MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF WOODEN BREAST MYOPATHY IN COMMERCIAL BROILER CHICKENS

by

Michael Papah Babak

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal and Food Sciences

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Michael Papah Babak

Approved: _____
Erin E. Connor, Ph.D.
Chair of the Department of Animal and Food Sciences

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

Approved: _____
Douglas J. Doren, Ph.D.
Interim Vice Provost for Graduate & Professional Education and Dean of the Graduate College

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Behnam Abasht, Ph.D.
Professor in charge of dissertation

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Jack Gelb Jr., Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Carl J. Schmidt, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Stewart-Brown Bruce, DVM
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Erin M. Brannick, DVM
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed: _____

Christopher M. Modlesky, Ph.D.
Member of dissertation committee

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ABSTRACT

The development of commercial broiler chickens through artificial genetic selection has been one of the greatest milestones in the poultry industry on the quest for a food secure world. Indeed, modern broiler chickens have high genetic merit for economically important production traits such as fast-growth rate, high muscle yield and high feed efficiency. The possession of these traits by the genetically selected broiler chickens has contributed immensely to a steady increase of chicken meat production in the U.S and around the world for the past 50 years. However, the development of modern broiler chickens has also been accompanied by the emergence of myodegenerative disorders targeting the fast-growing and high-feed efficient broiler birds. One of these disorders is Wooden Breast (WB) myopathy.

Wooden Breast myopathy is a relatively novel myopathy that frequently affects the pectoralis (P.) major muscles of modern broiler chickens resulting in their extreme firmness and microscopic changes in the muscle tissue thereby compromising meat quality. While significant knowledge about the gross, microscopic and molecular changes of WB in commercial broiler chickens has been gained through several studies on chickens around market age, information about time of onset, early pathogenesis of the myopathy including pathological and molecular changes in the P. major muscle is scarce. Consequently, the disorder continues to cause significant economic losses in the poultry industry unabated. To allow for development of mitigation strategies and prevention of losses attributed to WB in the poultry industry, it was imperative that a comprehensive understanding of the onset and progression of

the myopathy throughout the growth phase of broiler chickens be undertaken. This, therefore, formed the main objective of the present study project, that is to gain an in-depth understanding of the pathology and pathogenesis of WB in modern broiler chickens in commercial settings. To accomplish this major objective, three-pronged systematic studies aimed at assessing clinical/phenotypic, macroscopic, microscopic and molecular perturbations in pectoral muscles associated with the onset and development of WB in broiler chickens was conducted.

The first study involved a time-series assessment of the clinical/phenotypic features coupled with gross and microscopic changes of the pectoral muscles affected with WB in chickens raised from day-old to week 7 post-hatch. This study utilized chickens belonging to a high-breast-muscle-yield, purebred commercial broiler line, raised in standards that were similar to those in commercial settings. Upon weekly evaluation of chickens for phenotypic, gross and microscopic changes, it was established that WB exhibits an earlier onset than when detectable by clinical examination. Further, this study showed that the disease assumes a progressive course with acute vasculitis limited to small-caliber veins, lipid infiltration and deposition, and myodegeneration occurring in the earlier stages, followed by a chronic fibrotic phase.

The second study involved molecular evaluations focusing on the global gene expression in the P. major muscles between affected and unaffected chickens at week 2, 3 and 4 of age. The specific aim of this study was to identify the main genes that were differentially expressed between WB-affected and unaffected chickens, and subsequent establishment of their biological relevance to the pathogenesis of the myopathy. This particular study used the same experimental chicken samples as that

of the first study. However, unlike the first study which used necropsy samples, this study used muscle biopsy samples harvested from the cranial and caudal ends of the P. major muscles from selected chickens at the 3-time points and then processed for RNA-sequencing. The findings from this study revealed existence of molecular perturbations involving energy metabolism associated with lipid and carbohydrate metabolism, vasculature and extracellular matrix, as well as response to inflammation as being pertinent to the onset and early pathogenesis of WB in commercial meat-type chickens.

The third study, essentially a continuation of the second one, was based on our previous knowledge from transcriptomic studies that indicated possible evidence of perturbations in lipid metabolism and presence of slow myofiber-phenotype in the typically fast myofiber-type of P. major muscles in affected chickens. The objective of this study was, therefore, to evaluate and confirm the occurrence of altered lipid metabolism and occurrence of slow myofiber-phenotype during the development of WB in commercial broiler chickens. Specifically, the aim was to localize the cellular expression of specific lipid metabolism-related and slow muscle-related genes in the P. major muscle tissue in affected and unaffected chickens. To accomplish this objective, P. major muscle tissue samples from two chicken lines, namely slow growing Legacy chickens (not known to be affected by WB), and fast-growing Ross chickens (frequently affected by the muscle disorder), were harvested at around 3 weeks of age and subsequently processed for RNA *in situ* hybridization targeting the expression of specific genes. The specific genes examined included *lipoprotein lipase* (*LPL*) and *perilipin 1* (*PLIN1*) for lipid-related genes, and *myosin binding protein C slow-type* (*MYBPC1*), and *cysteine and glycine rich protein 3* (*CSRP3*) for slow-type

muscle-related genes. Concomitant to this, the global transcriptomic profile (RNA-seq) of 10 genes related to lipid metabolism and 7 genes related to slow-myofiber-type muscle genes were examined. The RNA-seq expression data was generated from a comparison between WB-affected and unaffected chickens at 3 weeks post-hatch (early stage of the myopathy) and 7 weeks post-hatch (late stage of the myopathy). From this study, *LPL* was revealed to be expressed in the vascular endothelial cells of capillaries and small-caliber veins of all chickens. As such, this became the first study to reveal the expression of *LPL* from the endothelial cells in chickens, a feature that is in stark contrast to that of mammals. Further, this study confirmed presence of dysregulation of lipid metabolism between the early and late stages of WB. On the other hand, cellular expression of slow myofiber-type genes were enhanced in mature myofibers of affected chickens suggesting the existence of appearance of slow myofiber-type phenotype in the face of WB. Similarly, analysis of global gene expression related to slow myofiber-type indicates a concomitant increase of the rate of expression of slow myofiber-type isoform genes with the severity of the disease.

In conclusion, the findings from the three studies have shown that WB is a complex myopathy that begins early in the life of chickens, with the initial sites or processes to be affected being the vasculature, energy homeostasis involving lipids and carbohydrates, and extracellular matrix. As a response to the homeostatic imbalance, recruitment of inflammatory cells and fibroblasts, together with the emergence of slow-myofiber phenotype occurs resulting in the various gross and microscopic changes that characterize WB.

Chapter 1

INTRODUCTION

Meat generally forms one of the main sources of high-quality protein diet to humans. While a variety of animal species that provide this highly regarded source of protein to humans exists, poultry meat is particularly desired by the majority of people. The preference for poultry meat, which is considered white meat, is largely driven by its high biological value protein (20-22%), as well as presence of microelements such as iron, copper and zinc, which have high bioavailability in lower quantities compared to red meats. Additionally, poultry meat has significantly high content of group B vitamins such as thiamin, riboflavin, pantothenic acid and vitamin B6. It has also been shown that the low total fat quantity and unsaturated lipids (mainly cutaneous fat that is easily removed) associated with poultry make the meat highly desired (Marangoni et al., 2015). Further, poultry meat is relatively inexpensive compared to meats from other animal species, hence, can easily be afforded by most households (Marangoni et al., 2015). As a consequence of all these, market demand for poultry meat and associated products in the U.S. and most parts of the world have been on a constant upward trend over the last five decades (Daniel et al., 2011; Magdelaine et al., 2008). This increased demand has led to development of modern broiler chicken lines, possessing high capacity for desired production traits such as fast-growth rate, high muscle yield and high feed efficiency (Havenstein et al., 1994, 2003; Lilburn, 1994).

While the development modern broiler chickens has resulted in an increase of poultry meat in the U.S. and around the world, novel muscular disorders targeting these group of birds in commercial broiler production systems appear to be emerging (MacRae et al., 2006; Mitchell and Sandercock, 2004). A full description of the various myopathic disorders affecting broiler chickens is found in Chapter 2. Owing to the importance of these disorders in the broiler industry, significant research attention is currently being undertaken in attempting to find insights into their underlying etiologies, pathogenesis and pathology, and possible prevention strategies. Of the several emerging myopathic disorders, Wooden Breast, and, to a large extent, White striping, are the most significant muscle defects since they affect the pectoralis (P.) major muscles, which is considered prime cuts in the poultry industry. Therefore, carcasses affected by Wooden Breast and/or White Striping have reduced quality grades, and severe carcasses are frequently condemned. Indeed, the economic loss due to WB with or without White Striping is estimated to be between USD 200 million to 1 billion (Kuttappan et al., 2016; Zanetti et al., 2018). This therefore calls for urgent management and preventive measures to forestall the huge economic losses associated with the WB syndrome in commercial broiler chickens. To achieve this, an in-depth understanding of time of onset, pathology and pathogenesis of the WB myopathy throughout the growth period of commercial broiler chickens is needed. Therefore, the primary objective of the present study was to characterize the development of WB disease in modern broiler chickens by examining the clinical/phenotypic, gross, microscopic and molecular changes associated with the pathology and pathogenesis of the disorder in commercial broiler chickens. Chapter 3 of this work focuses on the clinical and morphological changes comprising gross, microscopic and ultrastructural

perturbations in P. major muscles associated with the pathogenesis of WB myopathy. Similarly, Chapters 4 and 5 examine the gene expression patterns and associated biological pathways underpinning the development of WB disease in modern broiler chickens. It is envisaged that the findings from this study in its entirety, would be important not only in providing insights into the etiology of WB, a feature that has remained elusive for long, but also in the efforts geared towards formulation of management and preventive strategies against the myopathy.

An understating of the morphological and molecular alterations that occur in the P. major muscle as a result of WB requires an appreciation of the basic biology of the muscle tissue in the physiological state. The following section, therefore, examines the morphological organization and the physiology of the skeletal muscle.

The vertebrate musculature is composed of three types namely, skeletal muscles which forms the bulk of vertebrate musculature, cardiac muscles found in the heart, and smooth muscles found in walls of tubular organs and associated glands. The vertebrate musculature is often classified on the basis of structure, function or a combination of the two schemes (Kisia and Onyango, 2005). Structural classification relies on the morphological arrangement of myofilaments within a muscle tissue. Under structural classification, muscle tissue is categorized as striated or non-striated. Striated muscles have characteristic cross striations/banding of myofilaments within a myofiber when viewed under the light microscope and comprise skeletal and cardiac muscles. In non-striated muscles, the myofilaments do not exhibit typical cross-banding pattern within the muscle tissue, and this includes the smooth muscles. The functional classification of muscles, on the other hand, relies on the type of innervation. In this case, voluntary muscles in which all skeletal muscles belong, are

innervated by somatic nerves and the response is under conscious control by the individual. Conversely, involuntary muscles, which smooth and cardiac muscle belong, are innervated by autonomic nervous system, and their response is independent of individual's consciousness. Therefore, the combination of the two approaches leads to three basic types of muscles namely, striated voluntary comprising skeletal muscles; striated involuntary comprising cardiac muscles and non-striated involuntary comprising the smooth muscles (Kisia and Onyango, 2005). The present study focuses on the striated voluntary skeletal muscles, specifically, *pectoralis major* muscles in chickens.

1.1 Skeletal Muscle Fiber Types and their Characteristics

Skeletal muscle fibers in vertebrates are generally characterized on the basis of the speed of contraction, oxidative capacity, glycolytic metabolism and biochemical properties (Schiaffino et al., 2013). In this regard, mammalian skeletal muscle fibers have been conventionally grouped into type I and type II myofibers. Type I skeletal muscle fiber, also referred to as "red muscle" type, is typically distinguished by slow but sustained contraction (slow-twitch), oxidative phosphorylation, high number of mitochondria and myoglobin, with the later imparting the reddish coloration on this muscle group. On the other hand, type II myofibers, colloquially referred to as "white myofibers", are characterized by fast-contraction (fast-twitch), predominantly glycolytic metabolism; higher glycogen content, but less mitochondria, myoglobin content and capillary network, making them whitish in color (Schiaffino and Reggiani, 2011). Type II myofibers have further been divided into type IIA exhibiting intermediate characteristics and type IIB, which are primarily glycolytic (Peter et al., 1972). Unlike mammals, however, avian skeletal muscles are divided into 5

categories. These are type I slow-contracting "red" fibers, type IIA and IIB fast-contracting "white" fibers, and a type IIIA and IIIB which are slow, tonic "intermediate “fibers (Barnard et al., 1982; Peter et al., 1972; Verdiglione and Cassandro, 2013). In chicken, type I and IIA myofibers comprise muscles exhibiting sustained level of activity such as *soleus* and *sartorius* of the thigh muscles respectively; type IIB muscles, as in mammals, are the fast-contracting muscles, and they comprise exclusively the pectoral muscles (Barnard et al., 1982; Verdiglione and Cassandro, 2013). Type IIIA and IIIB myofibers, which are absent in mammals are slow-tonic and comprise the *plantar* and the *anterior latissimus dorsi* of the avian species (Barnard et al., 1982; Goldspink and Yang, 1999; Hník et al., 1985; Jan et al., 2016).

1.2 Structure of Skeletal Muscles

Embryologically, skeletal muscle is derived from the nucleated mesodermal cells, which undergo terminal differentiation to form myoblasts. The myoblasts then fuse to form myotubes that differentiate to form the mature multinucleated muscle fibers arranged in syncytial patterns. Therefore, mature skeletal muscles consist primarily of muscle cells, also referred to as myofibers, containing the contractile materials that forms the bulk of skeletal muscle tissue. Besides myofibers, skeletal muscles also contain connective tissue, blood vessels and nerves often localized in the extracellular matrix. Each myofiber is covered by an endomysium, a thin connective tissue layer that is in close contact with the sarcolemma. Several myofibers are arranged into a muscle fasciculus, which is surrounded by another connective tissue layer referred to as a perimysium. A group of muscle fascicles then forms a whole muscle, for example *Pectoralis major* surrounded by another thin connective tissue

layer referred to as the epimysium (Gillies and Lieber, 2011; Kraemer et al., 2011). Further, a fascia, which is a tough and thin connective tissue membrane consisting primarily of collagenous and elastic fibrils surround a muscle or groups of muscles. All the connective tissue coverings from the one surrounding each myofiber to the one surrounding the whole muscle are continuous with the myotendinous junction and eventually, the tendons (Mcnally et al., 2006).

Skeletal muscles have a nerve input that frequently contain motor, sensory and autonomic fibers. Motor neurons are involved in the transmission of impulses from the central nervous system (CNS) to the muscle for contraction, and they contact muscle fibers at the motor end plates. Sensory nerves, which originate from the intrafusal fibers of muscle spindles and tendon sheets, carry impulses to the CNS (Kisia and Onyango, 2005; Mcnally et al., 2006). Autonomic fibers, on the other hand, originate from the CNS and innervate blood vessels within the muscles, hence control blood supply in the muscle (Kisia and Onyango, 2005).

At the microscopic level, mature myofibers of a skeletal muscle contain numerous nuclei arranged in the periphery of individual myofiber, just beneath the sarcolemma. The sarcoplasm of myofibers is laden with rod-like contractile structures, called myofibrils, which are about 1mm in diameter. Myofibrils are made up of protein myofilaments (actin and myosin), which in striated (skeletal and cardiac) muscles, are arranged in microscopic units called sarcomeres (Goldspink and Yang, 1999). The sarcomeres, which measure 2-3 micrometer in length and about 1-2 micrometer in width, are the functional contractile units of all striated muscles. The sarcomeres, separated by the Z-discs from each other, are arranged in register within a myofibril. Each sarcomere within a myofibril consists of two principal structural

myofilament proteins namely actin and myosin, which form 70-80% of total protein content of a single myofiber (Frontera and Ochala, 2014). The arrangement of these myofilaments within the sarcomere results in two main distinct bands visible in the light microscope. The central darker portion of the sarcomere is designated the A-band, and it contains the M-line/band that runs across the central-most part of the sarcomere, as well as the H-band comprising only the myosin filaments. On the other hand, the I- band comprises the lighter areas of the sarcomere between the A-band and the Z discs (Frontera and Ochala, 2014; Goldspink and Yang, 1999; Hanson and Huxley, 1953; Squire, 1997). The thick myosin myofilaments exhibit anisotropy and are localized at the central portions of the sarcomere, making no contact with the Z-discs. As such, they extend from one end of the A-band, through the H-band, to the other side of the A-band, terminating at the A-I boundary of the sarcomere. Further, stretch or contraction of the muscle does not alter the length of myosin filaments (Huxley, 1953; Huxley and Hanson, 1954). Actin filaments, which exhibit isotropy, on the other hand, extend from the Z-discs/lines through the I-band region of the sarcomere into the A-band, where they lie between the myosin filaments to terminate on either side of the H-zone (Huxley, 1953; Huxley and Hanson, 1954). The Z-disc, therefore, defines the longitudinal boundaries of the sarcomere on either end. The structure of the Z-disc is defined by a filamentous lattice comprising several proteins such as α-actinin, γ-filamin, nebulin, FATZ, telothenin and the titin component of the Z-disc (Faulkner et al., 2000). In electron microscopy, the Z-disc lattice exhibit zigzag profiles whose structural proteins cross-link with the antiparallel actin filaments (Squire, 1997).

1.2.1 Other Proteins in Skeletal Muscle Tissue

Myofibers of skeletal muscles contain other proteins besides myofilaments. These proteins have been classified on the basis of their functions. They include actin regulatory proteins which comprise tropomyosin and calcium-dependent troponin complex, all of which are in close association with the double stranded actin polymer forming the thin filament of the skeletal muscle (Gunning et al., 2015). Tropomyosin is an important protein known for its role in regulation of contraction in striated muscles. It is composed of two-stranded α-helical coiled coil dimers forming a head to tail polymer along the length of actin filaments (Gunning et al., 2015). Troponin, on the other hand, is a heterotrimeric protein complex involved in the regulation of calcium in myofibers, specifically on actin filaments, and is located on each tropomyosin dimer. Troponin consists of three subunits namely, the calcium binding troponin C, the inhibitory troponin I and the tropomyosin-binding troponin T (Gomes et al., 2002). The coordinated interaction of myosin heads with actin filaments in association with regulatory proteins and Ca^{2+} results in the formation of actomyosin cross-bridges that ultimately causes muscle contraction (Canepari et al., 2010; Holmes and Geeves, 2000; Schiaffino and Reggiani, 2011).

Other important proteins that straddle the sarcomere length include the giant titin filament protein, also called connectin and nebulin. Titin spans an entire half of the sarcomere from the Z-disc to the M-line within the A-band where it interacts with a variety of proteins (Kötter et al., 2014). On the Z-disc, titin is anchored via nebulin, α-actinin and telothenin proteins, while in the A-band, it associates with myosin and myosin-binding protein C. The I-band portion of titin is elastic and therefore plays a role in sarcomere stretching (Kötter et al., 2014; Squire, 1997). Owing to its large size and longitudinal disposition within the sarcomere, titin has been shown to play several

functions. By acting as a scaffolding protein, titin aids in myofibrillar assembly during sarcomerogenesis, while its central position within the sarcomere allows it to act as a sensor for biomechanical stress and enhancement of sarcomere integrity, a role that is augmented by its interaction with telethonin-muscle LIM protein complex (Kötter et al., 2014). Nebulin, on the other hand, is a structural protein that associates with thin filaments along its entire length in the sarcomere. As such, it interacts with actin, tropomyosin, tropomodulin and capZ (β-actinin) proteins within the sarcomere. Nebulin has been shown to regulate and stabilize thin filaments as well as prevention of depolymerization of the actin filaments (Pappas et al., 2010). Tropomodulin and capZ are actin filament capping proteins located on the minus (slow growing/pointed) end (Weber et al., 1994) and plus (fast growing/barbed) ends (Pappas et al., 2008) of the actin filament respectively. As such, they are involved in the regulation of the length of actin filaments in skeletal muscles during nucleation.

1.2.2 Muscle Fiber Organelles

Besides the myofibrils which is the most abundant organelle in the muscle tissue, the sarcoplasm of skeletal muscle fibers also contains other organelles such as mitochondria, sarcoplasmic reticulae and the transverse (T) tubule system. These organelles are important for the execution of physiological functions of the muscle. Mitochondria generate energy through oxidative phosphorylation for use in running muscle functions. It is known that mitochondria form three-dimensional network throughout the sarcoplasm. Some population of mitochondria are located close to the sarcolemma while others are distributed at the intermyofibrillar space of the myofiber (Frontera and Ochala, 2014).

Sarcoplasmic reticulum (SR) in skeletal muscles are modified smooth endoplasmic reticulum that is organized into a network of tubules throughout the intermyofibrillar spaces, and are specialized in the storage, release and uptake of calcium. The longitudinal tubular network of the SR end in transverse tubules on either side referred to as terminal cisternae, where calcium is stored. Terminal cisternae of the SR harbor three important classes of proteins responsible for intracellular calcium homeostasis in the muscle. The first class of proteins are those involved in binding of calcium for storage, which include calsequestrin, histidine-rich calcium-binding protein, junctate, and sarcalumenin proteins (Beard et al., 2004; Rossi and Dirksen, 2006). The second class of proteins are those responsible for calcium release channels, and they comprise type 1 ryanodine receptor (RyR1) and IP_3 receptors. The third class of proteins include sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) responsible for calcium reuptake into the cisternae following muscle contraction (Lamboley et al., 2014; Melzer et al., 1995).

The T-tubule is an invagination of the sarcolemma that extend transversely into the A-I boundary of the sarcomere in mammals or Z-discs in other vertebrate muscles (Jayasinghe and Launikonis, 2013). The main function of the T-tubule is to conduct the nerve action potential generated upstream from the neuromuscular junction into the sarcoplasm to ensure fast and uniform propagation of excitation throughout the myofiber (Jayasinghe and Launikonis, 2013). The T-tubule membrane is in close apposition with 2 flanking terminal cisternae of the sarcoplasmic reticulum forming a structure referred to as the triad i.e. two cisternae and one T-tubule (Dowling et al., 2014; Frontera and Ochala, 2014).

The main function of the triad is to regulate excitation-contraction (EC) coupling in the muscle (Dowling et al., 2014). Regulation and coordination of the EC coupling by the triad is accomplished through a series of steps. First, the transmitted action potential from neuromuscular junction depolarizes the triad membrane, triggering the activation of the dihydropyridine receptors (DHPRs) in the T-tubule, which in turn activates the opening of the skeletal muscle ryanodine receptor (RyR1) calcium release channels in the terminal cisternae of SR. RyR1 activation causes the releases of calcium ions stored in the SR, which then bind to troponin C located in the thin filament, thereby triggering acto-myosin interactions during the cross-bridge cycling (Dowling et al., 2014; Melzer et al., 1995). During the cross-bridge cycling activity, actin filaments are pulled towards the center of the sarcomere by myosin heads, resulting in the shortening of the sarcomere and, hence, muscle contraction. Repolarization of the triad membrane following cessation of action potential stops the release of calcium ions, and instead, calcium reuptake into the SR through SERCA is initiated which ultimately reverses the process of muscle contraction (Dowling et al., 2014).

1.3 Satellite Cells

Satellite cells are the adult stem cells of skeletal muscles. They were discovered in 1961 by Mauro (1961) (Mauro, 1961), and since then, they have been identified as the only source of new myonuclei in post-neonatal skeletal muscles (Yin et al., 2013). Satellite cells are intimately apposed to myofibers sitting just outside the sarcolemma. Indeed, both the myofiber and its satellite cell are surrounded by the same basement membrane (Mauro, 1961). In adult mature skeletal muscles, satellite cells under normal conditions, assume a quiescent state at G_0 phase with no biological

activity except for sporadic fusion of satellite cells to account for muscle turn over due to wear and tear (Yin et al., 2013). However, in an event of a mechanical stimulus or myofiber injury, satellite cells become activated. Activation of satellite cells is marked by entry into the cell cycle to allow for proliferation through symmetric or asymmetric division. Asymmetric division often results in two populations of cells; one that is aimed at maintaining the stem cell lineage of satellite cells and which does not express Myf5 transcription factor, while another population, which expresses Myf5 is committed to myogenic differentiation. Hence, the myogenic satellite cells differentiate to myogenic precursor cells or adult myoblasts (Yin et al., 2013). Under regulated molecular events, the myogenic precursor cells then fuse to form multinucleated myotube. In histological sections, these new fibers appear as rowing myofibers with a series of conspicuous nuclei (Papah et al., 2017).

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Chapter 2

LITERATURE REVIEW

2.1 Genetic Selection in Broiler chickens and Related Implications

The chicken meat is one of the most important sources of proteins to humans. In particular, the chicken meat has been considered to be relatively inexpensive, largely free from social-cultural and religious aversions, and possess a good nutritional profile compared to other meat sources (Kuttappan et al., 2016; Petracci et al., 2015; Petracci and Cavani, 2012; Valceschini, 2006). Consequently, total consumption and market demand for poultry meat and associated products in most parts of the world have been on a constant upward trend over the last five decades (Valceschini, 2006). The increased demand for chicken meat has further been compounded by shifts in consumer trends, for example, increased preference for white breast meat over whole bird carcass (Henry and Rothwell, 1995; National Agricultural Statistics Service and USDA, 2016), as well as preference for further processed chicken-meat products (Berri et al., 2007; Petracci et al., 2015). As a consequence, there has been sustained pressure on the poultry industry to avail broiler chicken lines with capacity to meet the ever-increasing market demand. To this end, artificial genetic selection has been utilized to produce broiler chicken lines with high genetic merit exhibiting increased growth performance and improved body composition in chickens. The main driving force of genetic selection in modern broiler chickens are economically important traits such as growth rate, body weight, breast muscle yields, feed efficiency and conversion ratio, and abdominal fatness (Berri et al., 2001; Havenstein et al., 1994; Petracci et al.,

2015; Zuidhof et al., 2014). Indeed, it has been demonstrated that the growth rate of broiler chickens has increased by over 400% with a concurrent 50% reduction in feed conversion ratio compared to unselected chickens (Zuidhof et al., 2014). At the same time, genetic selection has resulted in a tremendous reduction in the growing time to attain market age by about half (Petracci et al., 2015). The overall effect of these genetic selection strategies has been increased production capacity of the modern broilers on a global scale compared to the unselected ones (Havenstein et al., 2003; Zuidhof et al., 2014).

Despite these apparent positive effects and gains in the poultry industry as a result of advancements in genetic selection of the broiler bird over the years, there has been, albeit inadvertently, concomitant development of unintended and undesired consequences in these chickens. Most of these conditions affects the skeletal muscles, which eventually result in reduction of meat quality traits. These include increased cases of skeletal disorders (Julian, 1998, 2005; Lilburn, 1994; Rath et al., 2000), altered immune functions (Cheema et al., 2003; Qureshi and Havenstein, 1994; Yunis et al., 2000) and metabolic disorders (Julian, 2005; Scheele, 1997), most of which have welfare implications (Bessei, 2006; European Commission, 2000). In this respect, the most pronounced negative and undesired effect witnessed so far is the development of muscle disorders (MacRae et al., 2006; Mitchell and Sandercock, 2004; Petracci and Cavani, 2012; Siller, 1985).

2.2 Muscle Disorders Affecting Commercial Broiler Chickens

Modern rapidly growing lines of broiler chickens, especially those exhibiting high breast muscle yields, have been increasingly associated with high susceptibility to a number of idiopathic and spontaneous stress-induced myopathies in comparison to

their slow growing counterparts (Mitchell, 1999; Mitchell and Sandercock, 2004; Petracci et al., 2015; Petracci and Cavani, 2012). This is especially so due to the increasing cases of new muscular disorders, which continue to be reported in these group of birds. These muscle disorders include deep pectoral myopathy (Grunder et al., 1984; Wight et al., 1981), focal myopathy (Mitchell and Sandercock, 2004), nutritional myopathy (Guetchom et al., 2012), white striping, (Alnahhas et al., 2016; Boerboom et al., 2018; Kuttappan et al., 2012a, 2013c), myodegeneration of *Anterior latissimus dorsi* (Zimermann et al., 2012) and Wooden Breast (Mutryn et al., 2015a; Sihvo et al., 2014, 2017, 2018; Velleman et al., 2018). It is suggested that these muscle disorders have profound consequence on the overall muscle composition and metabolism, leading to development of postmortem disorders such as pale, soft and exudative (PSE) (Barbut 1997, 2009) and dry firm and dark (Lesiów and Kijowski, 2003) muscle conditions. Indeed, some of these muscle disorders have been associated with poor meat quality characteristics such as extreme toughness, poor cohesiveness, color variations and water holding properties, all of which impart negatively on the final processed meat product (Dransfield and Sosnicki, 1999; Petracci and Cavani, 2012). Of the current diseases affecting fast-growing chickens, the most important one in the poultry industry presently, and which is associated with significant economic loss is Wooden Breast Disease.

