW) of embryos and pectoralis muscle weight (PMW) of the Pekin duck embryos were analyzed in SAS 9.3. An acceleration in BW at ED12 in the 38.5°C treatment was observed but not at later ages. MIXED model is performed to determine bands responding significantly to incubation temperature. Three proteins/bands are determined to significantly respond to temperature.
Acknowledgments

The completion of this thesis from the initial proposing stage to later experimental and analysis stage and the finale writing stage cannot be done without the resource and assistance from my committee, Dr. Lilburn, Dr. Wick and Dr. Pope. Dr. Lilburn had been active providing liaison with the poultry industry, Maple Leaf Farm that provide adequate and fertile Pekin duck eggs and provided endless guidance in the writings of this thesis. Dr. Wick had been assisting in providing poultry muscle biology knowledge and abundant and resourceful experience in coping with muscle-related experiment. Dr. Pope had been really helpful in providing distinguishable guidance in the formation of the proposal and the thesis. I need to thank Dr. Zapata in helping analyzing the Statistics. Also, I need to express my appreciation to work with the Maple Leaf Farm that has been working closely with Dr. Lilburn in providing Pekin duck resources. I felt deeply thankful with the help from Dr. Reddish and my colleague, Jacqueline Griffin, Stephanie Loeffler and Amanda Prickett in Dr. Wick’s lab and Jimsoo Ahn from Dr. Lee’s lab when they were bombarded with my endless questions. I also felt really thankful of being given a chance by Dept. of Animal Science and the Ohio State University to learn and explore the knowledge in every aspect. Last but not the least, I want to express my
gratitude to my parents and sister in Taiwan who have been always on my side and supported me.
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Fields of Study

Major Field: Animal Sciences
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PM</td>
<td>pectoralis muscle</td>
</tr>
<tr>
<td>k</td>
<td>kilo</td>
</tr>
<tr>
<td>m</td>
<td>milli</td>
</tr>
<tr>
<td>mol</td>
<td>mole(s)</td>
</tr>
<tr>
<td>L</td>
<td>litter(s)</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>ED</td>
<td>embryonic day</td>
</tr>
<tr>
<td>D</td>
<td>day (post-hatch)</td>
</tr>
<tr>
<td>PH</td>
<td>post-hatch</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>°F</td>
<td>degree Fahrenheit</td>
</tr>
<tr>
<td>MBP</td>
<td>mean band percentage</td>
</tr>
<tr>
<td>GLM</td>
<td>generalized linear model</td>
</tr>
<tr>
<td>SAS</td>
<td>statistical analysis system</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide agarose gel electrophoresis</td>
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Chapter 1: Project Description

Domestic duck breeds, descendants from the Muscovy duck and Wood Duck together with the Swan and Goose, form the order of Anatidae under the order Anseriformes. Among the various poultry species produced in the U.S., the Pekin duck is the fastest growing commercial species in terms of gain per day but is often undervalued by consumers. As the availability of duck products increases, it may offer an alternative option for consumers that are conscious of their diet. In view of market value, its unique characteristics such as rapid growth rate, improved feed conversion and value as an export product to Pacific-rim countries, duckling has the potential to become an increasingly important part of the U.S. commercial poultry industry. In countries outside the US such as France, China, Hong Kong, and Taiwan, duck is already valued as a versatile poultry species on par with chicken. A report on international egg and poultry demand from the United States Department of Agriculture states that world duck meat production increased approximately 7% from 2008 to 2010. In 2012, the Chilean Ministry of Agriculture agreed to include duck as a frozen and chilled poultry meat export commodity. The popularity of duck in Europe, Asia, and now South America underscores the growing commercial status of duck meat and the importance of studying myogenesis in commercial ducks (Baeza, 2006).
The PM represents up to 30 percent of total body mass and enables wild ducks to fly long distances, even in windy conditions. Similar to the domestic turkey and broiler chicken, selection for breast muscle has become an important practice in commercial meat-type Pekin ducks. In the literature, there are reports of different ratios of mixed fiber types between *Gallus gallus* and *Anas platyrhynchos* resulting in the former being commonly known as white meat while the latter appears to have increased pigmentation. The different mixed fiber types may result from the need in wild ducks to migrate (George and Berger, 1921).

The differences in muscle phenotype underscores the importance of characterizing the pattern of sarcoplasmic protein expression in ducks so as to better understand the mechanisms underlying the mixed fiber types and the rapid growth of commercial ducks. The growth and development of a muscle is a dynamic balance between protein synthesis and degradation. The synthesis of muscle can be classified into hyperplasia (increase in myocyte number) and hypertrophy (increase in myocyte size) within a given muscle fiber. Debate still exists on which synthesis pathway is more dominant at different points during muscle growth and development. In avian species, it is generally accepted that hyperplasia is the predominant mechanism of myogenesis during embryonic development with hypertrophy being more important post-hatch (Stockdale and Miller., 1987; Picard et al., 2002; Stickland et al., 2004).

Analysis of Pekin duck embryos in response to increased incubation temperature by Bowers et al. (unpublished, 2008) showed that accelerated growth occurred in those embryos exposed to the higher incubation temperatures during the early incubation
period (37.5 °C to 38.5 °C; 0 to 10 days). Based on these observations, our hypothesis is that small increases in incubation temperature (37.5 °C to 38.5 °C) during the first ten days of incubation will accelerate early embryonic growth and may be reflected by a differential expression pattern of sarcoplasmic proteins when compared with control embryos incubated at a standard temperature (37.5 °C) throughout the entire incubation period.

