RNA-SEQUENCING ANALYSIS OF WOODEN BREAST DISEASE: CHARACTERIZATION OF A NOVEL MUSCLE DISEASE IN CHICKENS THROUGH DIFFERENTIAL GENE EXPRESSION AND PATHWAY ANALYSIS

by

Marie F Mutryn

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Animal Science

Spring 2015

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Marie F Mutryn

Approved: _____
Behnam Abasht, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Limin Kung, Ph.D.
Interim Chair of the Department of Animal and Food Sciences

Approved: _____
Mark Rieger, Ph.D.
Dean of the College of Agriculture and Natural Resources

Approved: _____
James G. Richards, Ph.D.
Vice Provost for Graduate and Professional Education

ACKNOWLEDGMENTS

The project was funded and supported by Heritage Breeders, LLC. I would like to thank my advisor Dr. Behnam Abasht for taking me on as a graduate student in his lab and for his continued advice and motivation during my time at University of Delaware. I would also like to thank my committee members, Dr. Erin Brannick, Dr. Jack Gelb, and Dr. Bruce Stewart-Brown for their expertise and numerous suggestions through my entire thesis. Many thanks also to my lab members: Weixuan Fu, Nan Zhou, Zhu Zhuo, Michael Babak, and Abby Buurma. I cannot thank them enough for the help and support they provided me both in the lab and the writing process. I thoroughly enjoyed getting to know each and every person! Finally, I would like to thank my family and friends for their continued guidance, support, and love.

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ABSTRACT

Improvements in poultry production within the past 50 years have led to increased muscle yield and growth rate, which may in turn be contributing to an increased rate and development of new muscle disorders in chickens. Previously reported muscle disorders and conditions are generally associated with poor meat quality traits and have a significant negative economic impact on the poultry industry. Recently, a novel myopathy phenotype has emerged which is characterized by palpably “hard” or tough breast muscle. The objective of this study is to identify the underlying biological mechanisms that contribute to this emerging muscle disorder colloquially referred to as “Wooden Breast”, through the use of RNA-sequencing technology.

We constructed cDNA libraries from five affected and six unaffected breast muscle samples from a line of commercial broiler chickens. After paired-end sequencing of samples using the Illumina Hiseq platform, we used Tophat to align the resulting sequence reads to the chicken reference genome and then used Cufflink to find significant changes in gene transcript expression between each group. By comparing our gene list to previously published histology findings for this disorder and using Ingenuity Pathways Analysis (IPA®), we aimed to develop a characteristic

gene expression profile for this novel disorder through analyzing genes, gene families, and predicted biological pathways.

Over 1500 genes were differentially expressed between affected and unaffected birds. There was an average of approximately 98 million reads per sample, across all eight samples. Results from the IPA analysis suggested “Diseases and Disorders” such as connective tissue disorders, “Molecular and Cellular Functions” such as cellular assembly and organization, cellular function and maintenance, and cellular movement, “Physiological System Development and Function” such as tissue development, and embryonic development, and “Top Canonical Pathways” such as, coagulation system, axonal guidance signaling, and acute phase response signaling, are associated with the Wooden Breast disease. Some pathways that were also considered in depth were “Actin Cytoskeleton Signaling” and “Axonal Guidance Signaling”.

There is convincing evidence by RNA-seq analysis to support localized hypoxia, oxidative stress, increased intracellular calcium, as well as the possible presence of muscle fiber-type switching, as key features of Wooden Breast Disease, which are supported by reported microscopic lesions of the disease.

We also conducted a large-scale phenotypic study of over 2,500 commercial broiler chickens raised under commercial conditions for 29 days in which they were placed into experimental stations to measure individual feed efficiency until market-age at 47 days. This experimental study was designed to understand the incidence rates and phenotypic trait correlation with Wooden Breast disease. This newly

described myopathy has been reported to severely impact as much as 10% (per flock) of commercial bred chickens. During the feed efficiency trial, chickens were housed in an experimental station to measure individual feed intake, and ultimately scored for Wooden Breast disease by manual palpation and visual observation of macroscopic lesions under the groups: “hard”, “normal”, and “soft”. At the conclusion of this study, multiple phenotypic characteristics such as: initial body weight, final body weight, final breast muscle weight, feed consumption, final abdominal fat weight, and pH were recorded to use for statistical analyses. Records of initial and final body weights and feed consumption were used to estimate each bird’s residual feed consumption as a measure of feed efficiency. These traits were analyzed statically by Tukey’s HSD test to determine differences in mean phenotypic values using each phenotypic measurement as the independent variable and breast muscle hardness score as the dependent variable. From our data, we find evidence to suggest that hardness of the breast muscle is significantly associated with high performing birds that exhibit increased body weight, breast muscle weight, and feed efficiency, as well as lower abdominal fat weight. We also can conclude that breast muscle hardness is also associated with breast muscle that had higher ultimate pH values.

Chapter 1

BACKGROUND AND LITERATURE REVIEW

A cluster of commercial broilers in the Delmarva region has recently been found to exhibit a skeletal muscle myopathy in the pectoralis region causing the muscle to become extremely firm. The broilers' breast muscle has changed in texture and color, becoming tough and discolored, as well as pale. The "hard muscle" phenotype, colloquially referred to as "wooden breast", was relatively unknown in the literature until it was presented by Sihvo et al. 2014 [1]. Hereafter this syndrome will be referred to as Wooden Breast disease. Sihvo et al. 2014 [1] cite the issue's occurrence in Finland, which provides evidence for the global distribution of the problem. Specifically, they studied the microscopic and histologic properties of pectoralis major muscles of ten broilers between five and six weeks old. From their report, histological evidence supports multifocal degeneration and necrosis of the tissue, hypereosinophillia, infiltration of inflammatory cells, and myofiber degeneration. Although they speculate that nutritional deficiencies of vitamin E and selenium, exertional myopathy and hypoxia could cause the occurrence of these histologic changes, the localization of these lesions occurring solely in the breast muscle cannot be accounted for. Sihvo et al. (2014) [1] suggest the hardness of the breast muscle may be due to fibrosis, otherwise known as connective tissue buildup

within the tissue space. Interestingly, they also cite the correlation of the occurrence of white striping within birds affected with Wooden Breast disease. Sihvo et al. (2014) [1] report provides the first description of this muscular disorder, and maintains that the underlying etiology of this disorder remains unknown. [1]

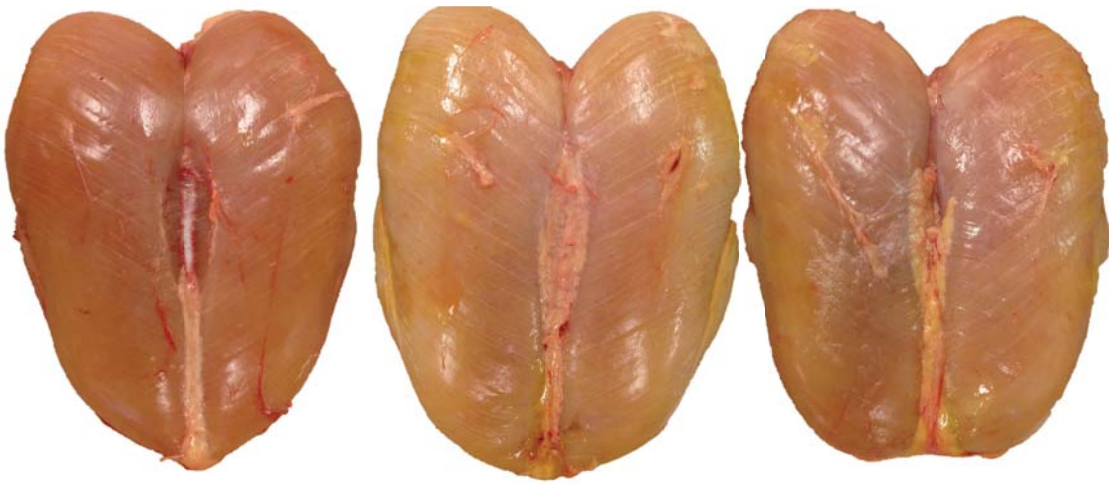


Figure 1 **Visual Appearance of Wooden Breast Disease**. Pictured above are pectoralis major ordered left to right in increasing severeness of Wooden Breast disease. From left to right: normal breast muscle, moderate to severe affected breast muscle, severely affected breast muscle.

The disease has had a significant implication for the industry as the changing meat quality has resulted in a product unpalatable for human consumption. To my knowledge, there have been no attempts to characterize the RNA sequencing profiles and related pathways of Wooden Breast to better uncover specific biological

mechanisms related to this problem. This research will reveal genes that are upregulated and downregulated in Wooden Breast affected birds. The data obtained by RNA-sequencing will allow comparison of gene expression profiles between affected vs. unaffected birds. From this work, it may be possible to gain insight into the specific genes and pathways that play a role in development of this muscular disorder.

Knowing the genetic components of various muscle disorders, such as Wooden Breast, will be key to identifying specific molecular markers, which will ultimately be beneficial to breeding programs around the world. Selection against undesirable characteristics will optimize breeding programs for the best possible output of traits. It is also likely that after analyses of RNA-sequencing data, new genes underpinning the disorder will be discovered and can be added to the annotation of the chicken genome. Understanding the genetic components of the Wooden Breast phenotype is crucial for the next step of research and may provide insight into possible solutions for this important problem.

1.1 The Importance of Meat Quality Traits

Meat quality traits in poultry have been historically studied and documented and continue to be of importance as the poultry industry maintains economic growth. Skeletal muscle damage is an issue of importance because of the influence and role of the broiler industry on global food production. According to the USDA, the United States broiler industry is the largest meat-producing segment in the United States and

is among the largest industries in the world [2]. As of 2005, it was responsible for raising approximately 9 billion broilers a year and generating close to 21 billion dollars in profits [2]. In 2007, broilers accounted for 39% of total meat consumption worldwide, with the United States representing the highest overall total consumption of broiler chickens [3].

Consumer demand has driven the poultry market and this demand has resulted in continual bird growth over the past 50 years. Historically, poultry was acquired in the form of a whole carcass. Recent demand has influenced a shift in production towards specific cuts of meat such as breast, thigh, and leg [4]. This has resulted in a variety of processed cuts that are available for purchase by consumers [4]. To consumers, meat tenderness and texture are the most important factors assessment of overall meat quality [5, 6]. Consumers also consider juiciness, drip-loss, cook-loss, and shelf-life of the meat in their purchasing decisions [7]. Therefore, one can assume that muscle diseases, such as Wooden Breast disease may impact consumer's choices. If consumers tend to choose their meat based on texture and quality, it is likely that they would not choose hard breast muscle meat, if available, because this is an undesirable characteristic. It is important to understand the cause and origin of the Wooden Breast myopathy so that resources and products do not go unused.

Genetic selection based mainly on phenotypic and pedigree information has led to improvements in poultry growth and overall muscle yield. The goal is to breed birds that produce large amounts of meat in a shorter amount of time [8]. The ever-increasing global human population and basic consumer preferences have largely

impacted the industry. These restraints require improvements in meat quality, production, and processing and are the focus of major research [8]. Understandably, significant changes in size and growth rate over a seemingly short time period (time it takes for birds to reach market age) may be a contributing factor to a rise in muscle myopathies in the modern broiler [8–11].

Myopathies causing low quality meat impact the economic welfare of the poultry industry. Currently, the industry faces an economic burden as the number of muscles disorders continues to rise [12], suggesting the importance of thorough research to understand these disorders. Muscle disorders such as deep pectoral myopathy (DPM) [13–15] and white striping [16], as well as muscle conditions such as pale, soft, and exudative condition (PSE), the dry, firm, and dark condition (DFD), have impacted output numbers worldwide [17]. Many resources go into the production and the maintenance of animals used for food production such as food, water, space, equipment, and labor. Thus it is important to understand disorders that may cause these resources to be unused or wasted to improve the efficiency of poultry production.

It could be suggested that unused meat due to myopathies also has an environmental impact. Waste and excrement produced by each bird has the ability to negatively impact the environment. Meda et al. 2011 [18] state that the production of ammonia from chicken waste has contributed to water pollution through eutrophication and soil pollution through acidification. Ammonia also produces methane waste, which is a green house gas [18]. Pelletier 2008 [19] found that among

the U.S. broiler supply chain, feed accounted for roughly 80% of the supply chain energy use. Essentially, it is detrimental to the poultry industry when waste is produced without the benefit of useful poultry products. The assessment and understanding of muscle disorders and myopathies affecting poultry is crucial for the efficient use of resources as they are imperative for poultry industry production.

Revealing genetic information regarding muscle disorders will likely have a significant impact on the field of poultry science by clinically lessening their economic and environmental impact. Performing this research, specifically on Wooden Breast disease, will begin to provide an answer to the problem and could offer insight into other muscle diseases. By better understanding these muscle disorders, improvements to poultry meat products can be made. This includes the establishment of correct diagnoses, improved treatment and prevention, and overall, alleviation of the economic burden that myopathies create. Through research of genetic information and mechanisms relating to the novel Wooden Breast disease, I hope to contribute new information regarding muscle disorders and possibly discover new genes that can improve the chicken genome annotation.

1.2 Important Factors to Muscle Quality

1.2.1 Environmental Factors

Environmental factors have been shown to affect muscle quality. These factors may include particular stressors that impact the slaughtering process. Particular

stressors outlined by Woelfel et al. 2002 [20], pertain to pre-slaughter handling, stunning methods, and chilling regimens. Woelfel et al. 2002 [20] found that these stressors have the ability to accelerate development of rigor mortis. Accelerating this process can be detrimental to meat quality and can cause the formation of myopathies ultimately leading to protein denaturing [21]. Birds exposed to various and frequent pre-slaughter stressors have darker muscle color and higher muscle pH levels compared to unstressed birds [22]. Specifically, in relation to PSE type meat, a problem occurs when there is acceleration in postmortem metabolism [20]. When the body is still at high temperatures and rapid glycolysis occurs, the result is low pH in the meat [23]. Protein denaturation then causes pale color and poor water holding capacity in poultry meat, both characteristic of PSE-type meat [20].

Previous environmental stressors affecting poultry have been scientifically tested and studied [17]. Studies have suggested that a significant stressor for birds is the transportation process and this potentially has adverse effects on meat quality [24]. If birds are transported in high heat or humid conditions, muscle damages can occur. This ultimately alters the physical attributes of the muscle [25]. During the pre-slaughter process, it has been found that transportation of three hours or more can result in stress levels that are high enough to accelerate metabolism to the point that muscle glycogen content is diminished. This results in higher muscle pH [24]. As cited above, many studies show that the environment in which most commercial broilers encounter impacts the formation and incident rate of myopathies.

1.2.2 Collagen, Muscle Fibers, and Capillaries in Skeletal Muscle

It is also important to consider collagen when discussing meat quality and tenderness. Collagen is found in three different regions of muscle and can take up to 19 different forms [26]. When discussing meat tenderness, collagen types I and III are the most relevant as they make up the major components of intramuscular collagen [27]. The fibrous connective tissue of breast muscle in the broiler chicken is composed primarily of type III collagen fibers. Guetchom et al. 2012 [28] suggest that muscle fibers surrounded by type III collagen are more susceptible to necrosis and degeneration because of "increased diffusion distances" for oxygen as well as poor development of capillary networks seen specifically in those chickens who are selected for fast growth rate and high breast muscle yield. Contrary to popular belief, collagen concentrations remain consistent as an animal matures [29]. In actuality, changes in tenderness are related to the maturation of collagen [29]. Collagen is structurally bound together by intermolecular crosslinking that provides its shape and strength. Overtime, these crosslinkages mature into temperature-stable, less soluble crosslinkages ([26, 30]. There is a correlation between collagen crosslinkage and tenderness; greater crosslinking results in tougher meat [26]. Collagen concentration and the maturation of collagen crosslinkages should be taken into consideration when examining properties of meat tenderness [31].