2.2.1 Wooden Breast Myopathy

This is a novel degenerative muscle disorder colloquially referred variously as "Wooden Breast Disease", "Hard Muscle Disease", or "Woody Breast Condition" currently affecting the modern broiler birds, while the unselected birds remain largely unaffected. This muscle condition was first reported in Finland in 2013 (Sihvo et al.,

2014), and has since been described and reported in the US, Europe, South America and several other regions around the world that practice commercial broiler production (Bailey et al., 2015; de Brot et al., 2016; Mutryn et al., 2015a; Tekeli et al., 2016; Velleman and Clark, 2015). This disorder primarily affects the superficial pectoral muscles of the modern broiler birds exhibiting fast-growth rate, increased body and breast muscle weights, all raised in standard industrial settings. It is pathognomonically characterized by palpably firm consistency of the affected breast muscles, extreme pallor and out-bulging of the cranial portions of the breast region (Mudalal et al., 2015; Mutryn et al., 2015a; Sihvo et al., 2014; Velleman and Clark, 2015). This disorder has emerged to be very significant globally due to its effect in causation of poor meat quality traits, leading to significant economic impact on the poultry industry globally (Abasht et al., 2015; Mudalal et al., 2015; Mutryn et al., 2015a; Petracci et al., 2015). Additionally, as a muscle disorder, this condition potentially impacts negatively on the welfare of the affected birds (European Commission, 2000).

2.2.1.1 Clinical Signs and Pathology of WB

Although the disorder is known to be largely asymptomatic, features such as enhanced angularity of the breast, as well as decreased wing motion have been noted in the severely affected birds. Macroscopically, the affected breast muscles appear to out-bulge, and exhibit varying degrees of lesions including multifocal to diffuse firmness and pallor, subcutaneous edema and petechial hemorrhages, all subject to degree of severity (Sihvo et al., 2014, 2017; Velleman and Clark, 2015). Histologically, the affected muscles show varying degrees of multifocal fiber degeneration and fragmentation, occasional fiber splitting, loss of fiber striations and

necrosis (Clark and Velleman, 2017; Sihvo et al., 2014, 2017; Velleman and Clark, 2015). These features are frequently accompanied by inflammatory cell infiltration into and around the myofibers, primarily by the heterophil and macrophage cell populations. Advanced cases are characterized by fibrosis, typified by thickened interstitial tissues, as well as endomysial and perimysial connective tissues, and irregular deposition of adipose tissue (Clark and Velleman, 2017; Sihvo et al., 2014, 2017; Soglia et al., 2016b; Velleman and Clark, 2015). Further, the pattern of fibrosis, often characterized by either parallel and dense, or diffuse and irregular deposition of collagen fibers has been shown to vary with broiler lines (Velleman and Clark, 2015). A rather unique histologic lesion frequently associated with this condition is phlebitis characterized by the infiltration of mononuclear inflammatory cells into the walls of veins, whereas the arteries remain largely unaffected (Mutryn et al., 2015a; Sihvo et al., 2014, 2017).

2.2.1.2 Molecular Presentation of WB

In the recent past, a limited number of studies have focused on elucidation of molecular dynamics associated with the occurrence of WB. These studies, which have examined gene expression profiles between affected and unaffected birds, have largely been undertaken on birds at market age (Mutryn et al., 2015a; Zambonelli et al., 2016). Functional analysis of differentially expressed genes between affected and unaffected chickens revealed localized hypoxia within the pectoral muscle, oxidative stress, increased intracellular calcium buildup, cellular repair and possible fiber-type switching as the main biological processes associated with the disease process (Mutryn et al., 2015a; Zambonelli et al., 2016). Additionally, attempts to determine the potential use of some of the differentially expressed genes as biomarkers for the

different stages of the condition have been advanced (Abasht et al., 2015). Similarly, another gene, *decorin*, was also found to be responsible in the enhancement of crosslinking of collagen fibrils during fibrosis, thereby positively contributing in imparting the firm consistency of muscles associated with the disorder (Velleman and Clark, 2015). Studies on the metabolomics profiles of the pectoral muscles associated with WBD have also been examined (Abasht et al., 2016). Metabolomics analysis revealed alteration of glucose metabolism, impaired redox homeostasis, increased oxidative stress, increased protein degradation, and decreased glycogen content in affected muscles in comparison to unaffected cases (Abasht et al., 2016).

2.2.1.3 Current Insights into the Pathogenesis of WB

Even though the etiology of WB continues to be elusive, a number of hypotheses have been put forth as likely factors contributing to the development of this condition. It is generally known that birds exhibiting increased body weight and breast muscle weights are highly predisposed to develop this condition (Kawasaki et al., 2018; Mudalal et al., 2015; Mutryn et al., 2015a, 2015b; Sihvo et al., 2014). Incidentally, myofibers of the breast muscles in these birds undergo unprecedented hypertrophy, indicative of increased metabolic activity and oxygen demand. The high rate of muscle hypertrophy has, however, been linked with vascular disruptions (Sihvo et al., 2018; Velleman, 2015). Additionally, it has been suggested that these pectoral muscles are associated with decreased capillarization in the face of an increasingly hypertrophic myofibers (Petracci et al., 2015; Sihvo et al., 2018; Velleman, 2015; Velleman and Clark, 2015). Consequently, decreased and compromised vascularization potentially results in buildup of hypoxic to near anoxic environment within the muscle tissue (Sihvo et al., 2018). In the event of hypoxia, the pectoral

muscles engage glycolysis leading to increased utilization of glycogen from the muscle tissues, thereby resulting into its significant depletion in the pectoral muscles of affected chickens (Abasht et al., 2016). On the other hand, arterial-sparing phlebitis coupled with insufficient capillary bed in the muscle tissue results in stagnant tissue perfusion, as well as decreased rate of drainage and clearance of metabolic waste. The continued accumulations of metabolic waste products, coupled with persistent hypoxic environment within the muscles have been postulated to contribute to the initiation of muscle damage and hence, degenerative changes associated with WB (Mutryn et al., 2015a; Petracci et al., 2015; Sihvo et al., 2014, 2018; Velleman, 2015).

Another school of thought regarding the potential initiation of WB implicates increased accumulation of intracellular calcium resulting from growth-related metabolic stress frequently associated with modern broiler chickens. In this case, increased intracellular calcium activates proteases (eg calpains) and lipases that target myofibrillar and sarcoplasmic proteins as well as sarcolemma, which collectively, results in myofiber degeneration and necrosis (Petracci et al., 2015). Degenerative muscle changes are considered to precede inflammatory response, frequently characterized by infiltration of inflammatory cells comprising heterophil and macrophage populations into damaged myofibers. Additionally, other factors may be thought to exacerbate the disease process such as dysregulation of energy metabolism involving carbohydrates and lipids, disruption of redox-homeostasis causing increased oxidative stress and increased degradation of muscles, as revealed by metabolomics studies (Abasht et al., 2016). Advanced stages of the disease, as characterized by fibrosis, increased lipid infiltration, and myoregeneration are associated with the reparative phase of disease (Clark and Velleman, 2017; Sihvo et al., 2014, 2017;

Soglia et al., 2016b; Velleman, 2015). However, the final phase frequently fails to reach complete repair, and it is therefore characterized by extreme firmness of the pectoral muscles (Velleman, 2015; Velleman and Clark, 2015).

2.2.1.4 Incidence and Predisposing Factors to WB

The incidence and prevalence rates of WB in broiler chickens are not fully established. However, a report indicated existence of anecdotal evidence to suggest that up to 10% of a flock exhibit the severe form of the condition (Mutryn et al., 2015a). Another study conducted on over 2500 broiler chickens randomly sampled from three different male broiler flocks in Delmarva region of the US, for a period of 17 days (from day 29 to day 47 post-hatch), indicated 0.7%, 8.7%, 81.3% and 9.3% of the experimental birds represented severely affected, moderately affected, normal and soft pectoral muscles respectively (Abasht et al., 2015; Mutryn et al., 2015b). Further, it has been suggested that mild to moderate clinical and subclinical cases may be more prevalent in the affected flocks. This follows the detection of subtle microscopic lesions of WB in clinically normal birds, as well as clustering of some clinically unaffected birds with clinically affected ones based on hierarchical clustering according to their genes (Abasht et al., 2015; Mutryn et al., 2015a). Indeed, a recent study indicated the possibility of the existence of an entire spectrum of the disease states in a flock of the same age. The disease states/stages include mild, moderate and severe disease, and are often presented on a case to case basis. This has been seen to present problems in the classification of the disease based on the clinical presentations as exhibited in the flock (Abasht et al., 2015; Mutryn et al., 2015a). Nonetheless, the existence of a strong correlation on the development and occurrence of WB with performance of birds is well established. This disorder has been observed to be highly

associated with birds exhibiting high body weight, breast muscle weight, feed efficiency, as well as decreased abdominal fatness (Kawasaki et al., 2018; Mutryn et al., 2015b; Sihvo et al., 2014, 2017). In addition, functional analysis of differentially expressed genes between low-feed and high-feed efficient broiler chickens revealed activation of biological processes and pathways in the high-feed efficient birds, which were largely similar to those of WB (Zhou et al., 2015). Further, it was determined that the degree of firmness was also correlated with higher ultimate pH in affected muscle tissue (Alnahhas et al., 2014; Mudalal et al., 2015; Mutryn et al., 2015b).

2.2.1.5 Effects of WB in the Poultry Industry

Wooden Breast disease has not been directly associated with mortalities among the affected chickens so far. However, it is considered as one of the most important disorders in the poultry industry causing an array of meat quality defects from freshly processed carcasses to processed products (Mudalal et al., 2015; Petracci et al., 2015; Soglia et al., 2016b, 2016a). Firstly, WB is often associated with gross features which impair the general aesthetic characteristics of meat, thereby reducing the consumer willingness and confidence for the affected fillets. Hence, carcasses from severely affected birds may be downgraded or condemned altogether (Mudalal et al., 2015). Likewise, moderate clinical to subclinical cases of WB, which often fail to be detected grossly and remain largely unaccounted for, may have detectable textural changes in the finished product, ultimately causing loss of consumer confidence. This is because WB interferes and changes the chemical and/or nutrient composition in affected cases in comparison to unaffected broiler meat. In this context, several studies conducted to assess the effects of WB on meat quality traits, as well as on raw freshly cooked and processed products showed presence of undesirable characteristics. These include

lower marinade uptake, poor water-holding capacity, poor textural properties, high ultimate pH, abnormal firmness and undesired muscle coloration ranging from extreme pallor to yellowness, which is frequently accompanied by gelatinous subcutaneous tissue (Mudalal et al., 2015; Petracci et al., 2015; Soglia et al., 2015, 2016a, 2016b; Trocino et al., 2015; Zhuang and Bowker, 2018). Additionally, WB has been shown to interfere with nutritive properties of meat as typified by low protein and high fat contents in affected fillets (Soglia et al., 2016b, 2016a). Incidentally, minerals such as calcium and sodium levels have also been reported to be higher in pectoral muscles affected with WB (Soglia et al., 2016b). Based on these observations therefore, it is evident that WB negatively impacts meat quality and also interferes with the quality of both raw and processed products (Mudalal et al., 2015).

All these may serve to decrease consumer acceptance, confidence and general appeal for breast meat, which in the long run, may impact the poultry industry negatively. Although no data is available so far showing the direct economic impact of WB on the poultry industry, the relatively high incidence rate of the disease, as indicated by the existence of all categories of the disease severity spectrum in a flock at a given time, may act as a pointer to the huge economic impact the disease may have in the poultry industry. Overall, the economic cost associated with this disorder in poultry may run into several millions of dollars per year including costs due to loss of employment and affiliated industries, which remain largely unaccounted for.

Previous studies have reported a number of muscle-related conditions afflicting broiler chickens. An assessment of the macroscopic and/or microscopic characteristics of WB shows some degree of overlap with features of other muscle disorders in

chickens, most of which are degenerative in nature. Incidentally, the occurrence of these muscle disorders was reported to be prevalent in the modern broiler chickens exhibiting high muscle yield and fast-growth rates. These disorders include White Striping (WS), Deep Pectoral Myopathy, Nutritional Myopathy, Focal Myopathy, Inherited Muscular Dystrophy, Spaghetti Disease and Toxic myopathies. The details of these muscle disorders will be reviewed in relation to WB in modern broiler chickens.

2.2.2 White Striping (WS)

This is an emerging myodegenerative disorder whose etiology is yet to be known, and exhibits features highly similar to those of WB. It affects mostly the pectoralis major muscles, and to a lesser extent, the thigh muscles (iliotibialis) of fast-growing broiler chickens. The condition is typically characterized by the presence of white fatty striations running parallel to the muscle fibers with varying degree of severity (Kuttappan et al., 2012c, 2012a, 2013a, 2013c; Lorenzi et al., 2014; Petracci et al., 2013b). The overall incidence of white striping in broiler breast meat in a commercial setting has been determined to be as high as 43% (Lorenzi et al., 2014). Additionally, the prevalence of the myopathy has been determined to be associated with body weight, with the heavier chickens having higher incidence and sex (males>females) (Lorenzi et al., 2014). Similar to WB, WS exhibits an early occurrence at the cranial regions of the pectoral regions where the muscle is considerably thicker, and so is the degree of severity (Kuttappan et al., 2013c, 2013a). A positive correlation of the severity of WS with increased lipidosis in the pectoral muscles has also been established (Kuttappan et al., 2012a), indicative of the possible link to the development of the disorder in chickens.

The major gross morphological feature characterizing WS is the presence of white striations observed on the pectoral muscle upon removal of the skin, as well as on cut sections (Cruz et al., 2016; Griffin et al., 2018; Kuttappan et al., 2017). Consequently, a scoring method that relies on visual appraisal of white striations on the affected muscles to deduce the degree of severity of WS in poultry namely normal, moderate and severe has been developed (Kuttappan et al., 2012c, 2013a). The degree of severity of WS is considered to be directly associated with the degree of muscle fiber damage (Kuttappan et al., 2013b, 2013c). Besides the typical white striations on the affected muscles, other lesions observed grossly in WS include petechial hemorrhage, subcutaneous edema, muscle discolorations or pallor, features that are highly similar to those of WB (Kuttappan et al., 2013c). The distinguishing feature between WS and WB is that the former affects skeletal muscles other such as thigh muscles besides the breast muscles, while WB occurs predominantly in the pectoral muscles. Additionally, WS lacks the firm phenotype of the breast muscle often associated with WB.

Microscopically, WS has been found to exhibit both acute and chronic phases (polyphasic) in the same tissue, indicative of continuous presence of the insult within the muscle tissue (Kuttappan et al., 2013c, 2013a). Major histopathological changes noted include loss of cross striations, variability in fiber size, floccular/vacuolar degeneration and fragmentation of fibers, mild mineralization, mononuclear cell infiltration, lipidosis, interstitial inflammation and fibrosis. These changes are often accompanied by myofiber regeneration as characterized by nuclear rowing and presence of multinucleated cells (Kuttappan et al., 2013c; Russo et al., 2015). Indeed, WS has been demonstrated to co-exist with WB (Bailey et al., 2015; Mudalal et al.,

2015; Sihvo et al., 2014; Trocino et al., 2015), and has been suggested to be a transitional disease-state for WB (Mutryn et al., 2015a). Hematological tests on affected birds have revealed no indication of infection with various microorganisms (Kuttappan et al., 2013c). However, evidence of muscle damage resulting from this disorder has been suggested owing to increased levels of plasma metabolites such as creatine kinase (CK), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) in severely affected birds. These enzymes are considered as key indicators of muscle damage (Kuttappan et al., 2013c).

2.2.2.1 Effects of WS in the Poultry Industry

Like WB, WS is a muscle quality defect, with no direct mortality cases being reported. Previous reports show that the disease interferes with visual attributes of affected fillets resulting in their rejection. The visual qualities implicated by WS include the white striations and discoloration ranging from pallor to redness or yellowness on affected (Kuttappan et al., 2013a). Similarly, the myopathy also impacts on the cooking quality properties, such as reduction in water holding capacity, reduction of proteins, increasing pH, drip loss and fat (Alnahhas et al., 2016; Kuttappan et al., 2017; Mudalal et al., 2015; Soglia et al., 2016a, 2016b). These characteristics have been demonstrated to be undesired and, therefore, contribute in reducing meat quality (Mudalal et al., 2014). This feature coupled with decreased consumer perception of the affected meat result in huge economic losses to the poultry industry (Kuttappan et al., 2012c; Petracci et al., 2013a, 2015). It should be noted that the economic implications of WS in chickens are similar to those of WB.

2.2.3 Deep Pectoral Myopathy

Deep Pectoral Myopathy (DPM) which was initially identified in turkeys (Harper et al., 1975), and later observed in chickens (Bianchi et al., 2006; Siller, 1985; Wight et al., 1981) is another muscle disorder that occurs in fast-growing chickens. DPM is variously referred to by other names such as the Green Muscle Disease (GMD), Oregon Disease (Siller, 1985) and Degenerative Myopathy of the *Supracoracoideus* (DMS) (Grunder et al., 1984). Unlike WB however, DPM affects primarily the *Supracoracoideus* (*Pectoralis minor*) muscles. Presenting itself either unilaterally or bilaterally, this condition is characterized by ischemic necrosis of the muscle arising from exertional events, causing widespread degenerative and hemorrhagic changes in the muscle. In advanced state, these pathological changes culminate in necrosis of the entire muscle resulting in the typical greenish discolorations of the affected muscle (Bianchi et al., 2006; Kijowski and Konstańczak, 2009; Wight et al., 1981).

As in WB, DPM does not present any obvious clinical signs nor impair the general health of birds. Therefore, the lesions associated with this disease are frequently manifested upon necropsy of the affected carcasses. The etiology of this disease remains to be known. However, the development of this disorder has been demonstrated to be as a result of an ischemic necrosis in the deep pectoral muscle (Lien et al., 2012; Martindale et al., 1979; Wight et al., 1981), often aggravated by risk factors. Several studies have shown that the location of the pectoralis minor muscle itself and the surrounding tissues play a crucial role in the initiation of ischemia (Lien et al., 2012; Siller et al., 1978; Wight et al., 1981). Inelastic fascia of the deep pectoral muscle, huge pectoralis major muscle and the sternum surrounding this muscle, create restriction and a limited space upon which it can expand freely during physiological

dynamics for example in exertional exercises involving wing flapping (Lien et al., 2012). Studies in turkeys and broilers have shown that the weight of deep pectoral muscle can increase up to 20% during exercise as a result of increased blood supply to the muscle (Martindale et al., 1979). In heavy poultry breeds, increases in weight are particularly high leading to increased pressure within the muscle itself (Kijowski and Konstańczak, 2009; Siller, 1985; Yalcin et al., 2018). The increasing pressure against the restricted space results in occlusion and strangulation of blood vessels within the muscle leading to ischemia. Consequently, the resultant ischemia initiates a cascade of the characteristic pathological features associated with DPM (Kuttappan et al., 2016; Martindale et al., 1979; Siller, 1985; Siller and Wight, 1978; Wight et al., 1981). The development of this disease is manifested by changes in coloration and texture of the affected muscle. A swollen reddish-brown hemorrhagic lesion characterizes the early phase, while in later stages, the tissue becomes shrunken exhibiting green or pale grey coloration (Bianchi et al., 2006; Kijowski and Konstańczak, 2009; Wight et al., 1981). In this condition therefore, inflammation has been thought not to play a role in its pathogenesis (Siller, 1985).

Histologically, the affected areas exhibit a composition of necrotized and regenerating fibers, which in early phases, are infiltrated with macrophages aimed to phagocytize necrotized tissue. Later stages are characterized by replacement of necrotic muscle fibers with fibro-adipose tissue (Siller and Wight, 1978; Wight et al., 1981; Wight and Siller, 1980). Ultrastructurally, affected areas have been reported to show irregular sarcolemma, disintegrated sarcoplasmic reticulum, loss of I bands, numerous large lipid globules in association with dense masses of collagen fibers, and in some cases, necrosis (Wight and Siller, 1980).

Although no known specific cause of this disease has been found yet, it is believed that increased bird activity characterized by flapping tendencies may induce the development of this disease, (Kijowski et al., 2014; Kijowski and Konstańczak, 2009; Lien et al., 2012). Further, the incidence of the disease, like in most myopathic diseases, has been associated with broilers selected specifically for fast-growth rate, body weight and breast muscle weight (Bianchi et al., 2006; Kijowski and Konstańczak, 2009; Siller, 1985). For this reason, it has been postulated that the disease poses a great challenge to the poultry industry owing to economic losses incurred due to downgrading of the affected muscles (Bianchi et al., 2006).

2.2.4 Dietary Deficiency Myopathies/Nutritional Myopathies

Disorders related to nutritional deficiencies in domestic animals have been widely investigated. The disorders range from deficiencies due to primary nutrients such as carbohydrates, proteins and vitamins to mineral salts including trace elements. In chickens, the most common dietary deficiency-related disorders are those that result due to insufficiency of Vitamin E (alpha-tocopheral) and Selenium (Cheville, 1966; Guetchom et al., 2012; Machlin and Gordon, 1962; Mitchell, 1999). These dietary components are known to exert their metabolic roles through their antioxidant properties against damaging effects of lipid peroxides and free radicals produced during normal metabolism. Their anti-oxidative properties are particularly required by tissues and cells, which are often involved in active and rapid oxidative metabolism, such as muscles (skeletal, cardiac and smooth), and blood cells (e.g. erythrocytes, phagocytes and lymphocytes). These tissues are generally characterised by generation of massive amounts of free radicals and other highly oxidizing species. Consequently, these tissues are considered to be highly susceptible to oxidative damage in the event

of a deficiency in one or both of these dietary components, (Machlin and Bendich, 1987; Mitchell, 1999).

The deficiency of Vitamin E and Selenium in chicken has been shown to have a multi-systemic involvement. As a result, deficient birds frequently develop a wide range of clinical signs, which is depended on the severity of the condition, as well as the system involved and the ensuing lesions. The most studied disorders resulting from the deficiency of these dietary components are encephalomalacia, exudative diathesis and muscular dystrophy/myopathy (Cheville, 1966; Machlin and Gordon, 1962). Lesions associated with exudative diathesis occur as a result of damage to the vasculature, resulting in generalized edema that is typical of the disorder. Grossly, exudative diathesis is distinguished by the presence of varying degree of diffuse subcutaneous, intermuscular, and interstitial edema in the early phase, which later progresses to involve multiple organs. Therefore, the systemic occurrence of exudative diathesis contrasts the vascular changes of WB often limited to the pectoral muscles.

Myopathies resulting from the deficiency of either one or both of these dietary components is observed in all three groups of muscles in the body namely; cardiac muscle, smooth muscles and skeletal muscles, with the latter being more likely to be affected (Avanzo et al., 2001; Hassan et al., 1990). Histologically, the muscular involvement is characterized by degenerative changes, which start as a single myofiber degeneration, loss of striations, central migration of nuclei, as well as hyalinization. Late phases of the muscle disorder exhibit splitting and curling of muscle fibers, complexed with infiltration by fat and inflammatory cells, vascular lesions, hemorrhages and calcium deposition (Cheville, 1966; Hassan et al., 1990; Mitchell, 1999). Ultrastructurally, this condition is characterized by accumulation of

glycogen, dilatation of sarcoplasmic reticulum, as well as mitochondrial swelling (Cheville, 1966), features that are closely similar to those of WB. Indeed, clinical signs and lesions associated with vitamin E and Selenium deficiency are often reversed or prevented following dietary supplementation with the respective component of either selenium, vitamin E or both, indicating their vital metabolic function in chickens (Cheville, 1966). However, while the muscle lesions arising from nutritional deficiency are similar to those of emerging myopathies such as WB and WS, the cause of the later appears to be different. In this respect, studies involving dietary supplementation with vitamin E did not alleviate the development of WS in modern broiler chickens (Kuttappan et al., 2012b). Additionally, Guetchom et al (2012) showed little effect in reduction of nutritional myopathies in broilers upon supplementation of feed with vitamin E (Guetchom et al., 2012).

2.2.5 Myodegeneration of *Anterior latissimus dorsi*

This is a relatively new muscle disorder in broiler chickens first detected in Brazil (Zimermann et al., 2012). The condition is observed primarily in the *anterior latissimus dorsi* (ALD) muscle of fast-growing modern broiler chickens. As in WB and WS, affected birds show no detectable clinical sign; therefore, the disease is diagnosed during processing of birds at slaughterhouses. Additionally, the males are more affected than the female chickens, features that are consistent with those of WB and WS. The highest incidence of the disease reported in Brazil was 6% and has consequently been touted as one of the most important poultry diseases in that country (Zimermann et al., 2012). Owing to its novelty and confusion with other conditions, the authors suggested renaming of the disease as Dorsal Cranial myopathy (DCM).

The main gross lesions of DCM include yellowish discoloration of the skin and swelling of the dorsal cranial region of the ALD muscle. On cut sections, affected muscle exhibit firmness, subcutaneous edema, superficial petechial hemorrhages, and pallor, adherence to adjacent muscles, and increased thickness and density. Microscopic examination present degenerative and polyphasic features, variation in myofiber size and splitting, hyalination, necrotic and regenerative myofibers evidenced by proliferation of satellite cells, sarcolemmal nuclei, and nuclear rowing of basophilic fibers. Additionally, it has been reported that the disease shows lymphohistiocytic infiltration accompanied by extensive fibrosis and accumulation of adipose tissue (Sesterhenn et al., 2017; Zimermann et al., 2012), features that are highly similar to those of WB and WS. Even though the etiology of this condition remains unknown, a correlation of the development of DCM with the weight of the broiler chickens have been reported (Sesterhenn et al., 2017; Zimermann et al., 2012), corresponding to WB and WS. Owing to DCM, the poultry industry in Brazil is reported to experience huge economic losses resulting from rejection or downgrading of the finished poultry products (Sesterhenn et al., 2017; Zimermann et al., 2012). Further it has been reported that the manifestation of the disorder in Brazil could be an indication of the occurrence of the condition in other countries practicing extensive broiler chicken production (Sesterhenn et al., 2017; Zimermann et al., 2012).

2.2.6 Toxic Myopathies

Use of toxic agents have been considered to be one of the possible causes contributing to the development of myopathies in animals. One of the groups of toxic agents often used in poultry are ionophores (Chalmers, 1981; Dowling, 1992), and plants such as *Cassia occidentalis* (Graziano et al., 1983). In poultry, the use of

monensin, an anticoccidial ionophore antibiotic has been frequently linked to the development of myopathies among other toxic effects exhibited in other tissues (Chalmers, 1981; Dowling, 1992; Hanrahan et al., 1981; Mitchell, 1999; Weisman et al., 1994). Even though ionophores have broad spectrum of activity, the narrow safety margin demonstrated in this antibiotic has been touted as the reason associated with their myotoxicosis (Dowling, 1992; Mitchell, 1999). It has been suggested that monensin, which is a sodium ionophore, imparts its toxicity by disrupting the sodium-potassium balance across the sarcolemma resulting in increases in intracellular calcium by the sodium-calcium exchange initiating the process of cellular damage (Mitchell and Sandercock, 2004; Sandercock and Mitchell, 2004; Trump et al., 1989).

In poultry, monensin damage to muscles has generally been indicated by the presence of elevated levels of circulating plasma creatine kinase (CK) and Aspartate Aminotransferase (AST) activity (Dowling, 1992; Mitchell, 1999). Gross lesions associated with ionophore toxicity in chickens is frequently systemic as typified by generalized congestion, myocardial enlargement and pallor, ascites, hydropericardium, myocardial streaking and dilatation of the ventricles. Histopathological lesions on the other hand, are accentuated in skeletal muscles, with a predilection to red myofibers compared to the white fibers (Dowling, 1992; Mitchell, 1999). The main microscopic lesions include myofiber hyalinization, degeneration and necrosis, fatty infiltration and in severe cases, infiltration by macrophages and heterophils are observed (Dowling, 1992; Hanrahan et al., 1981), features that are common to those of modern broiler myopathies. Further, interaction of ionophores with other drugs such as macrolides antibiotics has been reported to exacerbate its toxicosis (Dowling, 1992; Roder, 2011). Therefore, in comparison to WB which is largely limited to breast muscles, toxic

myopathies appear to present generalized effect in all muscle tissues and is primarily associated with the use of toxic agents.

2.2.7 Pale Soft and Exudative Condition in Chickens

Pale, soft, and exudative (PSE) myopathy is a post-mortem quality condition in which the meat exhibits pale coloration, soft consistency and poor water-holding capacity. First described in swine (Bendall and Swatland, 1988; Wismer-Pedersen, 1959), and later in turkeys (Barbut, 1993, 1996, 1997a) and broilers (Barbut, 1997b, 1998; Van Laack et al., 2000), PSE is thought to develop following accelerated glycolytic process, induced by acute ante-mortem stress or heat. This ultimately results in the decrease of the meat pH, consequently denaturing myofiber proteins (McKee and Sams, 1998; Owens et al., 2009; Van Laack et al., 2000). In chicken, the normal pH values estimated from the breast muscles are approximately 5.8-5.9 (Alnahhas et al., 2014; Qiao et al., 2001). Hence, broiler meat with pH values (<5.7) are likely to develop PSE-like condition.