Significance

In Asia, an increasing amount of duck meat is consumed annually because of its’ easy acquisition from local markets and the juiciness of duck meat is favored by the Asian market as an alternative to red meat. As the population in Asia continues to grow, there will certainly be an increasing demand for foods with more protein that are also of high quality. The genetic selection for improved growth of commercial poultry post-hatch has had little effect on the length of the incubation period. The optimal incubation temperature for all commercial poultry species was determined decades ago and this has not changed (Romanoff, 1936). What has changed, however, is the recognition that within commercial incubators there are small differences in temperature and what has not been determined is the extent to which these small changes can interact with the development of whole systems such as the skeleton and skeletal muscle. This study is novel to the extent that it investigated the effect of early differences in incubation temperature on embryonic muscle growth and development, specifically the temporal
expression of sarcoplasmic proteins. There were several unique aspects of the research:

1) Through the combination of one dimensional sodium dodecyl sulfate polyacrylamide agarose gel electrophoresis (1D SDS-PAGE) and image analysis, proteins/peptide bands important for growth were identified according to changes in their expression level or the appearance of new proteins.

2) The similarity or differences in the expression pattern of breast muscle sarcoplasmic proteins in response to differences in incubation temperature may identify potential proteins exclusively affected by temperature.

3) This proteins could potentially be used as markers for other environmental changes that influence embryonic development. Since genetic selection is based on post-hatch performance traits, the incubation environment has yet to be considered as a potential selection tool. A proteomic analysis on the early post-hatch sarcoplasmic protein profile when combined with BW may shed light on the genetics of post-hatch muscle growth.

4) Commercial Pekin ducks are being selected for BW and breast muscle. Duck meat is often favored by consumers due to its tenderness and juiciness (Baeza, 2006) and proteomic studies with duck muscle could contribute to our understanding of the mechanisms underlying its specific physiochemical features.

5) Duck breast muscle is reported to have mixed muscle fiber types, the majority of which is type IIa or fast-twitch oxidative and with a few of type IIb or fast-twitch glycolytic. Breast muscle selection in chickens and turkeys involves mostly type IIb and the current study with duck embryonic muscle will necessarily include proteins.
unique to both type IIa and type IIb.
Chapter 2: Myogenesis in Avian Embryogenesis

By definition, myogenesis is the process of muscle development and growth that begins in early embryonic development. Myogenic precursors or muscle progenitor cells, in concert with myogenic regulatory factors, give rise to myoblasts that first proliferate followed by a differentiation phase. Differentiated myoblasts eventually fuse together and align to form multinucleated myotubes and subsequently myofibers. The myogenesis pathway is directed by the previously mentioned myogenic regulatory factors such as MyoD, Myf-5, myogenin and Mrf-4 which are, in turn, regulated by the up-stream factors such as Wnt and Pax proteins (Gilbert, 1949). Skeletal muscle in vertebrates comes from the dermamyotome of the somite, of which the ventro-lateral portion will form the myoblasts that eventually give rise to the breast and limb. The aforementioned upstream proteins such as Wnt and Pax guide the differentiation by activating the family of MRFs.

At the end of incubation, the number of active myoblasts was found to be lower with total nuclei less than 5% when compared to the period of maximal embryonic growth (Allouh et al., 2008; Halevy et al., 2004).

The total number of myofibers is fixed at birth in bovine, swine and avian species (Stickland, 1978; Wigmore and Stickland, 1983; Stockdale and Miller, 1987). For avian species, myogenesis occurs primarily during incubation. In chickens, primary myofibers are formed at ED6 followed by a group of secondary myofibers that are believed to
originate from the primary myofibers. These secondary myofibers are reported to
differentiate between approximately ED12 and ED16. (Stockdale, 1992; Stickland et al.,
2004). Stockdale (1992) reported that myoblast numbers in chick embryos were most
abundant at ED5 and from ED15 onward. Adult myoblasts or satellite cells can be
distinguished by their morphology and location beneath the basal lamina of the myofibers
(Mauro, 1961). These myoblasts have been identified as the primary source of myogenic
precursors in postnatal muscle (Schultz et al., 1994). Chen (2012) reported that from
ED22 to ED28 (day of hatch), there was a 55% decline in myofiber size in the PM of
Pekin duck embryos. Satellite cells have been reported to be capable of re-entering the
cell cycle and initiate fusion into an existing or newly formed fiber (Hawke et al., 2001;
Schultz et al., 1994).