Connective tissue, which surrounds muscle fibers, plays an important role in muscle structure and development. Collagen and muscle fibers make up an entire

network in the body. Myofibers have been found to have a major role in meat tenderness and the structural variations found within this category [26, 32]. An et al. 2010 [32] studied the effects of myofiber and how it contributes to meat tenderness and meat quality. They tested factors such as breast muscle weight, pH, drip loss, cooking loss, fiber diameter, and myofiber density. They found that the smaller the myofiber size, the more tender the meat and also noted that the thickness of fiber diameter of the perimysium and endomysium, two of the collagen regions in skeletal muscle, play a contributing factor in tenderness. It is likely that breast muscle weight increase corresponds to an overall increase in myofibers number or size [32]. It should be considered that growth rate may thus impact meat quality due to physiological changes at the tissue level.

The poultry industry is focused on a high breast muscle yield. This has created a bird that has a wider and thicker pectoralis major muscle [8]. Genetic selection has also contributed to an increase in the diameter and length of muscle fibers [33]. A 2007 study by Berri et al. [8] researched the impact of fiber size on muscle properties. They note that as muscle fiber surface area increases, the expected post-mortem muscle pH decrease was hindered, as was glycolytic potential, leading to an increase in ultimate pH. Furthermore, there is an undesirable correlation, described below, between the number of breast muscle fibers and the density of capillaries. This is because the density of fibers inhibits the amount of oxygen the muscle is able to receive. These factors may have an effect on post-mortem processes potentially leading to variations in meat quality [8].

The area and density of muscle fibers affects the capillary supply in broilers. Capillaries are the primary sites of oxygen diffusion in cells; therefore, it is important to assess capillary function when discussing muscle disorders [34]. It has been shown that broilers with a higher percentage of breast muscle yield have lower capillary density within the breast muscle [34]. An increase in muscularity may decrease or shift the capillary to myofiber ratio found in the muscle; this ultimately increases the risk for a low oxygen supply to the muscle [34]. Due to the reduction of capillaries in broilers with higher percentages of breast muscle yield, any high metabolic load, which can occur from exercise, further decreases the supply of oxygen to the muscle [34]. This decreased supply of oxygen can result in conditions such as necrosis leading to poor meat quality characteristics.

1.2.3 Glycogen and pH

Glycogen content plays an important role in the processing performance of poultry meat. The total amount of glycogen found in the muscle at death correlates with ultimate pH [8]. Other properties specific to meat quality such as water holding capacity, color, and firmness are affected by ultimate pH [4, 8]. LeBihan-Duval et al. 2008 [4] discussed the genetic heritability and variability of these meat qualities traits in a 2008 study. They proposed that traits such as pH, ultimate pH, color, and drip loss are highly heritable, and noticed that ultimate pH of poultry meat has a significant impact on meat quality [4]. Essentially, measurements of ultimate pH correlate to

changes in meat color, water-holding capacity, and texture; a decrease in ultimate pH caused meat to be pale, more exudative, and tough [4]. They also found that the level of glycogen stored in the breast muscle at slaughter time is highly heritable. They conclude that ultimate pH may be a genetic control for other variables such as glycolytic potential and pH [4].

In a similar study, Sibut et al. 2011 [35] compared gene expression between birds exhibiting high glycogen content and low glycogen content. They did this by assessing fat and lean lines. The study revealed a difference in 197 transcripts for fat versus lean lines and 254 transcripts for high versus low glycogen content. Specifically, the authors maintained that there was a down regulation of the CEBPB and RGS2 genes; these genes are thought to impact glycogen turnover rates. Overall, the research provides a first step in assessing the molecular mechanisms affecting variations in poultry meat by studying glycogen content. [35]

The color of meat correlates with meat qualities such as pH, drip loss and cook loss [7, 22]. These various quality factors can predict meat quality and shelf life [7]. Dark coloration in the breast muscle of poultry is particularly common and may be caused by an increase in total color pigment [36]. This results from an increase in glycolysis; this increase can be induced by a variety of environmental factors [20, 22]. Increase in glycolysis leads to an overall increase in pH further resulting in a dark coloration of meat [37]. Boulianne & King 1998 [37] revealed that dark colored meat contains a higher pH, an increase in total pigment concentration, and an increase in myoglobin and iron content.

1.3 Known Muscle Disorders of Chickens

1.3.1 Nutritional Myopathy

Nutritional myopathy is a condition negatively affecting meat quality in most food animal species and is found frequently in the poultry industry [28]. The industry's interest in a larger bird, bred through genetic selection, may have increased the prevalence of this disease by producing a bird more susceptible to oxidative damages and antioxidant deficiencies [28, 38]. The primary lesion characteristics of this disease are found histologically. One characteristic is the presence of white striations in the skeletal muscle [39]. Other characteristics include cell-death (necrosis) and the degradation and regeneration of myofibers in the muscle [28].

Nutritional myopathy has been associated with low levels of vitamin E and/or selenium in the diet, but can also be asymptomatic [28]. Selenium performs an array of functions in both humans and animals. When selenium deficiency occurs, vitamin E may be used to prevent disease caused by this deficiency by enhancing the antioxidant defense system [38]. However, it appears selenium is a better preventative measure for nutritional myopathy than vitamin E [28, 38]. The necessity and importance of selenium in terms of maintaining stable health has been clearly established, but the underlying molecular mechanisms and properties of selenium are not yet fully understood [40].

Research surrounding nutritional myopathy is consistently concerned with diet supplementation. Selenium deficient turkeys experienced lower live weight gain and

feed intake [38]; thus, selenium can be considered necessary in preventing nutritional myopathy [38]. It has been histologically shown that vitamin E supplementation results in a decrease in the number of damaged fibers in the pectoral muscle by a significant amount [28]. Vitamin E supplementation has been shown to reduce breast muscle damages up to 28 days of age, but no significant effects after this time period have been established [28].

Clinically measuring creatine kinase in the muscle is widely used to diagnose nutritional myopathy because it is cost effective at a large scale, and may potentially aid in the early detection of muscle damage [28]. Studies suggest that increased amounts of creatine kinase in the blood may be related to a systematic malfunction in muscle tissue [8, 41]. This is dictated by levels of stress or protein turnover rates and may be related to muscle growth [8, 41]. Creatine kinase levels may also be related to selenium deficiency; for example, creatine kinase levels significantly increase after 35-days in turkeys that were selenium deficient [38]. Vitamin E supplementation, however, shows to have no impact on creatine kinase activity [28].

The study of nutritional myopathy at the molecular level provides information regarding the actions of the various biological mechanisms involved in the disease. Specific genes encode selenoproteins, which are mRNA sub-proteins regulated by selenium [42]. For example, the gene SPEN1 encodes selenoprotein N (Se1N) [43, 44]. Mutations within this gene have been linked to the early onset of muscular disorders in humans [43, 44]. In contrast, selenoprotein W (Se1W) is associated to muscular disease and in many cases is over expressed in response to oxidative stress

during muscle growth and cellular differentiation in early development [45, 46]. Muscle sensitivity is established by differences in Se1W and Se1N mRNA expression patterns between skeletal and cardiac muscles in response to levels of selenium in chickens [40]. Zhang et al. 2012 [40] suggests that the sensitivity of Se1N and Se1W expression may impact muscle tissue functioning and metabolism. Studying the general functions of proteins will not only aid in understanding nutritional myopathy, but also provide insight into a large number of muscle disorders and will hopefully lead to better knowledge of the mechanisms involved in their development.

1.3.2 White Striping

Kuttappan et al. 2013 [47] describe white striping as a new muscle condition found in chickens. It can be identified through observation of white fatty lines on the breast muscle running in the same direction as the muscle fibers of the breast muscle [47]. White striping is typically associated with broilers selected for breast muscle with heavier breast muscle weight [48, 49]. Most white striping is ventrally located where muscle stretching is greater, as opposed to areas that are dorsally located [47]. It is speculated that white striping is caused from a mineralization of fat on myofibers, and general necrosis of the muscle [47].

White striping is a fairly recent muscle disorder yet has already had a large influence on consumers. One study conducted by Kuttappan et al. 2012 [16] found that over 50% of consumers claimed they would not purchase breast fillets with any degree

of white striping, because they “looked fatty”. Recent consumer trends suggest that consumers are currently concerned with the amount of fat in the meat they purchase; thus, the white striping condition is likely to have a negative impact on the industry’s sales [16].

Kuttappan et al. 2013 [50] suggest that white striping is directly related to the increase in growth rate and weight of breast muscle of the modern broiler population. As described by Kranen et al. 2000 [51] selection for rapid growth rate may also lead to developmental flaws, potentially producing negative myopathic changes in the muscle. The growth rate of connective tissue cannot keep pace with the growth rate of the muscle, causing the development of muscle to exceed the development of supporting connective tissue [51]. This, paired with decreased oxygen supply caused by insufficient capillary density in birds with high breast muscle percentage, can likely lead to oxidative stress and tissue damages [34]. Providing evidence for this, Soike & Bergmann 1998 [52] suggest that selection for growth rate may cause white striping due to a decrease in capillary supply to skeletal muscle, found more often in heavier birds with thicker fillets. These birds represent the most severe cases of the disorder [47, 52].

White striping has not been found to be associated with nutritional myopathy. A study involving a supplemental diet containing vitamin E has shown that vitamin E supplementation does not affect the incidence rate of white striping [50]. Also, commercial handling and meat processing regimens do not appear to have an influence on the rate of this disease [50]. Meat quality measurements such as pH, cook

loss, and color do not seem to be a predictor for this condition either [50]. Overall, this muscle disorder is prevalent in the poultry industry and may be a result of increased growth rate; however, further research is needed to better understand its occurrence.

1.4 Important Myopathies in Other Species

Similar muscle disorders can be found in other agricultural animals such as horses, pigs, and sheep [53–55]. Therefore, it is possible to apply current research regarding similar muscle disorders in other species to the Wooden Breast. For example, research regarding nutritional myopathy in species such as turkey and sheep [38, 55, 56] has provided information on relevant blood parameters and enzyme levels that may be measured in order to correctly diagnose the disease. To take advantage of new technologies, and to continue to progress the field, we must also aim to identify tools that diagnose and treat muscle disorders, such as Wooden Breast. This will aid the industry in efficiency and reduce waste.

Recurrent exertional rhabdomyolysis (RER), a type of myopathy found in horses, results in major cramping and stiffness of the muscles after extreme exercise or work [53]. Clinical symptoms of this disease report high creatine phosphokinase and an increase in arginine succinyl transminase [53]. Barrey et al. 2012 [53] perform genetic expression profiles on horses with this disorder and find many genes that take part in fatty acid oxidation, the Krebs cycle, and the mitochondrial respiratory chain that were down regulated. These results suggest that there is an imbalance in the

muscle fiber calcium homeostasis [53]. Furthermore, when too many mechanical stressors are present in the skeletal system, muscle fiber structure is compromised, possibly explaining the flow of muscule enzymes into the blood stream [53].

Nutritional myopathy has also been observed in young sheep that show deficiencies in either selenium or vitamin E [55]. Fry et al. 1994 [55] have evaluated the onset of this disorder through enzymatic blood tests including creatine kinase. Although plasma creatine kinase is a common indicator of nutritional myopathy, it should be noted that this measure might not be accurate when testing for nutritional myopathy in sheep [56]. The primary advantage of performing blood tests rather than muscle sampling is that a larger number of subjects can be tested without conducting an invasive procedure, and can be run in a timely manner [55].

Chapter 2

INCIDENCE OF WOODEN BREAST DISEASE AND ITS CORRELATION WITH BROILER PERFORMANCE AND ULTIMATE pH OF BREAST MUSCLE

2.1 Introduction

Recently, a novel muscle myopathy, referred to as Wooden Breast disease, has emerged in commercial broiler chickens severely impacting the breast muscle. Previously described by Sihvo et al. 2014 [1], the central characteristic of this degenerative disease is extreme hardness of the breast muscle, which can be detected through manual palpation. Wooden Breast greatly decreases the meat quality of the breast muscle and is likely to have a significant impact on the poultry industry, as lesions can be viewed both clinically and microscopically. At times the incidence rate of this disease has been reported to be as high as 50% in some commercial flocks, but the most extreme cases likely impact roughly 10% of commercial populations [57].

The importance of thoroughly investigating the underlying biological components of this myopathy is great, as the disease has been established (reported) globally in areas other than the United States, such as Finland [1], and to the authors' knowledge is present in several other countries. It should also be noted, that the

underlying mechanisms, onset, and overall etiology of this disease remain largely unknown.

As poultry breeding has drastically progressed within the past 50 years, breeding systems focus mainly on important economic factors such as growth rate and feed efficiency. Feed efficiency measures the ability to convert feed intake into total body weight gain [58]. The significance of continually advancing these traits, mainly through genetic improvements, is of high importance as poultry meat is regarded as one of the most efficient protein sources in the world; this is extremely significant as the global population continues to climb. Although these advancements are largely beneficial, it is probable that they are also inadvertently contributing to an increase in the emergence and incidence of musculoskeletal disorders in broilers. Previous muscle disorders described in chickens, such as deep pectoral myopathy (DPM) and white striping, are typically associated with high-yield breast muscle birds with high growth rate [13, 59]. Some studies have found these muscle disorders are likely to be unfavorable in meat products based on consumer preference [16, 59], highlighting the importance of studying musculoskeletal disorders in commercial poultry.

It has been suggested that Wooden Breast disease is associated with rapid growth rate and high breast muscle yield in broiler chickens; however, to the best of our knowledge there is no published report analyzing and comparing phenotypic differences between Wooden Breast-affected and unaffected birds. In this study, a large number of more than 2,700 commercial broiler chickens were tested for feed efficiency, recorded for body weight, breast muscle weight, and pH of the breast

muscle after necropsy. Trained personnel subjectively evaluated and scored these birds for breast muscle hardness. The results of this study provide an estimate of the prevalence of this disorder in commercial broiler bird population, and also show clear phenotypic differences between clinically affected and unaffected chickens. We believe that this study can provide evidence that increased breast muscle weight and feed efficiency of modern-day broilers are key factors relative to Wooden Breast disease in commercial broilers.

2.2 Materials and Methods

A total of 2773 crossbred commercial broiler chickens were randomly sampled from seven different male broiler flocks in the Delmarva region of the United States. These birds were hatched, housed under commercial conditions, and then transferred to individual cages at day 29 to assess their individual growth rate and feed efficiency. The birds were weighed at the start of the feed efficiency trial, 29 days of age (BW_{29}), and at the end, 46 days of age (BW_{46}). BW_{29}, BW_{46}, and feed consumption from day 29 to 46 were used for feed efficiency measurements. All chickens had free (unrestricted) access to water and feed for the duration of the study. Chickens were euthanized via cervical dislocation at day 47, the day following the end of the test, and body weight was measured after euthanasia (BW_{47}). This measurement (BW_{47}) was not used for estimating feed efficiency, but was used for estimating breast muscle percentages and fat percentage.

The breast muscle (pectoralis major and minor) were dissected and weighed (BMW_{47}), and the hardness of the pectoralis major (PM) was subjectively scored by bilateral manual palpation and visual observation of macroscopic lesions, edema, etc., on the PM muscle by trained personnel. This hardness score was taken immediately post-euthanasia. The scores for subjective evaluation of the PM muscle hardness were as follows: 1) *hard*, moderately to severely affected, observance of macroscopic lesions in all cases, very tough, pale PM muscle; 2) *normal*, no detectable hardness, flexible, "normal looking" PM muscle; 3) *soft*, extremely flexible PM muscle. The whole carcass was then placed in cool storage at -4° C and after 24 hours, the abdominal fat was dissected and weighed (AFW_{47}). The pH (pHu) of the PM was also measured 24 hours after necropsy using the Testo® 205 pH/Temperature Meter.