Predisposing factors to this condition have been thought to be genetic mutations, environmental factors or both (Kuttappan et al., 2016; Owens et al., 2009). Environmental factors have been conveniently divided into two; those associated with ant-mortem stress factors and those associated with post-mortem factors (Owens et al., 2009). A genetic mutation on the ryanodine receptor protein has been identified as one of the factors contributing to the development of PSE (Strasburg and Chiang, 2009). The mutation of ryanodine compromises the regulation of calcium ion flow from the sarcoplasmic reticulum to the sarcoplasm of the myofiber. The excess calcium ions within myofibers causes stimulation of various enzymes in muscle resulting in denaturation of proteins, alteration of energy metabolism and muscle activity,

ultimately leading to reduced capacity of the animal to tolerate stress, and hence, development of PSE conditions (Alderton and Steinhardt, 2000; Owens et al., 2009; Strasburg and Chiang, 2009). On the other hand, environmental antemortem stress factors such as sustained high ambient temperatures, preslaughter handling practices, and transportation have been associated with porcine stress syndrome and PSE meat in swine (Owens et al., 2009).

In poultry, similar environmental factors have been suggested to contribute to the development of PSE in the post-mortem meat (Owens et al., 2009). In particular, the current commercial lines of birds, most of which have been genetically selected for rapid growth and increased body weights, have been shown to be highly susceptible to high humidity and temperature (Kuttappan et al., 2016; Owens et al., 2009). It has been suggested that PSE could affect 5 to 30% of the turkey (Barbut, 1997a) and chicken (Barbut, 1997b) flocks in the US. Subtle morphological changes associated with PSE in chickens muscles including disorganization of Z-lines, shrinking and depolymerization of myofilaments have been reported (Wilkins et al., 2000). Although not directly related to WB or WS, PSE is largely considered a meat quality defect, affecting carcass pH, water holding capacity and aesthesis (Wilhelm et al., 2010; Wilkins et al., 2000). Hence it is postulated to cause significant losses in the poultry industry (Owens et al., 2009; Petracci et al., 2015).

2.2.8 Capture Myopathy

Capture myopathy in birds is a disorder that results due to exertion, struggle, or stress occurring during capture, handling, immobilization, or transport in all groups of birds. As a result, variation in the extent of muscle damage alongside injuries involving other organs such as bone fractures, bruise and blisters have been frequently

observed (Mitchell, 1999). Although Capture Myopathy can occur in all types of birds, those that have higher body weights such as turkeys (Spraker et al., 2013) and modern broiler chickens have been suggested to be more prone to the condition (MacRae et al., 2006). In this case, limited reports on wild turkeys indicate that up to 48% of the birds exhibited lesions attributed to Capture Myopathy which included white striations within the breast and leg muscles (Spraker et al., 2013). Additionally, microscopic changes on skeletal muscles reported include rhabdomyolysis, as well as coagulative necrosis and myocardial nuclear proliferation in the myocardium (Spraker et al., 2013). These lesions were thought to develop due to stress arising from the trapping, transportation and housing of the birds.

2.2.9 Focal Myopathy

This is a muscle disorder frequently characterized by spontaneous development of localized lesions within a group or groups of muscles. First reported in pectoral and cervical muscles exhibiting poor health and lameness (Maronpot et al., 1968; Mitchell, 1999), and later in clinically normal commercial turkeys (Sosnicki et al., 1988; Wilson et al., 1990), Focal Myopathy is not known to be associated with obvious clinical signs. Nonetheless, the prevalence of Focal myopathies have been shown to increase in rapidly growing turkeys, with lesions frequently restricted to the breast (pectoralis) and leg (iliofibularis) muscles (Mitchell, 1999).

The main histopathological features characterizing this disorder are largely similar to those of the modern broiler chickens and include fiber segmentation, hyaline fiber formation, mononuclear infiltration, as well as local necrosis. Late stage of the disease is characterized by the involvement of fatty tissue replacement. In the recent times, the spontaneity in the occurrence of focal myopathy in poultry has been

associated with selection for fast growth rate, which is generally reflected by excessive myofiber hypertrophy and inadequate development of support tissues and vasculature (Mitchell and Sandercock, 2004). Further, even though Focal Myopathy has not been documented in modern broiler chickens, it is suggested that any form of stress, which these chickens are prone to, may initiate localized ischemia mediating the development of the muscle pathology (Mitchell and Sandercock, 2004; Sosnicki et al., 1991; Wilson et al., 1990).

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Chapter 3

EVIDENCE AND ROLE OF PHLEBITIS AND LIPID INFILTRATION IN THE ONSET AND PATHOGENESIS OF WOODEN BREAST DISEASE IN MODERN BROILER CHICKENS

(Michael B. Papah, Erin M. Brannick, Carl J. Schmidt & Behnam Abasht *Avian Pathology*, 46:623-643, (2017)). https://www.tandfonline.com/doi/full/10.1080/03079457.2017.1339346

3.1 Abstract

Wooden Breast, a myopathy that frequently affects modern broiler chickens, is a disorder that has been associated with significant economic losses in the poultry industry. To examine tissue changes associated with the onset and early pathogenesis of this disorder, a time-series experiment was conducted using chickens from a high-breast-muscle-yield, purebred commercial broiler line. Birds were raised for up to seven weeks, with a subset of birds sampled weekly. Breast muscle tissues were extracted at necropsy and processed for analysis by light microscopy and transmission electron microscopy. Histologic presentation indicated localized phlebitis with lipogranulomas in Week 1, focal single-myofibril degeneration in Week 2 preceding an inflammatory response that started in Week 3. Lesions in Week 4 were characterized by multifocal to diffuse muscle fiber degeneration, necrosis, interstitial edema accompanied by increased lipid and inflammatory cell infiltration. Lesions in Weeks 5 to 7 revealed diffuse muscle degeneration, necrosis, fibrosis and fatty infiltration with lipogranulomas. Ultrastructural examination showed myofibrillar splitting and degeneration, irregular, displaced and degenerated Z-lines, mitochondrial degeneration and interstitial fibrosis with dense regular collagen fibers. This study,

therefore, demonstrates that WB exhibits an earlier onset in modern broilers than when detectable by clinical examination. Further, this study shows that the disease assumes a progressive course with acute vasculitis, lipid deposition and myodegeneration occurring in the earlier stages, followed by a chronic fibrotic phase.

3.2 Introduction

The high global demand for poultry meat results in pressure on the poultry industry to produce broiler chickens with high genetic merit for specific production traits, such as fast growth rate and high muscle yields. Through intense artificial genetic selection on broiler chickens, a process spanning over 50 years, modern broiler chickens bearing high growth rate, feed efficiency and muscle yields were developed (Havenstein et al., 1994, 2003; Schmidt et al., 2009; Zuidhof et al., 2014). The emergence of these modern broiler chickens have been increasingly associated, albeit inadvertently, with concomitant development of undesired consequences (Hocking, 2014), especially myopathies (MacRae et al., 2006; Petracci et al., 2015; Petracci and Cavani, 2012). Recently, a novel muscle disorder, colloquially referred to as “Wooden Breast ”, and bearing a unique predilection for the superficial and occasionally deep pectoral muscles, has been identified in the modern broiler chickens (Sihvo et al., 2014). This disorder, which is characterized by palpably firm consistency of the breast muscles on physical examination, primarily affects broiler chickens exhibiting fast-growth rate and high body and breast muscle weights, raised under modern commercial conditions (Mudalal et al., 2015; Mutryn et al., 2015; Sihvo et al., 2014). This disorder has been found to be largely subclinical, that is asymptomatic. Often, the condition only becomes clinically apparent at market age around 40 to 50 days post-hatch or during slaughter in processing plants. Consequently, a number of prior studies

(Abasht et al., 2015; Mutryn et al., 2015; Sihvo et al., 2014; Soglia et al., 2016b; Velleman and Clark, 2015) conducted on this condition have focused on broiler birds at market age.

Although WB has not been associated with direct mortalities in the affected chickens, it is generally considered as one of the most important disorders causing meat quality defects within the poultry industry (Petracci et al., 2015). In this context, several studies conducted to assess the effects of WB on meat quality traits, as well as on processed products, showed presence of undesirable characteristics (Mudalal et al., 2015; Petracci et al., 2015; Soglia et al., 2016b, 2016a).

Further, studies focusing on the pathology of WB have been conducted (Sihvo et al., 2014; Soglia et al., 2016b). While WB is largely asymptomatic, clinical signs such as outbulging of the lateral forebreast and decreased wing movement have been noted in severely affected birds. Grossly, the affected breast muscles exhibit multifocal to diffuse firmness and pallor, subcutaneous and fascial edema and petechial hemorrhages. Histologically, in the affected muscles, varying degrees of multifocal myofibril degeneration, necrosis, occasional myofiber splitting and fragmentation have been described. These features are frequently accompanied by inflammatory cell infiltration into and around the myofibers, primarily heterophils and macrophages. Advanced cases are characterized by fibrosis, typified by thickened interstitial, endomysial and perimysial connective tissues and irregular deposition of adipose tissue (de Brot et al., 2016; Sihvo et al., 2014, 2017; Velleman and Clark, 2015). A rather unique histologic lesion frequently associated with this condition is phlebitis, characterized by the infiltration of mononuclear inflammatory cells into the walls of veins, whereas neighboring arteries remain largely unaffected (Sihvo et al.,

2014, 2017). On a molecular basis, the occurrence of WB has been demonstrated to be associated with several biological processes, such as localized hypoxia within the muscle, oxidative stress, increased intracellular calcium buildup and cellular repair (Mutryn et al., 2015; Zambonelli et al., 2016). Recent studies have also documented possible molecular biomarkers of the disease based on gene expression (Abasht et al., 2015), as well as biochemical signatures based on metabolomics profiles between unaffected and affected birds (Abasht et al., 2016).

To better understand the development and progression of WB in chickens, recent studies have focused on employing time-course evaluations of broiler chickens until market age (Radaelli et al., 2017; Sihvo et al., 2017). Consequently, WB myopathy has been found to manifest itself as early as the 2nd week of age, and then progressing in scope and severity towards market age (Radaelli et al., 2017; Sihvo et al., 2017). While the studies already undertaken on WB provide significant information towards advancing our understanding of this relatively novel myopathy in broiler chickens, a number of pertinent pieces of information still remain to be elucidated. Firstly, information on phenotypic correlates to development and progress of WB from the onset to market age (week 6-7) remains to be fully understood. Secondly, no information is available on the ultrastructural presentation of the disease over the growth period. Indeed, since a mosaic of overlapping lesions including muscle degeneration, inflammation, fibrosis, adipogenesis, vasculitis and muscle cell regeneration characterize this disorder in advanced stages, it is not possible to identify and pinpoint the primary initiating factor of the disease at the time of clinical detection (i.e. market age) or to determine the sequence of events in lesion development and progression.

Therefore, the main objective of this study was to determine the clinical/phenotypic, macroscopic, microscopic and ultrastructural changes characterizing the development and progression of WB in modern high-breast-muscle-yield broiler chickens through a time-course evaluation analysis. This study utilized time-series samples of broiler chickens raised from day-old to 7 weeks of age in conditions mimicking those of modern commercial settings.

3.3 Materials and Methods

3.3.1 Experimental Animals

This study utilized Pectoralis (P.) major muscle samples from a flock of 350 male birds of a high-breast-muscle-yield, purebred broiler line. The birds were brought in as day-old-chicks from Perdue Farms on October 28th, 2014, and subsequently placed and raised at the University of Delaware (UD) poultry houses for up to 49 days. Birds were raised in strict adherence to the guidelines provided by the UD College of Agriculture and Natural Resources' Animal Care and Use handbook and the UD College of Agriculture and Natural Resources' Animal Care and Use committee protocol (#44 07-08-14R). All birds were individually wing-banded and subsequently separated into 2 main groups for the entire experimental period, namely necropsy (N) and biopsy (B) groups comprising 200 and 100 birds respectively, while 50 additional birds served as a replacement stock. A subset of birds in the 'N' group were humanely euthanized and necropsied on weekly basis until the end of the experiment (Table 3.1). Birds in the 'B' group were subjected to biopsy of the breast muscle at week 2 and 4 or week 3 and 5 of the experimental period for molecular evaluation prior to necropsy at Week 6 and 7 for tissue collection; biopsy samples were not included in the present

analysis. Therefore, necropsy specimens used in the present study in week 6 and 7 comprised those from 'N' and 'B' groups (Table 3.1).

Table 3.1: Weekly necropsy of birds

Week	Number of birds necropsied
1	20
2	20
3	24
4	45
5	45
6	67
7	64

The number of bird samples necropsied on each week throughout the experimental period

Birds from both experimental groups were co-housed, and feed and water were provided *ad libitum* to allow the birds to express their full production potential, thus increasing the likelihood of any individual bird developing WBD. Throughout the experimental period, the birds were provided with standard commercially formulated broiler feed sourced from Perdue Farms, a private company. At placement, the chicks were introduced to chick feed for one week followed by grower feed until the 5th week, when they were introduced to finisher feed until the end of the experimental period at the 6th and 7th weeks. Throughout the experimental period, routine house inspections were performed daily, and weights of individual birds were measured weekly. All pertinent phenotypic and clinical data including muscle palpation scores were collected and recorded during these sessions; from the 1st week to the 7th week of age.

3.3.2 Housing Conditions

At initial placement, all birds were randomly distributed in three houses. Chicks were brooded at 90 to 95 °F (32.22 to 35 °C) upon placement, and house temperatures were decreased by 5 °F weekly to level at 70-75 °F (21.11 to 23.38 °C) at 4 weeks of age and then maintained at that level to the end of the experiment. Two of the houses were of standard closed-broiler house fully equipped with automatic ventilation, heating and temperature-monitoring systems, as well as a 24-h artificial electric lighting system. Conditions in the third house were not automated hence; environmental parameters in this house were manually regulated and closely monitored on daily basis to match those of the other two. Additionally, the chickens in this house were exposed to natural light during the day and appeared to be more active compared to those in the other 2 houses. Throughout the study, a minimum of 2.5 cm feeder space per bird was provided to prevent competition for resources, and birds were raised at a minimum stocking density of 30kg of BW/m^2 (Dozier et al., 2005). The effect of house on the prevalence of WB in chickens was also assessed statistically.

3.3.3 Detection and Scoring of Disease by Use of Clinical and Gross Evaluations

The scoring of WB was performed on live birds during routine clinical evaluations, as well as postmortem on birds at necropsy. At each routine examination, conducted weekly during weighing or biopsy sessions on the farm and prior to necropsy, all birds were evaluated physically for clinical changes related to WB. Clinical examination involved visual observations of the birds for demeanor, wing motion, gait and posture, as well as bilateral manual palpation of the breast (pectoralis major muscle) in a cranio-caudal direction. Manual palpation included qualitative

grading of clinical Wooden Breast Disease (cWBD) for degree of firmness using the following protocol: cWBD score 1 (Unaffected): no detectable increase in firmness of the breast area; cWBD score 2 (Mildly affected): breast length exhibiting localized mild firmness than normal; cWBD score 3 (Moderately affected): breast area moderately firm on palpation than normal in a focally extensive area; cWBD score 4 and 5 (Markedly affected): >75% of the breast muscle belly markedly firm on palpation exhibiting widespread/diffuse coverage, with the score of 5 being given to birds with exceptionally firm breast muscles.

Furthermore, the scoring system employed during gross evaluations of breast muscles for Wooden Breast Disease at necropsy, designated as gross Wooden Breast Disease (gWBD) in this study, included the following: gWBD score 0 (Unaffected): no detectable increase in firmness of the breast muscle; gWBD score 1 and 2 (Mildly affected): <25% of muscle belly palpably firmer than normal in focal or limited multifocal pattern; gWBD score 3 (Moderately affected): 25-75% of muscle belly palpably firmer than normal in focally extensive or coalescing multifocal pattern; gWBD score 4 and 5 (Severely affected): >75% of muscle belly palpably firmer than normal in widespread multifocal to diffuse pattern, with the score of 5 being given to specimens which were exceptionally firm on palpation. Unlike in the clinical evaluation where only one score per bird was recorded, serial gross evaluation involved palpating and scoring for degree of firmness, both on the cranial and caudal aspects of the breast muscle, with the average being taken as the final score. In addition to palpation, grading of the disease after necropsy also took note of the gross changes on the muscles. Further, the presence and degree of white striations, indicative of White Striping (WS) myopathy in the breast muscles was also

determined and scored. Lesion scoring for WS from both cranial and caudal aspects of the breast muscle at necropsy was performed subjectively using a score ranging from 0 to 5. In details, the WS score of (0) indicated no white striations across the whole breast muscle; the WS score of (1) was used when there was presence of thin white striations sparsely distributed and covering <20% of the cranial or caudal aspects; score of (WS2) was used when the white striations covered 20-40% of the cranial or caudal portions of the breast muscle; score of (WS3) was used when 40-60% of the breast muscle was occupied by the white striations; score of (WS4) was used when 60-80% of the breast muscle was covered by dense, thick multifocal striations, while the highest score of WS5 was given to samples that exhibited diffuse, dense, thickened white striations occupying >80% of the breast muscle. The WB *de novo* scoring system was developed by a veterinarian and certified veterinary anatomic pathologist (E.B.), and clinical examination and grading of all birds were performed by a veterinarian and anatomist (M.B.P.).

3.3.4 Necropsy Protocol

Throughout the experimental period, birds for necropsy were randomly sampled from all the three houses and then euthanized by cervical dislocation prior to necropsy. Tissues were collected during each necropsy to assess for myopathic lesions in Pectoralis major/breast muscle, red (*Iliotibialis lateralis*/thigh) muscle, smooth muscle (Ventriculus/gizzard) and cardiac muscle (heart). With the knowledge that WB presents itself bilaterally, in this study, the upper 1/3 or craniolateral aspect of right superficial pectoral muscle was used consistently as the sampling site on all samples. The choice for this specific location was guided by the knowledge that clinical, gross and histological changes associated with WB is largely known to start from the cranial

aspect of the muscle before becoming apparent caudally (Clark and Velleman, 2017). Further, muscle samples excised from this site was about 2-3 cm deep from the outer aspect of the muscle. The thigh muscles (*Iliotibialis lateralis*) were used to determine if there was concurrent expression of the disorder in other skeletal muscles other than the superficial breast muscles. Further, the gizzard and the heart muscle samples were used to determine concurrent existence of the disorder in the smooth muscles and the cardiac muscles respectively.

3.3.5 Sample Preparation for Microscopic and Ultrastructural Analysis

One set of tissues per bird including the breast muscle, thigh muscle, heart and gizzard were fixed by immersion into 10% neutral buffered formalin for microscopic analysis. Breast muscle tissues were trimmed, processed, paraffin-embedded, sectioned to 4 μm thickness and stained with Hematoxylin & Eosin (H/E) at the University of Delaware Comparative Pathology Laboratory (Newark, DE). Both transverse and longitudinal sections of superficial pectoral (P. major) muscles were prepared and subsequently examined using an Olympus BX40 light microscope and relevant photomicrographs collected using a Nikon DS-Fi2 camera and NIS Elements D software. Similarly, representative samples from the thigh muscle, gizzard and cardiac muscle from 8 birds belonging to the following categories n=3 unaffected (gWBD0); n=2; mildly affected (gWBD1 &2); n=2 Moderately affected (gWBD3); n=1 severely affected (gWBD 4) all from Week 6 &7 were also processed for H/E staining and evaluated for concurrent microscopic changes.

Microscopic analysis focused on the type, extent and severity of myopathic lesions (vascular damage, lipid infiltration, myodegeneration, myositis, interstitial edema, myoregeneration and fibrosis). Frozen muscle specimens from 11 birds

belonging to the following groups: n=4 unaffected (gWBD0) from Week 4 &5; n=2 mildly affected (gWBD2) from Week 6; n=1 moderately affected (gWBD3) from Week 4; and n=4 markedly affected (gWBD4 &5) from Week 5& 6, were also processed to examine for evidence of myofiber type switching, as was suggested in a previous study (Mutryn et al., 2015). Briefly, fresh muscle tissue specimens were collected and transported in physiologic saline to the Nemours-A.I. duPont Hospital for Children Histochemistry and Tissue Processing Core Laboratory (Wilmington, DE), where they were oriented on cork using tragacanth gum and snap frozen in isopentane. Following sectioning, representative slides were histochemically stained for ATPase and NADH activity to evaluate for potential myofiber type switching. The same sets of frozen samples (n=11) were also evaluated for lipidosis within myofibers using Oil Red O staining technique (Mehlem et al., 2013).

Additionally, breast muscle samples from 10 birds whose gross WB status consisted of the following: n= 6 unaffected (gWBD0) from Week 4, 5 and 6; n= 3 mildly affected (gWBD1 &2) from Week 4 and 6; and n=1 moderately affected (gWBD3) from Week 4 were fixed for transmission electron microscopy (TEM). Briefly, the tissues were cut into 1 mm^3 pieces and subsequently fixed by immersion into 2 % glutaraldehyde and 2% paraformaldehyde in 0.1M sodium cacodylate buffer at pH 7.4 and stored at 4 $^{\circ}$C for at least 24 h before further processing. Tissues for TEM were processed at Delaware Biotechnology Institute (Newark, DE) following routine protocol. Briefly, the tissues were rinsed in the same buffer, post-fixed in 1 % aqueous osmium tetroxide (OsO_4), rinsed in double distilled water, dehydrated in graded alcohol concentrations, cleared in propylene oxide, infiltrated and then embedded in epoxy resin mixture followed by polymerization at 60 °C overnight.

Semi-thin (1µm thick) sections were then obtained from a Reichert-Jung Ultracut E ultramicrotome, stained with Toluidine blue and examined for light microscopy using an Olympus BX40 light microscope and relevant photomicrographs collected using a Nikon DS-Fi2 camera and NIS Elements D software. Ultrathin (60–70 nm thick) sections were subsequently obtained from the same blocks, mounted on formvar/carbon coated 200 mesh copper grids, stained with standard uranyl acetate/Reynold's lead citrate protocol, examined and photographed using a Zeiss Libra 120 Transmission Electron Microscope.

3.3.6 Scoring of Microscopic Lesions

Grading of microscopic lesions of P. major muscle was performed using an ordinal scoring system (Gibson-Corley et al., 2013). In this case, the extent and distribution of myopathic lesions, as well as the degree of structural perturbations attributed to the lesion was used to assign scores to the lesion. The score scale ranged from 0 to 5, where a score of (0) indicated normal muscle tissue with no presence of myopathic lesion, fibrosis or lipidosis; a score of (1) was given when <20% of the tissue was occupied by lesions; score of (2) was used when the lesion involved 20-40% of the tissue; score of (3) was used when the lesion was distributed in 40-60% of the tissue and; score of (4) was used when the lesion/ histologic parameter was distributed in 60-80 % while a score of (5) was used when the distribution of the lesion was diffuse, covering > 80% of the tissue. Lesions scores then roughly correlate to the severity of myopathic or inflammatory changes in the tissue as absent (0), minimal (1), mild (2), moderate (3) or marked/severe (4/5). Tissues were also assessed for the presence or absence of typical lesions of WB, including phlebitis,

lipid deposition, myofiber swelling and degeneration, myositis, edema, myoregeneration and fibrosis.

3.3.7 Statistical Analysis

Assessment of different categories of cWBD scores in birds with their respective body weights during the growth period was performed using one-way ANOVA followed by Tukey's HSD test for mean differences. Descriptive statistics, 2-sample t-test and correlation analysis was used in the assessment of gWBD scores generated at necropsy. Additionally, comparison of the different microscopic lesion scores across the experimental period was performed using Tukey's HSD test, while all correlation analyses were done using Spearman's rho correlation for non-parametric tests. Further, verification of absence of house effect and biopsy effect on the prevalence of cWBD were determined at Week 6 using Fisher's exact test. In all statistical assessments, a p-value ≤ 0.05 was considered significant. All statistical tests were conducted using the JMP statistical software (JMP Version 12, SAS Institute Inc.).

3.4 Results

3.4.1 Phenotypic Analysis and Scoring of Wooden Breast Disease on Live Birds

Evaluation of all live birds for presence of WB during the experimental period was primarily accomplished through manual palpation of the breast region. Assessment of birds in the first 3 weeks revealed no changes in the firmness of the breast muscle. However, starting from Week 4 onwards, birds started to exhibit varying degrees of firmness of the breast muscles. Phenotypic evaluation for clinical breast firmness at Week 4 revealed 85.37% (210/246) as unaffected (cWBD1);

13.15% (33/246) as mildly affected (cWBD2); 0.81 (2/246) as moderately affected (cWBD3) and 0.41% (1/246) as markedly affected (cWBD4 & 5). However, it should be noted that 10 samples from Week 4 were not included in this analysis since 3 of the samples were outliers owing to very low body weights, while 7 samples missed to be evaluated clinically.

In Week 5, the proportion of the unaffected category (cWBD1) was 56.57% (99/185); mildly affected (cWBD2) birds rose to 28% (49/185); moderately affected category (cWBD3) comprised 14.86% (26/185), while markedly affected category consisted of 6.29% (11/185) of the chickens. In this week, 4 samples were outliers, while 6 missed to be evaluated clinically. In Week 6, the proportion of birds in the (cWBD1) category was 32.19% (47/136); (cWBD2) category contained 24.66% (36/136) and (cWBD4 &5) category consisted of 8.22% (12/136) of the chickens. As in other weeks, 1 sample was an outlier, while 3 samples missed to be evaluated clinically. Analysis of the house effect on the prevalence of cWBD for presence or absence of disease at Week 6 indicated no significance difference for all three houses ($p=0.109$).

Further, the body weight of birds in each cWBD category across the experimental period (from Weeks 4, 5 and 6) was significantly different (Table 3.2). In this case, mildly affected (cWBD2) birds in Week 4 were significantly heavier than the unaffected (cWBD1) ones. However, since there were only 2 samples in the moderate (cWBD3) and 1 in the markedly affected (cWBD4 & 5) categories, they were not included in the statistical calculations. In Week 5, birds in the unaffected group also appeared to have significantly lower body weights compared to the moderate and severe cases. In Week 6, although the mild and moderate groups

exhibited no significant difference in body weight from one another, the severe group, which exhibited the heaviest mean body weight, was significantly different from less-affected birds.

Table 3.2: Mean and Standard error (SE) of body weights (BW) of the different classes of hardness score of WB evaluated on live birds from Week 4 to 6.

Class of Hardness	Unaffected (cWBD1)		Mildly affected (cWBD2)		Moderately Affected (cWBD3)		Severely affected (cWBD4 & 5)		R-square
Week 4 BW (g)	N=210 (85.37%)		N=33 (13.15%)		N=2 (0.81%)		N=1 (0.41 %)		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	
	1257.08[a]	10.91	1364.58[b]	27.51	N/A		N/A	N/A	0.048
Week 5 BW (g)	N=99 (56.57%)		N=49 (28%)		N=26 (14.86%)		N=11 (6.29%)		
	1881.5[a]	19.4	1955.35[ab]	27.57	2072.69[b]	37.86	2112.27[b]	58.2	0.144
Week6 BW (g)	N=47(32.19%)		N=36 (24.66%)		N=41(28.08%)		N=12 (8.22%)		
	2570.04[a]	29.93	2740.14[b]	34.19	2739.1[b]	32.04	2920.75[c]	59.2	0.212

Body weight (BW) in grams, number (N). Means not sharing a common superscript letter within each class of WB are significantly different ($p<0.05$, Tukey's HSD test). R-square of one-way ANOVA analysis by breast muscle hardness.

In addition, assessment of the level of association between body weight and degree of severity of cWBD (R-square) appeared to increase with the weight and age of the birds (0.048, 0.144 and 0.212 for Weeks 4, 5 and 6 respectively) (Table 3.2). Overall assessment of live birds throughout the experimental period revealed an increase in the frequency in which birds were clinically affected by the disease, with a simultaneous increase in clinical severity with advancement in age (Figure 3.1a). Furthermore, comparison of mean growth rates of the cWBD categories of birds generated from phenotypic evaluation of live birds throughout the experimental period revealed all 3 affected categories (mild, moderated and marked) to be consistently heavier compared to the unaffected birds (cWBD1) from the outset. On the other hand, the mean weights of mildly (cWBD2) and moderately (cWBD3) affected groups were frequently found to be intermediate (Figure 3.1b).

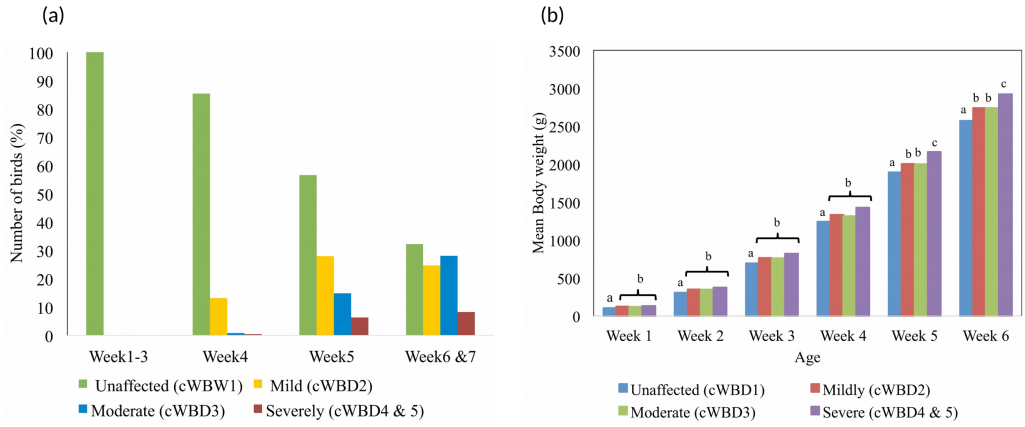

Figure 3.1: (a) Prevalence of clinical Wooden Breast Disease (cWBD) by disease severity in chickens, with bird age in weeks and (b) Weekly mean growth weights of live birds by clinical Wooden Breast Disease (cWBD) categories generated from Week 6.