The detailed myogenic pathway based on broiler embryos has been well
summarized (Stockdale and Miller, 1987; Figure 1). There is insufficient duck-specific
research to support the model based on broilers that was proposed by Stockdale and
Miller (1987) for chickens. With a longer incubation period (28 vs. 21 days), it’s
generally accepted that the timeline of duck embryo development is extrapolated from
broiler embryos and similar molecular events may be delayed.
As stated earlier, muscle growth in the postnatal phase is mainly an event of hypertrophy – elongation in the sarcomere followed by an increase in the diameter of myofibers. The increase in length of myofibers occurs via the addition of sarcomeres but it’s believed that this process ends before the increase in myofiber thickness (Moss, 1986; Swatland, 1980). The neonatal stage of growth is an important period used by scientists who study the transformation and finale composition of muscle fibers of animals. Unlike chickens and turkeys, ducks are found to have mixed - fiber types in the PM. Information on fiber type transformation of the PM in chickens and turkeys can be found in Ashmore and Doerr (1971) and Aberle and Stewart (1983). Hatchlings are found to have type IIa but within the first week a majority of type IIa fibers transform into type IIb or white muscle fibers; the same process occurs within the 2nd week post-hatch in turkeys. Gille (1998) reported that the transformation (type IIa $\rightarrow$ type IIb) occurs from 20 to 26 days
post-hatch in ducklings and only 75% of the type IIa fibers begin this transformation. After 26 days, the composition of type IIb fibers is maintained at approximately 15%. As stated in the previous paragraph, postnatal muscle growth is mainly the result of hypertrophy, an increase in the volume of myocytes by the proliferation of satellite cells (Leslie and Davidson, 1951; Helmi and Cracraft, 1977; Remignon et al., 1994). Confined to its physiochemical characteristics, i.e. the need for oxygen for metabolism, type IIa fibers have less capacity for increasing their diameter when compared to type IIb fibers. This results in an average size of 25-30 µm for type IIa fibers versus 50 µm for type IIb fibers at 10 weeks of age (Table 2; Figure 2). The combination of decreased diameter size of type IIa fibers and fewer total type IIb fibers both contribute to reduced PM size in ducks compared to PM in chicken with mostly type IIb fibers.

<table>
<thead>
<tr>
<th>Age [days]</th>
<th>FTO [µm]</th>
<th>FTG [µm]</th>
<th>FTG [%]</th>
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<tbody>
<tr>
<td>0</td>
<td>2.74 ± 0.25</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>4.79 ± 0.34</td>
<td>—</td>
<td>—</td>
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<tr>
<td>13</td>
<td>9.61 ± 1.1</td>
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<td>20</td>
<td>10.88 ± 4.0</td>
<td>23.97 ± 2.6</td>
<td>25.7 ± 8.8</td>
</tr>
<tr>
<td>26</td>
<td>13.83 ± 4.3</td>
<td>26.84 ± 3.3</td>
<td>14.0 ± 7.8</td>
</tr>
<tr>
<td>33</td>
<td>15.87 ± 10.4</td>
<td>29.11 ± 7.4</td>
<td>16.2 ± 6.8</td>
</tr>
<tr>
<td>47</td>
<td>20.64 ± 3.4</td>
<td>36.74 ± 8.21</td>
<td>14.2 ± 7.3</td>
</tr>
<tr>
<td>62</td>
<td>26.73 ± 3.2</td>
<td>47.37 ± 6.3</td>
<td>11.8 ± 6.1</td>
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<td>76</td>
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<td>47.30 ± 8.8</td>
<td>18.0 ± 10.6</td>
</tr>
<tr>
<td>104</td>
<td>28.53 ± 4.4</td>
<td>48.62 ± 7.6</td>
<td>15.6 ± 6.4</td>
</tr>
<tr>
<td>146</td>
<td>30.83 ± 5.0</td>
<td>50.51 ± 9.8</td>
<td>14.0 ± 5.3</td>
</tr>
</tbody>
</table>

Table 1: Myofiber diameter by fiber type (means +/- standard deviation) and percentage of fast-twitch glycolytic fibers in the pectoralis muscle of white Pekin ducks (Gille et al., 1998). Fast-twitch oxidative (FTO) myofibers are type IIa fibers while Fast-twitch glycolytic (FTG) myofibers are type IIb fibers.
Figure 2: Fiber diameter and resulting Janoschek growth and growth rate curves of both type IIb and type IIa fibers in the pectoralis muscle (Gille et al., 1998). Fast-twitch oxidative (FTO) myofibers are type IIa fibers while Fast-twitch glycolytic (FTG) myofibers are type IIb fibers.
Chapter 3: Thermal Manipulation of Incubation Temperature

During the process of commercial incubation, incubation temperature serves as the primary external energy source for avian embryonic development. In addition to the incubator (ambient) temperature, the humidity and concentrations of oxygen and carbon dioxide are also monitored at regular intervals. In commercial incubation, there is not a single temperature that is maintained throughout embryonic development and this has been investigated extensively. Romanoff (1936) was the first report that the optimal incubator temperature was 37.5°C and it is still considered to be the optimal average temperature used in commercial practice today.

At the start of incubation in chickens, an energy reserve of approximately 88 kcal is deposited in the yolk and albumen, the 70 kcal deposited in the yolk is primarily in the form of triglycerides. During late incubation, the yolk reserve is reduced to 28 kcal while total kcal deposited in the chick is 36 kcal. Approximately 24 kcal of energy is lost as metabolic heat during the process of embryo growth (Romanoff, 1967; Rahn, 1981). During the first nine days of incubation, embryonic heat production cannot maintain an egg temperature at 37.5°C so external, supplemental heat is needed. Metabolic heat production rises steadily during embryonic development and increases dramatically during the last 10 days. In broiler embryos, the shell temperature should be the same as the ambient temperature at ED10 and from ED11 through time of hatch, shell
temperature will progressively increase and reach a 2-degree differential by the end of incubation (Robert, 1996). This differential is the prerequisite for managing energy flow in and out as embryos transition through different stages of development (Briedis and Seagrave, 1984).