Prior to data and statistical analyses, 212 of the total 2773 birds were excluded from the study due to a variation of factors such as premature or natural death, sickness, injury, missing data, etc. Residual feed consumption (RFC) was estimated at the end of the study by subtracting the actual feed consumption (FC) by the expected feed intake. The following equation was used: $RFC = FC – (a + b1*BW_{29} + b2*BW_{46} + Location)$. In this equation FC is the actual measured feed consumption; BW_{29} and BW_{46} are the body weights at days 29 and 46, respectively; Location represents the fixed effect of cage location; and a is the intercept; b1 and b2 are the partial regression coefficients of BW_{29} and BW_{46} effects. Those birds found with weight gain or RFC values ± 3 standard deviations outside the mean values for these traits were excluded from the study as well. It should be noted that the instrument used to measure pH,

Testo 205 pH/Temperature Meter, was calibrated every 100 chickens. Therefore, pH was adjusted for pH meter calibration effect within each group, prior to data analysis. The data collected from this study was analyzed using a standard least square model by fitting each phenotypic measurement separately as independent variable and breast muscle hardness score as a dependent variable. Differences in mean phenotypic values between multiple categories were assessed using Tukey's HSD test. JMP Pro® (SAS) (Version 11.0.0) was used for all statistical analyses as well as for plotting phenotypic data.

2.3 Results

In the present study, 2561 of the original 2773 birds were used for statistical analyses of multiple phenotypic traits, which were ultimately compared with scored breast muscle hardness. Among the total number of birds, 233/2561 (91%) birds presented with the "hard" PM score, 2087/2561 (81.5%) birds presented with the "normal" PM score, and 241/2561 (9.4%) birds presented with the "soft" PM score. The mean initial body weight (BW_{29}) was 1.27 kg with a standard deviation (STD) of ± 0.13. The mean final body weight (BW_{46}) was 2.82 kg with a STD of ± 0.23. From this, we can estimate that the average bird in this study underwent a 2.2-fold increase in body weight over the duration of 17 days. The mean and standard deviation for all phenotypic trait measurements are presented in Table 1.

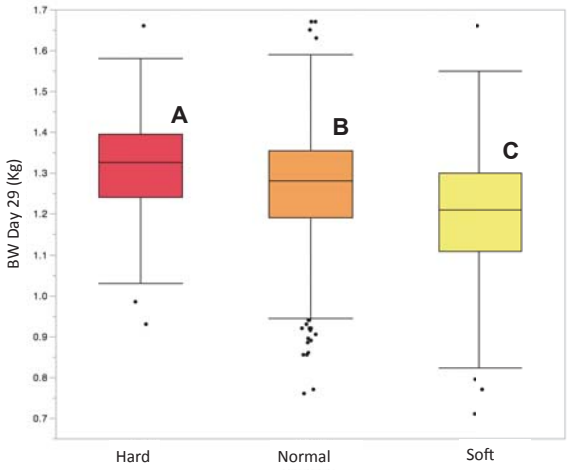
Table 1 **Means and standard deviations for all phenotypic traits**. This table provides the mean and standard deviation for all measured phenotypic traits in this study. All values except for pH are presented in kilograms. JMP was used for data distribution analyses.

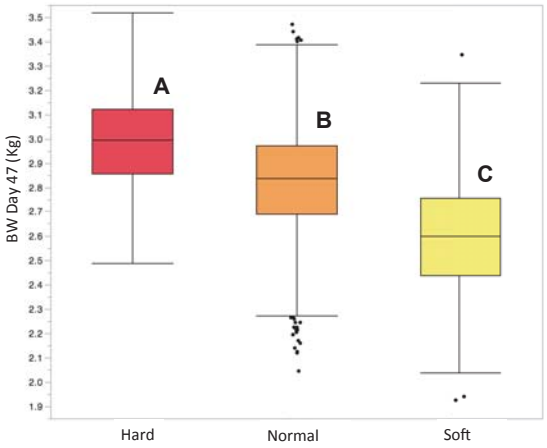
Phenotypic Trait	Mean	Standard Deviation
Initial Body Weight (BW_{29})	1.27 kg	± 0.13
Final Body Weight (BW_{46})	2.82 kg	± 0.23
Breast Muscle Weight (BMW_{47})	0.64 kg	± 0.08
Abdominal Fat Weight (AFW_{47}) [1]	0.055 kg	± 0.01
Feed Consumption[2]	2.83 kg	± 0.26
Weight Gain[2]	1.55 kg	± 0.16
pHu[3]	5.79	± 0.11

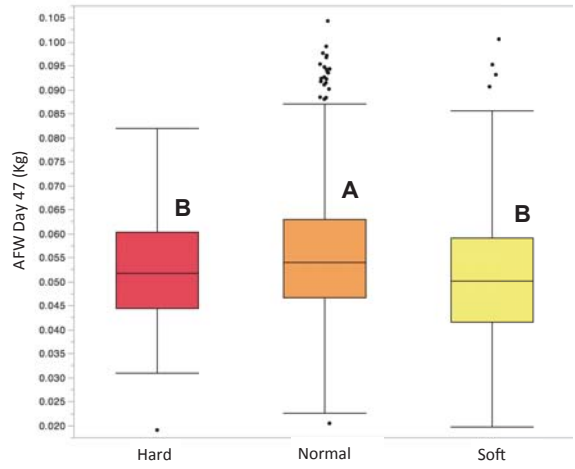
[1]Fat in abdominal cavity and around gizzard
[2]From 29 to 46 days
[3]pH at 24 hours post-euthanasia

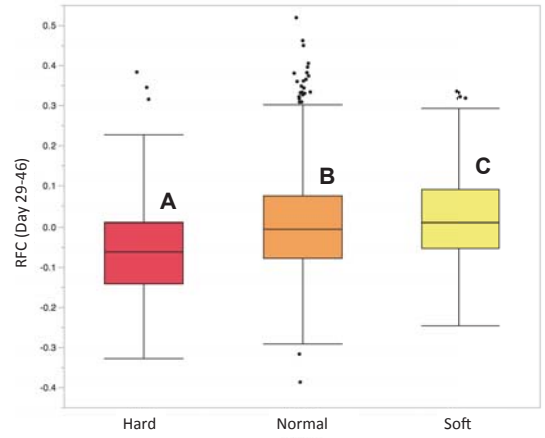
Tukey's HSD test (alpha = 0.05) was used to compare the three muscle hardness scores, "soft", "normal", and "hard" to six different phenotypic categories: initial body weight (BW_{29}), final body weight (BW_{47}), final deboned breast muscle weight (BMW_{47}), residual feed consumption (RFC), abdominal fat weight (AFW_{47}), and pHu. It was found that the means for all three hardness scores were significantly different from each other when initial body weight (BW_{29}), final body weight (BW46), breast muscle weight (BMW47), residual feed consumption (RFC), and pHu were fitted as the independent variable. The differences between hard vs. soft, normal vs. soft, hard vs. normal were highly significant for these five phenotypic traits; $p < 0.0001$ for all comparisons except between normal vs. soft for RFC, which was <0.02. One trait, abdominal fat weight was not significant for all three comparisons; the mean

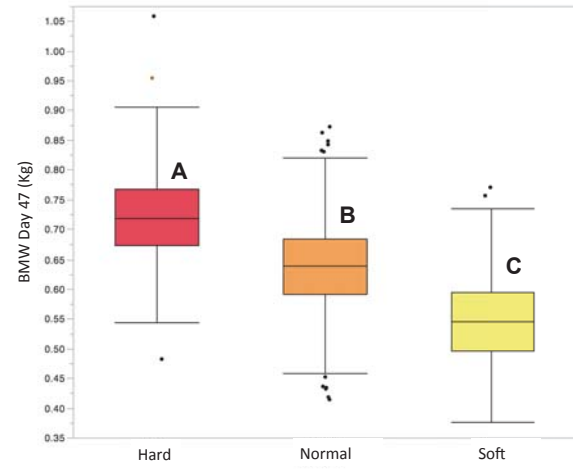
for “normal” scored breast muscle was significantly different ($p < 0.02$) from both “hard” and “soft” scored, but the mean for “hard” and “soft” scores was not significantly different from each other ($p= 0.26$). Figure 1 illustrates the results of the Tukey’s HSD test for each phenotypic category using box plots to also show a visual representation of the data for each category.


BW Day 29 (Kg)
A
B
C
Hard
Normal
Soft


BW Day 47 (Kg)
A
B
C
Hard
Normal
Soft


AFW Day 47 (Kg)
B
A
B
Hard
Normal
Soft


RFC (Day 29-46)
A
B
C
Hard
Normal
Soft


BMW Day 47 (Kg)
A
B
C
Hard
Normal
Soft

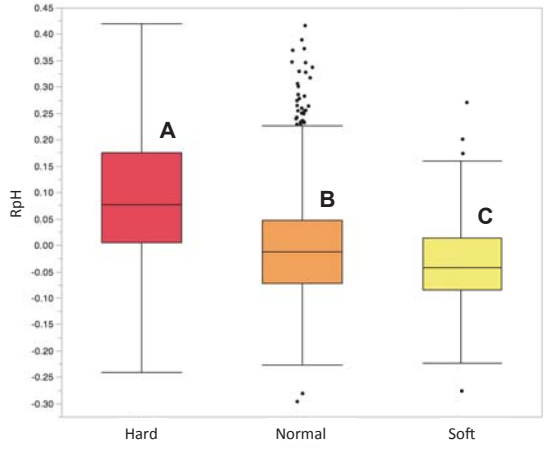

RpH
A
B
C
Hard
Normal
Soft

Figure 2 **Various bird phenotypic traits compared to muscle hardness score.** This figure represents analyses using Tukey's HSD test. All categories with different letters are considered to be statistically different from each other at an alpha level of 0.5. Those with the same letter are not statistically different from each other.

2.4 Discussion

For this study, we analyzed data from over 2,500 chickens to gain insight into the relationships between Wooden Breast disease and phenotypic traits such as body weight and breast muscle weight. Given the results of this study, we are able to better understand the correlation of multiple phenotypic traits and their occurrence with wooden breast disease in commercial broilers. There is evidence to conclude that birds that become affected with Wooden Breast have an overall higher initial and final body weight. This suggests that larger, more feed efficient birds are predisposed to develop Wooden Breast. Similarly, the data also support that there is a significant correlation for breast muscle hardness ("hard") and higher breast muscle weight, when compared to both "normal" and "soft" breast muscle. Previously reported work by Sandercock et al. 2009 [60] suggests that modern day commercial broilers—those that are genetically selected for feed efficiency and growth rate—can have altered meat composition, which negatively impact certain meat quality traits. Our data also seems to agree with this, since Wooden Breast is certainly regarded as a negative muscle/meat phenotype. It should also be pointed out that research by Petracci et al. (2013) [59] suggests that white striping, another meat quality condition correlated with Wooden Breast [1], may actually decrease the nutritional contents of the breast muscle.

The data also suggest that birds that are higher performing in terms of feed efficiency and abdominal fat amount/percentage can be correlated with breast muscle hardness. Although fat amount/percentage is typically considered a carcass component trait, rather than a performance trait, it should be considered important. The mean value for residual feed consumption (RFC) was significantly lower—which translates to higher feed efficiency—in birds with "hard" muscle scores as compared to birds with "normal" and "soft" breast muscle. It should also be noted that birds scored "hard" had significantly lower final abdominal fat weight than birds scored "normal". Although this is a key point, it remains to be elucidated as to why bird that develop Wooden Breast disease growth faster and more efficiency than those that remain unaffected.

We may also conclude that Wooden Breast or "hard" scored birds had a significantly higher pHu when compared to both "normal" and "soft" phenotypes. It is known that high pHu in poultry meat tends to lead to a muscle condition known as DFD-type (dark, firm, and dry), which cause changes in the meat, leading to reduced shelf life [17]. Although the DFD meat conditions may be somewhat related to Wooden Breast disease, the muscle color of Wooden Breast affected meat tends to be extremely pale in color [1], rather than dark. It is also interesting to note that in the authors' laboratory (data not shown), measurements for glycogen content of the breast muscle (mg/g) of Wooden Breast affected birds is markedly lower (roughly 2-fold) than unaffected or "normal" birds. It is also likely that ultimate pH and glycogen content of the muscle are negatively correlated with one another [4].

In conclusion, by analyzing this large data-set of commercial chickens, we are able to draw conclusions about phenotypic trends that correlate significantly with the observation of Wooden Breast disease. From this data, we can determine that birds that have high feed efficiency are more likely to develop Wooden Breast disease; birds with high initial and final body weight, as well as high breast muscle yield and low abdominal fat percentage, which are all key factors when considering feed efficiency, were significantly correlated with Wooden Breast disease. Therefore, the modern day fast growing, feed efficient, and high yield birds, may be more likely to acquire this myopathy. It is also interesting to note that our data suggests birds with Wooden Breast disease have a significantly higher breast muscle pH, which may be related to low glycogen content (data not shown) also found in the breast muscle. This information will be very valuable to industry breeders and producers, and we hope that we can use this information as a starting point in the planning of future research opportunities related to helping eliminate and study Wooden Breast disease in commercial chickens.

Chapter 3

CHARACTERIZATION OF A NOVEL CHICKEN MUSCLE DISORDER THROUGH DIFFERENTIAL GENE EXPRESSION AND PATHWAY ANALYSIS USING RNA-SEQUENCING

3.1 Background

In terms of consumption, poultry meat is highly regarded as one of the most efficient food sources; it contains high quality nutrients such as protein, and is relatively inexpensive for purchase. However, muscle disorders of commercial chickens such as deep pectoral myopathy (DPM) [13–15] and white striping [16], as well as other conditions such as pale, soft, and exudative condition (PSE) or dry, firm, and dark condition (DFD) have had an increasing negative impact on worldwide chicken meat production and quality [17]. Recently, flocks of commercial broiler chickens in the United States have developed a myopathy affecting the pectoralis major, and occasionally minor, muscle. Previously described by Sihvo et al. 2014 [1], this disease is colloquially referred to as “woody” or “wooden” breast, due to clinical and microscopic changes to the muscle resulting overall in palpable severe hardness of the breast muscle. Hereafter this syndrome will be referred to as Wooden Breast. The superficial area of the pectoralis muscle tends to be more affected than deeper portions

of the muscle. Lesions can be detected clinically through manual palpation of the breast muscle in live birds as early as 3 weeks in age. Owens (2014) [57] has recently reported that severe cases of Wooden Breast disease can likely affect about 10 percent of an entire flock; but it was also noted that some degree of Wooden Breast disease has been anecdotally reported to affect up to 50 percent of a flock. The disease is emerging on a global scale, already present in Finland [1] and to the authors' knowledge several other countries.

Sihvo et al. 2014 [1] specifically studied the microscopic and histologic properties of the pectoralis muscle from chickens affected with Wooden Breast. Although histological evidence indicated multifocal degeneration and necrosis of muscle tissue with infiltration of inflammatory cells, the underlying etiology of this disorder was not apparent [1]. Wooden Breast has also previously been reported in the United States by Bilgili 2013 [61] at the University of Auburn in Alabama with the general phenotypic properties of the myopathy described being in agreement with Sihvo et al. 2014 [1]. Lesions associated with the myopathy appear to be aseptic, superficially-located, and include muscle fiber fragmentation, hyalinization, and swelling with replacement by fibrous connective tissue, as well as an influx of macrophages and other immune cells and the occurrence of irregular adipose tissue throughout the muscle. Bilgili 2013 [61] hypothesize that these features may be associated with hypoxia due to a reduction in capillary supply.