Notice the increase in the presence of moderate to severe clinical disease as the birds advance in age. Unaffected (cWBD1); mildly affected (cWBD2); moderately affected (cWBD3); severely affected (cWBD4 & 5) and, (b) Means showing different letters in each week are significantly different. Unaffected (cWBD1); mildly affected (cWBD2); moderately affected (cWBD3); severely affected (cWBD4 & 5).

In addition to manual palpation, other clinical signs were also noted in some heavy and markedly affected birds, such as inability to right themselves from accidental dorsal recumbency and decreased wing movements during euthanasia (data not shown). Diminished wing movement in affected birds following euthanasia was subjectively determined through clinical evaluation by the veterinarians (authors MBP and EB) handling the birds and was not directly measured. Moreover, some of the affected birds exhibited resistance in overall movement and ambulation, preferring to feed while recumbent on their ventrum. These clinical signs were frequently observed at later stages (Week 5 to 7) of the experimental period. It was also noted that biopsy had no significant impact on the occurrence of cWBD in chickens necropsied at week 6 and 7 ($p=0.091$).

3.4.2 Gross Evaluation of Breast Muscle for WB

Weekly evaluation of the broiler chickens at necropsy revealed different combinations and degrees of gross lesions as the birds advanced in age. While birds necropsied in Weeks 1 and 2 showed no gross lesions on their breast muscles (Figure 3.2a), those in Week 3 through to Week 6/7 exhibited variable gross lesions (Figure 3.2c-e). Breast muscle samples from Week 3 ($n=24$) showed mild lesions such as localized petechiations ($n=1$) and white striping ($n=5$). Other samples also exhibited 1 to 5 pale, soft foci ranging from 1-5mm in diameter ($n=5$) distributed randomly on the P. major muscles. However, beginning from Week 4 onwards, gross evaluation at

necropsy revealed overt pathologic changes of WB condition at different degrees of severity and firmness (gWBD scores upon palpation) (Figure 3.2c-e).

In Week 4, palpation of all breast muscle samples (N=45) for degree of firmness revealed the following categories: normal consistency/unaffected (gWBD0) comprised 77% (35/45), mildly affected (gWBD1 &2) 16% (7/45), moderately affected (gWBD3) 2% (1/45), while 4 % (2/45) of the samples were missed to be graded (Table 3.3). Grossly, the affected breast muscles in Week 4 (gWBD1 to 3) were characterized by presence of white striations running along the longitudinal axis of muscle fibers, as well as subtle petechial hemorrhage (Figure 3.2c), compared to the normal breast muscle (Figure 3.2f). Additionally, pallor of the breast muscles, which assumed a focal to multifocal pattern, was a characteristic feature in this week (data not shown).

In Week 5, palpation for degree of firmness score revealed 42% (19/45) to be of normal consistency (gBWD0), 22% (10/45) were mildly firm (gWBD1 & 2), 9% (4/45) were moderately firm (gWBD3) and 4% (2/45) were markedly affected (gWBD4 & 5), while 22% (10/45) of the samples were missed to be graded (Table 3.3). The gross presentation of the affected samples (gWBD1 to 5) in this group was characterized by lesions, such as edematous subcutaneous fat; hemorrhage, most often of the petechial type; and diffuse pallor (Figure 3.2d), compared to the normal breast muscle (Figure 3.2g).

Table 3.3: Prevalence of chickens affected with gross Wooden Breast Disease (gWBD) by lesion severity

	Unaffected gWBD0 n (%)	Mild gWBD1&2 n (%)	Moderate gWBD3 n (%)	Severe gWBD4&5 n (%)	Ungraded samples n (%)	Total in group (n)
Week4	35 (77)	7 (16)	1 (2)	0 (0)	2 (4)	45
Week5	19 (42)	10 (22)	4 (9)	2 (4)	10 (22)	45
Week6	16 (24)	27 (40)	17 (25)	6 (9)	1 (2)	67
Week7	7 (11)	34 (53)	12 (19)	11 (17)	0 (0)	64

Note that the prevalence and degree of severity increases with age. Ungraded samples include birds which were missed to be evaluated grossly.

Evaluation for degree of firmness of breast muscle in birds necropsied in Week 6 revealed 24% (16/67) as gWBD0, 40% (27/67) as gWBD1 &2, 25% (17/67) as gWBD3 and 9% (9/67) as gWBD4 & 5, while one sample was missed to be graded (Table 3.3). Similarly, the samples that were necropsied in Week 7 (N= 64) revealed 11% (7/64) as gWBD0, 53% (34/67) as gWBD1 & 2, 19% (12/64) as gWBD3, and 11% (7/64) belonging to gWBD4 & 5. A statistical analysis to compare gWBD scores (0 to 5) from Week 4 to Week 7 revealed a significant difference ($p \leq 0.05$) of the scores in Week 4 and 5, while Week 6 and 7 were not found to be significantly different (Table 3.4).

Further analysis of the breast muscles grossly, revealed the cranial aspects to be firmer compared to the caudal aspect especially in the mildly (gWBD1 &2) and moderately (gWBD3) affected groups. Using a score range of (0 to 5), the cranial aspect was firmer in both mildly (gWBD1 & 2) ($n=61$ $P<0.0001$) and moderately (gWBD3) ($n=29$ $P<0.0001$) affected categories. In addition to the gross presentations indicated in previous weeks, the markedly affected (gWBD4 and 5) samples in Week

6 and 7 exhibited diffuse pallor, edema within subcutaneous fat, multifocal to diffuse white striping and petechial to ecchymotic hemorrhages (Figure 3.2e). These features were in complete contrast to the gross presentation of normal breast muscles at this time point (Figure 3.2h).

Table 3.4: Comparison of mean histologic lesion scores and gross Wooden Breast Disease (gWBD) scores by age in weeks

Weeks Necropsied	Lipid deposition	Fibrosis	Myositis	Degeneration and necrosis	gWBD scores at necropsy
Week 1	0.20^{c}	0^{c}	0.00^{d}	0.45^{d}	NA
Week 2	0.05^{c}	0^{c}	0.05^{cd}	1.17^{d}	NA
Week 3	0.75^{abc}	0.79^{bc}	0.83^{bc}	2.04^{abc}	NA
Week 4	1.13^{ab}	1.18^{ab}	0.91^{b}	1.87^{bc}	0.31^{a}
Week 5	0.67^{bc}	0.91^{b}	1.11^{ab}	1.82^{bc}	1.12^{b}
Week 6	1.22^{a}	1.22^{ab}	1.47^{a}	2.36^{ab}	1.85^{c}
Week 7	0.94^{ab}	1.66^{a}	1.60^{a}	2.61^{a}	2.05^{c}

Mean histologic lesion scores (0 to 5) and gWBD scores (0 to 5) at necropsy compared across the experimental period using Tukey's HSD test. Means showing different superscript letters in each lesion category or gWBD score across the 7 weeks are significantly different at $p < 0.05$.

It was also noted that some of the lesions of WB were frequently seen together with those of White Striping (Figure 3.2c and e), as was exemplified by the occurrence of muscle firmness and white striation lesions on the same sample. Analysis of degree of firmness and white striping (score scale of 0 to 5), exhibited a Spearman's correlation coefficient score of 0.42 in Week 4 (n=45), 0.44 in Week 5 (n=45) 0.47 in Week 6 (n=66) and 0.59 in Week 7 (n=64), all being highly significant at ($p<0.0001$). In addition, the white striations lesions were generally more prominent on the cranial

aspects of P. major muscle, becoming thinner and less apparent caudally. This was further indicated statistically by significantly different (t-test; P<0.0001) white striping mean scores (from 0 to 5) between the cranial and the caudal aspects of the breast muscle in all three categories gWBD1 &2, gWBD3 and gWBD 4 &5 at the end of the experiment (week 6 and 7).

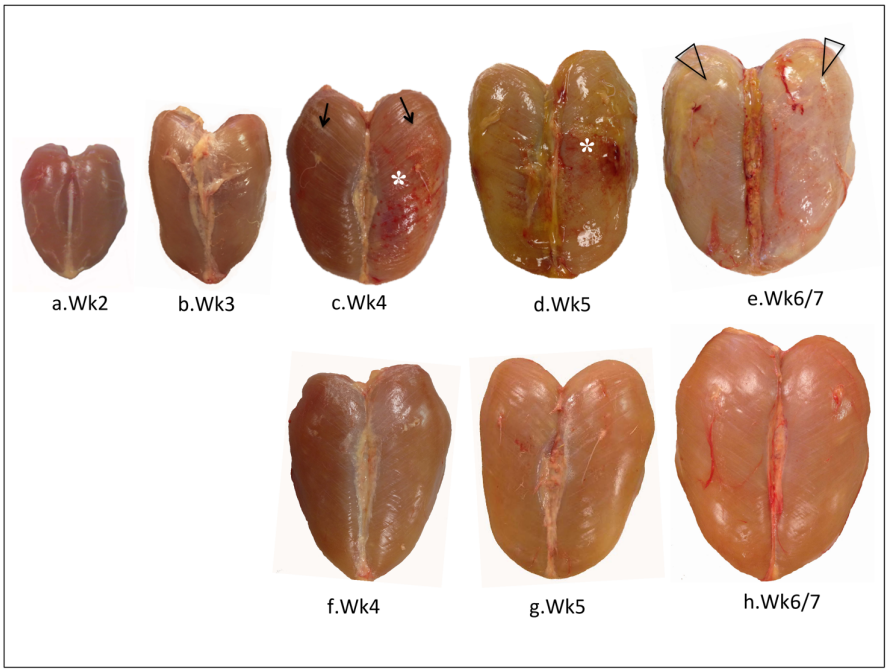

Figure 3.2: Breast muscle samples from Week 2 to Week6/7 showing gross changes associated with progression of WB.

Notice that (a) and (b) show no discernable gross lesions, while (c) shows white striations (arrows) and subcutaneous hemorrhage (asterisks) that in Week 5 (d) shows subcutaneous edema, ecchymotic hemorrhage and pallor, while in Week 6/7 (e) shows diffuse pallor (open arrowheads) with multifocal hemorrhagic areas. Pallor generally corresponds with areas of enhanced firmness typical of WB. Figures f–h indicate gross

representation of samples with normal consistency devoid of gross lesions for the respective weeks. Wk: Week.

3.4.3 Microscopic Evaluation of WB

A total of 285 H&E stained pectoral muscle specimens from tissue collected at 1-7 weeks of age, as well as those from the thigh muscle, cardiac and the gizzard were evaluated microscopically for pathologic changes associated with Wooden Breast Disease. Microscopic examination focused on presence of vasculitis, lipid infiltration, myofiber degeneration and necrosis, myositis, myofiber regeneration and fibrosis. Microscopic examination of the thigh muscle, cardiac muscle and the gizzard revealed no morphological changes across all samples. On the other hand, all but 40 (40/285, 14%) of the P. major muscle specimens exhibited one or more significant microscopic lesions of inflammation or degeneration/necrosis at different prevalence rates (Figure 3.3). The histologic lesions observed are described by time point below.

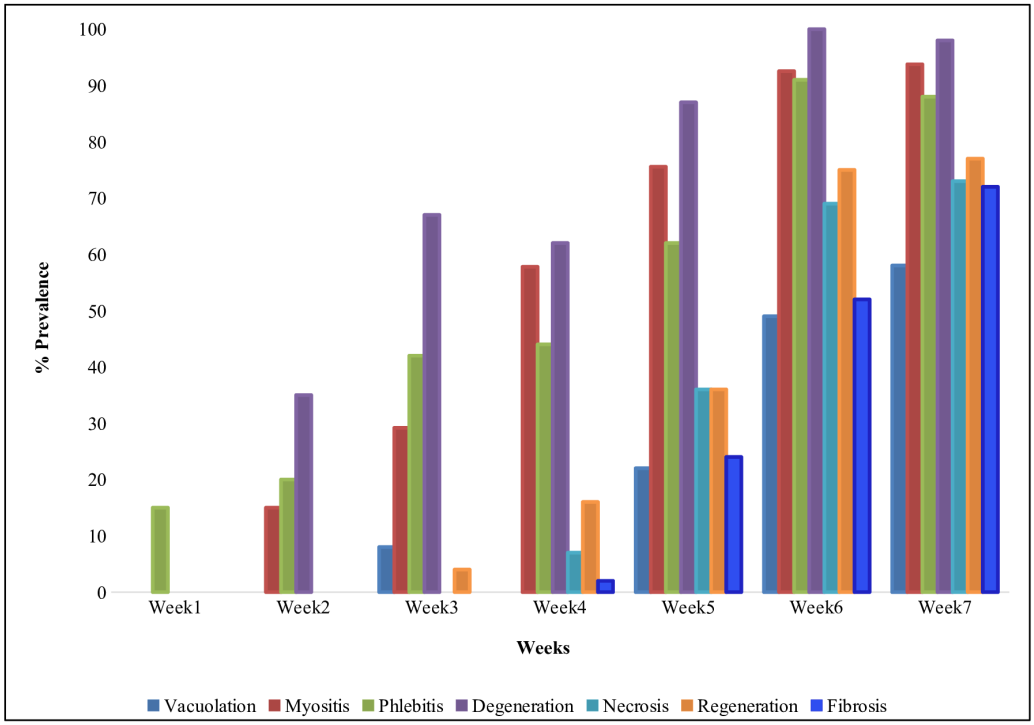

Figure 3.3: Prevalence of microscopic lesions of the WB in broiler chickens by age (weeks).

By Week 1 post-hatch, there was histologic evidence of lymphoplasmacytic inflammation of small caliber veins and venules (phlebitis) in 3 pectoral muscle specimens (n=20, 15%), which resulted in focal venous stenosis, congestion and edema in one bird (Figure 3.4a). Other than rare myofiber hypereosinophiliax and rounding, indistinguishable from sampling artifact, no significant myofiber lesions were observed in conjunction with phlebitis. The prevalence of phlebitis increased in successive weeks to 6 weeks of age (prevalence of 20, 42, 44, 62 and 91% in weeks 2-6, respectively) and then appeared to plateau at 7 weeks of age (88%) (Figure 3.3). The severity and scope of phlebitis also appeared to change over time. At early time points, inflammation was mild, focal to multifocal in distribution, and often

eccentrically located along the vessel wall (Figure 3.4a). In later weeks, inflammatory infiltrates invaded the entire vascular wall circumferentially and coalesced to form thick perivascular cuffs that extended along the length of entire veins (Figure 3.4b). In some specimens (9/185, 5%), multifocal vein lumina were entirely occluded by inflammatory infiltrates and, occasionally, there was evidence of attempted recanalization in affected vessels (Figure 3.4b-d). Phlebitis was limited to small to medium-sized caliber veins/venules; large veins were unaffected when present in specimens. Incidentally, while the venous vessels were largely affected from the outset, arterial vessels across all time points remained unaffected (Figure 3.4d).

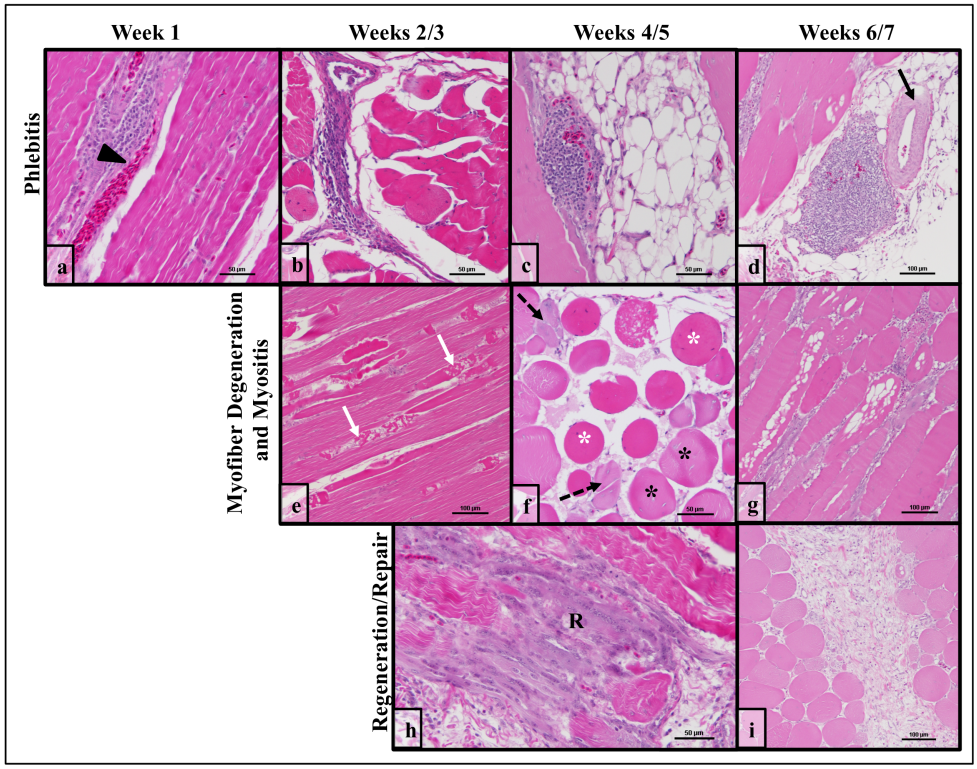

Figure 3.4: Time progression of histologic lesions of Wooden Breast myopathy in Pectoral muscle of broiler chickens, 1 to 7 weeks of age

Representative microscopic lesions are presented at each time point observed. Lymphoplasmacytic phlebitis is observed as early as 1 week of age, beginning as occasional eccentric mural lymphoid infiltrates (arrowhead (a)) with secondary venous congestion and edema (a) and progressing to complete venous occlusion (b–d), while adjacent arterial vasculature is unaffected (dark arrow: (d)). Myofiber degeneration and necrosis characterized by segmental myofibrillar disruption ((e), white arrows), myofiber rounding and hypereosinophilia ((f), white *), hyalinization ((f), dark *), fiber splitting ((f), broken arrow) and vacuolation (g) is observed from 2 weeks of age with concurrent development of heterophilic infiltrates within and around affected myofibers (myositis) (g). Myofibre degeneration and myositis progresses in lesion distribution and severity over time with acute degenerative changes observed alongside reparative lesions (multifocal multiphasic myopathy, (g)). Attempted regeneration and repair is evident by myotube formation with nuclear rowing by Week 3 (R, (h)) and early fibrosis from Week 4 with progression to scarring ((i), centre) in pectoral muscle by market age (5–7 weeks of age). All microscopic lesion images were captured in subclinical cases with no (gWBD0) to mild (gWBD1) gross evidence of disease. H&E: a, b, c, f and h, bar = 50 μm; d, e, g and i, bar = 100 μm

Edema, corroborative evidence of impaired venous return, was variably observed at all time points in 44 - 92% of the muscle specimens. Frequently, edema was observed in the same tissue region with inflamed vessels as indicated in 78% (145/185) of all samples. In addition to edema fluid, the perivascular connective tissues were also often expanded by aggregates of lipid-laden macrophages and occasionally by free lipid. Furthermore, lipogranuloma formation was visualized by cuffing of veins with concurrent lesions of phlebitis. Lipid infiltration and lipogranuloma formation was evident in 65% (13/20 birds) in Week 1 and increased in prevalence to affect ~90% of specimens in later weeks (Weeks 5-7) (Figure 3.4c).

Segmental myofiber degeneration was first observed beginning in the second week (Figure 3.4e) (7 of 20 birds, 35%) and then increased in prevalence and severity in subsequent collection time points (from 62-67% prevalence at 3-4 weeks to 98-100% prevalence by 6-7 weeks of age (Figure 3.3) and (Figure 3.4f and g). As in the first week, scattered myofibers in Week 2 specimens exhibited hypereosinophilia,

shrinkage, and rounding, interpreted as myofiber hypercontraction, in 13 of 20 birds (65%). In Week 2, however, hypereosinophilia was associated with rare fibers also showing evidence of segmental myofibrillar degeneration. In these fibers, myofibrils fragmented and formed irregular horizontal bands separated by clear space (Figure 3.4e and f).

Hypercontraction and segmental myofibrillar degeneration were the most common myofiber changes observed histologically throughout the study with 60 to 100% and 35 to 100% prevalence, respectively. On the other hand, myofiber hyalinization, in the author's experience, the predominant degenerative change found in field cases of Wooden Breast, was identified much less frequently and was only observed in specimens collected during Weeks 3-6. Hyalinization, or myofiber swelling with glassy to lightly eosinophilic amorphous conglomerations of degenerate myofibrils (Figure 3.4f), was observed in 29% (7/24) of specimens in Week 3 and then in <10% of specimens in Weeks 4-6.

Starting in the third week, a small proportion of degenerating myofibers in 2 specimens (2/24, 8%) also exhibited single to multiple variably sized, discrete, clear vacuoles suggestive of localized electrolyte abnormalities (i.e. vacuolar degeneration). There was no evidence of myofiber lipidosis by Oil Red O staining of a subset of the 11 frozen muscle specimens (n=11, gWBD0=4, gWBD2=2, gWBD3=1 and gWBD4 &5=4) collected alongside specimens for H & E staining (data not shown), therefore, indicating that the open spaces in the myofibers in H &E were indeed cytoplasmic vacuoles and not lipids. Similar vacuolation was also inconsistently observed in 22-58% of specimens in weeks 5-7 (Figures. 3.3 and 3.4g). Necrosis and loss of entire myofibers was present starting in Week 4 in 3 of 45 birds (7%). Myofiber necrosis

also increased in prevalence (Figure 3.3) and progressed from loss of isolated individual fibers to involve widespread loss of fibers within a given fascicle. Myofiber degeneration and necrosis appeared to occur randomly and did not seem to preferentially localize to either the center or the periphery of affected fascicles. In fact, degenerating myofibers were frequently observed adjacent to apparently healthy, unaffected fibers even in advanced cases of the disease.

Inflammation surrounding and invading affected myofibers (i.e. myositis) was first observed in pectoral muscle specimens starting at Week 2. Myositis prevalence increased from 15% in Week 2 to 94% by Week 7 (Figures 3.3 and 3.4g). Heterophils and histiocytes (macrophages) predominated in inflamed muscle, in contrast to venous and perivascular infiltrates which were primarily lymphoid in nature. Attempted tissue repair was observed alongside muscle inflammation as early as Week 3 (Figure 3.4h). At this time-point, myofiber regeneration, evidenced by myotube formation and myofiber nuclear rowing, was observed in a single bird (Figure 3.4h; gWBD0, Week 4). Myofiber regeneration then increased in prevalence for the remainder of the study (16, 36, 75, and 77% prevalence in weeks 4-7, respectively) (Figure 3.3). Likewise, interstitial fibrosis was observed in a single bird the following week (Week 4); whose gross presentation included petechiations, white-striping lesions (score of 2 out of 5) prominent on the cranial aspect and mildly firm (gWBD1). The prevalence of interstitial fibrosis increased (24, 52, and 72% prevalence from weeks 5-7, respectively) (Figure 3.3) in distribution (from multifocal perifascicular fibrosis to diffuse fibrosis surrounding fascicles and individual myofibers) and severity over time (Figure 3.4i). From Week 3 to the conclusion of the study, myopathic lesions were multifocal and multiphasic in nature, with repair occurring simultaneously with

ongoing myofiber degeneration, inflammation, and necrosis. Interestingly, in week 7, acute myofiber injury appeared to subside even as reparative and inflammatory lesions escalated in scope and severity. There was no evidence of myofiber-type switching by ATPase and NADH staining performed on a subset of muscle samples (n=11, gWBD0=4, gWBD2=2, gWBD3=1 and gWBD4 &5=4). Type II myofibers predominated (data not shown).

While phlebitis and myositis frequently occurred together, the two processes were sometimes observed independently of one another. For instance, in 51 cases (51 of 285 total specimens, 18%), phlebitis/perivascular inflammation was observed without simultaneous muscle inflammation. Conversely, roughly the same proportion of birds (49/285, 17%) exhibited myositis without concurrent inflammation of the vessels observed in the plane of section.

Analysis of histologic scores for different lesions across the entire experimental period, by and large, revealed an increase in severity with age (Table 3.4). Weeks 1 and 2 showed no significant difference in lesion scores. Conversely, statistical analysis of the histologic scores starting from Week 3 all through to Week 7 indicated increase in the severity of lesions (Table 3.4). This was reflected by the sequential overlap of histologic scores for lipid deposition, fibrosis, myositis, and degeneration and necrosis from Week 3 to Week 7.

Analysis of the prevalence of the different microscopic lesions on samples originally categorized under different gWBD classes also revealed an increase in prevalence of the myopathic lesions with the degree of firmness of breast muscle (Table 3.5). In this regard, even though some birds were not graded for degree of firmness, the prevalence of birds, in percentage, for myopathic lesions evaluated

namely, degeneration, myositis, fibrosis, phlebitis, necrosis and regeneration, all appeared to increase with advancing clinical/gross disease- from gWBD0, gWBD1 &2, gWBD3 to gWBD4 &5. Further, it was also evident that birds grouped grossly as being of normal consistency (gWBD0), exhibited microscopic lesions albeit at a lower prevalence compared to the mild (gWBD1&2), moderate (gWBD3) and markedly affected birds (gWBD4 &5). On the other hand, samples in the markedly affected group showed 100% prevalence for all the histopathologic lesions (Table 3.5).

Table 3.5: Prevalence (%) of myopathic lesions by categories of gross Wooden Breast Disease (gWBD) scores at necropsy from Week 4 to 7

		Prevalence of microscopic lesions in (%)				
		Unaffected gWBD0	Mild gWBD1&2	Moderate gWBD3	Severe gWBD4&5	Lesion % across gWBD groups
Week 4 (n=45)	Myositis	51	86	100	NA	58
	Regeneration	6	43	100	NA	16
	Fibrosis	0	14	0	NA	2
	Phlebitis	46	43	0	NA	47
	Degeneration	20	86	100	NA	33
	Necrosis	3	29	0	NA	9
Week 5 (n=45)	Myositis	53	80	75	100	71
	Regeneration	16	40	50	100	33
	Fibrosis	5	10	100	100	24
	Phlebitis	32	60	100	100	58
	Degeneration	68	90	100	100	82
	Necrosis	21	20	75	100	31
Week 6	Myositis	88	93	94	100	93
	Regeneration	38	89	94	100	78
	Fibrosis	19	37	88	100	51

(n=67)	Phlebitis	81	96	100	100	94
	Degeneration	100	100	100	100	100
	Necrosis	31	89	94	100	78
	Myositis	71	94	100	100	94
Week 7	Regeneration	43	74	92	100	77
(n=64)	Fibrosis	43	68	83	100	72
	Phlebitis	86	82	100	100	89
	Degeneration	86	100	100	100	98
	Necrosis	43	65	100	100	73

Notice that prevalence of histologic lesions appears to consistently increase with the severity of gross Wooden Breast Disease (gWBD) across the 4 weeks. NA in prevalence indicates that there were no gross samples in that gWBD category.

Additionally, a pairwise correlation analysis of the microscopic lesion scores across the entire period was conducted using the Spearman's rho correlation test for non-parametric test. This test revealed variations of correlation coefficients for the different myopathic lesions and degree of firmness (gWBD) across the 7-week experimental period (Table 3.6). While all the tests were significant ($P<0.0001$), correlation coefficient of fibrosis with degeneration and necrosis was the highest at (0.78). Similarly, relatively strong correlations were observed between, myositis with fibrosis, fibrosis with lipid infiltration, and myositis with degeneration and necrosis, all at (0.7). On the other hand, correlations of degeneration and necrosis with lipid infiltration, and myositis with lipid infiltration exhibited a correlation coefficient of about (0.6) among the histologic parameters (Table 3.6). Furthermore, a pairwise correlation of microscopic lesion scores (range 0 to 5) with those of gross changes (score of 0 to 5) indicated relatively lower correlation coefficients compared to those analyzed between histopathologic lesions only (Table 3.6). In this case, the highest

correlations of gWBD score were found when analyzed with degeneration and necrosis, at (0.56); and myositis at (0.51). Conversely, the lowest correlations were found when analyzed with fibrosis and lipid infiltration; at (0.46) and (0.3) respectively (Table 3.5).

Table 3.6: Correlation between histologic lesions of Wooden Breast Disease with one another and with gross Wooden Breast Disease (gWBD) scores at necropsy throughout the 7-week experimental period.