In in-vitro studies with primary myoblasts from broiler embryos at ED17, short-term and direct exposure of these myoblasts to increased temperature (3 or 6 hrs at 39.5°C) was reported to increase cell proliferation. However, proliferation was not detected when the cells were exposed to 40.5°C. The authors suggested that IGF-1 contributed to the increased muscle cell proliferation (Yoge et al., 2009). Persistent stimulation of IGF-1 expression through 2 weeks post-hatch was recorded in broiler embryos and chicks after thermal manipulation during late-term incubation (Yoge et al., 2009). In work reported by Bowers (2008), Pekin duck embryos exhibited accelerated development through approximately ED20 when incubated at 38.5°C versus 37.5°C for the first 10 days of incubation. It is hypothesized that thermal manipulation during this early period of embryonic development may be a driver of accelerated embryonic development which may express itself in a differential expression of muscle proteins which can be visualized via 1D SDS-PAGE electrophoresis.
Chapter 4: Sarcoplasmic Proteins

In this study, our hypothesis is that the pattern of breast muscle sarcoplasmic proteins will be different in Pekin duck embryos that are exposed to an early increase in incubation temperature and this can be documented via a combination of 1D SDS-PAGE and image analysis software. A single muscle fiber is composed of three protein fractions: sarcoplasmic, myofibrillar and stromal. Sarcoplasmic proteins are soluble in a low salt buffer while myofibrillar proteins are high-salt-soluble. Stromal proteins are primarily insoluble collagen which can be dissolved in acid and myofibrillar proteins are generally believed to be those proteins that constitute the contractile apparatus (i.e. myosin, actin, and tropomyosin) and the scaffolding materials providing support for the myocytes. The sarcoplasmic proteins are mainly the proteins involved in metabolism and may include soluble enzymes involved in anaerobic metabolism and mitochondrial enzymes comprising the tricarboxylic acid cycle and electron transport system. In meat processing, muscle pigment proteins and proteases are especially important for their effect on the physical characteristics of meat. Based on previously published studies and preliminary data, we hypothesize that the proteins present in the sarcoplasmic fraction may be uniquely expressed as a result of differential incubation temperatures. One of the reasons we did not include contractile proteins in our research is that some of these proteins are not within the linear dynamic range of those proteins we could identify with
our research approach. The separation and isolation of sarcoplasmic proteins from the muscle is a simple but effective approach. The separation approach used is outlined in Figure 3 below.

Figure 3: Flowchart of sarcoplasmic proteins separation.
Chapter 5: Characteristics of Duck’s Meat

Duck meat production is primarily derived from the commercial practice of crossbreeding different Pekin duck strains (Anas platyrhychos; Pingel, 1997; Zeidler, 1998; Md. Shawkat et al., 2007). Duck breast is considered to be red meat in that adult ducks are reported to have mixed fiber types with the majority being type IIa fibers when compared to chicken breast muscle (Smith et al., 1993; Md. Shawkat et al., 2007). It has been reported that a one-week-old duckling has 84% type IIa fibers a proportion of total breast muscle fibers (Smith, 1993). Shawkat (2007) reported that the meat from a 45-day-old broiler had a higher percentage of protein and ash while duck meat had a significantly higher proportion of fat. Smith (1993) also reported that duck breast meat had increased moisture and lipid and was lower in protein, ash, and calories compared with broiler breast muscle. Increased muscle pigmentation as reported by Shawkat (2007) supports the observations by Smith (1993) that duckling breast muscle has a higher proportion (84%) of type IIa muscle fibers.

In terms of muscle fiber type, it’s fair to compare duck meat to beef in terms of storage method and time. The physical characteristics of duck meat: lightness, redness and yellowness increase with length of storage time (Feldhusen et al., 1995; Insausti et al., 1999). Tenderness decreases with storage time in beef (Morgan et al., 1991) which is the same pattern observed in ducks as evidenced by increased shear force (Shawkat, et
Shawkat et al. (2007) also reported that there was an increase in percentage cooking loss and increased sheer force (kg/cm2) in duck versus chicken breast muscle. This supports the previous observations by Smith and Fletcher (1992) that duckling breast had higher Allo-Kramer shear force values than chicken meat during the 0 to 24 hr. postmortem period. Alvarado and Sams (2000) reported similar observations of increased cooking loss and shear force value at different post-mortem deboning times in broilers, turkeys and ducks. Higher cooking loss and less emulsion stability in duck breast meat may be attributed to the reduced water-holding capacity due to its increased lipid content (Joseph et al., 1972). However, the higher lipid content is thought to vaporize more readily than water during cooking and may result in a higher cooking loss and possibly lower emulsion stability. An emulsion activity test done with patties from duck, broiler and spent hen meat showed that there was enhanced emulsion stability because fat and water can be retained inside the meat matrix in the latter two species. (Biswas et al., 2006).
The most common duck used for meat production in the U.S. is the white Pekin duck which originated in northern China and imported into the U.S. in the 1870s (Cherry and Morris, 2008). It was after the importation into the U.S. that the Pekin duck started producing purely white feathers or down and was developed for improved market weight. The BW of a mature male is approximately 10 - 11 lbs. and a female 7 - 9 lbs. Pekin drakes can be sexed by the sex feather in their tail. The present day commercial duck is known for it fast growth rate and can reach a market weight of approximately 7.3 lbs. at 8 weeks. However, the Pekin duck has a lower percentage of PM and excessive subcutaneous and intramuscular fat which cause it to be less popular in certain regions in Asia.

In addition to Pekin ducks, the Muscovy duck is also a meat-type duck that originated in Central America. This species is known for its flightiness and sexual dimorphism. Males grow slower than females and the market weight for male is 11 lbs. in 12 weeks. The Muscovy duck has less fat and better meat quality than the Pekin duck (Baeza et al., 1998). In terms of the global market for duck meat consumption, ducks bred for meat are either purebred or crosses. In China and south Asia, the market for duck meat is served primarily by Pekin ducks, Tsaiya ducks, Muscovy ducks and the crossbreds of these three breeds. In Southeast Asia, ducks are widely raised by local
farmers because their maintenance cost (i.e. housing, equipment) is low. As a forager on weeds and pests, farmers often find them to be beneficial in rice production systems.