Previously described muscle disorders in chickens, such as white striping and nutritional myopathy, have been shown to affect the integrity of the pectoralis major

muscle in chickens. White striping is identified through the observation of white fatty lines on the breast muscle running in the same direction as the muscle fibers of the breast muscle [47]. It is speculated that white striping is caused from a mineralization of fat on myofibers, along with general necrosis of the muscle [47]. Interestingly, there may be a correlation between white striping and Wooden Breast, as birds affected by Wooden Breast are likely to show gross features of white striping [1]. It is also possible that the two disorders represent a disease spectrum, with white striping cases being the less severe form of the myopathy than Wooden Breast. Nutritional myopathy also affects the breast muscle of broiler chickens and is generally associated with low levels of vitamin E and selenium in diet [28]. The main clinical features of nutritional myopathy can be observed grossly as white striations in the skeletal muscle [39] and histologically as degradation, necrosis, mineralization, and regeneration of myofibers in the muscle [28]. Although some of the characteristics of white striping and nutritional myopathy overlap with lesions of Wooden Breast, the grossly palpable hardness of the pectoralis muscle remains unique to Wooden Breast. Additionally, unlike in nutritional myopathy, no lesions are observed in other muscle groups, such as the gizzard or heart, beyond the pectoralis major and minor in Wooden Breast.

The overall objective of this paper is to use RNA-sequencing to identify biological and molecular pathways that are involved in the underlying etiology and pathogenesis of this emerging muscle disorder of the pectoralis muscle in commercial broiler chickens. We use RNA-sequencing as this powerful technology provides comprehensive and accurate gene expression data, performing better than previous

technologies such as microarrays [62]. Also, useful tools have been generated for RNA-sequencing data analyses that make for user-friendly and efficient analysis of the data.

3.2 Methods

Chickens used in this study were all males from a high breast meat yield purebred line of broiler breeder chickens raised on the floor, in pens of 300 by Heritage Breeders in the Delmarva region of the United States. Birds were given free access to both feed and water, and were housed according to optimal industry growing standards. Birds were euthanized at 47 days of age by cervical dislocation. After euthanasia, pectoral muscle samples were collected at necropsy. Based upon clinical examination and manual palpation of pectoral muscle for evidence of palpable "hardening," eight affected and eight unaffected birds were identified; affected birds were those deemed to have "severe" to "moderately-severe" lesions, while unaffected birds showed no apparent gross lesions. Samples from the pectoralis major were taken from the caudal aspect of the right superficial pectoral muscle, perpendicular to myofibers and to the keel bone. Samples were taken with the Keyes biopsy punch (8 mm) manufactured by HNM. Roughly one to two grams of tissue was extracted from each sample location and immediately flash frozen in liquid nitrogen. The protocols were submitted to, and the use of the collected samples for research was approved by,

the University of Delaware Agricultural Animal Care and Use Committee (protocol number: 44 12-15-13R).

Total RNA was extracted from the breast muscle tissue samples using the *mir*Vana™ miRNA Isolation Kit according to the manufacturer's protocol (Life Technologies®). Multiple quality checks were performed to assess the RNA quality and integrity using NanoDrop 1000 and Angilent Bioanalyzer 2100 to measure RNA concentrations following the established manufacturer protocol. The RNA integrity number of samples was larger than 8, which is considered acceptable for cDNA library preparation.

Eleven samples, five affected and six unaffected, were prepared to create cDNA libraries for RNA-sequencing. All samples used for RNA-sequencing were also strictly verified for correct identity (Appendix A). cDNA libraries were constructed following the TruSeq Stranded mRNA Sample Prep Kit following the protocol for low sample (LS). After construction, the 11 cDNA libraries were normalized as suggested by the manufacturer, to 10nm/µl using Tris buffer (Tris-Cl 10mM, 0.1% Tween 20, pH 8.5). The 11 uniquely indexed, normalized libraries were pooled together in 2 tubes and submitted to the Delaware Biotechnology Institute (DBI) for 101 nucleotides paired-end sequencing on 2 lanes of a flow cell using the Illumina Hiseq 2000 system and on 1 lane of a flow cell using the Illumina Hiseq 2500 system.

The quality of the data received from DBI was checked with FastQC v0.10.1 [63]. RNA sequencing reads that passed this quality control measure were then mapped to the chicken reference genome (Galgal4.0 Nov 2011) using TopHat v2.0.4

[64]. Parameters of TopHat were set to only report read alignments if 1) both reads in a pair could be mapped, 2) there were no more than two mismatches per read, 3) concordant mapping occurred for both reads in a pair, 4) no more than one mismatch per segment (--segment-mismatches), and 5) mean inner distance between mate pairs set at 110. TopHat cut up input reads into smaller segments and map these segments independently. The default parameter (25 nucleotides) was used for segment length. Cufflinks v2.1.1 [65] was used for the differential expression analysis: parameters of Cuffdiff were set to allow normalization of the number of fragments mapping to individual loci to improve the strength of differential expression analysis (change in default-- added –N/--upper-quartile-norm parameter). Cuffdiff labeled genes as significant or not based on whether the p-value of statistical test for differential expression is greater than the false discovery rate (FDR) after Benjamini-Hochberg correction for multiple testing [65]. Genes with a q-value or FDR-adjusted p-value of < 0.05 and a fold change greater than 1.3 were considered statistically significant and submitted to Ingenuity Pathway Analysis (IPA®) [66] for functional annotation and identification of significant biological pathways and upstream regulatory factors. The fold change for each gene was provided by Cuffdiff as $\log_2$(fold change) and was then calculated as $2^{\log_2(\text{fold change})}$ for genes with a positive $\log_2$(fold change) and $-1/(2^{\log_2(\text{fold change})})$ for genes with a negative $\log_2$(fold change) before submission to IPA.

To verify the gene expression data we used NanoString nCounter® technology previously described by Geiss et al. 2008 [67]. We selected 192 target genes, along with 12 housekeeping genes across various RNA-sequencing projects within our lab to

be measured. The11 samples used in this paper were sent to NanoString Inc. (Seattle, WA, USA), where they custom designed 204 individual probes for target sequences. NanoString Inc. hybridized 100ng of each RNA sample to the 204 designed probes. After incubation, the samples went through the nCounter® Prep Station and the Digital Analyzer for final transcript counting. Control factors and housekeeping genes were used to normalize the data for further analysis [67]. Finally, the $\log_2$(fold change) for gene expression between affected and unaffected birds were calculated from the NanoString results to be compared with the $\log_2$(fold change) of gene expression from the RNA-sequencing results.

3.3 Results and Analysis

The total number of reads per sample provided from RNA-sequencing can be found in the Figure 3. The average number of reads across all 11 samples was 97,890,272.

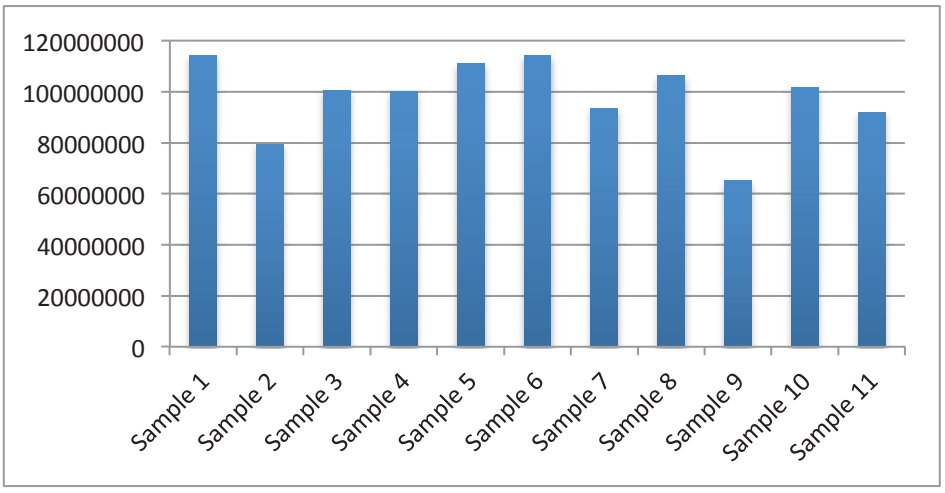


Figure 3 **Total number of reads per sample**. Total number of reads per sample for all (11) samples with an average of 97,890,272 reads per sample.

Expression data for each gene within each sample was used to create a heatmap for cluster classification. The heatmap was generated using the R package "plots" (Figure 4). This cluster analysis revealed a clear separation between affected and unaffected samples. It should be noted that one sample (Sample 51 unaffected) was seemingly clustered in the incorrect group. We believe that this inconsistency is likely due to the broad range of disease severity possible in Wooden Breast. It is reasonable to suspect that the syndrome was in fact developing in Sample 51, but not yet expressed in terms of the palpable muscle firmness. This suggests clinicopathological heterogeneity in the classification of this disease.

We verified our RNA-sequencing data using the Nanostring nCounter gene expression assay. Comparison of the $\log_2$(fold change) for normalized NanoString count data against our RNA-sequencing data using the Pearson pair-wise correlation coefficient were very high, with a value of 0.857 across all overlapped genes tested in RNA-seq.

Ensembl ID of differentially-expressed (DE) genes, i.e., genes with a q-value<0.05 and a fold change higher than 1.3 or lower than -1.3, were submitted to IPA® for functional annotation as well as mapping to canonical pathways and identifying upstream regulators. The differentially expressed genes with positive or

negative fold change values are considered to be, respectively, upregulated or downregulated genes in affected birds relative to their expression in unaffected birds.

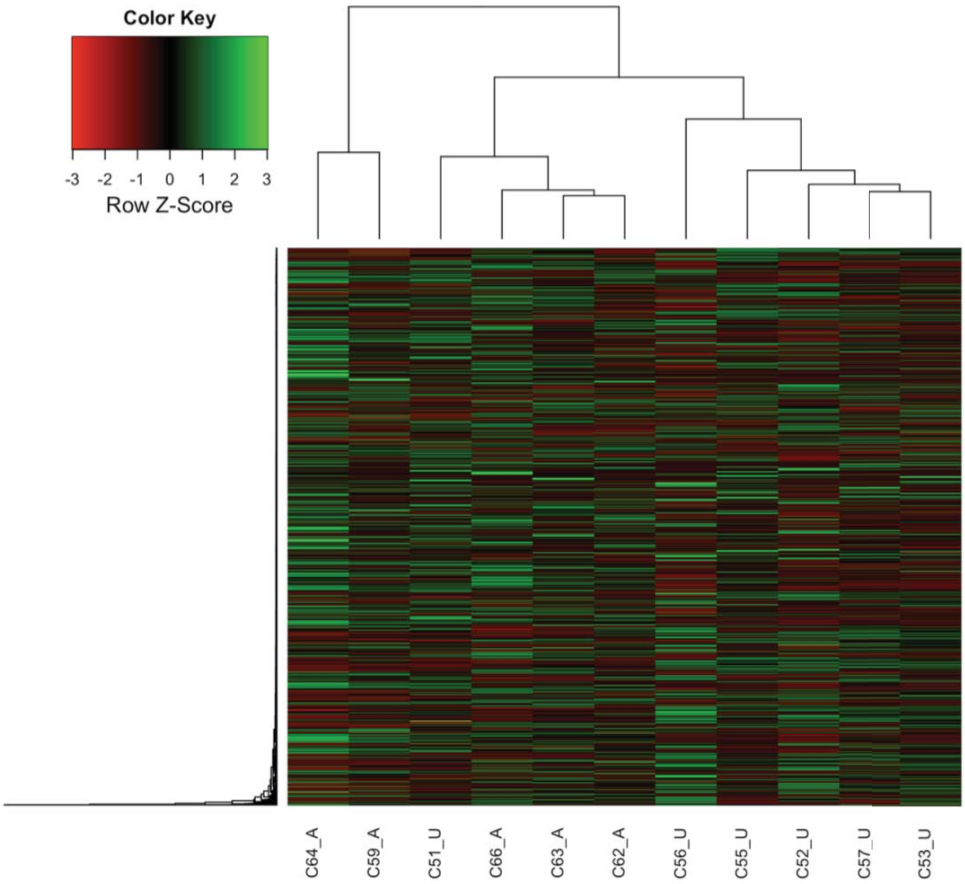


Figure 4 **Heatmap gene cluster classification for Wooden Breast and unaffected muscle samples.** Using the expression for each gene (in rows) and sample (in columns), the heatmap is generated by the R package "plots". The expression levels of each gene across samples are shown as Z-scores scaled by their FPKM from RNA-seq. The scaled expression values are color-coded according to the legend. The dendrogram depicting hierarchical clustering is based on the expression of all genes.

Among the 1637 significant DE genes identified, IPA recognized 1390 of these genes for analysis. The Ingenuity Knowledge Base®, which supports the mapping of genes, relies on ortholog information for only human, mouse, and rat. Some genes went unmapped if there is no known ortholog with either of these species (human, mouse, rat). A summary of the top ten up-regulated and top ten down-regulated genes in the affected birds, along with the fold change for each gene is presented in Table 2.

A summary of the genes identified in the IPA® category of "Top Biological Functions" is presented in Table 3 and contains the subcategories of "Diseases and Disorders", "Molecular and Cellular Functions", "Physiological System Development and Function" and "Top Canonical Pathways". Also presented in Table 3 is the significance or P-value listed for each subcategory. IPA determines the P-value for each subcategory by dividing the number of DE genes (# of Genes) for each subcategory by the total number of known genes within that specific subcategory present in the IPA software database "Query Ingenuity Knowledge Base" (IPA website). The P-value for the top canonical pathways is also determined in the same manner. The "Genes" and "Ratio" columns in this subcategory were determined by dividing the number of observed DE genes by the total number of genes within that specific canonical pathway present within the IPA software database: "Query Ingenuity Knowledge Base" [66].

Table 2 **Top ten upregulated and downregulated genes with fold change for Wooden Breast myopathy-affected birds.** This table provides a summary of both the top ten upregulated and downregulated genes found in among affected chickens within the sample population. Positive and negative fold change values support, respectively, upregulation and downregulation of gene expression in affected birds relative to the gene expression in unaffected birds. The fold change for each gene is also presented along with the fold change for each gene that was tested using the NanoString nCounter® Technology to verify our expression data.

Gene	Description	RNA-seq Fold Change	Nanostring Fold Change
ENSGAL00000009603	Prolactin-like protein precursor	43.9	N/A
CA3	Carbonic anhydrase III	28.5	25.5
CLEC3A	C-type lectin domain family 3, A	25.3	N/A
KPNA7	Karyopherin alpha 7	24.4	8.0
CSRP3	Cysteine and glycine-rich protein 3	23.2	27.3
CRISP1	Cysteine-rich secretory protein 1	21.5	N/A
FAM132A	Family sequence similarity 132, A	18.0	N/A
MB	Myoglobin	14.3	12.4
CRH	Corticotropin releasing hormone	14.3	13.3
PHGDH	Phosphoglycerate dehydrogenase	11.6	5.4
GC	Group Specific Component	-48.0	N/A
PIT54	PIT 54 protein precursor	-46.0	N/A
FGA	Fibrinogen alpha chain	-20.8	-3.0
FGG	Fibrinogen gamma chain	-18.1	-2.8
OGCHI	Orosomucoid 1	-16.9	N/A
ALB	Albumin	-16.8	-4.0
ENSGAL00000016859	Uncharacterized protein	-14.9	N/A
FGB	Fibrinogen beta chain	-13.3	-3.8
APOH	Apolipoprotein H	-13.1	N/A
YFV	MHC I antigen precursor	-12.9	N/A

Table 3 **Top biological functions of genes differentially expressed between Wooden Breast myopathy-affected and unaffected birds as presented by Ingenuity Pathway Analysis (IPA®).** Top Biological Functions of DE genes associated with affected chickens from the sample population within this study provided by Ingenuity Pathway Analysis (IPA®). The P-value provided by this table was determined by IPA by comparing the number of genes found within each category (# of Genes) with the number of known genes within the IPA software system. The P-value for the "Top Canonical Pathways" is also determined by IPA in the same manner.