Variable 1	Variable 2	Spearman's ρ correlation	Prob>|ρ|	Sample size
Fibrosis	Lipid infiltration	0.7	<.0001	202
Myositis	Lipid infiltration	0.56	<.0001	202
Myositis	Fibrosis	0.73	<.0001	285
Degeneration and necrosis	Lipid infiltration	0.6	<.0001	202
Degeneration and necrosis	Fibrosis	0.78	<.0001	285
Degeneration and necrosis	Myositis	0.7	<.0001	285
Degeneration and necrosis	WB score at Necropsy	0.56	<.0001	283
gWBD score at Necropsy	Lipid infiltration	0.3	<.0001	202
gWBD score at Necropsy	Fibrosis	0.46	<.0001	283
gWBD score at Necropsy	Myositis	0.51	<.0001	283

Pairwise correlation analyses of various histologic and WB categories scores conducted using the Spearman's rho correlation test. There was a strong correlation between degeneration and necrosis with fibrosis and with myositis, while the lowest correlation was is seen between gWBD with lipid infiltration

3.4.4 Phlebitis and Lipid Infiltration

Multifocal perivascular, and more specifically perivenous aggregates of lipid-laden macrophages (i.e. foam cells) were observed in 13 of 20 birds (65%) in Week 1 (Figure 3.5a). Similar lipogranulomas were found perivascularly, as well as between myofibers present in the majority (58-91%) of specimens at all time points (Figure 3.5b). These fatty lesions were thought to correlate to white striping striations often associated with white striping (WS) myopathy, as observed grossly in experimental specimens. Interestingly, at the later stages starting from Week 4 to the end of the experimental period, the affected veins presented cellular infiltrates comprising lymphocytes and plasma cells occupying the entire vascular wall including the valve leaflets (Figure 3.5c). This presentation was confirmed by the examination of semi-thin sections (processed with osmium tetroxide which stains fat), and ultrastructural sections starting from Week 4 to 6. Indeed, histological examination of the semi-thin sections from 3 birds (2 belonging to gWBD0 from Week 4 and 6, and 1 at gWBD1 from Week 4) revealed veins with presence of perivenous lipid droplets, as well as a mixture of inflammatory cell infiltrates across the entire wall including the valve leaflets of veins (Figure. 3.5d). The identity of these inflammatory cell infiltrates as revealed by ultrastructural examination indicated they were of both lymphocytic (Figure 3.5e) and histiocytic populations, interlinked with collagen fibers (Figure 3.5f). Thus, the tunica adventitia and tunica media of these veins appeared to be completely effaced by the infiltrating immune cells and fibrosis, potentially compromising their functional capacity significantly. Evaluation of the tunica intima of these veins, exhibited an unaffected endothelial layer as typified by the presence of intact tight junctions, as well as desmosomes between adjoining endothelial cells

(Figure 3.5g). Further, endothelial cells appeared unaffected and contained typical organelles such as centrosomes, Golgi apparatus, and ribosomes (Figure 3.5g).

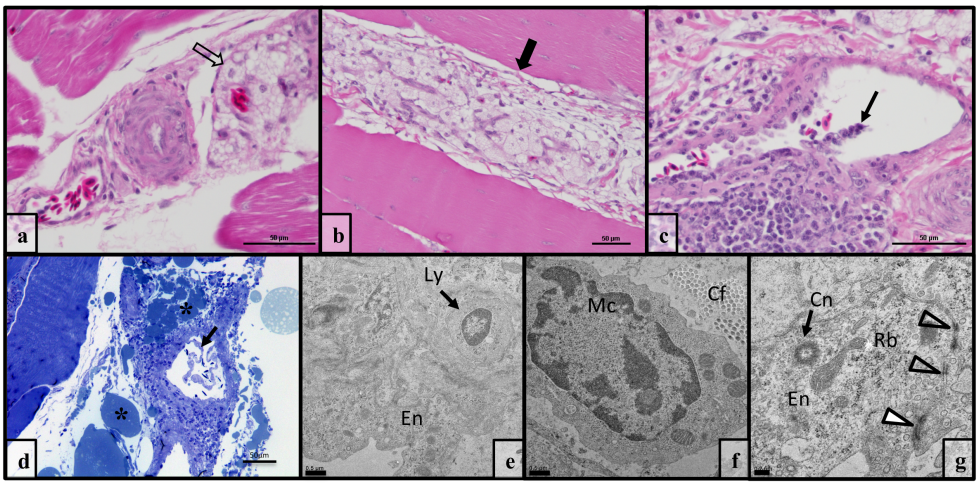

Figure 3.5: Vascular lesions of Wooden Breast myopathy overlap lesions of WS in pectoral muscles of broiler chickens.

Perivenous aggregates of lipid-laden macrophages circumferentially cuff small veins (open arrow, (a)) and between myofibres (closed arrow, (b)), which contribute to white linear streaks observed grossly. Lymphocytes, macrophages and plasma cells invade the wall and valve leaflets (arrows) of small veins potentially leading to valvular insufficiency, impaired venous return and oedema (c,d). Perivascular lipid infiltration is demonstrated by lipid droplets (d, *). A lymphocyte (Ly) adjacent to the endothelium (En) in (e) and a macrophage (Mc) interlinked with collagen fibres (Cf) in (f), represent the components of intramural infiltrates in affected veins. The endothelial cells (En) of the affected veins remain healthy with intact tight junctions (white arrowhead) desmosomes (open arrow- heads), centrosome (Cn) and free ribosome (Rb) in (g). All microscopic lesion images were captured in subclinical cases with no (gWBD0) to mild (gWBD1 & 2) gross evidence of disease from weeks 1–7. H&E: a–c, bar = 50 µm; Toluidine blue: d, bar = 50 µm; electron micrographs: e, f, bar = 0.5 µm, g = 0.2 µm.

3.4.5 Lipid Infiltration into Muscles and Extracellular Matrix

A subset comprising 10 samples from Week 4, n=3 (gWBD0, gWBD1 and gWBD3) Week 5 n=2 (gWBD0), and Week 6, n=5 (3 gWBD0 and 2 gWBD1) were examined for semi-thin ultrastructural evaluation. All of these samples but one (gWBD0, Week 5) were confirmed to have histologic lesions related to WBD. Further examination of the semi-thin sections from the 9 affected specimens from this group revealed lipid infiltration in all but one sample (gWBD0, Week 6). Random infiltration and deposition of fat droplets of varying sizes between myofibers and muscle fascicles especially in the areas beneath the perimysium were observed. Lipid droplets were also observed prominently in the extracellular matrix (ECM) (Figure 3.6a, gWBD1 Week 4). Within the ECM, some lipid droplets appeared to freely coalesce forming larger fat droplets while others appeared to be contained within variably-sized, randomly distributed nodular lipogranulomas (Figure 3.6a).

Ultrastructural examination of these nodular lipogranulomas revealed presence of giant macrophages characterized by the prominence of their nuclei lying contiguous with each other (Figure 3.6b, gWBD1 Week 4). Additionally, these macrophages appeared to be contained within a network of bundles of dense collagen fibers winding within and around the wall of the nodular lipogranulomas (Figure 3.6b). This structure, therefore, appeared to form a formidable wall enclosing fat droplet localized within the interstitium. Additionally, traversing the interstitium especially at the interfascicular junctions were thick bundles of dense regular collagen fibers, which also traversed between lipid droplets (Figure 3.6c and d, gWBD1 Week 4).

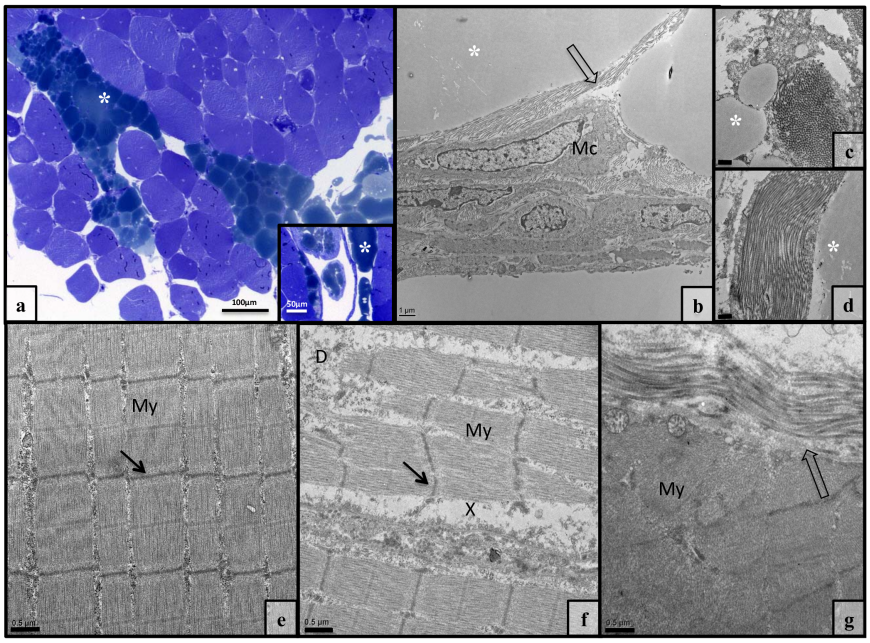

Figure 3.6: Semi-thin and TEM micrographs of pectoral muscles with WB

(a) Lipid infiltration in a semi-thin section (asterisk) between muscle fibers, as well as ECM. Notice the presence of lipids beneath the perimysium as well as lipogranulomas (inset) whose electron micrograph (b) shows giant macrophages (Mc) and fibrosis (open arrow). Within the ECM are bundles of dense regular collagen fibers around lipid droplets (asterisk) (c,d). (e) shows normal ultrastructure of skeletal breast muscle with regular Z-bands (arrow). Affected muscles exhibit irregular and disconnected Z-bands, degeneration and dissolution (D) of myofibrils (My) and separation of myofibrils (X, (f)) and endomysial fibrosis (open arrow (g)). Notice the loss of Z-bands in (g). All microscopic lesion images were captured in subclinical cases with no (gWBD0) to mild (gWBD1) gross evidence of disease from Week 4 (a–d), 5 (e) and 6 (f, g), respectively. [(a) and inset: Toluidine blue: a, bar = 100 µm, inset = 50 µm; electron micrographs: b, bar = 1 µm; c, d, bar = 0.2 µm; e–g, bar = 0.5 µm.

3.4.6 Ultrastructure of Affected Myofibers

The structural presentation of the unaffected skeletal muscle, as observed in one sample (gWBD0, Week 5) exhibited myofibers oriented longitudinally and

comprising sarcomeres arranged in register with each other, typical of the normal skeletal muscles. Sarcomeres were bound by clearly distinct Z-bands, running parallel with each other and perpendicular to contiguous myofibrils, within myofibers. Each sarcomere comprised clearly distinct contractile elements (actin and myosin filaments) characteristic of the skeletal muscles (Figure 3.6e).

On the other hand, 9 of the samples, as indicated above: Week 4, n=3 (gWBD0, gWBD1 and gWBD3) Week 5 n=1 (gWBD0), and Week 6, n=5 (3 gWBD0 and 2 gWBD1), initially examined histologically, revealed disruptions of myofibers ultrastructurally, consistent with muscle damage. The myofibers of these samples revealed disturbances of the normal architecture of Z-band as exhibited by irregular, disconnected or dissolution of Z-lines (Figure 3.6f). Additionally, the ultrastructural presentation of the affected samples showed disruptions and disorganization of the myofibrillar apparatus as depicted by degeneration and splitting of myofilaments, including localized losses of the myofibrils (Figure 3.6f). Endomysial fibrosis in affected samples was also a prominent feature, as typified by the accumulation of layers of collagen fibers on the endomysium of affected muscle fibers (Figure 3.6h). In contrast to the normal morphology of the mitochondria often depicted by presence of well-defined cristae and matrix (Figure 3.7a, gWBD0, Week 5), those in the affected myofibers presented degeneration and dissolution of their cristae, the matrix, and to some extent, degeneration of entire mitochondria (Figure 3.7b, gWBD0, Week 6).

Examination of advanced cases based on ultrastructural evaluation of myofibers, and whose myofibrils had distorted architectural presentation; their sarcolemma including those of contiguous/apposing myofibers appeared to undergo active folding and shrinkage, thereby increasing the interstitial space between adjacent

myofibers (Figure 3.7c gWBD1, Week 4). Additionally, cristae of mitochondria within the degenerating muscle fiber appeared to undergo disintegration (Figure 3.7c). On the other hand, myofibril degeneration of other samples seemed to start from the periphery of the myofiber, beneath the sarcolemma, spreading to the interior (Figure 3.7d gWBD2, Week6). In this case, mitochondria found within degenerating myofibrils appeared to undergo atrophy (Figure 3.7b). Ultrastructural presentation of the muscle in affected cases, therefore, shows that disruption and/or degeneration of the mitochondrion morphology, myofibrillary apparatus, as well as that of the general sarcomeric structure may play a role in the development of the WB myopathy.

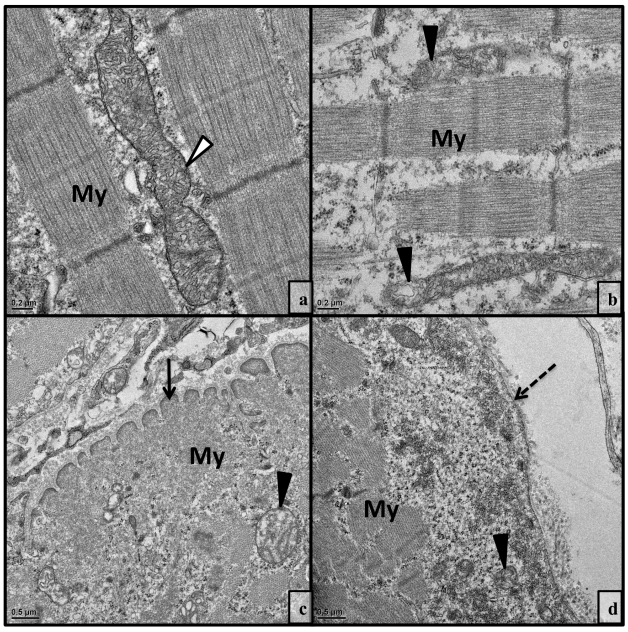

Figure 3.7: Mitochondrial and myofibrillar degeneration in WB in broiler chickens

(a): gWBD0, Week 5 indicates normal mitochondrion with clearly defined cristae (open arrowhead), while (b): gWBD0, Week 6 shows degenerating mitochondria with matrix undergoing dissolution (filled arrowheads) in the vicinity of degenerating

myofibrils (My). (c): WBD1, Week 4 shows an advanced case of muscle degeneration. Notice the folded nature of the sarcolemma (arrow), the degenerating mitochondrion (arrowhead) and the absence of z-bands. (d): gWBD2, Week 6 shows a myofibre with completely degenerated myofibrils beneath the sarco- lemma (broken arrow), which appears to spread towards the interior of the myofibre (My) with atrophying mitochondrion (filled arrowhead). All TEM lesion images were prepared from subclinical cases with no (gWBD0) to mild (gWBD1 & 2) gross evidence of disease, belonging to age range 4–6 weeks. Electron micrographs: a, b, bar = 0.2 μm; c, d, bar = 0.5 μm.

3.5 Discussion

The present study critically examines the phenotypic, clinical and pathologic features associated with the onset and progression of WB in commercial broiler chickens from hatch to market age. While this study did not find an apparent direct etiologic agent that could be implicated in the initiation of WB in broiler chickens, it has brought attention to evidence of phenotypic, histologic and ultrastructural perturbations that may be contributing to the initiation and progression of the myopathy.

Increased body weight gain and breast muscle weight have been suggested and confirmed to be among the main predisposing factors for a number of breast muscle disorders including WB (Baeza et al., 2002; Berri et al., 2007; Kuttappan et al., 2012, 2013a; Sihvo et al., 2014; Siller, 1985). The present study demonstrates that birds with high weight gain from the outset (Week1 or 2 of age) have high potential to develop WB as they approach marketable weight at week 6 to 7. Indeed, this observation further confirms that the modern broiler chickens, which have been genetically selected for rapid growth rates and increased breast muscle yield, may have a predisposition to develop breast myopathies (Mitchell and Sandercock, 2004; Velleman, 2015).

Wooden Breast was not clinically evident in birds in the present study until 4 weeks of age. While this disorder has not previously been associated with overt clinical signs beyond palpable muscle firmness, some affected birds in this study were unable to right themselves from accidental dorsal recumbency. Additionally, affected birds exhibited resistance to general movement. Although no mortalities were directly associated with this disorder, these clinical signs could indicate welfare concerns and reduced quality of life in WB-affected birds (Bessei, 2006; European Commission, 2000). Another possible impact of WB on animal welfare could also be associated with the demonstration of tissue pathology involving the vasculature, as well as the musculature of the breast region in affected birds. Even though this study did not specifically assess for pain in the affected chickens, similar tissue pathology has been frequently associated with painful episodes in humans (Zebracki and Drotar, 2008). Therefore, it may be possible that affected chickens with WB also experience similar pain levels. Additionally, with the protracted nature of the disorder as demonstrated in this study, it is likely that chickens exhibiting WB have reduced welfare compared to unaffected birds (Bessei, 2006).

The findings in this study have also brought to the forefront the key lesions characterizing the early onset and progress of WB in modern broilers. The initial gross changes were observed from Week 3 of age, while the overt firm consistency of the breast muscle typical of WB was detected beginning from Week 4. This observation is closely in line with recent findings, which indicated the earliest detection of macroscopic changes of the disease on the P. major muscles at 18 days of age (Radaelli et al., 2017; Sihvo et al., 2017).

While distinct difference on gross changes and degrees of firmness of the breast muscles allowed categorization of the chickens based on severity in this study, there was appreciable overlap of microscopic lesions across all gWBD categories including gWBD0, "grossly normal" birds. This observation is consistent with those in the recent study which found degenerative changes in macroscopically unaffected breast muscles (Sihvo et al., 2017). This, therefore, indicates that microscopic perturbations precede gross changes, as evidenced by microscopic lesions of WB and WS (i.e. phlebitis, perivascular lipid accumulation) from the first week of life. Further, the presence of microscopic lesions even in grossly normal muscle as observed in this study could explain the relatively weak correlation (0.3-0.5) between histologic lesions and gWBD scoring, despite their highly significant associations ($p<0.0001$). Nonetheless, the prevalence of microscopic lesions (lipid deposition, myodegeneration, myonecrosis, myositis and fibrosis) appeared to increase with the severity of gross changes, which is consistent with other studies (Radaelli et al., 2017; Sihvo et al., 2017). The absence of concurrent microscopic lesions in the other muscle types namely red (*Iliotibialis lateralis*/thigh) muscle, smooth muscle (ventriculus/gizzard) and cardiac muscle (heart) confirms that WB is largely limited on the P. major muscle in the modern broiler chickens, consistent with previous studies (Sihvo et al., 2014, 2017). It should be noted that P. major comprises type II skeletal muscle (Barnard et al., 1982; Verdiglione and Cassandro, 2013), suggesting a possible predilection of the disorder to this type of muscle while sparing other types.

Analysis of specific microscopic lesions in the P. major muscle in this study provides possible insights into the pathogenesis of WB in chickens. Firstly, this study has found evidence of hemodynamic perturbations, (transmural infiltration and

extravasation of inflammatory cells, impaired venous outflow visible as congestion/edema, valvular insufficiency) as exemplified by the various degrees of phlebitis in the course of development of WB over the study period. While vascular involvement of WB has been reported previously at marketable age (Sihvo et al., 2014), as well as on day 18 of age (Sihvo et al., 2017), this study is the first to establish vascular involvement before the development of myopathic (i.e. myofiber degeneration and necrosis) lesions. In fact, from this work, it appears that the sequellae of phlebitis, such as congestion, edema, and to some extent, venous stenosis, may, in turn, produce physiologic and/or structural perturbations to muscles drained by the affected veins, subsequently leading to myopathic lesion development. For instance, impaired venous drainage may lead to local accumulation of metabolic waste and buildup of reactive oxygen species, secondarily triggering myofiber degeneration. It is, therefore, not surprising to note that muscle changes such as degeneration and fragmentation appear shortly after vascular changes from Week 2. In addition to the observed vasculopathy of WB, it has previously been hypothesized that the unprecedented increase in muscle fiber size observed in the modern broilers is not concomitant with the rate of vascularization (Velleman, 2015). This may lead to, not only generation of hypoxic conditions in the breast muscle tissue over time, but also inadequate nutrient supply, poor drainage and retention of metabolic waste, which if taken together, would result in muscle damage (Petracci et al., 2015; Velleman, 2015). In view of the above, therefore, early phlebitis as demonstrated in this study, may serve to accentuate degeneration and necrosis on the muscle that is already prone to these tissue changes owing to increased rate of myofiber hypertrophy or inadequate vascularization. Molecular studies conducted previously on WB indicated evidence of

hypoxia at market age (week 6-7) (Abasht et al., 2016; Mutryn et al., 2015). Based on the findings of the present study, therefore, it is possible that hypoxic conditions begin early (from Week 1) due to vascular involvement, and that the effects increase over time as the animal advances in age.

Phlebitis in this study was also accompanied with lipogranulomas comprising lipid-laden macrophages (foam cells) adjacent to affected vein walls. Such foam cells are generally considered pathognomonic for atherosclerosis when observed in arteries (Valledor et al., 2015; Yuan et al., 2012). While chickens previously have been shown to have either spontaneous or induced atherosclerosis, mainly in response to high cholesterol diets, vascular injury on the endothelium or Marek's Disease Virus (Ayala et al., 2005; Fabricant and Fabricant, 1999), atherosclerosis is unlikely in the broilers in this study since lipid accumulation and foam cell infiltration were localized to veins alone without involvement of arterial vessels. Additionally, the venous lesions observed in this study are not typical of those of atherosclerosis. For example, the intimal layer generally remained intact during intramural infiltration, except in severe cases where the entire venous wall is completely infiltrated, and the lumen completely obstructed. However, the presence of ectopic free lipids in the vicinity of the venous vasculature is likely to be important in the initiation of reactive lesions (i.e. phlebitis, myositis and fibrosis), and hence, the progression of WBD. Corroborating our findings, previous investigations have indicated increased levels of lipids in breast muscles affected with WBD (Abasht et al., 2016; Zambonelli et al., 2016). Furthermore, lipid peroxidation products such as 13-HODE and 9-HODE (derived from lipids) have been found to be associated with WBD, suggesting presence of free radical exposure of potential damage to tissues, ultimately, resulting in oxidative stress

to muscles (Abasht et al., 2016; Mutryn et al., 2015). It is known that even a small amount of lipid peroxides can result in a number of tissue pathologies and disease states (Mylonas and Kouretas, 1999). Further, lipid peroxides have been implicated in blood vessel damage (Kipiani et al., 2006) and, thus, could incite progressive tissue damage and inflammation in chronic WB.

In this study, the severity and scope of phlebitis appeared to increase with age (from Week 1 to 7), accompanied by perivascular lipid and inflammatory cell infiltration, reaching its severe form (including complete obliteration of the veins) at the end of the experimental period. This presentation is consistent with a recent study on WBD, which also indicated high association of phlebitis with the severity of the myopathy (Sihvo et al., 2017). However, while Sihvo, *et al* (2017) did not find phlebitis in (WB0) samples, our study revealed presence of phlebitis in all gWBD categories (gWBD0 to gWBD5). The increase in scope and severity of phlebitis could indicate the presence of an inciting agent or a recurrent tissue injury that continuously elicits inflammatory reactions on the veins. Therefore, it is conceivable to consider the increased lipids and lipid peroxides due to lipid peroxidation as the inciting agents on the venous injuries. Consequently, injury to veins is often accompanied by an inflammatory response within the venous wall, beginning within the tunica adventitia and progressing towards the endothelium of the tunica intima, with the latter being the last to be affected in severe cases as exhibited in this study. The localized nature of the artery-sparing venous lesions informed the authors argument that the inciting component/agent, potentially lipid peroxides from free lipids, is not systemic but rather emanates locally within muscle from the regions where lipids surround the veins. It is known that lipid peroxidation is frequently a self-propagating chain-

reaction that causes damage to tissues, inevitably eliciting an inflammatory reaction (Mylonas and Kouretas, 1999). Additionally, inflammatory cells, for example phagocytes produce inflammatory products including free radicals as part of their inflammatory response (Forman and Torres, 2002; Rosen et al., 1995), whose lack of specificity exacerbates oxidative stress on the vasculature, as well as the surrounding tissue (myofibers). This could, therefore, partly explain the increase in severity and scope of the vascular lesions in the breast muscles with time, as shown in this study.

The effects of phlebitis in this study can easily be appreciated by the extent of damage, not only on the veins themselves, but also on the muscles being served by the vessels. Indeed, the complete intramural and valvular infiltration by inflammatory cells (lymphocytes and macrophages) including fibrosis would typically render the affected veins dysfunctional. A study in humans involving the greater saphenous vein indicated that monocyte and macrophage infiltration into the entire venous wall including the valves could contribute to the shortening of the venous valves leading to valvular insufficiency and poor venous return (Ono et al., 1998). Similarly, effects such as congestion, decreased venous return, valvular insufficiency, vascular extravasation, decreased metabolic waste clearance from tissues, and edema due to blood stasis are possible sequelae of phlebitis demonstrated in this study. Ultimately, tissue damage such as myodegeneration and myonecrosis observed in this study might be arising partly due to retention of metabolic waste as previously suggested. Additionally, similar to the present study, Ono et al. (1998) reported no observable damage to the venous endothelium.

Lipid infiltration in this study was also a component observed within the extracellular matrix around myofibers and muscle fascicles. As in previous studies

(Bailey et al., 2015; Sihvo et al., 2014, 2017), lipid infiltration in this study was demonstrated grossly by presence of white streaks along the longitudinal axis of the breast muscles. It should be noted that the white fatty streaks are considered the principal pathognomonic presentation for WS in chickens (Kuttappan et al., 2013b, 2016; Petracci et al., 2013; Russo et al., 2015). Therefore, the finding of the Spearman's correlation coefficient score of up to 0.59 ($P<0.0001$) in Week 7 ($n=64$) between WS and gWBD in this study corroborates and reinforces the hypothesis that WS myopathy has a common cause with WB, or that WS and WB represent a spectrum/continuum of lesions for a single disease process (Bailey et al., 2015; Kuttappan et al., 2013b; Mutryn et al., 2015; Sihvo et al., 2017). Microscopically, the present study showed a combination of ectopic free lipid droplets in the extracellular matrix especially within the interfascicular space and perivascularly, or as lipogranulomas as noted above. Lipid infiltration in WS has been demonstrated to comprise largely intracellular accumulations within adipocytes (Kuttappan et al., 2013b), which contrasts the acellular lipid droplets or intracellular lipid droplets found in macrophages in the present study. The identification of lipogranulomatous lesions were based on structural features comprising acellular fat droplets enclosed by a formidable wall of prominent elongated macrophages (multinucleated giant cells) with conspicuous nuclei wrapped together with bundles of collagen fibers (Delladetsima et al., 1987; Williams and Williams, 1983). Thus, we speculate that the presence of lipogranulomatous lesions represent an attempt to minimize cellular damage from ectopic extracellular ("free") lipids within the muscle. In this case, the ectopic fat seems to be highly inflammatory, inciting a strong immune reaction to contain it. Histologically and ultrastructurally, it is possible that both the lipogranulomatous

reaction and myositis (i.e. lymphoplasmacytic and heterophilic response within and around affected myofibers) could be occurring in response to lipid infiltration.

Besides the lesions that occurred primarily on the vasculature and ECM, there were also lesions associated with myofibers. In this respect, the main lesions observed on myofibers in the early phase (Week 1 and 2) were hypercontracted fibers (hypereiosinophilic) and myodegeneration often distributed in focal to multifocal manner. Previous studies have shown presence of hypercontracted fibers at marketable age (weeks 6 to 8) in chickens (Kuttappan et al., 2013b; Sihvo et al., 2014; Velleman et al., 2010) and turkeys (Sosnicki et al., 1991). It has been hypothesized that in modern broiler chickens, hypercontraction occurs due to unmatched growth rate between muscle fibers, which often exhibit rapid radial growth compared to its respective endomysium. Hence, rapid growth rate against the limiting connective tissue membrane causes myofiber damage (Mazzoni et al., 2015). Therefore, this may explain the occurrence of these fibers very early at week 1, and their continued presence to the end of the experiment.

Myodegeneration, hyalinization and myonecrosis are lesions observed in breast muscles of modern broilers presenting various conditions indicating muscle pathology (Kuttappan et al., 2013b; Mazzoni et al., 2015; Soglia et al., 2016b). Similarly, in this study, the presence of myodegeneration and myositis observed as early as 2 weeks of age signals the onset of muscle pathology, which then increases in scope and severity with age. This is in line with recent findings on WB, which found myopathic changes in broiler chickens beginning from the second week of age (Radaelli et al., 2017; Sihvo et al., 2017).

Presence of myositis, as depicted by the infiltration of inflammatory cells as early as 2 weeks of age can also be thought of as the body's immune response to clear off the debris of degenerating and necrotizing muscle tissue and other cellular components (Kozakowska et al., 2015; Maxwell and Robertson, 1998). While heterophils predominated the acute phase, macrophages were the predominant cells in the chronic phase. It is known that macrophages not only promote phagocytosis of cellular debris, but also control and promote muscle regeneration and extracellular matrix remodeling, by acting directly on myogenic and fibroadipogenic precursors (Chazaud, 2016; Karalaki et al., 2009; Saclier et al., 2013; Tidball and Wehling-Henricks, 2007). Most importantly, it has been established that the pro-inflammatory macrophages (M1 macrophages) promote proliferation of myogenic precursor cells while anti-inflammatory macrophages (M2 macrophages) promote differentiation of myogenic precursor cells (Bentzinger et al., 2013; Saclier et al., 2013; Sciorati et al., 2016). In this study, myoregeneration was observed from Week 3, and it increased in intensity as the disorder gradually entered the chronic phase.