As stated earlier, Pekin ducks originated in northern China and Tsaiya ducks were first found in southern China and south Asia. Tsaiya ducks were initially reared for both egg and meat production. The white feather type of Tsaiya duck was crossbred with a Pekin drake to form a new line, Kaiya duck, which was later bred with Muscovy drakes to form a Mule duck. Mule ducks produced from Kaiya ducks and Muscovy drakes can grow to 6.5 lbs. in 10 weeks, faster than mule ducks derived from breeding Tsaiya ducks straight with Muscovy drakes. The reason for using the Tsaiya duck in this type of breeding system is due to consumer preference in Asia for less carcass fat than what is observed for pure Pekin ducks while the Kaiya progeny still retain the fast growth characteristics of Pekin ducks. The progeny from Kaiya ducks and Muscovy drakes will produce more edible meat in same amount of time.
Chapter 7: Statistic Modelling of Muscle Growth Using MBP as Independent Predictor

Proteomic studies done previously in our lab have shown that the MBP from individual bands within an electrophoretic gel can be incorporated sequentially into a multiple linear regression model which can be used to predict the dynamic growth of muscle (Updike, 2006; Reddish, 2008). Using the image analysis software Totallab™, the value of MBP is defined as individual band’s pixel volume divided by total pixel volume within an individual lane. It is hypothesized that determining the MBP of individual proteins/peptides can be a valuable tool in studying the expression level of certain proteins within a myocyte. This is the basis of a statistical model that predicts the BW and PMW using proteomics in which the muscle mass of animals during growth is correlated with the MBP of sarcoplasmic proteins/peptides as effected by environmental factors such as diet (Reddish, 2008; Zapata, 2011).

The statistical analysis of the data from this experiment is based on embryonic BW, PMW and the MBP of individual proteins/peptides as determined by 1D SDS-PAGE. The BW, PMW and MBP will be the response or dependent variables with environment (incubation temperature) and age (ED12, ED18, ED25, Hatch) serving as the independent or predictor variables.
Chapter 8: Experiment Design

The ultimate goal is to identify the effect of incubation temperature (37.5 °C or 38.5 °C) during early incubation (0 to 10 day) on myogenesis in Pekin duck embryos by profiling PM sarcoplasmic protein expression. A simplified graph of the experimental design is shown in Figure 4. The sarcoplasmic proteins were separated by 1D SDS PAGE according to their molecular weights and the individual protein bands within the PM were visualized using Coomassie blue staining. The raw images were digitized on a flatbed scanner and subsequently analyzed using TotalLab™ 1D software from which the molecular weight and MBP was estimated. The MBP value has already been established as a representative way of determining the relative expression of individual proteins (Sawdy et al., 2004; Updike et al., 2006; Reddish et al., 2008)

The MBP of individual bands was incorporated into a prediction model which indicated which, if any, proteins are specifically associated with the overall increase in embryonic yolk-free BW resulting from differences in incubation temperature.
Figure 4: Experiment design.
Chapter 9: Methodology

The approach to this experiment was based on an understanding of commercial incubation practice and similar proteomic studies in chickens and turkeys. The design of the experiment was discussed in the Chapter 7. Similar proteomic studies have been reported for other poultry species (Zapata, 2011, 2012; Huffman et al., 2013). The exception is the image analysis step in which modifications were made on the data processing when compared with previous reports.
Incubation and the Collection of Pekin Duck Eggs

Fertile Pekin duck eggs (n = 200) were all obtained from Maple Leaf Farms, Leesburg, IN. Through the first ten days of incubation, half the eggs were incubated at either 37.5 °C or 38.5 °C in each of two incubators with humidity set at 56%. On day 11, all eggs were moved to the same incubator and incubated at 37.5 °C for the remainder of incubation (28 days).

Ten eggs were removed from the control (37.5 °C) and high temperature (38.5 °C) treatments, respectively, at ED12, ED18, ED25, Hatch and PH8. Not all eggs were fertile so the actual number of embryos or ducklings sampled are shown below in Table 1.

<table>
<thead>
<tr>
<th>Sample Age</th>
<th>Embryonic Day 12 *</th>
<th>Embryonic Day 18 *</th>
<th>Embryonic Day 25 *</th>
<th>Hatching</th>
<th>8-day post-hatch**</th>
<th>Total samples collected at each incubation temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample No.</td>
<td>37.5°C</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>38.5°C</td>
<td>6</td>
<td>10</td>
<td>9</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Total samples collected at each sampling age</td>
<td>14</td>
<td>17</td>
<td>16</td>
<td>20</td>
<td>18</td>
<td>85</td>
</tr>
</tbody>
</table>

Table 2: The number of samples was collected and included in the following sarcoplasmic protein preparation and the analysis step (Sampling age with superscript *).
Sarcoplasmic Preparation of Muscle Sample

Pekin duck embryos and ducklings were euthanized by cervical dislocation and samples of the PM were immediately collected. All tools used during the dissection process were cleaned with 70% ethanol and kept inside a laminar flow cabinet. Sarcoplasmic proteins were prepared according to Zapata et al. (2009). Muscle tissue was homogenized in a rigor buffer [10 mM trismaleate, 60 mM KCl, 5 mM MgCl₂, 1mM EGTA, and 0.4 mM Pefabloc SC Plus, (Boehringer Mannheim Corp., Indianapolis, IN), pH 6.8; Ishiwata and Funatsu, (1985)] and the homogenate centrifuged at 10,000 x g for 5 minutes at 4°C. Sarcoplasmic proteins were removed from the supernatant fraction and combined with sample buffer (8 M urea/2 M thiourea, 75 mM dithiothreitol, 50 mM Tris, 3% SDS, 0.004% bromophenol blue, pH 6.8). The mixture was kept on ice for 30 minutes.