Diseases and Disorders:	**P-value**	**# Molecules**
Cancer	3.18E-31 – 4.08E-05	1208
Gastrointestinal Disease	9.84E-26 – 3.06E-05	890
Cardiovascular Disease	7.45E-21 – 4.06E-05	293
Hepatic System Disease	1.86E-20 – 3.06E-05	593
Neurological Disease	1.95E-17 – 3.92E-05	407
Molecular and Cellular Functions:		
Cellular Assembly and Organization	7.25E-19 – 2.06E-05	321
Cellular Function and Maintenance	7.25E-19 – 1.14E-05	402
Cellular Movement	1.80E-17 – 3.43E-05	322
Cell Morphology	1.51E-16 – 2.53E-05	387
Cellular Growth and Proliferation	2.81E-16 – 3.82E-05	476
Physiological System Development and Function:		
Nervous System Dev./Function	9.74E-14 – 3.90E-05	195
Tissue Development	9.74E-14 – 3.82E-05	501
Cardiovascular System Dev./Fun.	2.75E-13 – 3.70E-05	237
Organismal Development	5.44E-13 – 3.63E-05	432
Embryonic Development	9.51E-13 – 3.10E-05	306

Top Canonical Pathways:	**P-Value**	**Genes**	**Ratio**
Hepatic Fibrosis/Stellate Cell Activation	2.83E-12	43/197	0.218
Epithelial Adherens Junction Signaling	2.39E-08	30/146	0.205
Actin Cytoskeleton Signaling	3.18E-07	36/217	0.166
Axonal Guidance Signaling	1.27E-06	56/433	0.129
Coagulation System	1.39E-06	12/35	0.343

The resultant RNA-seq profile for Wooden Breast indicated by these analyses includes evidence of altered intracellular calcium, hypoxia, oxidative stress, potential fiber-type switching, and cellular repair within affected muscles. Additionally, there is direct correlation between RNA-sequencing data and the histologic lesions observed in the Wooden Breast myopathy.

3.4 Discussion

3.4.1 Intracellular Buildup of Calcium

Through analysis of DE genes in this study, there is ample evidence to suggest that intracellular calcium overload with the potential to impact membrane integrity is occurring in the pectoralis major muscle of broilers affected with Wooden Breast. The ATPase, Ca++ transporting, cardiac muscle, slow twitch 2 (*ATP2A2)* gene, which is upregulated in birds affected with Wooden Breast, encodes for sarco(endo)-plasmic reticulum Ca^{2+} -ATPase isoform 2a and 2b (SERCA2a/SERCA2b) [68]. *SERCA2a* and *SERCA2b* differ in the location where they are generally expressed with *SERCA2a* showing high levels of expression in the heart and in slow-twitch skeletal and smooth muscle, whereas *SERCA2b* is expressed in all tissues [68]. SERCA2 is a critical component of the endoplasmic reticulum (ER) and sarcoplasmic reticulum (SR) and acts as a pump to sequester [69] and translocate Ca^{2+}[70]. SERCA pumps also have a role in contraction and relaxation of myofibrils, maintaining calcium homeostasis by sustaining the correct calcium levels required for relaxation and as well as maintaining

"reloading" calcium levels needed for contraction [68, 70]. Increased expression of *SERCA2* leads to quicker calcium uptake and larger amounts of calcium loading within the ER [71]. Previously, a study in chickens showed that *SERCA* expression could be influenced by extrinsic factors such as disease [72]. The upregulation of *ATP2A2* in Wooden Breast birds may be occurring in response to increased amounts of intracellular calcium within muscle cells, potentially leading to up-take and loading calcium within the sarcoplasmic reticulum at faster than normal rates in affected birds.

Parvalbumin (*PVALB*), another upregulated gene in Wooden Breast birds, is essential to calcium buffering and regulating calcium concentrations within the cell [70]. PVALB acts to insure relaxation of the muscle through rapidly sequestering calcium from the sarcoplasm of the cell [70]. The upregulation of *PVALB* in birds affected within the current study may be compensatory to avoid a significant rise in intracellular calcium levels. This type of compensatory change has also been hypothesized in other muscle disorders such as Duchenne dystrophy in humans, in which excess calcium negatively impacts the muscle [73]. Therefore, it seems that increases in *PVALB* expression may act to stop hypercontraction of the muscle by inducing relaxation through calcium buffering and binding.

Abnormal accumulation of intracellular calcium can occur because of damaged cellular membranes of muscle cells or during episodes of metabolic imbalance between calcium and other ions [74]. The resultant damage to muscle cells can occur in various ways; it is hypothesized that extra calcium may activate proteases or lipases within the cell which eventually leads to muscle fiber breakdown and an increase in

calcium influx, initiating a “vicious cycle” of further muscle damage and calcium release [74]. It is also thought that excess calcium can impede mitochondrial performance ultimately decreasing the available energy supply when calcium cannot be effectively pumped out of the cell [74, 75]. Calcium overload can result in the activation of calpains and phospholipase A_2 (PLA_2), increase the overall production of ROS, and may also lead to excess mitochondrial calcium [75]. Damage to the sarcolemmal membrane occurs after activated PLA_2 disrupts mitochondrial function causing the formation of lysophospholipids [75] that in turn disrupt the sarcolemmal membrane [76]. In the current study, 2 membrane-associated phospholipase genes, the *PLA2G4A* gene, encoding cytosolic phospholipase A_2 (PLA2), and phospholipase B1 (*PLB1*) were upregulated in birds affected with Wooden Breast. *PLA2G4A* has previously been implicated in broad spectrum skeletal muscle myopathies in broiler chickens [77]. Overall, the upregulation of *PLA2G4A and PLB1 and* increased intercellular calcium may be causing changes in membrane integrity in Wooden Breast chickens.

3.4.2 Hypoxia

A number of significant differentially expressed genes in the present study have been found to be regulated by HIF-1 (Hypoxia-inducible factor- 1) (HIF-dependent expression), suggesting a hypoxic state in Wooden Breast. These genes include procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*), transforming

growth factor beta 3 (*TGFB3*), asparagine synthetase (*ASNS*), matrix metallopeptidase 2 (*MMP2*), transient receptor potential cation channel, subfamily A, member 1 (*TRPA1*), muscle-specific carbonic anhydrase III (*CA3*), and 6-phosphofructo-2-kinase (*PFKFB3*). Hypoxia-inducible factor 1 (*HIF-1*) is an important gene to consider when discussing hypoxia. *HIF-1* regulates many genes involved in angiogenesis, energy metabolism, vasomotor regulation, and cell proliferation as well as cell survival, and it is critical in maintaining oxygen homeostasis within the cell [78–80]. Although *HIF-1* itself is not differentially expressed between normal birds and those affected by Wooden Breast, our significant gene list suggests the activation of this gene in affected birds. It has been previously reported that in skeletal muscle *HIF-1* is highly expressed in both hypoxic and normoxic conditions [81], which may give insight into why *HIF-1* is not differentially expressed between affected and unaffected birds in the present study, though there is specific evidence for hypoxic conditions in the Wooden Breast profile.

For example, *PLOD2*, which is upregulated in wooden breast-affected birds is an important gene associated with extracellular matrix composition, specifically extracellular matrix stiffening and collagen alignment, such as with fibrosis [82]. *PLOD2* expression is essential for correct collagen formation in response to hypoxic events [83]. *HIF-1* increases *PLOD2* expression during hypoxia, which increases collagen content and impacts the extracellular matrix by increasing cell adhesion, elongation, and motility, creating a stiff cellular environment [83]. The upregulation of *PLOD2* in affected birds correlates with the histological characterization of fibrosis

previously reported in chickens with Wooden Breast [1] and observed histologically in our laboratory (data not shown). Furthermore, this gene may play an integral role in the accompanying stiffness of Wooden Breast birds, possibly related to changes within the extracellular matrix surrounding muscle cells in the breast muscle.

TRPA1 is another HIF-dependent gene upregulated in affected birds in our study. Through the formation of calcium and zinc ion channels, *TRPA1* acts as a sensor to reactive compounds, such as oxidative and thiol compounds, which are mainly produced during oxidative stress [84]. *TRPA1* also mediates inflammation [85]. Hatano et al. 2012 [86] recently provided evidence for the upregulation of *TRPA1* by *HIF*. Their findings included the induced expression of *TRPA1* by *TNF-alpha* and *IL1-alpha* primarily regulated through HIF. Although *TNF-alpha* is absent in chickens [87, 88], many *TNF* family members are differentially expressed between unaffected and affected birds in our study. Also, inflammation is a key characteristic of the breast muscle in Wooden Breast birds, which is critical as inflammatory cytokines and mediators induce *TRPA1* expression [86]. It should also be noted that within the current study, additional inflammatory cytokines IL-15, IL-16 and IL-18 are all upregulated in affected birds.

Matrix Metallopeptidase-2 (*MMP2*), characterized as a *HIF*-dependent gene, is also upregulated in birds affected with Wooden Breast. *MMP2* is a member of a large family of matrix metalloproteases and is typically associated with the degradation of cellular components such as the basement membrane and type IV collagen [89]. MMP2 also plays a role in regulating vascularization and response to inflammation

[90]. Milkiewicz & Haas 2005 [91] observed that during instances of mechanical stretch to muscle, both the expression of *HIF-1* and *MMP2* increased. More specifically, they detected increased gene expression of *MMP2* in capillary endothelium of muscle cells. Although the likely activation of *HIF-1* within the current study may be the primary cause for the upregulation of *HIF*-dependent *MMP2*, it must also be considered that *MMP2* expression may be increased due to vascular smooth muscle stretch and remodeling secondary to venous damage or inflammation in birds affected with Wooden Breast.

Other differentially expressed genes within our study such as transforming growth factor, beta 3 (*TGFB3*), asparagine synthetase (*ASNS*), and muscle specific carbonic anhydrase III (*CA3*) were previously reported as regulated by *HIF-1*. *TGFB3*, which is upregulated in affected birds in our study, has been previously found to be upregulated in traumatized muscle during instances of chronic inflammation and regenerating muscle tissue as evident microscopically in this study (data not shown), which stimulates a hypoxic microenvironment [92]. In the current study, *ASNS* is upregulated in affected chickens. Cui et al. 2007 [93] detail the impact that both hypoxic conditions and insufficient glucose levels have on *ASNS*, in relation to tumor growth. They reported a significant increase in expression of *ASNS* in response to inadequate glucose levels. The upregulation of *ASNS*, possibly due to low glucose levels, also correlates with previous observations, such as the downregulation of *PFKFB3*, suggesting deficient glucose metabolism. *CA3* is also differentially expressed between the two groups of chickens in the present study, and is upregulated

in affected birds. CA3 has previously been found to increase in muscle tissue of human hypoxia-trained athletes [94]. It is suggested that the observed increase in CA3 may be a mechanism to increase the rate of H^+ ions delivery to the interstitial fluid of the cell in order to combat muscle fatigue [94]. However, as discussed under the "oxidative stress" section, the upregulation of *CA3* in affected muscle may be primarily associated to its antioxidant role [95].

PFKFB3, a *HIF*-dependent gene, is found to be downregulated in birds affected with Wooden Breast in the current study. This is contrary to previous work, which demonstrated that under hypoxic conditions *PFKFB3* expression typically increases [96]. During hypoxic conditions, cells may switch from oxidative phosphorylation to glycolysis in order to maintain energetic homeostasis [96]; PFKFB3 plays a major role in this process as an activator and regulator of glycolysis and glucose consumption [97]. Since *PFKFB3* is downregulated in our study, it may be possible that glucose metabolism is regulated via a different mechanism under the hypoxic conditions associated with Wooden Breast in chickens. Further explanation for this finding remains to be studied.

Additionally, hypoxic conditions tend to stimulate satellite cell proliferation [81], which is extremely important for skeletal muscle regeneration [98], highlighting the significant role of oxygen concentrations and oxygen as a signaling molecule regulating satellite cells [99]. Hypoxic conditions can also influence the phenotype and differentiation ability of satellite cells [81]. Satellite cells constitute a supply of nuclei for muscle fibers during muscle growth and significantly contribute to

compensatory muscle hypertrophy. Muscle growth in chickens, after hatch, can also largely be attributed to hypertrophy [100]. In broilers, satellite cells have high proliferation and differentiation rates just after hatch [101]. After this time, however, proliferation rates tend to drop off [102], but satellite cells continue to contribute to myofiber hypertrophy [101]. Myogenesis, or muscle formation, is regulated in part by the transcription factor paired-box 3 (*Pax3*), which is upregulated in birds affected with Wooden Breast in the present study. It has been suggested that Pax3 activates genes associated with myogenic regulatory factors and after muscle injury, *Pax3* expression increases within cells that express myogenic regulatory factors [103]. Overall, the stimulation of satellite cell expansion under hypoxic conditions has the ability to lead to muscle hypertrophy during myogenesis and in myoregeneration, which has been observed microscopically in Wooden Breast muscle tissues in diagnostic cases analyzed in our laboratory (data not shown). Since all of the birds collected in this study were at the same developmental stage, it is likely that the upregulation of *Pax3* in affected birds is due to myoregeneration following muscle damage, rather than generalized muscle growth, though it is possible that this expression difference could also be linked with excessive muscle growth characteristic of birds with Wooden Breast.

3.4.3 Oxidative Stress

Oxidative stress caused by an increase in reactive oxygen species (ROS), may be a major contributor to the Wooden Breast phenotype. Many DE genes between the two groups of broilers being studied suggest an increase in ROS in birds affected with Wooden Breast. Increases in ROS within the skeletal muscle are typically generated as a response to increased contractile activity or after periods of major muscle disuse [104]. Increases in ROS can also occur following mitochondrial dysfunction as a result of oxidative phosphorylation [105]. A major increase in ROS is detrimental to muscle cell due to cytotoxicity and can impact cells by damaging or altering proteins and membrane lipids, thus altering cellular integrity [104]. It is also known that increased concentrations of ROS may impact calcium myofibril sensitivity and calcium release from the sarcoplasmic reticulum, resulting in damage to the contractile ability of muscle cells specifically [106]. Furthermore, increases in ROS also have the ability to impact and regulate cellular signaling pathways that are critical to gene expression and ultimately act on skeletal muscle remodeling and adaptation [104].

Oxidative stress can elicit high amounts of stress within the body and to specific tissues that undergo direct increases in ROS. Because of oxidative stress, the activation of stress response-related pathways and genes are appropriate reactions to prevent further damage. In the current study, the differential expression of many genes within the heat shock family and other stress response-related genes suggests the occurrence of oxidative stress within the breast muscle of affected chickens. The genes

within the heat shock family that are upregulated in affected chickens include: heat shock transcription factor 2 (*HSF2*), heat shock 27kDa protein family, member 7 (*HSPB7*), heat shock 70kDa protein 4-like protein (*HSPA4L*), and heat shock 105kDa/110kDa protein 1 (*HSPH1*). Another gene within this family that is also differentially expressed but found to be downregulated in affected chickens is FK506 binding protein (*FKBP51*). Heat shock proteins are broadly regulated, and a fairly large gene family, meaning that members respond to different stimuli [107]. This may explain why some heat shock protein family members in our study are upregulated while simultaneously another is downregulated. Heat shock proteins serve many functions such as molecular chaperones, guiding protein folding, and preventing protein buildup. During stressful events such as oxidative stress, the expression of heat shock proteins is known to increase greatly [108]. On the whole the heat shock proteins are activated in order to stimulate a pro-survival response during oxidative damage [109]. Therefore, the upregulation of most heat shock proteins in affected birds, indicates the possibility of oxidative stress damages within muscle cells, and also confirms the likelihood of cellular stress in birds with Wooden Breast. In addition, corticotrophin-releasing hormone (*CRH*), a major factor important to stress response pathways, was identified as a top upregulated gene in affected birds (fold change= 14.31) in the current study. *CRH* expression has been previously found to aid in the protection of neurons against oxidative stress [110]. CRH is found typically expressed in tissues outside the brain during inflammatory events [111]. The

accumulation of immune cells in Wooden Breast birds may be a potential source of CRH, as some immune cells are known to secrete CRH [111].