However, it should be emphasized that even though the severe form of WB disorder at marketable age (Weeks 6 and 7) is often characterized by a mosaic of lesions all occurring on the same individual, earlier phases of the disease present with varying lesion combinations that differ in severity. For instance, an individual bird could exhibit marked myofiber degeneration with little inflammatory response or could exhibit marked myositis with little concurrent myofiber degeneration. Generally, however myopathic lesions appeared to increase in prevalence, scope and severity as the birds advanced in age, reaching the peak at marketable age. Additionally, most lesions first appear to assume focal to multifocal distribution before becoming diffuse

in severe cases. While tissue destructive processes such as myodegeneration, myonecrosis and myositis occurred in affected muscles, simultaneous reparative processes such as fibrosis and myoregeneration were also observed indicating multiphasic nature of the disease. Moreover, it is likely that the combination of both myofiber degeneration (fiber swelling and disintegration of myofibrillar proteins) and fibrosis that contribute to gross muscle firmness and changes in meat quality rather than fibrosis alone as suggested previously (Sihvo et al., 2017).

Fiber-type switching from the type II to type I has been proposed as one of the responses of the affected breast muscle to disease presence based on gene expression profile (Mutryn et al., 2015). The present study, which aimed to test this hypothesis using histochemical assays with bird P. major samples from Week 4 to 6, revealed no change of myofiber-type. This difference could be attributed to the fact that fiber-type histochemistry assays in the current study tests for specific myofibrillar or muscle mitochondrial enzymes (Barnard et al., 1982), while the previous study used gene expression profile (Mutryn et al., 2015). Additionally, the proteins of the respective genes presented by the previous study are not the targets of the enzymatic testing of the present study. Therefore, the lack of fiber-type switch demonstrated in this study does not refute the findings of gene expression reported previously.

Ultrastructural examination of the breast muscles revealed presence of muscle fiber abnormality with myofibrillar apparatus and mitochondria being the main target organelles in WB. Besides muscle fibers, other areas that were affected included the endomysium, which showed fibrosis; the venous vasculature, which exhibited cellular infiltration and fibrosis; and extracellular matrix that revealed structural remodeling owing to fibrosis and lipid infiltration. Indeed, myofibrillar degeneration and splitting,

disruption of Z-bands and to some extent, dissolution of the sarcomere in this study is a clear deviation from the normal ultrastructure of the skeletal muscles (Hanson and Huxley, 1953; Huxley, 1953; Lauritzen et al., 2009). Previously, an ultrastructural study conducted on the P. major and gastrocnemius muscles in highly selected chickens raised in different production systems showed similar ultrastructural abnormalities (Polak et al., 2009). In mammals, the presence of these lesions in skeletal muscles are thought to signify myofibrillar myopathies (Fernandez et al., 2005; Nakano et al., 1996). Therefore, it could be postulated that the appearance of these ultrastuctural perturbations in the myofibrillar architecture signifies development of a pathological process.

A number of myopathies that have been associated with ultrastructural disruptions of myofibers in chickens, have been reported to be frequently accompanied by alterations of the ultrastructure of the mitochondria ranging from subtle nonspecific changes to overt mitochondrial specific disruptions (Polak et al., 2009; Van Vleet and Ferrans, 1976; Wight et al., 1981). Similarly, in this study, myofibrillary lesions were accompanied by mitochondrial architectural changes such as degeneration of cristae, and to some extent, dissolution of the matrix, or the entire mitochondrion. These changes could indicate compromised mitochondrial function and output especially bioenergetics. Previous studies have also related the occurrence of myofibrillary disruptions, disorganization and dissolution of Z-lines, as well as mitochondrial ultrastructural perturbations with hypoxia within muscles (Polak et al., 2009; Soike and Bergmann, 1998). Additionally, focal lesions on the mitochondria were also observed in a case of oxidative stress induced experimentally by muscle ischemia in the rat (Carmo-Araujo et al., 2007), as well as turkey (Sosnicki et al.,

1991). Based on these observations, it is therefore, possible that hypoxia and oxidative stress in muscles as earlier suggested could be involved in the muscle pathology in this study. This observation corroborates previous studies on molecular assessment of WB, which reported similar findings (Abasht et al., 2016; Mutryn et al., 2015; Zambonelli et al., 2016). Along these lines, a recent study provided evidence of a strong negative relationship between mitochondrial content within skeletal muscles with breast muscle yield and carcass yield in broiler chickens (Reverter et al., 2017). Therefore, with the knowledge that modern broilers have generally high breast muscle yield, and hence, lower mitochondrial content in their breast muscles (Reverter et al., 2017), any damage to the available mitochondria would be expected to have a significant effect on the general bioenergetics of the breast muscles, thereby increasing susceptibility to muscle damage.

The other notable ultrastructural feature observed in this study was fibrosis of the endomysium, as well as remodeling of the extracellular matrix with dense regular connective tissue. A study by Velleman and Clark (2015) on WB demonstrated presence of two forms of fibrosis whose occurrence depended on the chicken line, namely, highly interlinked collagen fibers, and highly ordered (regular) collagen fibers. In this study, it was evident the type of fibrosis was of highly dense regular type. The extensive fibrosis of the breast muscle in this study is therefore suggested to contribute to the firm phenotype associated with the disease.

3.6 Conclusion

It is demonstrated from this study that the occurrence of WB in modern broiler chickens is associated with rapid increase in body weight from the outset of the disease. It may be concluded that clear morphologic changes are present as early as the

first week of life preceding a clinical phase in subsequent weeks (>3 weeks). In this respect, WB appears to be first, and foremost, a vasculopathy with perivenous lipid infiltration, and secondarily a myopathy. While it currently appears that perivenous lipid accumulation and lipogranuloma formation may be the inciting cause for phlebitis, the converse may also be true; local lipid infiltration may occur surrounding veins compromised by vasculitis. Once the myopathy has been initiated, abnormalities in the myofibrillar apparatus and the mitochondria within myofibers may play an important role in the pathogenesis and progression of WB in chickens. Further, it is shown that myositis, fibrosis, and myofiber regeneration appear to be occurring to direct tissue repair rather than as underlying drivers for initial WB development. Overall, WB demonstrates a progressive disease, presenting first as phlebitis with concurrent lipid infiltration in an acute inflammatory phase, progressing to myofiber degeneration and myositis, followed by a chronic fibrotic phase with myofiber regeneration in subsequent weeks.

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Chapter 4

GENE EXPRESSION PROFILING OF THE EARLY PATHOGENESIS OF WOODEN BREAST DISEASE IN COMMERCIAL BROILER CHICKENS USING RNA-SEQUENCING

- (Michael B. Papah, Erin M. Brannick, Carl J. Schmidt & Behnam Abasht PLoS ONE 13(12): e0207346). https://doi.org/10.1371/journal.pone.0207346

4.1 Abstract

Wooden Breast (WB), a myopathy in commercial broiler chickens characterized by abnormally firm consistency of the pectoral muscle, impacts the poultry industry negatively due to severe reduction in meat quality traits. To unravel the molecular profile associated with the onset and early development of WB in broiler chickens, we compared time-series gene expression profiles of Pectoralis (P.) major muscles between unaffected and affected birds from a high-breast-muscle-yield, purebred broiler line. P. major biopsy samples were collected from the cranial and caudal aspects of the muscle in birds that were raised up to 7 weeks of age (i.e. market age). Three subsets of biopsy samples comprising 6 unaffected (U) and 10 affected (A) from week 2 (cranial) and 4 (caudal), and 4U and 11A from week 3 (cranial) were processed for RNA-sequencing analysis. Sequence reads generated were processed using a suite of bioinformatics programs producing differentially expressed (DE) genes for each dataset at fold-change (A/U or U/A) >1.3 and False Discovery Ratio (FDR) <0.05 (week 2: 41 genes; week 3: 618 genes and week 4: 39 genes). Functional analysis of DE genes using literature mining, BioDBnet and IPA revealed several biological processes and pathways associated with onset and progress of WB. Top

among them were dysregulation of energy metabolism, response to inflammation, vascular disease and remodeling of extracellular matrix. This study reveals that presence of molecular perturbations involving the vasculature, extracellular matrix and metabolism are pertinent to the onset and early pathogenesis of WB in commercial meat-type chickens.

4.2 Introduction

The poultry industry is one of the key players contributing to sustainable food sources in the world. Through the utilization of artificial genetic selection, commercial broiler lines with high merit for traits such as increased muscle yield, fast growth rates and feed efficiency have been produced (Schmidt et al., 2009; Zhou et al., 2015; Zuidhof et al., 2014). Despite the gains brought about by the use of genetic selection in modern broiler chickens, these chickens have also developed, albeit inadvertently, negative attributes, top among them being increased myodegenerative disorders (Baldi et al., 2017; Siller, 1985). One such myodegenerative disorder is Wooden Breast (WB), a myopathy that is uniquely characterized by extreme palpable firmness of the Pectoralis (P.) major muscles in severe cases (Clark and Velleman, 2017; Papah et al., 2017; Sihvo et al., 2014, 2017). This disorder has been determined to compromise meat quality traits especially in severe cases (Mudalal et al., 2015; Petracci et al., 2015; Tasoniero et al., 2016), resulting in significant economic loss to the poultry industry globally.

The gross and histopathological presentation of this disorder in broiler chickens has also been characterized. WB presents itself palpably as localized foci of firmness starting from the cranial aspects of the P. major muscle, progressing to multifocal and then diffuse distribution (Clark and Velleman, 2017; Papah et al., 2017;

Sihvo et al., 2014). Grossly, lesions such as outbulging of the lateral forebreasts, muscle hemorrhage and pallor, subcutaneous edema, and in most cases, white striations, are common characteristics of the myopathy. Microscopically, the disorder features lesions of myodegeneration, myonecrosis, inflammatory cell infiltration, characteristic arterial-sparing vasculitis of small caliber venous vessels, and in chronic or severe cases, interstitial fibrosis and myoregeneration (Clark and Velleman, 2017; Papah et al., 2017; Sihvo et al., 2014, 2017). Ultrastructural examination undertaken on the affected muscles have revealed degeneration of mitochondria and myofibrillar apparatus, complexed with formation of lipogranulomatous and dense fibrotic tissue straddling the extracellular matrix compartment (Papah et al., 2017).

Molecular evaluations involving both gene expression and metabolite profiling associated with WB have been conducted on chickens at market age (week 6 to 8) (Abasht et al., 2016; Mutryn et al., 2015; Zambonelli et al., 2016). Indeed, mRNA-gene expression analysis and metabolomics profiles between affected and unaffected chickens suggested presence of altered redox homeostasis and oxidative stress including compromised glucose metabolism as possible biological processes associated with the disease process (Abasht et al., 2016; Mutryn et al., 2015; Zambonelli et al., 2016). In addition, *decorin*, a gene responsible for collagen cross-linking was found to be increased in P. major muscle of affected chickens, suggestive of its role in promotion of fibrosis phenotype in advanced WB (Clark and Velleman, 2017; Velleman and Clark, 2015). While the studies already undertaken on Wooden Breast myopathy provide vital clues regarding the end stage of the fulminant disease in commercial broiler chickens, the molecular aspects involving its onset and early progression still remain to be elucidated.

This study, therefore, is aimed at characterizing the molecular profile associated with the onset and progression of WB in chickens throughout the early growth period (up to 4 weeks of age) before the myopathy is typically detectable by palpation. This was achieved by utilizing RNA-sequencing technique on muscle biopsy samples from P. major muscles between affected and unaffected birds belonging to the high-breast-muscle-yield, purebred broiler line. Gene expression analysis, evaluated through Illumina high-throughput sequencing platform used previously (Mutryn et al., 2015), has shown great potential in elucidation of the molecular dynamics associated with the disease at market age. This study, therefore, employed the same technique to discern gene expression profiles associated with the disease progression during the typical growing period for broilers. The results from this study are important not only in advancing our understanding of WB in modern broiler chickens, but also in efforts directed towards management and prevention of the condition in chickens.

4.3 Materials and Methods

4.3.1 Birds and Housing Conditions

Chicken husbandry and sample collection methods including experimental procedures used in this study were approved by the Animal Care and Use committee of the College of Agriculture and Natural Resources, University of Delaware (UD), under protocol number 44 07-08-14R. This study utilized muscle biopsy samples collected from a subset of 350 male chickens belonging to the Heritage Breeders line B, a high-breast-muscle-yield, purebred commercial broiler line whose background and genetic features has been described by Fu *et al*. (Fu et al., 2015, 2016). The birds

were raised from day-old to a maximum of 49 days post-hatch at UD's poultry houses following the protocol explained in a related study (Papah et al., 2017). Briefly, at placement, all birds were randomly designated to either necropsy or biopsy groups, with the latter being specifically utilized in the current study. The biopsy group initially comprised 100 birds, of which a total of 85 birds were successfully subjected to biopsy regardless of WB status at weeks 2 to 5 of age as described below; 13 birds were found dead of unknown causes while 2 were euthanized due to development of leg pathologies during the growth period. At the conclusion of the study (weeks 6 and 7), all birds in both biopsy and necropsy groups were necropsied and samples from the P. major muscle harvested for microscopic analysis to confirm the status of the muscle with respect to the disease.

4.3.2 Biopsy Protocol

The biopsy protocol used in this study involved performing two pectoral microbiopsies (Patel et al., 2012) on each bird either at weeks 2 and 4 (denoted as B1) or at week 3 and 5 (denoted B2). Thus, collectively, muscle samples collected represented timepoints across the typical broiler growing period. Microbiopsy was accomplished by sampling through the skin into the craniolateral (week 2 or 3) and caudolateral third (week 4 or 5) of the left pectoral muscle respectively. This second sampling location was selected to remove the potential for confounding factors from repeated sampling at or near the original biopsy site (i.e. resolving hemorrhage, inflammation, or fibrosis at the healing biopsy site). Prior to biopsy, the selected site was plucked of feathers if present, anaesthetized locally via topical application of lidocaine cream (2-4ml) covering the entire cranial or caudal portion of the pectoral muscle, and aseptically prepared with betadine scrub using cotton wool balls. Each

biopsy specimen was collected with a sterile Bard Max-Core disposable biopsy instrument with a 16-gauge biopsy needle and 22mm sample notch directed to the P. major muscle at ~ 45 degrees angle to the keel. This orientation facilitated sampling several muscle fibers over the length of the biopsy needle sampling notch. Once collected, the skin and any visible fat tissue was removed from the biopsy core, before the remaining muscle specimen was flash-frozen individually in liquid nitrogen and stored at -80° C for RNA-seq analysis. Only the left P. major muscle was biopsied, while the right was spared for internal control to be sampled for necropsy at week 6 or 7 for histological diagnosis of WB disease status. Following biopsy, bleeding was controlled by application of direct pressure at the site which was then cleaned and covered with iodine cream prior to release of birds. In addition, to allow for a close observation of the chickens and prevent wound contamination immediately after the biopsy procedure, biopsied birds were kept separate from the rest of the flock in clean area covered by a brown Manilla paper for 2 hours prior release. All biopsied birds were then allowed to grow until the end of the experimental period (week 6 and 7) where they were necropsied and evaluated both at gross and microscopic levels for status of Wooden Breast Disease.

4.3.3 Selection of Biopsy Samples for RNA-Seq Analysis

Identification of specific samples from the 85 stored muscle biopsy samples to be processed for RNA-seq analysis was accomplished retrospectively using several parameters previously used for scoring WB (Papah et al., 2017). Briefly, the parameters comprised: (1) Evaluation of gross presentation of the pectoral muscle including presence and distribution of lesions such as hemorrhage, subcutaneous edema, changes in muscle color or pallor were used. At this stage, the WB disease

scores of the biopsied chickens at necropsy (denoted “gross WBD”), score range (0 unaffected, to 4/5 markedly affected) were used. In addition, live bodyweights of the birds prior to necropsy were considered; those whose bodyweights were lower than 3 standard deviations from the average were excluded from the study. (2) Analysis of microscopic presentation and respective lesion scores of Wooden Breast after necropsy at week 6 and 7, score range (0 unaffected, to 4/5 markedly affected) (Papah et al., 2017). From all these assessments, biopsied birds were able to be defined as either “affected” or “unaffected” for WB at market age (week 6 and 7). Parameters and scores used to define and categorize affected and unaffected birds in this study are summarized under (Appendix B). Therefore, with the knowledge of the disease states of the biopsied birds at market age, the biopsy muscle samples previously harvested and stored were used to trace back on the early molecular presentation of WB disease states at their respective time points i.e. weeks 2, 3 and 4 in the selected birds. A sample size comprising 6 unaffected and 10 affected muscle samples from both weeks 2 and 4 were identified for RNA-seq analysis, using paired samples from the same individual birds at each time point (i.e. 32 total samples from 16 individual birds at 2 time points). Likewise, 4 unaffected and 12 affected (6 moderately and 6 severely affected) samples were identified from weeks 3 and 5 categories for RNA-seq evaluations. To decipher genes associated with early pathogenesis of WB, the key focus of this study, only sample categories from weeks 2, 3 and 4 were processed for RNA sequencing (Table 4.1). It should be noted that the number of samples in A and U groups in week 2 and 4 differ from those of week 3. This difference in sample size resulted from the low number of unaffected samples identified for week 3 compared to

those for week 2 and 4, following the application of the multi-parametric method indicated above.

Table 4.1: Number of broiler chicken superficial pectoral muscle samples used for RNA-sequencing analysis by bird age.

Bird Age (Week)	Biopsy site	Unaffected	Affected
2	Cranial	6	10
3	Cranial	4	12
4	Caudal	6	10

4.3.4 RNA Extraction and RNA-Seq Protocol

Total RNA was extracted from all selected samples using mirVana^{TM} miRNA Isolation Kit (Thermo Fisher Scientific) following manufacturer's protocol. The isolated RNA was stored at -80° C until the next stage of cDNA library preparation. Concentration and quality of the RNA samples were measured using NanoDrop 1000 (Thermo Fisher scientific) and Fragment Analyzer at Delaware Institute of Biotechnology (DBI), Newark, DE. For all RNA samples, the RNA quality integrity number (QIN) from fragment analysis was above 6, which was acceptable for cDNA library preparations.

Library preparation of cDNA from the samples was performed using the TruSeq Stranded mRNA Sample Prep Kit for low sample protocol (Illumina) following manufacturer's instructions. During processing, each sample of cDNA was barcoded with a unique index to allow pooling at the final stage of the cDNA library preparations. The constructed cDNA libraries were assessed for concentration and

quality using Nanodrop 1000 (UD) and Fragment analyzer (DBI) respectively following the manufacturer's protocol. All the cDNA libraries passed the quality test and were subsequently normalized to 10 nM/µl using Tris buffer (Tris-Cl 10 mM, 0.1 % Tween 20, pH 8.5). From the normalized samples, 10µl of each sample (total of 16 samples per week) was pooled into one tube for each week (total of 3 weeks) of the experiment and subsequently submitted to DBI for paired-end 2x75-nucleotide sequencing using Illumina HiSeq 2500 sequencer. The resulting sequence reads were checked for quality using FastQC program (v0.11.3) (Andrews, 2010). All the sequences passed the quality check, (namely basic statistics, per base sequence quality, per sequence GC content and per base K content), and were subsequently used for mapping to the chicken reference genome (Ensembl Gallgall5.0 May 2017 and gene transfer format (GTF), file release 88) using HISAT2 v2.0.5, software package (Pertea et al., 2016). Further, Cuffdiff v2.2.1 (Trapnell et al., 2012) was used for identification of differentially expressed (DE) genes between affected and unaffected groups. Genes were considered to be statistically significant, and hence, differentially expressed when the test statistic for the mean difference of Fragments Per Kilobase of transcript per Million mapped reads (FPKM) between unaffected and affected groups yielded a q-value or FDR adjusted p-value of <0.05 and fold-change >1.3.

Additionally, one severely affected sample (sample ID: 1683_wk3_Sev2) from week 3 dataset was detected as misclassified in the affected group and therefore, the entire set was re-analyzed in Cuffdiff to identify DE genes without this sample (4 unaffected and 11 affected sample). Further, an analysis consisting of the 6 moderately and 5 severely affected samples from week 3 dataset was performed and DE genes were analyzed to determine whether gene expression profiles at week 3 of the affected

group of chickens (moderate and severe) were consistent with their respective phenotypic presentations at week 6 and 7. To ensure that all DE genes used in this study were free from potential skin contamination inadvertently obtained during the biopsy sampling procedure, a list of skin-derived DE genes was generated from week 3 samples (Appendix A). Any gene with expression level of log2FC ≥1 from this skin-derived gene list was considered as a potential skin contaminant. Therefore, DE gene-sets obtained from all 3 time points were first scrutinized for potential skin contamination by comparing with the skin-derived gene list, and any contaminant subsequently removed if present. The resulting DE genes across all 3 timepoints were then used for downstream functional analysis.

4.3.5 Analysis of DE Genes

Functional analysis of DE genes from week 2 and 4 was accomplished using BioDBnet software (Mudunuri et al., 2009) and literature mining. Functional analysis of DE genes from week 3 dataset was accomplished using Ingenuity Pathway Analysis (IPA) program. Briefly, all DE genes were submitted to IPA for functional annotation and identification of canonical pathways and upstream regulators.

4.4 Results

4.4.1 Differentially Expressed Genes

Sequencing of cDNA libraries produced an average of 34 million reads per sample in week 2; 33 million reads in week 3 and 39 million reads in week 4, all counted as paired-end. Raw sequence reads in each week were processed and analyzed producing DE genes between affected and unaffected categories across the 3-week experimental period. In this case, there were 41 and 39 DE genes in weeks 2 and 4

respectively, with directionality of the genes shown in (Table 4.2). Week 3 dataset comprising 4 unaffected vs 11 affected yielded 618 DE genes (Table 4.2). Of the 618 DE genes, 504 genes were identified as mapped by IPA software and were therefore used for functional analysis downstream. Moreover, within the affected group in week 3, there were 30 DE genes between 6 moderately and 5 severely affected muscle samples (Table 4.2).

Table 4.2: Differentially expressed (DE) genes from week 2,3 and 4 sample sets comparing Wooden Breast unaffected (U-AFF) to affected (AFF) or moderately-affected (M-AFF) to severely-affected (S-AFF) broiler chicken pectoral muscle samples.

	Pectoral major muscle sample			Number of DE genes[2]		
Week[1]	Location	Condition 1 (n)	Condition 2 (n)	Total	Up	Down
2	Cranial	U-AFF (6)	AFF (10)	41	18	23
3	Cranial	U-AFF (4)	AFF (11)	618	251	367
3	Cranial	M-AFF (6)	S-AFF (5)	30	17	13
4	Caudal	U-AFF (6)	AFF (10)	39	18	21

[1] Week 2 and week 4 samples were taken from the same birds. Moderately (n=6) and severely (n=5) affected samples at week 3 were combined as one condition (n=11 affected) to be compared with the other condition (unaffected). [2]Upregulated (Up) and downregulated (Down) genes in the affected or severely affected group.

Overlapping Genes

Comparison of DE genes between week 2 and 4, whose biopsy samples were extracted from the same birds revealed 11 overlapping genes as shown in (Table 4.3). Of all 11 overlapping genes, 5 genes were upregulated, and the same number of genes were also downregulated, while only one gene (*ENSGALG00000044239*) showed

opposing directionality (Table 4.3) in WB-affected chickens. Among the top upregulated genes in both weeks included *fibromodulin* (*FMOD*), *crumbs 2* or *cell polarity complex component* (*CRB2*) and *collagen type XII alpha 1 chain* (*COL12A1*). Conversely, the top downregulated genes for week 2 and 4 included *sidekick cell adhesion molecule 1* (*SDK1*), *ENSGALG00000019175, kyphoscoliosis peptidase* (*KY*), and *stratifin* (*SFN*). Similarly, the overlap of DE genes in all 3 weeks showed only 5 genes, namely *CRB2, COL12A1, KY*, *ENSGALG00000030512* and *SDK1* (Table 4.4). The number of overlapping genes between week 2 and 3 was 15, where 9 genes showed consistency of expression pattern in both weeks while 6 genes showed opposing expression pattern (Figure 4.1). On the other hand, 26 DE genes were found to be unique in week 2 while 603 genes were unique in week 3. The gene overlap between week 3 and week 4 was 23 (Figure 4.1).

Table 4.3: DE genes between Wooden Breast-affected and unaffected pectoral muscle samples showing overlap between age groups at weeks 2 and 4 of age

GENE ID	Gene symbol	Gene name	Log2FC Week2	Log2FC Week4
ENSGALG00000034067	*FMOD*	*Fibromodulin*	↑0.66	↑1.25
ENSGALG00000001169	*CRB2*	*Crumbs 2,*	↑1.45	↑1.12
ENSGALG00000015908	*COL12A1*	*Collagen type XII alpha 1 chain*	↑0.77	↑1.08
ENSGALG00000041266	*CNN1*	*Calponin 1*	↑0.68	↑1.01
ENSGALG00000005024	*EFCC1*	*EF-hand and coiled-coil domain containing 1*	↑1.02	↑0.87

ENSGALG00000044975	*KY*	*kyphoscoliosis peptidase*	↓0.86	↓0.75
ENSGALG00000004420	*SDK1*	*Sidekick cell adhesion molecule 1*	↓1.09	↓0.79
ENSGALG00000044239	-	-	↑0.80	↓0.87
ENSGALG00000030512	-	-	↓0.86	↓1.09
ENSGALG00000019175	*LOC418544*	*Cystathionine beta-synthase-like*	↓1.03	↓1.43
ENSGALG00000028115	*SFN*	*Stratifin*	↓0.81	↓2.41

Log2FC of a gene is determined by log2 (FC= affected $_{FPKM}$/unaffected $_{FPKM}$). The dashes (-) indicate gene symbols and names are still unknown.
↑ indicates upregulation in affected group
↓ indicates downregulation in affected group
Log2FC values are negative for downregulated genes

Table 4.4: DE genes between Wooden Breast-affected and unaffected pectoral muscle samples showing overlap among age groups at weeks 2.3 and 4 of age.

Gene ID	Gene symbol	Gene name	Log2FC Wk2	Log2FC Wk3	Log2FC Wk4
ENSGALG00000004420	*SDK1*	*Sidekick cell adhesion molecule 1*	↓1.09	↓0.51	↓0.79
ENSGALG00000044975	*KY*	*Kyphoscoliosis peptidase*	↓0.86	↓0.73	↓0.75
ENSGALG00000001169	*CRB2*	*Crumbs 2, cell polarity complex component*	↑1.45	↓0.56	↑1.12
ENSGALG00000030512	-	-	↓0.86	↓0.93	↓1.09
ENSGALG00000015908	*COL12A1*	*Collagen type XII alpha 1 chain*	↑0.77	↑1.05	↑1.08

Log2FC of a gene is determined by log2 (FC= affected $_{FPKM}$/unaffected $_{FPKM}$). The dashes (-) indicate gene symbols and names are still unknown.
↑ indicates upregulation in affected group
↓ indicates down regulation in affected group
Log2FC values are negative for downregulated genes

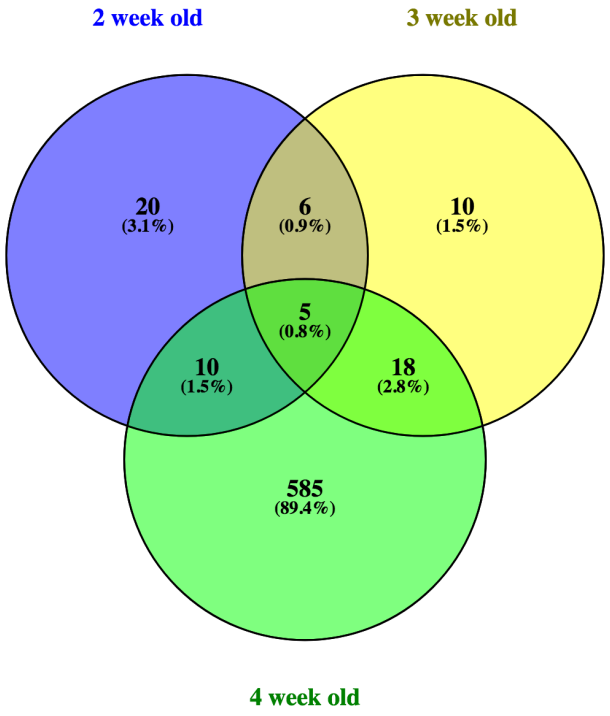

Figure 4.1: Overlapping DE genes expressed across 3-week period.