Measurement of Total Sarcoplasmic Protein Concentration

Prior to electrophoresis, the total protein concentration of each sample was measured using a Micro BCA Protein Assay (Thermo Scientific Pierce, Rockford, IL). The determination of unknown sample protein concentrations using a BCA assay is based on a linear regression model with a bovine serum albumin (BSA) standard. The effective measurement is made from the linear dynamic range of the total protein concentration between 0.5 – 20 micrograms per milliliter. The sarcoplasmic protein portion of each sample was subsequently diluted to assure equal protein concentrations for each electrophoresis measurement. A standard curve was plotted for each respective
sarcoplasmic protein prep and there were no significant differences observed between standard curve plotted by BSA and sarcoplasmic proteins (Figure 5).

Figure 5: Standard curve of total sarcoplasmic protein concentration using BCA assay.

Sodium Dodecyl-Sulfate Polyacrylamide Agarose Gel Electrophoresis

Sarcoplasmic proteins were resolved on SDS-PAGE according to the protocol reported by Updike et al. (2006). Electrophoresis was performed using a 1.5 mm x 12 cm x 14 cm polyacrylamide slab gel consisting of a 5-20% gradient resolving gel (30:0.8, acrylamide: N,N’-bis-methylene acrylamide) and 3% stacking gel containing 1% SDS.
The amount of sarcoplasmic proteins to be loaded on the gel was adjusted through serial
dilution in order to obtaining better resolution. As mentioned previously, the protein
concentration of each sample was determined using a BCA test so a constant amount of
protein could be applied to each lane within a gel. The same volume of each sarcoplasmic
protein extract was loaded onto each lane of a 10-lane gel. Based on preliminary results,
this volume gave the best resolution across samples. A broad range molecular weight
standard was also loaded in each gel and sarcoplasmic proteins were separated by
applying a constant voltage of 10 V.cm\(^{-1}\) until the dye front reached the bottom of the gel.
After electrophoresis, the gel was stained with Coomassie blue and subsequently de-
stained in 10% acetic acid according to Huffman et al. (2012). Staining and washing
were repeated until a desirable level of background was achieved.

Image Analysis

Image analysis was conducted according to (Zapata, 2009). Gels were scanned
using a Typhoon 9400 scanner and raw images were subsequently imported into
Totallab\textsuperscript{TM} TL 120 1D software (Nonlinear Dynamics, Newcastle upon Tyne, UK). The
bands were identified and analyzed to determine the MBP or percentage contribution of
each band to the total band volume in the same lane.
Confidence of Single Concentration SDS-PAGE Gels

In the present study embryonic myogenesis was viewed through the expression of and changes in a variety of sarcoplasmic proteins on 10% SDS-PAGE gels. The use of mini-size (1.5 mm x 12 cm x 14 cm) 10% discontinuous SDS-PAGE gels has already been established as a reliable approach to proteomic studies. The gels were further analyzed by TotalLab™ software for image analysis. Although mini-size SDS-PAGE gel electrophoresis is a proven preliminary tool, a discontinuous and single concentration SDS-PAGE gel was conducted to test for the linear dynamic range of the commercial broad range protein standards. Proteins prepared from animal muscle are often separated based on the differences in migration rate and their size can be extrapolated by imputing their relative mobility value (Rf) into a linear equation of relative mobility regressed with log-transformed molecular weight from the commercial molecular weight standard mix present on all gels. Previous research using proteomic studies conducted with SDS-PAGE have reported that protein electrophoresis is stable although there are concerns over gel-to-gel differences and the co-migration happening from proteins of similar molecular weight. Electrophoresis with a single gel concentration will show a better resolution with proteins within a particular range of molecular weights. In the studies reported herein, 10% polyacrylamide gels were used to resolve proteins in the range of "20–200" kD. As seen in Figure 6, bands that fall outside the 95% confidence interval range are found on the edge of gels and were not included in the calculation of MBP.
Figure 6: Probability plot of relative mobility value (R_f) of broad range protein ladder (PageRuler, Thermo Scientific™) from all one dimensional sodium dodecyl-sulfate polyacrylamide agarose gels (Dots with R_f near 0 or 1 are proteins whose molecular weight is below 20 kD or above 200 kD).