Other well-known myopathies, such as Vitamin E/Selenium deficiency-related (nutritional) myopathy, render skeletal muscle to be more susceptible to oxidative damages by depleting or overwhelming the antioxidant defense system [28, 38]. Such nutritional myopathies show overlapping microscopic lesions to Wooden Breast [1], such as muscle cell death, degradation and regeneration of myofibers [28], and white striations (adipose) within the musculature [39], vascular damage, and fibrosis [112]. These myopathies are frequently found in agricultural species, such as poultry, and negatively impact meat quality [28, 39, 113]. Most research surrounding nutritional myopathies, such as studies mentioned above, focus strongly on diet supplementation and feed intake [28, 38]. However, we can likely rule out feed as a major factor in Wooden Breast development because both affected and unaffected birds are exposed to the same diet. Additionally, unlike in nutritional myopathies, Wooden Breast appears to only affect the pectoral muscle rather than multiple muscles, gizzard, and heart, and myofiber mineralization is minimal to absent.

Nevertheless, selenoprotein-related deficiencies are strongly associated with oxidative stress damage, as selenoproteins are essential for counteracting oxidative stress [114]; the selenium-protein complex includes oxidative stress regulatory proteins such as glutathione peroxidase and thioredoxin reductase [114]. Within this study, selenoprotein O (*SELO*) is found to be downregulated in Wooden Breast affected broilers. SELO was recently characterized by Dudkiewicz et al. 2012 [115],

and it is suggested that it has important roles in oxidative stress response and may be a factor in molecular transport and efflux systems [115]. Interestingly, gene expression for chickens with dietary selenium deficiency (i.e. nutritional myopathy or White Muscle Disease) shows a downregulation of *SELO*, along with other selenoprotein-related genes [116]. The downregulation of this gene within the context of this study may either indicate a deficiency in SELO among birds impacted with Wooden Breast or that seloprotein concentrations are locally diminished within breast muscle of affected birds. In contrast to the downregulation of *SELO*, another family of antioxidative enzymes glutathione peroxidase 7 and 8 (*GPX7*, *GPX8*) are observed to be upregulated in birds affected with Wooden Breast, potentially because of elevated ROS levels and also to compensate for changes to SELO. In past studies, GPX7 has been shown to provide anti-oxidant functions against high levels of ROS in order to combat oxidative damages in humans [117]. It was also suggested that the presence of GPX7 is needed in order to maintain low ROS levels in specific cells types like oesophageal cells found in the human throat [117]. Another gene that is upregulated in affected birds and likely serves an anti-oxidative function is carbonic anhydrase 3 (*CA3*). Within the current study, *CA3* is one of the top differentially expressed genes with a fold change of 28.47. It has been proposed that CA3 has the ability to serve as an anti-oxidizing agent against damaging molecules, has both regulatory and reparative functions, and is a critical player in the antioxidant defense system within skeletal muscle [95].

3.4.4 Fiber-Type Switching

In the current study, one of the most differentially expressed genes in affected sample was myoglobin (*MB*). Myoglobin is mainly distributed within Type I or aerobic muscle (slow twitch) fibers and serves to store oxygen. High myoglobin content is typically correlated with "red" meat, which has a high density of Type I fibers [118]. However, the pectoralis, a "white" muscle in chickens, consisting almost entirely of type II, or anaerobic (fast twitch) fibers, in which myoglobin has previously either not been detectable [119] or observed at very low levels [36, 120]. In fact, the lack of myoglobin and increased glycogen content in Type II fibers account for the white color of pectoral meat [118]. Therefore high expression of myoglobin in the pectoralis muscle of broilers is unexpected and may possibly be explained by the phenomenon fiber-type switching, or a change from fast twitch to slow twitch fibers in response to myofiber degeneration and necrosis observed in Wooden Breast myopathy. Muscle fibers are extremely dynamic and tend to adapt to environmental cues [121]. The phenotypic composition of muscle fibers can be altered by various stressors such as activity rate, mechanical stress, hormones, aging, response to muscle damage, and/or changes in muscle oxygenation [121]. These events have the capacity to modify the functional properties of a given muscle fiber, possibly leading to fiber-type switching [121].

Fiber type switching from fast-to-slow twitch fibers has also been suggested in turkeys exhibiting PSE muscle conditions [122]. Upregulation of troponin I type I,

skeletal slow, (*TNNI1)* in the present study may support the hypothesis of fast-to-slow switch in Wooden Breast myopathy. *TNNI1* expression likewise differs by muscle type and, similar to *MB*, is also limited to expression in only slow skeletal fibers [123]. The *TNNI* subfamily has further been found to play a role in the mediation of skeletal muscle relaxation and contraction [124]. Other slow skeletal fiber-type isoforms upregulated in this study include myozenin 2 (*MYOZ2)* and myosin binding protein C, slow type (*MYBPC1)*. *MYOZ2* expression has been reported in only cardiac and slow-twitch skeletal muscle, and acts to control for calcineurin, which is a positive regulator of slow-type fibers [125, 126]. Similarly, *MyBPC1* is one of three MyBPC isoforms, and interacts with slow skeletal muscle to regulate muscle contraction [127]. In this study *TNNI1*, *MYOZ2*, and *MyBPC1* are upregulated in affected birds, potentially indicating fiber-type switching to type I fibers following myofiber damage in the Wooden Breast myopathy.

Jordan et al. 2004 [128] suggest that ryanodine receptor 1 (*RyR1*) in the pectoralis muscle in birds may act to suppress slow muscle specific genes. This is supported by high levels of reactive oxygen species (ROS) or nitric oxide (NO), which can irreversibly impact RyR1 subunits as a consequence of oxidation [129]. Consequently, the inhibition of *RyR1* by ryanodine may lead to a fast-to-slow fiber type switch [128]. Mutation to the *RyR1* gene in pigs have been previously correlated with the occurrence of malignant hyperthermia; a skeletal muscle myopathy, which leads to defects in Ca^{2+} channel functioning [130], PSE-type meat [130], and rigidity of the muscle caused by hypermetabolism [131]. It has also been suggested that

inositol trisphosphate receptor 1 [132], protein kinase C alpha and theta [133] and muscarinic acetylcholine receptor (M1) [134], possess critical roles in fiber-type switching, although in the current study these genes remain insignificant. Fiber-type switching has also been characterized in the callipyge sheep muscle condition. This condition in sheep involves muscles within the pelvic region that exhibit an increase in muscle mass as well as leaner muscle and is associated with an increase in feed efficiency [135]. Callipyge sheep show some similar symptoms to birds affected with wooden breast such as hypertrophy of the affected muscle and localization to specific muscle regions; however, it is suggested that the sheep actually undergo a slow-to-fast fiber type switch [136]. Thus, though there is indication that fiber-type switching may be occurring during Wooden Breast in chickens, the pathways altered for fiber-type switching are likely different in broiler chickens than those exhibited in myopathies of other species, such as the callipyge phenotype in sheep.

3.4.5 Cellular Repair

Many genes involved in cellular repair and transport are differentially expressed within our dataset. It is highly probable that these genes are linked to the Wooden Breast myopathy in an effort to compensate for muscle damage in this degenerative muscle disease.

One of the top genes upregulated in affected chickens is cysteine and glycine-rich protein 3 (*CSRP3*) (fold change= 23.17), which is also referred to as muscle LIM

(Lin11, Isl-1, and Mec-3) protein within the literature [137, 138]. *CSRP3* expression is present only in striated muscle, including skeletal muscle, and it plays a large role in myogenic differentiation [139]. The expression of *CSRP3* also correlates with myotube growth and formation [139], which is a key feature of muscle repair. Barash et al. 2005 [140] studied *CSRP3* knockout mice and found that deficient mice displayed an array of skeletal muscle defects such as shorter sarcomere length and muscle atrophy. They suggested that CSRP3 has a role in the maintenance of skeletal muscle and also supports skeletal muscle regeneration following injury through structural repair and gene regulation [140]. *CSRP3* may significantly increase in expression after periods of exercise in humans [141]. This may occur in order to promote muscle growth as CSRP3 has also been linked to involvement in muscle hypertrophy and regeneration through the calcineurin/NFAT signaling pathway and Myogenic Differentiation (MYOD) expression [139]. It is also interesting to note that *CSRP3* is mainly expressed in slow skeletal muscle, compared to its low expression in fast skeletal muscle [142]. Similar to this, *CSRP3* expression was upregulated during fast-to-slow fiber type switch in rats [142]. Therefore, as discussed above, fiber-type switching may be occurring in birds with Wooden Breast as a secondary response to the disease, acting as a mechanism of myofiber repair and/or regeneration under an altered redox homeostasis. Xu et al. 2009 [138] also suggest that the expression of *CSRP3* could have an impact meat quality in a study of porcine muscle since *CSRP3* is involved in both myofiber generation and fiber-type switching.

Along a similar line, another highly upregulated gene in this study is nephroblastoma overexpressed (*NOV*) also referred to as *CCN3* [143] (fold change= 7.33). *NOV* is a member of the CCN family of proteins, which contains cysteine-rich angiogenic protein 61 (*CCN1*), connective tissue growth factor (*CCN2*), and nephroblastoma overexpressed (*CCN3*) [144]. This protein family is important role players for many biological functions such as cell migration, proliferation, differentiation, and extracellular modification [144]. It is also thought that the *CCN* gene family take part in critical roles during cell development, wound healing, tissue homeostasis, and varying conditions including cancer and fibrosis [143]. Heath et al. 2008 [143] conclude from their results that *NOV* is necessary and required for normal muscle maintenance and use, showing that the over-expression of *NOV* leads to increased cell proliferation and promotes the survival of cells expressing myogenin. Myogenin is an essential regulator of myogenesis, and crucial to muscle maintenance, regeneration, and repair [145], as well as being overexpressed in musculoskeletal tumors [146]. *NOV* expression has also been previously linked with multiple components of cellular repair, as well as wound healing and endothelial adhesion and migration [147]. It is likely that *NOV* is involved in the Wooden Breast disease acting as a regulator of cellular and tissue repair through myoregeneration and cellular movement.

Other differentially expressed genes within the current study, such as musculoskeletal embryonic nuclear protein 1 (*MUSTN1*) also contribute to cellular repair and regeneration in skeletal muscle. In the current study *MUSTN1, which* is

considered essential for skeletal muscle growth and regeneration [141, 148], is upregulated in affected birds. Increased expression of *MUSTN1* has also been reported in domestic chickens; its expression is higher in broiler chickens than it is in layer chickens. This observation supports the notion that *MUSTN1* may be involved in the regulation of muscle hypertrophy [149]. *MUSTN1* expression was also upregulated in correlation to exercise-induced muscle damage, supporting its possible involvement in muscle damage repair [141]. As both affected and unaffected birds in the present study were from a similar genetic lineage of broiler chickens, upregulation of *MUSTN1* in affected birds likely indicates compensatory hypertrophy or muscle repair secondary to muscle damage rather than physiologic hypertrophy during bird growth.

Other key contributors to the actin cytoskeleton system, such as members of the Rho GTPase family, could also indicate cell differentiation or movement in the reparative phase of the disease. Within our dataset there are four members of this family that are upregulated. These include: Rho GTPase activating protein 10 (*ARHGAP10*), Rho GTPase activating protein 20 (*ARHGAP20*), and Rho GTPase activating protein 40 (*ARHGAP40*). Another significant member of this family that is downregulated is Rho-related BTB domain containing 3 (*RHOBTB3*). The Rho GTPase family is important regulators of the organization of the actin cytoskeleton [150]. Rho activation is necessary to maintain strong cellular and focal adhesion between cells [150], and Rho-GTPase proteins are involved in many general functions such as cell migration, cell proliferation, and cytoskeleton reorganization [151]. As an example, it is thought that the upregulation of *ARHGAP10* is essential to cell

differentiation within the cytoskeleton [152]. Although some of the functions of Rho GTPase genes remain unknown, it is possible that the expression of these genes within the current study is due to disruption within the actin cytoskeleton and subsequent repair from the damage manifested by the Wooden Breast myopathy.

Results from IPA show that the canonical pathways "Actin Cytoskeleton Signaling" (p-value= 3.18E-07) and "Axonal Guidance Signaling" (p-value= 1.27E-06) have probable functions in tissue repair during Wooden Breast muscle disease. The "Actin Cytoskeleton Signaling" pathway contains 36 significant differentially expressed genes from our gene list. Most of the genes within this pathway relate to specific growth factors such as, epidermal growth factor (*EGF*) and fibroblast growth factor (*FGF*), and also include many cytoskeleton related genes such as, actin related protein complex (*ARPC*) and many member of the myosin heavy and light chain families (*MYH*/*MYL*). As shown in Figure 5 the major endpoints of this pathway deal with the assembly, polymerization, and the stabilization of both actin and myosin. We hypothesize that the "Actin Cytoskeleton Signaling" pathway is mainly dealing with secondary response to injury in the form of cellular repair in affected muscle. It is important to point out the growth factors associated with this pathway include epidermal growth factor (*EGF*) and fibroblast growth factor 10 (*FGF10*) which are upregulated, and fibroblast growth factor 1 (*FGF1*) which is downregulated. It is more likely that growth factors are expressed differently in affected birds due to repair and regeneration occurring in the diseased muscle. The upregulation of *EGF* is important since *EGF* is previously known to promote myoblast proliferation to aid in muscle

repair [153]. However, the downregulation of FGF1 is unexpected, since it is known that *FGF1* contributes greatly to skeletal muscle proliferation [154]. Mitchell et al.