Differentially expressed genes between Wooden Breast-affected and unaffected pectoral muscle showing overlap across broiler chickens at 2, 3 and 4 weeks of age**.** Only 5 DE genes were found in common at all time points

4.4.2 Functional Analysis of DE Genes from Week 2 and 4 Post-Hatch

In week 2, the main biological terms whose genes were upregulated in the pectoral muscles of affected chickens included skeletal muscle differentiation, ECM receptor interaction stress/oxidative stress and response to inflammation. Conversely, the biological terms whose genes were downregulated in pectoral muscles of affected chickens included cell adhesion and metabolic pathways (Table 4.5). In week 4, the biological pathways whose genes were upregulated in affected birds included cell adhesion while those that were downregulated included ATP-binding and metabolic pathways (Table 4.6).

Table 4.5: Top pathways/ biological functions from DE genes upregulated or downregulated in Wooden Breast-affected pectoral muscles as compared to unaffected muscles in broiler chickens at 2 weeks post-hatch

Biological term/Pathway	Gene symbol	Gene Full Name	RNA-seq-Log2FC
Cell adhesion	*COL12A1*	*Collagen type XII alpha 1 chain*	↑0.77
	SFN	*Stratifin*	↓0.81
	SDK1	*Sidekick cell adhesion molecule 1*	↓1.09
	GJD2	*Gap junction protein delta 2*	↓1.08
Metabolic pathways	*CHAC1*	*ChaC glutathione specific gamma-glutamylcyclotransferase 1*	↑0.84
	TMEM68	*Transmembrane protein 68*	↓0.82
	PDP1	*Pyruvate dehydrogenase phosphatase catalytic subunit 1*	↓0.54
	G0S2	*G0/G1 switch 2*	↓0.86
	LOC418544	*Cystathionine beta-synthase-like*	↓1.03
Stress/Oxidative stress	*FMOD*	*Fibromodulin*	↑0.66
	ATF3	*Activating transcription*	↑0.82

		factor 3	
	ANKRD1	*Ankyrin repeat domain 1*	↑0.87
ECM-receptor interaction	*FMOD*	*Fibromodulin*	↑0.66
	ATF3	*Activating transcription factor 3*	↑0.82
	ANKRD1	*Ankyrin repeat domain 1*	↑0.87
Response to inflammation	*ATF3*	*Activating transcription factor 3*	↑0.82
	ANKRD1	*Ankyrin repeat domain 1*	↑0.87
	G0S2	*G0/G1 switch 2*	↓0.86
Skeletal muscle differentiation	*ASB2*	*Ankyrin repeat and SOCS box containing 2*	↑0.56
	ATF3	*Activating transcription factor 3*	↑0.82
	ANKRD1	*Ankyrin repeat domain 1*	↑0.87
Transport	*AQP4*	*Aquaporin 4*	↑0.80
	ALS2	*Amyotrophic lateral sclerosis 2 (juvenile)*	↑0.58
	SLC20A1	*Solute carrier family 20 member 1*	↓0.75

↑ indicates upregulation in affected group
↓ indicates down regulation in affected group
Log2FC values are negative for downregulated genes

Table 4.6: To pathways/biological terms from DE genes between Wooden Breast-affected compared to unaffected pectoral muscle in broiler chickens at 4 weeks of age.

Biological term/Pathway	Gene symbol	Gene Full Name	RNA-seq-Log2FC
Cell adhesion	*ITGB2*	*Integrin subunit beta 2*	↑0.87
	TNC	*Tenascin C*	↑0.82
	SPP1	*Secreted phosphoprotein 1*	↑1.75
	COL12A1	*Collagen type XII alpha 1 chain*	↑1.08
	CNTN1	*Contactin 1*	↓0.92

ATP binding	*KIF21A*	*Kinesin family member 21A*	↓0.55
	ASNS	*Asparagine synthetase (glutamine-hydrolyzing)*	↓0.81
	ABCA12	*ATP binding cassette subfamily A member 12*	↓3.12
Metabolic pathways	*SLC16A9*	*Solute carrier family 16 member 9*	↓0.86
	ABCA12	*ATP binding cassette subfamily A member 12*	↓3.12

↑ indicates upregulation in affected group
↓ indicates down regulation in affected group
Log2FC values are negative for downregulated genes

4.4.3 Functional Analysis of DE Genes from Week 3 Post-Hatch

Differentially expressed genes whose gene symbols were identified and mapped numbering 504 of the total 618 were used to perform functional analysis on IPA program. Several functional categories associated with the DE genes as revealed by the IPA program included top canonical pathways, diseases and biological functions, as well as top upstream regulators of the DE genes.

4.4.3.1 Top Canonical Pathways

Evaluation of DE genes at week 3 for top canonical pathways (Z-scores >1.8) with Benjamin-Hochberg (B-H) multiple testing correction P-value ≤ 0.05 revealed pathways predicted to be activated and inhibited in affected chickens. Pathways predicted to be activated included complement system (Z-score=1.89) and acute-phase response signaling (Z-score= 2.12). Conversely, the pathways predicted to be inhibited in the pectoral muscles of affected chickens included EIF2 signaling pathway (Z-score

-2.89), oxidative phosphorylation (Z-score -3.87) and tRNA charging (Z-score -2.24) (Figure 4.2).

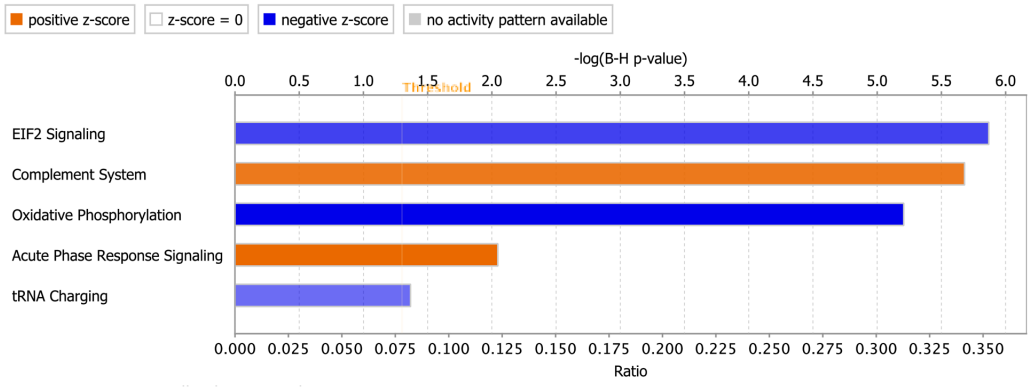

Figure 4.2: Top canonical pathways as predicted by IPA for pectoral muscles at week 3 of age.

Activated pathways (Z-scores > 1.8) are represented by orange-colored bars (color shade increasing with increasing strength of activation) while inhibited pathways are shown by blue-colored bars (color shade increasing with the strength of inhibition).

4.4.3.2 Diseases and Biological Functions

Analysis of DE genes for disease and biological functions in IPA revealed a number of biological processes as depicted by significant (Z-scores ≥ 2) for those predicted to be activated or (Z-scores ≤ -2) for those predicted to be inhibited in pectoral muscles of affected chickens. For convenience purposes, the biological and disease processes identified are grouped into the following disease and functional categories: vascular disease; inflammatory response; metabolic dysregulation; extracellular matrix (ECM) remodeling and excitation-contraction coupling (Figure 4.3).

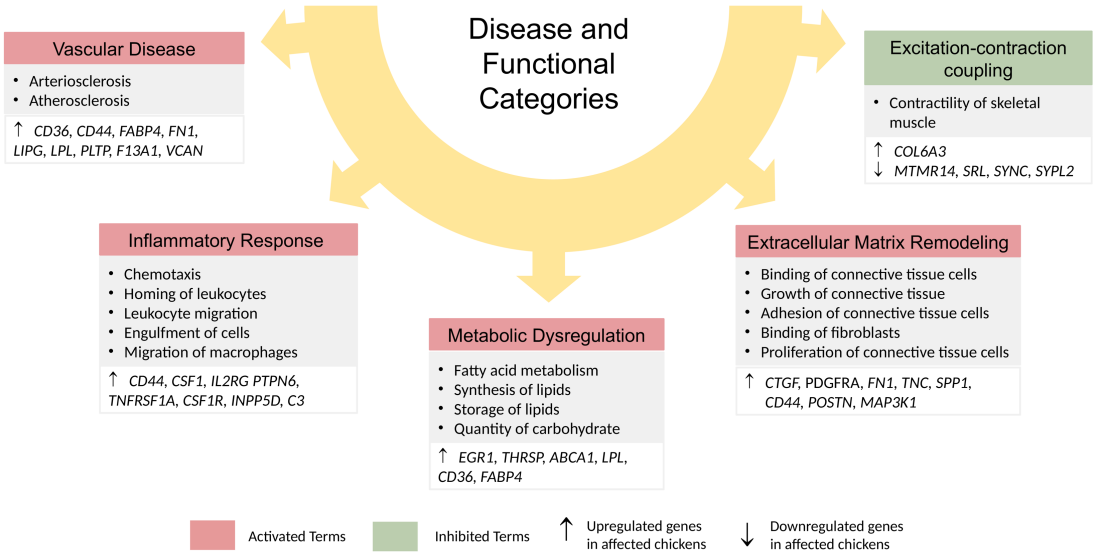

Figure 4.3: Disease and functional characterization of DE genes from the pectoral muscles at week 3 of age as predicted by IPA

Activated terms have (Z-scores ≥ 2) while inhibited terms have (Z-scores ≤ -2). Each biological term has cluster annotation(s) associated with it. In addition, the main genes associated with the cluster annotation(s) including their expression states with respect to affected chickens are shown

Vascular disease

Vascular disease process was predicted to be activated (Z-score of >2) in the breast muscles of affected chickens based on the identification of genes enriched for atherosclerosis and arteriosclerosis processes. The genes associated with these processes included *CD36 molecule or fatty acid translocase (CD36), CD44, fatty acid*

binding protein 4 (FABP4), FN1, *lipase G* (*LIPG*), *lipoprotein lipase* (*LPL*), *phospholipid transfer protein* (*PLTP*) and *versican* (*VCAN*) (Figure 4.3).

Inflammatory responses

Analysis of DE genes from week 3 samples revealed activation of several biological processes under inflammatory response cluster category in affected birds (Figure 4.3). These processes included, chemotaxis of immune cells, homing of leukocytes, leukocyte migration, engulfment of cells and migration of phagocytes (Figure 4.3).

Metabolic dysregulation

Metabolic dysregulation was predicted to be increased in the pectoral muscles of the affected chickens as depicted by 4 cluster annotations, namely fatty acid metabolism, synthesis of lipid, storage of lipid and quantity of carbohydrates. The 4 clusters were enriched with several genes related to metabolism such as *early growth response 1* (*EGR1*), *thyroid hormone responsive* (*THRSP*), *ATP binding cassette subfamily A member 1* (*ABCA1*), *LPL*, *CD36* and *FABP4* (Figure 4.3).

Extracellular matrix remodeling

Extracellular matrix (ECM) remodeling was predicted to be activated based on the significant Z-scores (≥2) of each of the 5 related clusters. The clusters included binding of connective tissue cells, growth of connective tissue, adhesion of connective tissue cells, binding of fibroblasts and proliferation of connective tissue cells (Figure 4.3). Several enriched genes such as *secreted phosphoprotein 1* (*SPP1*), *fibronectin*

(*FN1*), *CD44 molecule (CD44*), *periostin* (*POSTN*), *connective tissue growth factor* (*CTGF*) and *mitogen-activated protein kinase kinase kinase 1* (*MAP3K1*) were associated with all the cluster annotations (Figure 4.3).

Excitation-contraction coupling

Functional analysis of DE genes at week 3 of age using IPA revealed inhibition of contractility in affected muscles (Z score < -2). Several genes related to this pathway were downregulated in affected chickens including *myotubularin related protein 14* (*MTMR14*), *sarcalumenin* (*SRL*), *syncoilin, intermediate filament protein* (*SYNC*) and *synaptophysin like 2* (*SYPL2*) (Figure 4.3).

4.4.3.3 Upstream Regulators

Analysis of DE genes from P. major muscles at week 3 of age for upstream regulators of expressed genes and related functions in IPA revealed several significant biological molecules predicted to be activated (Z-scores ≥2) (Appendix C**)** or inhibited (Z-scores ≤ -2) (Appendix D**).** The upstream regulators identified based on the DE gene profile from week 3 belonged to a diverse group of biological molecules including enzymes, transcription regulators, transmembrane receptors, translation regulators, growth factors, cytokines, and endogenous chemicals (Appendix C and Appendix D). Of all the upstream regulators detected, 8 were part of the DE gene list submitted to IPA where 7 were upregulated and also predicted to be activated in affected chickens, while 1 gene namely, *VCAN*, which was upregulated in affected chickens, displayed opposing prediction state (Table 4.7). The 7 upstream regulators

included *CCAAT/enhancer binding protein alpha* (*CEBPA*), *CD44*, *complement C3* (*C3*), *mitogen-activated protein kinase kinase kinase 1* (*MAP3K1*), *Spi-1 proto-oncogene* (*SPI1), colony stimulating factor 1* (*CSF1*) and *FN1* (Table 4.7).

Table 4.7: Differentially-expressed (DE) upstream regulators and their respective target genes in the gene set of Wooden Breast-affected and unaffected pectoral muscle from broiler chickens at 3 weeks of age

Upstream Regulator symbol	Upstream regulator name	Expression Log2FC	Molecule Type	Target molecules in dataset
*CEBPA**	*CCAAT/enhancer binding protein alpha*	↑1.37	transcription regulator	*CSF1, CSF1R, FABP4, LPL, PLIN1, C3, C3AR1*
*CD44**	*CD44 molecule*	↑1.24	other	*CD36, CD44, CLU, FN1, SDC4, SPP1*
*C3**	*Complement C3*	↑1.08	peptidase	*C1QA, C3AR1, CSF1, FN1, UCP3*
*MAP3K1**	*Mitogen-activated protein kinase kinase kinase 1*	↑0.88	kinase	*EGR1, FOS, NOV, PTGS2, TNC*
*SPI1**	*Spi-1 proto-oncogene*	↑0.87	transcription regulator	*CEBPA, CSF1, CSF1R, KLF4, PTGS2*
*CSF1**	*Colony stimulating factor 1*	↑0.72	cytokine	*CEBPA, CSF1R, EGR1, FN1, THBS1*
*FN1**	*Fibronectin 1*	↑0.59	enzyme	*ACP5, FN1, PDGFRA, SPP1, THBS1*
VCAN†	*Versican*	↑0.80	other	*C3, CIDEA, CLU, COMP, DAG1*

The symbols show prediction activation states of the upstream regulators as provided by IPA, where asterisk (∗) indicates activated, while obelisk (†) indicates inhibited prediction states. Notice that one upstream regulator namely *Versican* (*VCAN*) shows contrasting prediction state with its expression pattern. The prediction states of the rest of the upstream regulators are consistent with their expression profiles.
↑ indicates upregulation of the gene in affected group

4.5 Discussion

This study examined the gene expression profile associated with the onset and progression of Wooden Breast in modern broiler chickens over the early growth period (week 2 to 4). While knowledge on the molecular profile of WB at market age (week 6 to 8 post-hatch) is available (Mutryn et al., 2015; Zambonelli et al., 2016), it is not sufficient to understand the early pathogenesis of the disease due to the advanced state of inflammation and fibrosis in affected birds of that age. Therefore, by evaluating the pectoral muscle biopsy samples harvested from affected and unaffected 2 to 4-week-old broiler chickens using RNA-seq analysis, it was possible to discern pertinent molecular features highly likely to be associated with the early pathogenesis of the WB syndrome. It should be noted that the selection of biopsy muscle samples in this study was correlated to gross and histologic WB disease status of the same birds at market age (week 6 and 7).

The current study showed relatively low number of differentially expressed genes between affected and unaffected birds from week 2 with 41 DE genes (cranial pectoral region) and week 4 with 39 DE genes (caudal pectoral region). Therefore, the disease may be thought to assume a spatiotemporal distribution whereby the caudal pectoral muscle at week 4 of the pectoral regions exhibits a similar stage of the disease process as the cranial muscle at week 2. This observation is further supported by similar directionality of the overlapping genes (n=10/11) between week 2 and week 4 observed in the current study.

4.5.1 Functional Analysis of DE Genes of Biopsy Muscles at Weeks 2 and 4 Post-Hatch

Functional analysis of DE genes from week 2 indicated enrichments for biological processes such as oxidative stress, ECM receptor interaction, skeletal muscle differentiation and response to inflammation. The observation of enrichment in skeletal muscle differentiation in particular suggests an earlier occurrence of myoregeneration than previously encountered (3 weeks of age), likely indicating the molecular activity preceding microscopically discernible evidence of regenerative myotubes (Papah et al., 2017). Even though the gene sets supporting the biological processes are fewer, these genes are indicative of their potential significance to the disease. In addition, a previous study reported fewer lesions as well as lower incidence of the lesions in the early stages of the disease such as localized phlebitis at week 1 followed by focal myositis, degeneration and phlebitis at week 2 (Papah et al., 2017; Sihvo et al., 2017), which further corroborates the low number of DE genes at week 2 in this study, and correlates with the early phases of the myopathy. Further, the finding of inflammation and oxidative stress in muscles of affected chickens at week 2 is a confirmation of the early subclinical onset of the myopathy which then increases in scope, clinical significance, and lesion severity towards market age (week 7) as demonstrated by the gene expression profile (Mutryn et al., 2015; Zambonelli et al., 2016).

Functional analysis of gene expression profile at week 4 (caudal pectoral muscles) showed enrichments for cell adhesion in chickens with WB. On the other hand, affected chickens showed decreased functions in ATP binding and metabolic pathways. These processes are in tandem with features of the early phase of the WB myopathy. For example, increase in cell adhesion activity, which serve to prime

inflammatory response through interaction of endothelial cells and leukocytes (Albelda et al., 1994), may be associated with the initiation of the vasculopathy (phlebitis) reported in the early stages of Wooden Breast (Papah et al., 2017).

4.5.2 Transcriptomic Analysis of Biopsy Muscles at Week 3 Post-Hatch

Analysis of cranial pectoral muscle biopsy samples between unaffected and affected chickens from week 3 showed higher numbers of DE genes (n=618) compared to both week 2 (n=41) and week 4 (n=39) muscle biopsy samples. This observation supports the findings of histological analysis of the pectoral muscles on the same group of birds that reported advancement in pathology of WB and increase in incidence of muscle lesions associated with WB for birds at week 3 compared to those at week 2 post-hatch (Papah et al., 2017). Based on these observations, it is evident that increasing scope and severity of pectoral muscle pathology as a result of WB is also manifested by divergence of the transcriptome profile between unaffected and affected phenotypes over time with advancing disease. Indeed, in the current study, a comparison of DE genes in the cranial pectoral region between week 2 and 3 revealed 26 unique genes (13 upregulated and 13 downregulated) at week 2 compared to 603 unique genes (243 upregulated and 358 downregulated) at week 3, with only 15 genes being common at both weeks. This finding also suggests that stage-wise progress of Wooden Breast in chickens, parallels increasing recruitment of gene expression associated with the myopathy. The low number of overlapping DE genes (n=5) among the 3-week-experimental period further confirms that the pectoral muscle transcriptome is not necessarily similar for a given severity classification across different stages of the myopathy.

Evaluation of the differential transcriptome between the moderately and severely affected biopsy muscle samples at week 3 of age revealed low number of DE genes (n=30) as opposed to the comparison between unaffected and the combined "affected" bird category (comprising moderate and severely affected birds) which yielded (n=618). Even though there were distinct phenotypic and histologic differences between the moderate and severely affected chickens that necessitated their grouping at market age (week 6 and 7), the same birds did not exhibit much variation of the breast muscle transcriptome at 3 weeks of age. This is an indication that the moderate and severely affected chickens were at the same stage of the myopathy at week 3. However, in subsequent weeks, differential rates of development of the disease among birds appeared to have occurred resulting in discernable differences in severity of the disease at market age (week 6 and 7). This phenomenon shows an apparent existence of time-dependent, multiphasic pattern involving the pathogenesis of WB in modern broiler chickens. Based on this finding, a time frame at or before 3 weeks of age could be targeted for mitigation strategies for the myopathy.

4.5.3 Functional Analysis of DE Genes of P. Major Muscles at Week 3 Of Age

Analysis of top canonical pathways by IPA predicted inhibition of the eukaryotic initiation factor 2 (EIF2) signaling pathway as well as tRNA charging, suggesting a potential occurrence of translation attenuation in affected chickens. The fast-growth rate and increased breast-muscle-weight phenotype frequently exhibited by modern broiler chickens suggests that protein synthesis is also accelerated in comparison with unselected chickens. Consequently, it may be argued that the unprecedented increase in protein synthesis potentially puts more strain on the endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR) to optimize protein folding.

Eukaryotes generally monitor protein synthesis and folding in the ER to maintain homeostasis especially for proteins that are processed in the ER (Kaufman, 1999). Accordingly, eukaryotic cells activate unfolded protein response (UPR) in the event of increased accumulation of unfolded proteins within the ER, frequently occasioned by elevated protein synthesis (Kaufman, 1999).

One way in which UPR is activated is through phosphorylation of the eukaryotic translation initiation factor 2 alpha (eIF2a), resulting in translation attenuation of mRNA, thereby allowing the unfolded proteins to be cleared by the cell and subsequent restoration of ER homeostasis (DuRose et al., 2009). While we did not examine phosphorylation events of any molecules in our current study, the downregulation of several genes involved in the EIF2 signaling pathway may serve to attenuate translation of mRNAs, whose effect would be more or less similar as those of phosphorylation of eIF2a. Based on this observation, the downregulation of genes involved in the EIF2 signaling pathway in affected chickens, may be a regulatory response employed by the P. major muscles following increased protein synthesis that is beyond the capacity of the ER/SR to handle. Therefore, attenuation of translation at the gene expression level may aid in preventing the potential buildup of the deleterious unfolded proteins in ER/SR of the muscles of affected chicken. It is also worth noting that several genes involved in the ubiquitin-proteasome pathways were downregulated in affected chickens. These genes include *ubiquitin specific peptidase 2* (*USP2*), *valosin containing protein* (*VCP*), *proteasome 26S subunit, ATPase 5* (*PSMC5*), *ubiquitin fusion degradation 1 like* (*UFD1L*) and *proteasome subunit beta 4* (*PSMB4*). The downregulation of these genes suggests a potential reduction of ubiquitin-proteasome activity in affected chickens, which may lead to a buildup of damaged,

misfolded or dysfunctional proteins possibly triggering the inhibition of the EIF2 signaling pathway. The decreased ubiquitin-proteasome activity could also be reflective of the increasing levels of oxidative stress (Shang and Taylor, 2011) in the pectoral muscles of affected chickens that was initially reported in chickens at market age (Abasht et al., 2016; Mutryn et al., 2015).

Inhibition of oxidative phosphorylation suggests a compromised energy homeostasis function in the pectoral muscles of affected chickens. This observation is in agreement with previous studies which showed mitochondrial damage in affected chickens beginning from the 4th week of age (Papah et al., 2017), as well as alterations in energy metabolism in affected chickens at market age (Abasht et al., 2016). Additionally, the expression of the gene *citrate synthase* (*CS*) (log2FC -0.56), considered as an important biomarker of mitochondrial content in skeletal muscles (Hakamata et al., 2018) was downregulated in affected chickens, indicating decreased mitochondrial content, and hence, reduced muscle bioenergetics capacity in affected chickens. Indeed, in line with the study by Hakamata *et al.* (2018) who revealed a lower mitochondrial CS activity, and hence, decreased mitochondrial content in pectoral muscles of fast-growing modern broiler chickens (Hakamata et al., 2018), it is conceivable that dysregulation of muscle bioenergetics will be exacerbated in Wooden Breast-affected chickens.

Analysis for disease and biological processes showed evidence of vascular disease in affected chickens, inflammatory response, metabolic dysregulation, ECM remodeling and impairment of excitation-contraction coupling. However, there are significant overlapping pathways and biological processes owing to the overlapping

roles of associated genes. Consequently, some of these biological pathways and processes would be analyzed together in the context of WB.

4.5.3.1 Evidence of Vascular Disease

Analysis of DE genes from pectoral muscles of chickens at week 3 of age revealed evidence of vascular pathology of the arterial end in affected chickens as depicted by arteriosclerosis and atherosclerosis using IPA. Although arteriosclerosis and atherosclerosis conditions in WB have not been described in histology at market age, arterial-sparing phlebitis has been extensively observed in previous studies (Papah et al., 2017; Sihvo et al., 2014, 2017). Therefore, the current observation suggests that besides the veins, the arteries are also likely affected during the early phase of WB in chickens. Alternatively, the unique venous lesions that frequently characterize WB, could be exhibiting molecular signatures similar to those of atherosclerosis and arteriosclerosis. Indeed, Papah *et al*. (2017) reported close resemblance of the histological presentation of the arterial-sparing phlebitis to atherosclerosis (Papah et al., 2017).

4.5.3.2 Early Inflammatory Response

Analysis of genes at week 3 showed occurrence of early inflammatory response in the pectoral muscles of affected chickens as evidenced by a number of pathways (see Figures 4.2 and 4.3. Firstly, the complement system was predicted to be activated by IPA in affected chickens with Z-score=1.89, owing to upregulation of several genes in the complement system namely *complements C3, C7, C1R, C1S, C1QA, C1QB, C1QC* including *complement C3a receptor 1* (*C3AR1)* involved in this pathway. The complement system comprises plasma proteins as well as membrane-

bound regulators and receptors that interact with cells and mediators of both innate and adaptive immune system (Markiewski and Lambris, 2007). The activation of the complement system has not only been observed to be a prerequisite for an inflammatory reaction, but also as part of the earliest events of an inflammatory reaction (Markiewski and Lambris, 2007). Additionally, complement system activation, which is known to be activated by a variety of insults including aseptic injury, is widely linked with acute inflammation (Markiewski and Lambris, 2007). Therefore, the activation of the complement system in the current study suggests the existence of early inflammatory reaction in affected chickens.

Secondly, the present analysis revealed activation of acute phase response system (Z-score= 2.12) in pectoral muscles of affected chickens at week 3 of age. This is evidenced by the upregulation of acute phase proteins such as the chicken transferrin (*TF*) *ovotransferrin* (*ovo-TF*), *complement C3* (*C3*), *serpin family member 2* (*SERPINF2*) and *TNF receptor superfamily member 1A* (*TNFRSF1A*) in affected chickens. It is known that the levels of positive acute phase proteins frequently increase in response to inflammation (Murata et al., 2004). Therefore, like the complement system, the activation of acute phase response system in the present study is an indication of initiation of an inflammatory reaction at an earlier age. In support of this observation, the current study revealed upregulation of specific genes highly linked with inflammatory reaction in the pectoral muscles of birds at week 3. These genes include *interleukin2 receptor subunit gamma* (*IL2RG*) also known as *common gamma chain*, and *TNF receptor superfamily member 1A* (*TNFRSF1A*), both of which were upregulated in affected chickens at 3 weeks of age.

IL2RG gene encodes the cytokine receptor of interleukin-2 (IL-2) receptor subunit gamma (common gamma chain), which is an IL-2 receptor subunit common to several interleukin receptors such as IL-4, IL-7, IL-9, IL-15 and IL-21 (Suzuki et al., 2012). IL2RG plays a key role in the lymphoid development (Suzuki et al., 2012), and it is therefore an important component in inflammatory responses. *TNFRSF1A* gene, on the other hand, encodes the soluble or membrane-bound tumor necrosis receptor 1 (TNF-R1), which interacts with tumor necrosis factor (TNF) alpha, an important pro-inflammatory cytokine considered as one of the key mediators of inflammation (Zelová and Hošek, 2013). Hence, the upregulation of both *IL2RG* and *TNFRSF1A* genes in affected chickens evidenced in the current study supports existence of an early inflammatory reaction in WB. Additionally, the upregulation of other genes in affected chickens at week 3 associated with inflammatory reaction including *prostaglandin-endoperoxide synthase 2* (*PTGS2*), *colony stimulating factor-1* (*CSF-1*) and its receptor *CSF-1R* serve to augment this observation. Taken together, the transcriptomic assessment of the pectoral muscles at week 3 suggests the occurrence of early inflammatory response in the course Wooden Breast. Previous studies on Wooden Breast reported development of artery-sparing vasculitis in the early phase of disease process (Papah et al., 2017; Sihvo et al., 2017). Based on this observation, it is likely that the early inflammatory events demonstrated in the present study are directed towards the venous walls and perivascular lipids, causing the phlebitis phenotype reported previously. This observation is also in agreement with the previous study on the disease which demonstrated the initiation of acute inflammatory reaction beginning from week 3 of age as typified by focal infiltration of inflammatory cells into degenerated myofibers (Papah et al., 2017). From this time point (3 weeks of

age), it is postulated that the scope and intensity of inflammation (vasculitis and myositis) increases as the severity of the disease increases towards market age (Papah et al., 2017; Sihvo et al., 2017).