Statistical Analysis

The statistical model has already been described in Chapter 6. BW and PMW of embryos were analyzed by PROC GLM in SAS (9.3). MBP was analyzed by factorial analysis using PROC MIXED in SAS (9.3). A simplified flowchart is provided here to summarize the statistical approach used for the current experiment.
Experiment Model:

- Generalized linear model –
  
  a. Response variables – yolk-free body weight/ pectoralis muscle weight
  
  b. Predictor variables – Incubation temperature and incubation age

- Analysis of MBP from the effect of incubation temperature and age.
  
  a. MIXED procedure in SAS 9.3
  
  b. Factorial analysis.
  
  c. Solid when model response is in linear relationship to predictor variables.
Chapter 10: Results

Effect of Incubation Temperature on Yolk-free Body Weight and Body Weight of Pekin Duck Embryos and Ducklings

Statistical analysis of the effect of incubation temperature and incubation period (Age) and the interaction of both on log-transformed yolk-free embryo BW was performed by employing SAS 9.3 GLM with a significance level ($\alpha$) at 0.05. The PROC GLM procedure was chosen because this takes into consideration the unequal-sample-sizes associated with our data at different incubation ages. With logarithm transformation we believe that differences in embryonic BW are more readily detected as these changes are relatively small in term of a larger scale of incubation period.

The result of the GLM analysis shows that yolk-free BW was significantly affected by embryo age ($p < 0.001$). There was no significant overall effect of incubation temperature on embryo yolk-free BW ($p = 0.334$) but there was a significant interaction of incubation temperature and age ($p = 0.0385$) with temperature effects observed at ED12 (3.09 vs. 2.79 grams; $p = 0.0415$; Figure 7). No differences in embryo yolk-free BW were observed at ED18 and ED25 (Figure 7). At hatch, ducklings in the control temperature were heavier than ducklings exposed to the higher temperature ($p = 0.071$;
Figure 7). At 8D post-hatch, ducklings from the control group had the same BW as those in the treatment group (p = 0.6485; Figure 8).
Effect of Incubation Temperature on pectoralis muscle weight of Pekin duck embryos and ducklings

The statistical analysis of the effect of incubation temperature, age and the interaction of temperature and age on PMW was performed using the GLM model of SAS 9.3, similar to the analysis done on yolk-free embryo BW. The data from 8D post-hatch was analyzed independent of the embryonic data. The log-transformation of PMW, which is even less than BW, allow us to eliminate confounding influence form incubation period. Among predictors, incubation age had a significant effect on PMW \( (p < 0.0001) \). There were no significant differences in the PMW due to incubation temperature at ED12, 18, 25, Hatch (Figure 9), or 8 days post-hatch (Figure 10).
Figure 9: Pectoralis muscle weight of Pekin duck embryos during incubation.

Figure 10: Pectoralis muscle weight of Pekin ducks on 8th day post hatch.
One-dimensional Sodium Dodecyl Sulfate Polyacrylamide Agarose Electrophoresis

Sarcoplasmic proteins extracted from each sample were mixed with sample buffer (urea/thiourea), quantified by BSA assay and analyzed after protein on 1D SDS-PAGE. The electrophoresis migration pattern was visualized with Commassie Brilliant Blue G-250 over night and washing with 10% acetic acid as shown in Figure 11. The raw images were then inputted into the Totallab™ software which designates the proteins/peptides bands with a pixel volume value that was unique to each individual sample. The following procedure was adapted to assure that sarcoplasmic protein portions of similar quantities from the same sample after multiple electrophoresis runs show observable intensity. One lane from three of the most consistent runs were selected and inputted into the image analysis.

The results showed that samples across multiple individuals, ages and temperature have a variable number of bands as observed by the image analysis – Totallab™ software. In order to facilitate the proper statistical analysis based on enough replicates, a synthetic lane was formed as shown in Figure 12. A total of 34 bands are shown in the synthetic lane. To be more specific, if a band is not detected in one embryo but in all other embryos, a manual band-picking step is performed at the same pixel position (longitudinal axis) as shown in Figure 12. In this way, essentially adding a band of this “zero” MBP can allow the statistics to proceed and also not affect the original MBP values of the other bands.
Figure 11: One-dimensional sodium dodecyl sulfate polyacrylamide agarose gel electrophoresis pattern. L1 is commercial broad range protein ladder; L2, 3 are ED12 embryos; L4, 5 are ED18 embryos; L6, 7 are ED25 embryos; L8, 9 are Hatch embryos. * stands for treatment group (38.5°C).

Figure 12: Lane profile of ED12 samples after manual band-picking from Totallab™
Values from the MBP of all samples were compiled in an Excel spreadsheet that was later processed into SAS 9.3. The statistical model was constructed on the assumption that the MBP will be reflective of total band numbers, age and incubation temperature effects on the Pekin duck embryos. The MIXED procedure of SAS 9.3 was performed using the type III variance to calculate the partial effect on MBP due to either age or temperature. The results showed that the interaction effects of band x age or band x temperature were both significant whether or not we used the arithmetic mean (band x age: p < 0.0001; band x temperature: p < 0.005).

There were 18 bands that were determined to be responsive to age and 3 bands that were responsive to increased early incubation temperature. However, as several hypotheses were being tested in this statistical model, the multiplicity might be the result of accumulated error and lead us to reject the null hypothesis when the null hypothesis is true (type I error). In order to address this, a Bonferroni correction was used to lower the incidence by dividing the significance level (p = 0.05) by band and treatment number, 4 for age and 2 for temperature. Therefore, the significance level will be 0.00036 for age and 0.00073 for temperature. After justification, bands responding significantly to age or temperature are listed in Table 3.
<table>
<thead>
<tr>
<th>Band Index (No.)</th>
<th>Effected by Age</th>
<th>Effect by Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>*</td>
<td>NS</td>
</tr>
<tr>
<td>10</td>
<td>*</td>
<td>NS</td>
</tr>
<tr>
<td>12</td>
<td>*</td>
<td>NS</td>
</tr>
<tr>
<td>13</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>14</td>
<td>*</td>
<td>NS</td>
</tr>
<tr>
<td>15</td>
<td>*</td>
<td>NS</td>
</tr>
<tr>
<td>19</td>
<td>*</td>
<td>NS</td>
</tr>
<tr>
<td>20</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
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<td>*</td>
<td>*</td>
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<td>22</td>
<td>*</td>
<td>NS</td>
</tr>
<tr>
<td>23</td>
<td>*</td>
<td>NS</td>
</tr>
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<td>24</td>
<td>*</td>
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<td>32</td>
<td>*</td>
<td>NS</td>
</tr>
<tr>
<td>33</td>
<td>*</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 3: Result of MIXED proc. in SAS 9.3 (* = significant; NS = non-significant).