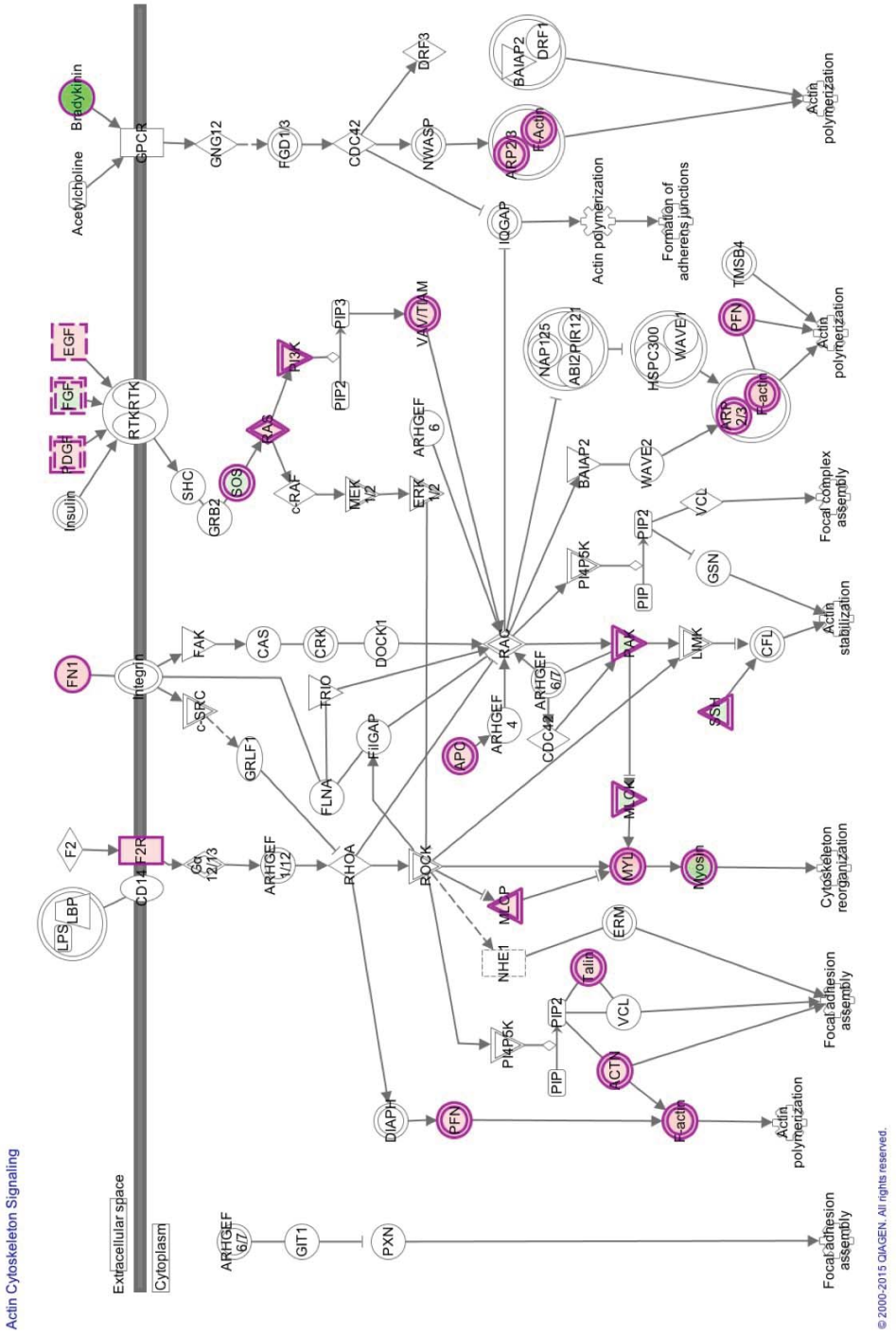


Figure 5 **The "Actin Cytoskeleton Signaling" pathway highlighting differentially expressed genes of birds affected with Wooden Breast myopathy.** Canonical pathways were identified and depicted by Ingenuity Pathway Analysis (IPA®) software. This pathway shows the "Actin Cytoskeleton Signaling" as related to significant differentially expressed genes in affected birds from the sample population. Genes that are highlighted in green are considered to be downregulated in affected birds. Genes that are highlighted in red are considered to be upregulated in affected birds

1999 [155] also found a downregulation of *FGF1* when studying the response to muscle stretch, or stretch hypertrophy, in chickens. Mitchell et al. 1999 [155] were inconclusive as to why this phenomenon was occurring, but hypothesized that stretch itself may contribute to downregulation of FGF1. Also seven members of the myosin family are differentially expressed within the "Actin Cytoskeleton Signaling" pathway including three myosin heavy chain genes (*MYH2, MYH6, MYH10*), and four myosin light chain genes (*MYL1, MYL9, MYL12B, MYLK2*). Myosin is essential for muscle contraction and comprises most of the myofibrillar proteins within muscle cells [156]. Within this pathway myosin is functioning to create "actomyosin assembly contraction" (Figure 5, IPA®). The assembly and contraction of the actomyosin complex is essential as both actin and myosin are responsible for muscle contraction [157].

The other canonical pathway related to cellular repair presented by IPA is "Axonal Guidance Signaling" (Table 5). This pathway contains 56 significant

differentially expressed genes from our gene list. Some of the gene families within this pathway include the A Disintegrin and Metalloproteinase (ADAM) metallopeptidase

Table 4 **Summary of the differentially expressed genes in the "Axonal Guidance Signaling" Pathway from IPA®.** Summary of significant DE genes their respective fold change expressed in affected birds in found within the "Axonal Guidance Signaling" canonical pathway. This table is a product of Ingenuity Pathway Analysis (IPA®).

Symbol	Entrez Gene Name	Fold Change
ABLIM2	actin binding LIM protein family, member 2	1.534
ACTR3	ARP3 actin-related protein 3 homolog (yeast)	1.591
ADAM8	ADAM metallopeptidase domain 8	2.651
ADAM11	ADAM metallopeptidase domain 11	-2.568
ADAM12	ADAM metallopeptidase domain 12	2.214
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	-1.514
ADAMTS2	ADAM metallopeptidase with thrombospondin type 1 motif, 2	1.516
ADAMTS6	ADAM metallopeptidase with thrombospondin type 1 motif, 6	2.557
ADAMTS7	ADAM metallopeptidase with thrombospondin type 1 motif, 7	1.720
ARPC5	actin related protein 2/3 complex, subunit 5, 16kDa	1.592
ARPC1A	actin related protein 2/3 complex, subunit 1A, 41kDa	2.597
BMP7	bone morphogenetic protein 7	1.994
C9orf3	chromosome 9 open reading frame 3	2.153
COPS5	COP9 signalosome subunit 5	-1.653
CXCR4	chemokine (C-X-C motif) receptor 4	1.973
EGF	epidermal growth factor	1.594
EPHA3	EPH receptor A3	2.422
EPHB3	EPH receptor B3	1.801
FZD1	frizzled class receptor 1	1.990
GNA14	guanine nucleotide binding protein (G protein), alpha 14	-5.738
GNG2	guanine nucleotide binding protein (G protein), gamma 2	1.850
HHIP	hedgehog interacting protein	-2.551
HRAS	Harvey rat sarcoma viral oncogene homolog	1.823
LINGO1	leucine rich repeat and Ig domain containing 1	-3.306
LRRC4C	leucine rich repeat containing 4C	-5.124
MET	MET proto-oncogene, receptor tyrosine kinase	1.815
MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	2.183
MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	2.337
MMP11	matrix metallopeptidase 11 (stromelysin 3)	2.545
MMP13	matrix metallopeptidase 13 (collagenase 3)	2.461

MRAS	muscle RAS oncogene homolog	2.989
MYL3	myosin, light chain 3, alkali; ventricular, skeletal, slow	5.358
MYL9	myosin, light chain 9, regulatory	1.732
MYL10	myosin, light chain 10, regulatory	4.976
MYL12B	myosin, light chain 12B, regulatory	1.941
PAK1	p21 protein (Cdc42/Rac)-activated kinase 1	1.687
PAK3	p21 protein (Cdc42/Rac)-activated kinase 3	-1.504
PDGFD	platelet derived growth factor D	1.949
PFN2	profilin 2	2.077
PIK3R6	phosphoinositide-3-kinase, regulatory subunit 6	1.688
PLXNA2	plexin A2	-1.590
PLXNC1	plexin C1	1.585
PRKCH	protein kinase C, eta	-2.143
ROBO1	roundabout, axon guidance receptor, homolog 1 (Drosophila)	2.144
ROBO3	roundabout, axon guidance receptor, homolog 3 (Drosophila)	4.484
SEMA3A	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	1.560
SEMA3G	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3G	-1.874
SEMA7A	semaphorin 7A, GPI membrane anchor (John Milton Hagen blood group)	-2.359
SLIT3	slit homolog 3 (Drosophila)	3.103
SOS1	son of sevenless homolog 1 (Drosophila)	-1.655
TUBA8	tubulin, alpha 8	-2.487
TUBA1B	tubulin, alpha 1b	2.399
TUBA1C	tubulin, alpha 1c	-1.714
TUBB6	tubulin, beta 6 class V	1.791
WNT11	wingless-type MMTV integration site family, member 11	2.511
WNT2B	wingless-type MMTV integration site family, member 2B	2.380

family, myosin light chain family, and the SEMA (Semaphorins) domain family. Since the muscle and surrounding connective tissues are undergoing injury, inflammation, and edema in Wooden Breast, it is possible that there is a simultaneous disruption and repair in axons of nerve fibers within the muscle. As reported through IPA some of the possible functions of this pathway include, cytoskeleton reorganization, actin filament reorganization, axon outgrowth, and axon guidance. Several genes within this pathway are regulators of cellular repair and regeneration in the muscle and axon systems. For example, *ADAM12*, which is upregulated in affected

birds, is most often expressed in adult muscle during regeneration, and may take part in reducing necrosis and inflammation [97]. Secondly, *SEMA3A*, which is upregulated in affected birds, acts to promote intramuscular re-innervation in damaged muscle fibers in order to restore muscle contraction [158]. It is also interesting to note that, clinically, birds with Wooden Breast typically display a drastic decrease in the amount of muscle fasciculations after death compared to normal birds (data not shown). Although this phenomenon remains to be studied more fully, it is possible that damage to either muscle or nerve fibers have led to loss of function within the muscle, though there is no significant histologic evidence of primary nerve damage within affected muscle. From the results given through IPA we postulate that both the "Actin Cytoskeleton Signaling" and "Axonal Guidance Signaling" canonical pathways are not likely primary causes of the Wooden Breast myopathy, but are secondary to the disease, differentially expressed during the repair phase of the illness.

3.4.5.1 Coagulation System Pathway and Related Genes

The inflammation observed microscopically in birds affected with Wooden Breast suggests that genes involved in the coagulation system pathway would likely be upregulated due to vascular (venous) damage and inflammation. It is highly regarded that inflammation promotes the activation of coagulation [159]. However, our data supports the downregulation of genes within the coagulation system pathway in affected brids as given by IPA® (Figure 6). It should be noted that the genes involved

in the coagulation system pathway are mainly expressed in the liver, where they are synthesized, rather than in muscle. The DE genes involved within the coagulation pathway identified by the present study include: alpha-2-macroglobulin (*A2M)*, fibrinogen alpha chain *(FGA),* fibrinogen beta chain *(FGB),* and fibrinogen gamma

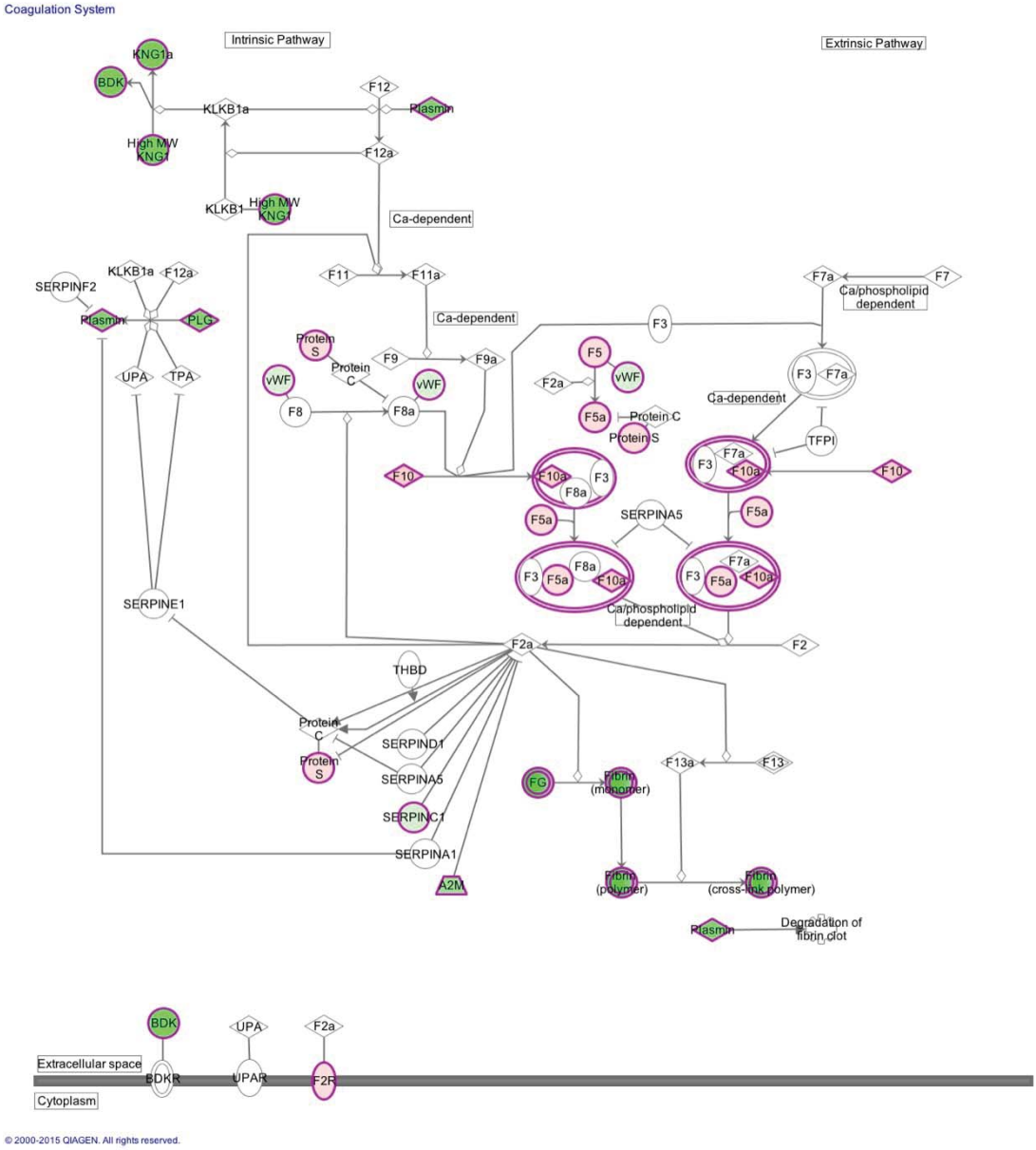


Figure 6 **The "Coagulation System" pathway highlighting differentially expressed genes in birds affected with Wooden Breast myopathy.** Canonical pathways were identified and depicted by Ingenuity Pathway Analysis (IPA®) software. This pathway shows the "Coagulation System" as related to significant DE genes in affected birds from the sample population. Genes that are highlighted in green are considered to be downregulated in affected birds. Genes that are highlighted in red are considered to be upregulated in affected birds.

chain (*FGG)*, kininogen 1 (*KNG1)*, plasminogen (*PLG)*, serpin peptidase inhibitor, clade A, member 1 (*SERPINC1)*, and von Willebrand factor (*VWF),* which are all found to be downregulated (Figure 2). Other DE genes within this pathway which are upregulated include: coagulation factor V (*F5)*, coagulation factor X (*F10)*, coagulation factor II (*F2R)*, and protein S (alpha) (*PROS1)*. The reasons for the involvement of the coagulation pathway and its overall downregulation in Wooden Breast remain to be elucidated.

3.4.6 Biological Significance of Gene Expression Profile:

Features of the gene expression profile (increase in intracellular calcium, hypoxia, and oxidative stress, etc.) brought to light by this study have strong correlation to the histologic lesions reported previously by Sihvo et al. 2014 [1] and observed in diagnostic specimens evaluated by our laboratory (data not shown).

A major characteristic of increased intracellular calcium levels is myofiber hypercontraction. Hypercontraction associated with skeletal muscle degeneration has been previously identified across species [160, 161]. It is suggested that the

accumulation of Ca^{2+} within the cell leads to hypercontraction either as a result of poor Ca^{2+} -ATPase performance [162] or because of interference with sarcolemma integrity [163]. Ultimately hypercontraction results in cell degeneration and necrosis [162]. Histologically hypercontraction can be established through the observation of irregular Z bands or as hyaline fibers, which form as a result of excess calcium [164, 165] and leads to the coagulation of the contractile proteins within the cells [165]. Although irregular Z bands have not been a prominent feature of Wooden Breast cases, swelling and hyalinization of fibers is the predominant myofiber lesion present in affected Delmarva poultry (data not shown) and has also been reported by Bilgili 2013 [61]. The "hypereosinophilic" nature of myofibers described by Sihvo et al. 2014 [1] would also correlate to hyaline degeneration of myofibers. Furthermore, it is highly likely that the microscopic swelling and coagulation of proteins observed to be widespread within affected muscle fibers may be contributing to clinically and grossly evident hardening of the pectoral muscle as a whole. These observations support the hypothesis of hypercontraction and/or contractile protein denaturation, potentially due to increased levels of intracellular calcium. It should also be noted that no significant mineralization within the muscle was observed grossly or microscopically to suggest dystrophic calcium deposition in degenerating or necrotic myofibers. As such, it appears that calcium overload, as supported by gene expression profiling results, mainly affects the contraction state of myofibers in Wooden Breast.