The charts of MBP of bands No. 13, 20 and 21 are shown in Figure 13-15. This experiment can be helpful in categorizing protein expression during embryonic development by looking at bands responsive to the effect of age. However, the purpose of this experiment was to identify proteins that were responsive to the early incubation temperature change. With that being said, charts of bands analyzed being responsive to age will not be shown and those responsive to temperature are listed in Figure 13-15.
Figure 13: Change of mean band percentage of Band No.13 over time and treatment.

Figure 14: Change of mean band percentage of Band No.20 over time and treatment.
Figure 15: Change of mean band percentage of Band No.21 over time and treatment.
Chapter 11: Discussion

The yolk-free body weights of Pekin duck embryos were approximately 0.2 to 0.3 grams at ED12, 20 to 21 grams at ED18, 46 to 47 grams at ED25 and 56 to 60 grams at hatch. The respective body weights are similar to the values reported by Bowers (2008; ED 18) and Gu (2013; ED 12, 18 and 25). The pectoralis muscle was similar in weight to what was reported by Bowers (2008) and Gu (2013). Under commercial incubation temperature conditions (37.5°C), Gu (2013) reported an increasing phase of pectoralis muscle growth from ED10 to ED20 followed by a plateau phase starting at ED22 to ED27, which might suggest a decline in the effects of selected myogenic growth factors (i.e. MyoD, Mrf-4). Myoblast numbers may be fixed at or before ED20 and the transition from proliferation to the fusion of myoblasts is suspected to occur between ED14 and ED18 based on differences in morphology. A late atrophy in pectoralis muscle mass as documented by Gu (2013) and Chen (2012) suggests that the Pekin duck embryonic pectoralis muscle undergoes a reduction in mass and cross section area of muscle fiber bundles between ED22 and hatch. In late-term turkey embryos, Moore (2005) also observed a similar reduction in myofiber cross-section area followed by decreased mitotic activity. The present results also show that the weight of the pectoralis muscle is significantly lower at hatch compared with ED25 (0.64 g vs. 0.72-0.76 g at ED25). This reduction in the weight of the pectoralis muscle may be due to protein mobilization
needed for energy prior to hatch (Christensen et al., 2001; Uni et al., 2005), proliferation of lymphocytes (Rudrappa and Humphrey, 2007) and intestinal development (Uni et al., 2003). In-ovo injection of nutrients has been reported to spare the mobilization of pectoralis muscles (Uni et al., 2005).

In order to analyze the MBP data, a manual band-picking process was performed for sufficient replicate comparisons. The PROC MIXED procedure of SAS with a SLICE EFFECT was performed to identify candidate bands with MBP that were affected by incubation temperature. The analysis showed a significant interaction of band*temperature (P < 0.005) and three bands were significantly affected by temperature (P < .001; Figures 13-15). The PROC MIXED procedure is a factorial analysis method that is robust and allows us to answer our hypothesis: some embryonic proteins will be affected by incubation temperature and age.

Within commercial incubators, there are isolated areas where the optimal incubation temperature (37.5°C) can fluctuate and small increases in temperature can occur which can influence embryonic development. Bowers (2008) showed that accelerated embryonic growth in Pekin duck embryos when exposed to a higher incubation temperature (38.5°C) from 0 to 10 days resulted in an overall increase in the length of tarsal bones. The current experiment SDS-PAGE further unveiled the complex interaction of proteins during embryonic growth.

The objective of the present study was to see if individual proteins would be responsive to increases in early incubation temperature. In samples from both the control and treatment temperature, the majority of the proteins were primarily responsive to
developmental age. The MIXED model was used to determine if there were candidate proteins that responded to early thermal manipulation and three bands were identified based on their respective MBP values. Further research needs to be done to identify which aspect of growth these bands (proteins) represent.

Sarcoplasmic proteins are involved in both aerobic and anaerobic metabolism. With most of the sarcoplasmic proteins having molecular weights below 200 kD, 1D SDS-PAGE can effectively separate proteins based on their size. In Zapata et al (2009; 2012), the feasibility of using 1D SDS-PAGE and Totallab™ to analyze the sarcoplasmic proteins of avian muscle extracts was established. In the present study, a standardized approach was used to monitor the quality of SDS-PAGE gels. Regression analysis formulated on $\log_{10}(\text{MW})$ and $R_f$ from commercial protein standard run on all gels were tested for equal variance and those bands on upper and bottom edges of gels (20-200kDa) were excluded from the calculation of MBP due to the fact that their extrapolated M.W. from Totallab™ was outside the linear dynamic range. This standardized approach is innovative and can improve the efficacy of protein sequencing at some later stage.

The study confirmed the accelerated growth in embryos due to temperature manipulation in early stage of incubation. Protein sequencing would be an important future step in identifying which proteins are most sensitive to potential environmental effects on embryonic development.
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