Birds affected with Wooden Breast also show characteristics of histologic changes and clinical symptoms associated with hypoxia [1, 61], potentially due to

vascular damage or inadequacy [61]. From previous histologic reports [1, 61] it is unclear whether poor vasculature is the inciting cause of hypoxia or if there are other contributing factors, which establish a hypoxic environment. Hypoxia and oxidative stress have been continually linked in research, as in the present study. As mentioned, from RNA-seq results, it is likely that the pectoralis major muscle undergoes hypoxic events in Wooden Breast. Although vascular damage is present in affected birds, this is only seen on the venous side, rather than the arterial side of the vascular system; as such, there is little microscopic evidence to suggest impairment of oxygenated blood delivery to the tissues unless there is a subtle difference in vascular density between affected and unaffected birds. However, venous damage, supported by histologic evidence of an accumulation of immune cells surrounding and within veins in affected muscle, as reported by Sihvo et al., 2014 [1] and as observed in our laboratory (data not shown), could ultimately impair the removal of cellular metabolic waste products, lead to build-up of oxidative metabolites, and trigger further damage to surrounding cells. Inflammation in the muscle may also be contributing to or responding to a hypoxic state in the tissue.

To study the impact of hypoxic environment in mice, Lopes-Ferreira et al. 2001 [98] investigated skeletal muscle degeneration using fish venom and reported histological outcomes associated with the hypoxic environment including, inflammation, infiltration of macrophages and leucocytes, fiber necrosis, variations in fiber size, hypercontracted fibers, as well as fibrosis, microscopic findings similar to those in Wooden Breast. Fibrosis in particular is a common sequela of muscle repair

under hypoxic conditions, as the occurrence of fibrosis aims to replace lost muscle tissue with collagen and fibrocytes [98]. Fibrosis is evident microscopically in Wooden Breast muscle, and several genes within the collagen family have been determined to be upregulated in the present study, including: *COL11A1*, *COL12A1*, *COL14A1*, *COL16A1, COL21A1*, *COL24A1, COL25A1*, *COL27A1*, *COL3A1, COL 4A3, COL4A4*, *COL4A5*, *COL5A1, COL5A2*, *COL6A1-3, COL8A1*, *COL8A2*, and *COLEC12*. Furthermore, there is additional microscopic evidence to corroborate a hypoxic state; beyond fibrosis, the accumulation of lipid within affected muscle is also apparent histologically as variably-sized discrete vacuoles within the sarcoplasm of affected muscles (data not shown). Hypoxia has been previously linked with increases in lipid storage in cultured cells [166], cardiac muscle [167], and also in macrophages [168]. In support of the previous studies [1, 61], the present study revealed various differentially-expressed genes between affected and unaffected birds that may be correlated with a hypoxic state within the pectoralis major muscle of chickens with Wooden Breast. Hypoxia appears to be a critical factor in the Wooden Breast myopathy, though it is not apparent whether it is a primary cause for muscle lesions or is occurring secondary to inflammation and myofiber swelling from another cause.

Previous muscle disorders described in chickens, such as deep pectoral myopathy (DPM), have been associated with hypoxia resulting from restricted blood flow to the muscle as the predominant cause of muscle degeneration [13, 169]. The incidence rate of DPM is typically associated with high-yield breast muscle birds with high growth rate [13]. In the authors' experience, Wooden Breast has also a high

correlation with the growth rate and breast muscle weight of commercial broilers; most cases occur in breeding lines associated with fast growth, high feed efficiency, and high breast muscle yield. However, unlike in DPM, necrosis is limited to individual myofibers rather than widespread, and the superficial pectoral muscle is more affected than the deep pectoral muscle. Regardless, as Sihvo et al. 2014 [1] mention, it is highly likely that the high growth rate and increased breast muscle weight of modern-day broilers are key factors in myodegenerative disorders, including Wooden Breast and deep pectoral myopathy.

The gene expression profile brought to light within this study and previously reported histology of Wooden Breast suggests a pro-inflammatory response is taking place in affected birds, which involves the influx of immune cells. In the human muscular disease, Duchenne muscular dystrophy (DMD), cellular membrane damage caused by high intracellular calcium levels and inflammation leads to further immune responses observed by a dramatic rise in inflammatory cell concentration within the affected area [170, 171]. Immune cells are known to generate ROS during inflammatory events in order to stimulate phagocytosis [172]; in mammals, ROS is formed by neutrophils and macrophages through NADPH-oxidase, a membrane-bound molecule [173]. ROS produced by immune cells has the ability to cause direct damage to muscle tissue or to act indirectly through the activation of catabolic signaling through immune molecule interaction with muscle receptors [174]. Histologic lesions for Wooden Breast suggest signs of oxidative stress with observations such as fiber degeneration, variation in myofiber size, membrane and cellular rupture,

inflammation, immune cell response, and replacement of myofibers with connective tissue and/or fat [1, 61]. Sihvo et al. 2014 [1] suggest that broiler selection for large breast muscle size coupled with an increased growth rate may cause broilers to be more vulnerable to oxidative stress.

Histologically, it is seen that tissue repair is occurring through mechanisms such as inflammation, myofiber regeneration, and fibrosis [1], as well as possibly through fiber-type switching. Canonical pathways reported by IPA such as "Actin Cytoskeleton Signaling" and "Axonal Guidance Signaling" also support attempted regeneration and repair of the muscle. The infiltration of immune cells to the area may support these efforts to repair damaged tissues. Macrophages are essential for removing damaged muscle proteins to allow regenerative fiber replacement; some macrophages have pro-regeneration qualities helping with muscle regeneration associated with their function [175]. Fibrosis and myofiber regeneration, both of which are also supported by the differentially expressed genes in our study, are other examples of microscopic observations that support a reparative phase is taking place. Ultimately, genetic expression profiling undertaken in the present study has strong correlation to known microscopic lesions present in Wooden Breast.

3.5 Conclusion

Using RNA-sequencing and pathway analysis tools such as IPA, we were able to define possible primary and secondary factors affecting the breast muscle of

affected chickens. We were further able to link RNA-sequencing data to histologic findings in our lab and previously published reports of Wooden Breast myopathy [1] to show potential biologic significance for the characteristic gene expression profile observed in Wooden Breast. Through the analyses of differentially- expressed genes significant differences were observed in genes associated with intracellular calcium, possible fiber-type switching, hypoxia, and oxidative stress (Table 6). Although it remains difficult to characterize features of the disease are primary or secondary, it is likely that all play a significant role in the pathogenesis of this disease. Also, we were able to establish a trend of significant genes and pathways related to cellular repair, which are likely occurring secondary to tissue damage in Wooden Breast myopathy.

The present work should be considered a first step towards elucidating the underlying genetic fingerprint of the Wooden Breast myopathy in commercial broilers. With the RNA-sequencing profile identified by this work, there is a potential to find specific genetic markers characteristic for this disease for use in diagnosis and detection of subclinical or carrier birds. Future work with RNA-sequencing across the time course of disease development would be beneficial. Since birds in the present study were all collected at market age, performing a time-series study using RNA-sequencing could help determine the specific age of onset of this disease and may help distinguish primary from secondary mechanisms contributing to Wooden Breast and associated related gross and histologic lesions. Histochemical staining for specific

Table 5 **Summary of main categories of genes involved in Wooden Breast myopathy separated by category**. For each gene category, the complete gene name, symbol, fold change from RNA-seq data, and fold change from Nanostring® data are provided. Positive and negative fold change values support respectively upregulation and downregulation of gene expression in birds affected by Wooden Breast myopathy relative to the gene expression in unaffected birds.

Gene Category	Gene Name	Symbol	RNA-seq Fold Change	Nanostring Fold Change
Fiber-Type Switching:				
	Myoglobin	MB	14.3	12.4
	Myosin Binding Protein C, slow type	MYBPC1	9.1	16.2
	Myozenin 2	MYOZ2	7.8	7.7
	Troponin I type I, skeletal slow	TNNI1	4.0	N/A
Intracellular Calcium:				
	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	ATP2A2	2.1	N/A
	Cytosolic phospholipase A2	PLA2G4A	2.3	N/A
	Parvalbumin	PVALB	8.1	7.2
	Phospholipase B1	PLB1	3.4	N/A
Hypoxia:				
	6-phophofructo-2-kinase	PFKFB3	-4.1	N/A
	Arylhydrocarbon Receptor Nuclear Translocator 2	ARNT2	2.2	1.7
	Asparagine synthetase	ASNS	4.4	N/A
	Matrix metallopeptidase 2	MMP2	2.2	N/A
	Muscle-specific carbonic anhydrase III	CA3	28.5	25.5
	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	PLOD2	2.5	N/A
	Transforming growth factor beta 3	TGFB3	3.9	3.7
	Transient receptor potential cation channel, subfamily A	TRPA1	6.7	N/A
Oxidative Stress:				
	Corticotrophin-releasing hormone	CRH	14.3	13.3
	FK506 binding protein	FKBP51	-1.9	N/A
	Glutathione peroxidase 7	GPX7	2.3	N/A
	Glutathione peroxidase 8	GPX8	2.1	N/A
	Heat shock 27kDa protein family, member 7	HSPB7	3.2	4.3
	Heat shock 70kDa protein 4-like protein	HSPA4L	1.5	N/A
	Heat shock 105kDa/110kDa protein 1	HSPH1	2.2	N/A
	Heat shock transcription factor 2	HSF2	2.9	N/A
	Muscle-specific carbonic anhydrase III	CA3	28.5	25.5
	Selenoprotein O	SELO	-2.1	N/A
Cellular Repair:				
	ADAM metallopeptidase domain 12	ADAM12	2.2	N/A
	Cysteine and glycine-rich protein 3	CSRP3	23.2	27.3
	Epidermal growth factor	EGF	1.6	N/A
	Fibroblast growth factor 1	FGF1	-2.1	-1.9
	Fibroblast growth factor10	FGF10	2.4	1.3
	Musculoskeletal embryonic nuclear protein 1	MUSTN1	4.9	6.0
	Myosin, heavy chain 13, skeletal muscle	MYH13	-4.7	1.0
	Myosin, light chain 2, skeletal slow	MYL3	5.4	1.5
	Myosin, light chain 12A regulatory	MYL12A	1.9	N/A
	Myosin light chain kinase 2	MYLK2	-2.6	N/A
	Nephroblastoma Overexpressed	NOV	7.3	3.0

	Rho GTPase activating protein 10	ARHGAP10	2.2	N/A
	Rho GTPase activating protein 20	ARHGAP20	2.4	N/A
	Rho GTPase activating protein 40	ARHGAP40	3.0	2.8
	Rho-related BTB domain containing 3	RHOBTB3	-11.9	-5.1
	Sema domain, Ig, short basic domain, secreted, 3A	SEMA3A	1.6	N/A

fiber type could further be implemented to confirm if a fiber type switch is occurring in affected birds. Since it is also possible that a single gene or a few candidate genes are principle causal factors in this disorder; conducting a genome-wide association study (GWAS) could also be greatly beneficial.

To our knowledge, this is the first study on the Wooden Breast disease in commercial broiler chickens to focus on RNA-sequencing data and gene expression profiling. Although the underlying cause of this disease still remains unknown, this work has brought forward many factors associated with the pathogenesis of this muscle disease. This work further shows the importance of using RNA-sequencing in biologic correlation with gross and microscopic disease lesions to study and compare muscle disorders across species. While more research must be performed to gain insight into the specific origin of Wooden Breast myopathy in chickens, RNA-sequence profiling has the potential to directly contribute to disease diagnosis and research for myopathies across animal species, including humans.

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Appendix

SAMPLE IDENTITY VERIFICATION

To verify the identity of the samples used in our RNA-seq experiment, we applied both genotyping of genomic DNA (gDNA) with high-density SNP arrays and SNP genotype calling from the RNA-seq data. For genotyping with SNP arrays, gDNA was isolated from blood samples by QIAGEN® DNeasy Blood and Tissue Kit following the manufacturer's protocol for nucleated blood. The gDNA samples were genotyped using the high-density 600k chicken SNP array from Affymetrix ® [1] in which 580,961 SNPs were included.

For SNP calling from the RNA-seq data, we applied a 2-step variant calling method to reduce reference allele bias. First, reads from RNA-seq of all samples were initially mapped to the latest assembly of the chicken reference genome (Galgal4.0) with 2-pass alignment in STAR [2], and then we followed the workflow of GATK for variant calling [3]. To reduce false positive calls, we eliminated the low quality variants (with read depth (DP) < 100, quality by depth (QD) < 2 or strand bias (FS) >30) and clusters with more than 2 SNPs within a window of 15 bp. By applying these criteria, we obtained 701,067 SNPs and INDELs (insertions and deletions) in the first step of variant calling. Secondly, we created a masked chicken genome in which all variant positions from the first step were masked to N for reads alignment, and then followed the same workflow of variant calling by GATK [3] and the same criteria for quality control. To further reduce reference allele bias, we excluded INDELs and the

SNPs in less than 100bp from nearby INDELs because the reads carrying INDELs have less chance to be mapped to the reference genome [4]. After all, we obtained 201,079 SNPs on exonic regions of the chicken genome, of which 14,887 were overlapped with the SNPs from the 600k SNP array based on their physical positions. Of these overlapped SNPs, we only used homozygous loci for the identity verification of the samples used in our RNA-seq experiment, as heterozygous SNPs may express in allele specific ways. For each chicken, we matched its 600k SNP genotypes of homozygous loci with those in variant calling from RNA-seq data and used the percentage of matching genotypes for the identity verification of the samples.

We found the percentages of matching genotypes between SNP arrays and RNA-seq from the same birds were higher than 99.2 but between different birds, the percentages ranged from 58.4% to 68.5%. This information allowed us to verify the identity of all samples used in our RNA-seq experiment (see below Table 1).

Table 6 **Matrix of percentages (%) of matching genotypes between 600K SNP array and RNA-seq.**

SeqID*	Samples genotyped by 600k SNP chips										
	C51	C52	C53	C55	C56	C57	C59	C62	C63	C64	C66
C51	99.4	59.7	59.5	60.0	61.4	59	58.9	59.7	60.7	60.5	60.3
C52	62.8	99.4	63.6	62.4	62.6	64.5	64.3	64.3	64.8	65.4	63.8
C53	61.0	62.1	99.4	61.2	62.9	61.5	63.5	63.6	62.8	64.0	60.9
C55	60.8	60.3	60.5	99.2	62.6	66.4	62.4	63.4	62.7	60.1	62.0
C56	64.7	63.1	64.7	64.9	99.3	62.4	65.5	65.5	63.4	62.7	65.1
C57	60.9	63.2	61.9	67.6	60.8	99.4	61.5	63.2	65.0	62.5	63.7
C59	59.3	61.5	62.2	61.9	62.5	60.1	99.4	63.6	62.6	65.1	61.2
C62	61.1	62.4	63.3	63.7	63.3	62.7	64.5	99.5	63.2	63.2	68.5
C63	60.7	61.7	61.3	61.6	59.8	63.1	62.2	61.7	99.5	62.4	60.7
C64_1	60.9	62.2	63.2	63.6	63.1	62.5	64.5	99.5	62.9	63.1	68.3
C64_2	61.7	63.4	63.5	60.4	60.8	61.8	66.0	63.1	63.7	99.5	61.7
C66	59.5	59.6	58.4	60.1	60.4	60.6	59.9	65.9	59.7	59.5	99.4

Note: *: ID from RNA-seq samples. C64_1 stands for sample C64 in our first batch of cDNA library preparation for RNA-seq, which shows that its genotypes from RNA-seq are matched to sample C62's genotypes on SNP array, marked red color in the table. Thus, we repeated the RNA isolation, cDNA library preparation and RNA-seq for C64 labeled as C64_2 in the table. Its percentage of matching genotypes between 600K SNP array and RNA-seq is 99.5%, marked green color in the table. Since NanoString data for this sample was obtained using C64_1 RNA sample, we excluded this sample from log2 (fold-change) estimation from Nanostring data.

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