4.5.3.3 Dysregulation of Lipid Metabolism

The present study revealed increased expression of genes associated with lipid metabolism, as depicted by activation of clusters in fatty acid metabolism, lipid synthesis and storage in affected birds. Specifically, these clusters contained several genes associated with lipid metabolism that were upregulated in the pectoral muscles of affected chickens. They include *CD36*, also known as *fatty acid translocase*, a transmembrane protein primarily involved in the transportation of free fatty acid (FFA) into the cytoplasm (Abumrad et al., 1993); *fatty acid binding protein 4* (*FABP4*), also referred to as *AP2*, is an intracellular chaperone or an adipokine belonging to the family of intracellular fatty acid binding proteins (FABPs) (Zimmerman and Veerkamp, 2002). FABP4 is involved in binding and intracellular trafficking of hydrophobic molecules such as saturated and unsaturated fatty acids, retinoids, eicosanoids, prostaglandins and fat-soluble vitamins to specific compartments within the cell (Zimmerman and Veerkamp, 2002).

Another important gene involved in lipid metabolism which was upregulated in the pectoral muscles of affected individuals is e*xtracellular fatty binding acid protein* (*EX-FABP*) (Log2FC 1.9). *EX-FABP*, also referred to variously as *Ch21* or *p20K*, is a lipocalin involved in selective binding of long chain unsaturated fatty acids (Cancedda et al., 1996). Other lipid-related genes that were expressed in higher levels in affected chickens include *lipase G* (*LIPG*), *lipoprotein lipase* (*LPL*), *perilipin 1* (*PLIN1*), *thyroid hormone responsive* (*THRSP*) also known as *spot14* and *ATP-*

binding cassette, sub-family A, member 1 (*ABCA1*). Functional analysis of these lipid-metabolism-associated genes in the present study suggests elevated transport, uptake, translocation and consequently deposition and greater availability of lipids across various extracellular and intracellular domains in the P. major muscles during the disease process. This observation agrees with previous studies which demonstrated presence of lipid infiltration in various components of affected P. major muscle at microscopic level, and grossly as lipid-laden white striations on the muscle at market age (Papah et al., 2017; Sihvo et al., 2014, 2017). Similarly, the findings of the current study corroborate the metabolomics evaluations of Wooden Breast at market age, which reported elevated lipids in affected muscles resulting in lipid dysregulation (Abasht et al., 2016). These observations suggest that lipid metabolism dysregulation frequently associated with Wooden Breast starts early in life and may be linked with the pathogenesis of the disease in chickens.

4.5.3.4 Altered Carbohydrate Metabolism

This study showed evidence of increased amount of carbohydrates in the pectoral muscles of affected chickens following functional analysis of the DE genes at week 3. Similarly, upstream regulator analysis of the same gene-set demonstrated elevated levels of glucose and fructose in the pectoral muscles of affected chickens at week 3 post-hatch (see Appendix C). In spite of the likely higher levels of carbohydrates (including glucose and fructose) in the P. major muscles of the affected chickens, assessment for the utilization of the respective chemical compounds show evidence of potential alterations and/or shifts from bioenergetic pathways to other metabolic pathways, an observation that was first suggested by Abasht *et al*. (Abasht et al., 2016). Firstly, the gene expression of *citrate synthase* (*CS*) (Log2FC -0.56),

encoding for the enzyme citrate synthase, and *oxoglutarate dehydrogenase* (*OGDH*) (Log2FC -0.65), encoding for alpha-ketoglutarate dehydrogenase complex (KGDHC), both of which are essential enzymes in the mitochondrial tricarboxylic acid (TCA) cycle (Akram, 2014), were downregulated in the pectoral muscles of affected chickens. This suggests a potential reduction of utilization of glucose or fructose in the TCA cycle, and hence, reduced output in the bioenergetic capacity of the P. major muscle. This observation is line with the reduced oxidative phosphorylation pathway, a major canonical pathway detected by IPA as being inhibited in the affected group in the current study. Conversely, the gene *glutamine-fructose-6-phosphate transaminase 2* (*GFPT2*) (log2FC 0.89), which encodes for an enzyme that utilizes glucose/fructose was upregulated in muscles of affected chickens at week 3. *GFPT2* is the first and the rate-limiting step of the hexosamine biosynthesis pathway (HBP) (Yamazaki, 2014; Yang and Qian, 2017). *GFPT2* converts D-fructose-6-phosphate (Fru-6-P) and L-glutamine to L-glutamate and D-glucosamine-6- phosphate (GlcN-6-P), an important precursor of hexosamines such as uridine diphosphate-N-acetyl-D-glucosamine (UDP-GlcNAc) (Yamazaki, 2014). UDP-GlcNAc, on the other hand, is a substrate for multiple biological processes including biosynthesis of glycans for subsequent glycosylation events, as well as for protein O-GlcNAcylation, i.e. post-translational modification of proteins with O-linked β-N-acetylglucosamine (O-GlcNAc) on their serine and threonine residues (Bond and Hanover, 2015; Yang and Qian, 2017). Therefore, the upregulation of *GFPT2* indicates an increase in HBP flux in affected chickens, possibly for use in various processes such as biosynthesis of components of the extracellular matrix including proteoglycans, glycoproteins and glycosaminoglycans through glycosylation. Indeed, the gene *mannosyl* (*alpha-1,6*)-

glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase, isozyme B (*MGAT5B*) (Log2FC 0.9), which encodes the enzyme beta (1,6)-N-acetylglucosaminyltransferase involved in the biosynthetic pathway of N-glycans from UDP-GlcNAc (Stanley et al., 2017), was upregulated in the pectoral muscles of affected chickens. Consequently, the upregulation of *MGAT5B* gene suggests enhanced glycosylation events in affected muscle tissue. This finding is further supported by the upregulation of *galectin-1* (*LGALS1*) gene in affected chickens in the current study, whose protein galectin-1, is a glycan-binding protein (Brinchmann et al., 2018).

Glycosylation has been shown to modify the functions of proteins. For example, positive acute phase proteins such as complement C3 (McCarthy et al., 2014) and ovotransferrin (Xie et al., 2002), whose respective gene expression levels were upregulated in affected chickens in the current study, have been shown to undergo glycosylation thereby modulating their inflammatory responses (McCarthy et al., 2014). This observation, together with enhancement of receptor recognition functions of glycosylation (McCarthy et al., 2014), shows a possible link of glycosylation with early inflammatory response in the pathogenesis of WB in chickens.

Besides glycosylation, the presence of UDP-GlcNAc from HBP in the affected muscles also suggests potential increase of O-GlcNAcylation modification of P. major muscle proteins in the course of Wooden Breast disorder. O-GlcNAcylation modification, which is closely related to phosphorylation events, has been shown to influence the functional properties of proteins. For example, O-GlcNAcylation of diverse contractile and structural proteins of skeletal muscles in rodents, has been shown to modulate their physiology including calcium signaling pathway (Cieniewski-

Bernard et al., 2014). Accordingly, disruption of O-GlcNAc homeostasis has been linked with development of many diseases in humans including insulin resistance, diabetes, neurodegeneration and cancer (Cieniewski-Bernard et al., 2014; Gong et al., 2012; Yang and Qian, 2017). Hence, in the present study, it is likely that O-GlcNAcylation impacts the functional properties of P. major muscle potentially contributing to the pathogenesis of WB. However, the exact role of O-GlcNAcylation in P. major muscles in the pathogenesis of Wooden Breast warrants further investigation.

Taken together, HBP, which subsequently leads to glycosylation and/or O-GlcNAcylation processes in tissues, appears to be one of the important pathways driving the Wooden Breast disease process in chickens. HBP has been shown to be sensitive to changes in nutrient flux, metabolite availability and enzyme activities (Bond and Hanover, 2015). It is therefore plausible to hypothesize that the elevated carbohydrate levels demonstrated in the P. major muscles of the affected chickens, activates HBP. This observation agrees with Abasht *et al*. (2016) and Zambonelli *et al*. (2016) who indicated a potential shift of glucose metabolism from glycolysis to HBP in affected chickens at market age (Abasht et al., 2016; Zambonelli et al., 2016). This finding further suggests that this alteration starts earlier in the development of WB than initially suspected. HBP has also been found to be associated with increased endoplasmic reticulum stress, lipid accumulation and inflammation in the liver (Sage et al., 2010), features that are replicated in the pectoral muscles of affected chickens in current study. Besides HBP, other pathways that were implicated in extensive utilization of glucose in the P. major muscles of affected chickens at market age

include sorbitol biosynthesis, glucuronic acid and pentose-phosphate pathways (Abasht et al., 2016).

4.5.3.5 Remodeling of Extracellular Matrix

Extracellular matrix (ECM) plays a key role in provision of physical framework upon which critical molecular events that necessitate cellular interactions, differentiation, proliferation, migration, growth and survival take place (Hynes, 2009). Consequently, a wide range of physiological responses, and to some extent, pathological changes are coordinated by the ECM (Hynes, 2009). Remodeling of the ECM leading to fibrosis is arguably one of the most conspicuous features of WB, frequently characterizing the chronic phase of the disorder. Fibrosis in WB, primarily arising from elevated and persistent deposition of collagen fibers by fibroblasts within the P. major muscles, has been demonstrated both at microscopic (Clark and Velleman, 2017; Papah et al., 2017; Sihvo et al., 2014, 2017) as well as at gene expression (Mutryn et al., 2015; Velleman and Clark, 2015; Zambonelli et al., 2016) levels. The earliest histological detection of fibrosis at week 4 in this group of birds (Papah et al., 2017), and the current identification of the same process at week 3 through transcriptome analysis demonstrates the progression of fibrosis from molecular (at week 3) to cellular changes (at week4 and onwards). Indeed, the current study revealed upregulation of several genes directly involved in ECM remodeling such as *CTGF*, *PDGFRA*, *FN1* and *TNC* in WB-affected chickens.

Genes associated with proliferation and function of fibroblasts, such as *fibroblast activation protein alpha* (*FAP*), *fibroblast growth factor receptor-like 1* (*FGFRL1*) and *fibroblast growth factor binding protein 1* (*FGFBP1*) were upregulated in affected chickens. Additionally, genes related to collagen synthesis such as *collagen*

type XII alpha 1 chain (*COL12A1*), *collagen type VIII alpha 1 chain* (*COL8A1*), *collagen type VI alpha 3 chain* (*COL6A3*) and *collagen type XIV alpha 1 chain* (*COL14A1*) were upregulated in the pectoral muscles of affected chickens. These molecules may be thought to work in concert in mediation of changes of the ECM with respect to composition, architectural and mechanical disposition, as well as biochemical cues and signaling responses in the course of Wooden Breast disease process. This observation is exemplified following examination of the role of individual genes associated with ECM remodeling. In this case, *CTGF,* a profibrotic cytokine, has been demonstrated to possess both physiological functions as evidenced in tissue developmental processes as cartilage and bones, as well as in the pathogenesis of several biological disorders through enhancement of fibrosis (Kubota and Takigawa, 2015). Similarly, the upregulation of *CTGF* in the affected chickens is thought to be involved in the initiation of fibrosis frequently associated with WB. *PDGFRA*, a key molecule in the PDGFR signaling pathway, has also been shown to cause increased and aberrant deposition of extracellular matrix in fibrotic muscle diseases in humans (Uezumi et al., 2014). Accordingly, blocking of PDGFR signaling have been shown to reduce fibrogenesis in several fibrotic diseases in humans (Andrae et al., 2008). In the current study, the upregulation of *PDGFRA* in affected chickens suggests activation of the PDGFR signaling, subsequently enhancing deposition of ECM, which leads to fibrosis.

TNC has also been implicated in several fibrotic diseases, where it stimulates profibrotic responses in fibroblasts and maintains persistence of fibrosis in tissues (Bhattacharyya et al., 2016). Similarly, the upregulation of *TNC* in affected chickens as evidenced in the current study may be thought to be associated with the initiation

and maintenance of fibrosis in the course of WB in chickens. *FN1* gene, encodes for fibronectin, a major extracellular protein besides collagen, involved in several extracellular signaling pathways and remodeling of the ECM by induction of fibrosis (Hynes, 2009; To et al., 2011). Hence, the upregulation of *FN1* together with other related genes in the current study, demonstrates that the process of ECM remodeling during the development of WB in chickens begins early in life than previously thought.

4.5.3.6 Impaired Excitation-Contraction Coupling

Functional analysis of DE genes at week 3 showed evidence of alterations of excitation-contraction (EC) coupling in the pectoral muscles of affected chickens, as indicated by inhibition of skeletal muscle contractility (Z-score <-2). This observation is in line with the previous histological study, which showed elevated hypercontracted myofibers in affected chickens at the same age (Papah et al., 2017). Optimal calcium homeostasis and metabolism is critical in the maintenance of EC coupling in skeletal muscles (Calderón et al., 2014). It is, therefore, likely that impairments in EC coupling in this study could also be linked to dysregulation of calcium homeostasis and metabolism.

Previous studies in WB have demonstrated aberrations of calcium metabolism in affected chickens at market age (Mutryn et al., 2015; Zambonelli et al., 2016). Similarly, examination of the genes implicated in muscle contraction in the current study exhibit association with calcium metabolism. *Myotubularin-related protein 14* (*MTMR14*), also known as *muscle-specific inositol phosphatase* (*MIP*), which is downregulated in affected chickens, is a phosphoinositide phosphatase expressed primarily in striated muscles (Shen et al., 2009). *MTMR14* is involved in maintenance

of calcium homeostasis and regulation of excitation-contraction coupling in skeletal muscles. Further, deficiency of *MTMR* in mice has been shown to result in muscle disease through disruption of calcium metabolism (Shen et al., 2009) as well as metabolic dysregulation and inflammation (Lv et al., 2015). Therefore, the downregulation of *MTMR* in affected chickens in this study may be contributing to impairment of EC coupling through alteration of calcium signaling cascade. Like *MTMR14, Sarcalumenin* (*SRL*) is primarily expressed in striated muscle cells, where it functions as a calcium binding protein within the sarcoplasmic reticulum (SR), similar to calsequestrin. *SRL* has also been found to buffer calcium in the SR lumen as well as maintenance of calcium pump proteins (Yoshida et al., 2005). In mice, deficiency of *SRL* was found to alter EC coupling (Yoshida et al., 2005). Similarly, the downregulation of *SRL* in the affected muscles may be thought to cause alteration of calcium metabolism, hence, compromising EC coupling. *Synaptophysin-like 2*, (*SYPL2*), also known as *mitsugumin 29* (*MG29*), is a transmembrane protein expressed in the triad junction of skeletal muscles (Takeshima et al., 1998). *SYPL2* is also involved in EC coupling where its deficiency in mice was reported to result in interference of intracellular calcium homeostasis and muscle fatigability (Brutto et al., 2004). In the current study, the downregulation of *SYPL2* in the affected muscle is consistent with the disruption of EC coupling and alteration of calcium homeostasis in the pectoral muscles affected chickens. *Syncoilin* (*SYNC*), a gene that was downregulated in the pectoral muscles of affected chickens, is also expressed in the striated muscles (Poon et al., 2002). Found at the neuromuscular junction, sarcolemma and Z-lines, and as a member of dystrophin-associated protein complex, *SYNC* is involved in the maintenance of myofiber integrity during EC coupling (Poon et al.,

2002). Therefore, the downregulation of this gene in affected chickens suggests disruption of muscle integrity, which could play a role in myodegeneration observed in Wooden Breast.

4.5.4 Role of Upstream Regulators in the Development of WB

Upstream regulator analysis by IPA predicted activation of several upstream regulators involved in regulation of cell growth, ECM remodeling, induction of fibrosis, vascular injuries, inflammatory response and lipid metabolism. *Spi-1 proto-oncogene* (*SPI1*), also referred to as *hematopoietic transcription factor PU.1,* was predicted as one of the activated upstream regulators in affected chickens. *SPI1*, a transcriptional factor, is specifically expressed in cells of the hematopoietic lineage (McIvor et al., 2003). *SPI1* is primarily involved in the development of myeloid and lymphoid lineages (McIvor et al., 2003), and therefore considered to play crucial roles in the inflammatory and immune cell responses observed during the early phase of WBD in chickens. Indeed, this role is appreciated by the direct interaction of *SPI1* with genes such as *colony stimulating factor-1* (*CSF-1*) and its receptor *CSF-1R* (see Table 4.7), which are primarily involved in inflammatory/immune cell development and functions. It is, therefore, not surprising that *CSF-1* was predicted as one of the upstream regulators in the pectoral muscles at week 3. *CSF-1* and its receptor (*CSF-1R*), both of which were upregulated in the affected birds, are involved in the regulation of macrophage proliferation, differentiation, migration and survival (Stanley and Chitu, 2014). This phenomenon agrees with the observation of infiltration of inflammatory cells into various components of the pectoral muscles at week 3 as reported previously (Papah et al., 2017; Sihvo et al., 2017). *CSF-1* is also known to directly induce the expression of *EGR1*, which was upregulated in affected

birds in the current dataset. *EGR1* gene is involved in wide cellular processes such as vascular wound response, differentiation, proliferation and fibrosis. As a transcription factor, *EGR1* is involved in the induction of a number of genes observed in the current study. This include *CD44* (Fitzgerald and O'Neill, 1999), a multifunctional transmembrane glycoprotein involved in both physiological and pathological processes (Mylona et al., 2006). In the current study, the upregulation of *CD44* in the P. major muscles of affected chickens at week 3 may be linked to the initiation of myoregeneration (Mylona et al., 2006), which was first reported at the same age in affected chickens (Papah et al., 2017). In addition, by interacting with its major ligand hyaluronan, *CD44* possibly plays other roles in the affected chickens such as coordination of inflammatory responses (DeGrendele et al., 1997) and remodeling of ECM (Mylona et al., 2006).

Another key upstream regulator observed in the dataset was *mitogen-activated protein kinase kinase kinase 1* (*MAP3K1*), a member of the mitogen-activated protein kinase kinase kinase (*MAP3K*), which control MAPKK-MAPK signaling cascades (Suddason and Gallagher, 2015). Equipped with both a kinase domain and homeodomain motifs, and upregulated in affected chickens at week 3, *MAP3K1,* also known as *MEKK1,* is involved in regulation of a wide range of biological responses including wound healing, growth, cellular migration, immune cell differentiation and function, and vascular remodeling (Suddason and Gallagher, 2015). Following activation by external stimuli such as growth factors, cytokines, cellular stresses or ligands for heterotrimeric G protein-coupled receptors (GPCRs), *MAP3K1* interacts with any of its numerous binding partners such as Mapk1 or c-Jun to activate NF-kB or JNK signaling pathways, thereby transducing their downstream effects (Cargnello

and Roux, 2011; Suddason and Gallagher, 2015). Similarly, the upregulation of *MAP3K1* in P. major muscles of affected chickens may be linked to initiation of myoregeneration as reported earlier (Papah et al., 2017), as well as associated pathologies such as inflammatory responses on the vasculature and myofibers observed in the current study.

CCAAT/ enhancer-binding protein alpha (*CEBPA*), one of the key upstream regulators and also upregulated (log2FC 1.37) in affected chickens, encodes a protein belonging to the enhancer binding protein family. The proteins in this family are known to function as transcription factors in regulating several biological processes such as differentiation, metabolism, and immune cell differentiation and maturation (Ramji and Foka, 2002). Based on IPA analysis, C*EBPA* exhibited direct interactions with the largest number of target molecules in the gene list, making it an important upstream regulator for the transcriptome associated with development of Wooden Breast at week 3. *CEBPA* has been shown to play a role in inflammatory and immune responses (Radomska et al., 1998). Similarly, in the current analysis, IPA analysis revealed direct interaction of *CEBPA* with its target genes encoding complement *C3* and *complement C3a receptor 1* (*C3AR1*), as well as inflammatory-related genes namely, *PTGS2*, *TNFRSF1A*, *CSF1* and its receptor *CSF1R* (see Table 4.7). Since all the named target genes were upregulated in affected chickens, it is likely that *CEBPA,* as an upstream regulator, plays a mediation role in the initiation of early inflammatory response observed in affected chickens at week 3 of age. *CEBPA* is also known to play important roles in adipogenesis as well as mediation of lipid and glucose metabolism and energy homeostasis, as evidenced in studies involving humans (Olofsson et al., 2008) and genetically modified mice (Matsusue et al., 2004). In agreement with these

observations, the functional analysis of DE genes in present study showed direct interaction of *CEBPA*, as an upstream transcription regulator with its lipid-related target genes namely, *stearoyl-CoA desaturase* (*SCD*), *ATP citrate lyase* (*ACLY*), *perilipin 1* (*PLIN1*), *lipoprotein lipase* (*LPL*) and *FABP4* (see Table 4.7).

4.6 Conclusion

The findings in the current study provide significant insights into the molecular mechanisms driving the early pathogenesis of WB in broiler chickens. Major molecular changes observed to be associated with onset and early progression of WB include vascular changes, primarily phlebitis. Vascular perturbations in the pectoral muscles could be considered critical for initiation of pathology owing to their direct impact on drainage of metabolic waste including nitrogenous and acidic compounds and carbon dioxide from tissues. Other key molecular features observed in the affected muscles include early inflammatory responses as evidenced by activation of complement system and acute-phase response signaling possibly directed towards venous walls as well as in response to myodegeneration that sets in early in life. Metabolic dysregulation primarily involving utilization of carbohydrates and lipid metabolism, and remodeling of the extracellular matrix which lays ground for fibrosis, were other significant processes associated with the disease development. This study also showed association of Wooden Breast with changes in the muscle physiology as evidenced by impairment of excitation-contraction coupling potentially compounded with dysregulation of calcium metabolism. The findings in the current study show that major cellular and molecular perturbations in the face of Wooden Breast are already present by 3 weeks of age, before the disease is even clinically evident by palpation.

Therefore, mitigation strategies of the myopathy should be targeted to a time frame at or before 3 weeks post-hatch in the future.

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Chapter 5

DYSREGULATION OF LIPID METABOLISM AND APPEARANCE OF SLOW MYOFIBER-SPECIFIC ISOFORMS ACCOMPANY THE DEVELOPMENT OF WOODEN BREAST MYOPATHY IN MODERN BROILER CHICKENS

(Michael B. Papah and Behnam Abasht: Submitted to *Scientific reports* journal)

5.1 Abstract

Previous transcriptomic studies have hypothesized the occurrence of slow myofiber-phenotype, and dysregulation of lipid metabolism as being associated with the development of Wooden Breast (WB), a meat quality defect in commercial broiler chickens. To gain a deep understanding of the manifestation and implication of these two biological processes in health and disease states in chickens, cellular and global expression of specific genes related to the respective processes were examined in pectoralis major muscles of modern fast-growing and unselected slow-growing chicken lines. Using RNA *in situ* hybridization, *lipoprotein lipase (LPL*) was found to be expressed in endothelial cells of capillaries and small-caliber veins in chickens. RNA-seq analysis revealed upregulation of lipid-related genes in WB-affected chickens at week 3 and downregulation at week 7 of age. Cellular localization of slow myofiber-type genes revealed their increased expression in mature myofibers of WB-affected chickens. Similarly, global expression of slow myofiber-type genes showed upregulation in affected chickens at both time points. To our knowledge, this is the first study to show the expression of *LPL* from the vasculature endothelium in chickens. This study also confirms the existence of slow myofiber-phenotype and

provides mechanistic insights into increased lipid uptake and metabolism in WB disease process.

5.2 Introduction

Wooden Breast (WB) a muscle quality disorder that imparts a firm feel on the pectoral (P.) major muscles of commercial broiler chickens upon palpation, continues to cause significant losses in the poultry industry unabated. Consequently, a number of studies on WB have been conducted to decipher its pathology and pathogenesis (Papah et al., 2017; Sihvo et al., 2014, 2017; Velleman et al., 2018) as well as molecular dynamics (Abasht et al., 2016; Mutryn et al., 2015; Papah et al., 2018; Zambonelli et al., 2016) characterizing the disease process in commercial broiler chickens. Histopathological studies of the early phases of the disease have revealed onset of phlebitis targeting smaller-caliber blood vessels and perivascular lipid infiltrations that increase in scope and intensity with disease process over time (Papah et al., 2017; Sihvo et al., 2017). Similarly, gene expression and biological functional analyses of WB disorder have revealed perturbations in lipid metabolism, remodeling of extracellular matrix and dysregulation of excitation-contraction coupling distinguishing the early stages of the WB myopathy (Papah et al., 2018). On the other hand, molecular analysis of WB in the later stages, have showed evidence of myoregeneration and occurrence of fast-to-slow muscle switch intermixed with enhanced fibrosis (Mutryn et al., 2015). Additionally, disturbances in lipid metabolism complexed with oxidative stress as well as compromised carbohydrate metabolism have been observed following metabolomic studies of WB in affected chickens at market age (Abasht et al., 2016).

Based on these previous studies, there appears to be an association of WB disease process with dysregulation of metabolism, especially relating to lipid metabolism, as well as the appearance of slow myofiber phenotype (type I or oxidative myofibers) in the P. major muscles in chickens. It is generally accepted that P. major muscles of chickens are comprised almost entirely of fast-twitch (type IIB) myofibers (Barnard et al., 1982; Ono et al., 1993; Verdiglione and Cassandro, 2013). With fewer mitochondria, capillaries and myoglobin content compared to type I fibers, fast-twitch myofibers rely heavily on glycolysis to meet its metabolic energy demands (Schiaffino and Reggiani, 2011). It follows, therefore, that P. major muscles in chickens would generally have limited capacity to accomplish oxidative phosphorylation as well as utilization of ß-oxidation of fatty acids for its bioenergetics. In contrast, slow-twitch (type-1 or oxidative) myofibers express slow sarcomeric protein isoforms, have higher mitochondrial content, myoglobin, capillaries as well as lipids, and primarily utilize oxidative phosphorylation for its metabolic functions (Bassel-Duby and Olson, 2006; Schiaffino and Reggiani, 2011). Based on these observations therefore, the occurrence of lipid dysregulation and emergence of slow-type myofiber genes in the P. major muscles of WB-affected chickens raises pertinent biological questions. 1. What is the cellular localization of some of the genes associated with lipids and slow myofiber-phenotype within the P. major muscle of chickens? 2. Why does the P. major muscle, a predominantly glycolytic muscle tissue exhibit increased expression of lipid metabolism as well as slow-type muscle genes during the development of WB myopathy? 3. What are the possible implications of lipids and slow myofiber proteins in the general muscle metabolism with respect to energy source and utilization?

To answer these questions, we evaluated the cellular localization, as well as global expression of specific genes highly related to lipid metabolism and myofiber-type switching during the early and late phases of WB disorder in chickens. This was achieved by utilizing RNA *in situ* hybridization technique to localize the expression of specific genes on the P. major muscles of slow-growing Legacy chickens (a line not known to develop WB disease), and WB-affected and unaffected Ross birds. We also used RNA-seq expression data from two commercial broiler chicken lines; one at 3 weeks of age (early phase of WB) (Papah et al., 2018) and the other at 7 weeks of age (late phase of WB) (Mutryn et al., 2015). From the RNA-seq datasets, we focused on genes related to lipid metabolism and slow-skeletal muscle phenotype. Results from the current study have brought to the forefront new insights into the cellular expression of *lipoprotein lipase* (*LPL*) in chickens that was not known before. Additionally, pertinent knowledge showing the relationship between changes in lipid metabolism and muscle-type transition in the P. major muscles with the development of WB in commercial broiler chickens have been revealed in this study.

5.3 Materials and Methods

5.3.1 Experimental Birds and Processing of Muscle Samples

The experimental animals (chickens), and experiments used in this study were approved by the University of Delaware's Animal and use committee under protocol number 72R-2017-0. Further, birds were raised in strict adherence to the guidelines provided by the Agricultural animal care and use in research and teaching handbook of the College of Agriculture and Natural Resources, University of Delaware.

The chicken lines used in this study were from two sources namely Ross 708 and Legacy birds also referred to as Heritage line (from the University of Illinois). The Legacy chickens were used in this experiment as a baseline group for RNA *in situ* hybridization protocol on the selected genes. Legacy chickens have not been subjected to genetic selection for fast-growth rate and high muscle yield since 1950s, in contrast to Ross birds which are modern commercial broiler chickens genetically selected for fast-growth rate, high feed efficiency and breast muscle yield (Schmidt et al., 2009). Consequently, the Legacy lineage of chickens do not develop WB myopathy and have no history of the myopathy so far. Commercial broiler chicken lines such as Ross birds, on the other hand, are known to be susceptible to WB, and have been reported to develop the myopathy (Sihvo et al., 2017).

Eggs from the respective chicken lines were incubated and hatched 2 days apart at the University of Delaware; the eggs of Ross birds were the first to hatch. The difference in day of hatching was occasioned by a 2-day delay in acquisition of eggs of Legacy birds. All the chickens were raised in the same pen within the chicken houses at the University of Delaware from June to July 2018. The birds were allowed free access to water and feed until they were euthanized. Throughout the experimental period, the birds were provided with standard commercially formulated feed for broiler chickens. This study utilized chickens at day18 post-hatch for Legacy line (n=7) and at day 20 post-hatch for Ross birds (n=8). The chickens were euthanized by cervical dislocation and pectoral major muscle samples harvested from the cranial aspect of the right pectoral region and immediately fixed by immersion into 10% neutral buffered formalin until further processing for microscopic analysis.

To identify suitable samples for RNA *in situ* hybridization, all harvested samples were first processed routinely for histological examination with Hematoxylin & Eosin (H/E) as outlined in a previous study (Papah et al., 2017). Briefly, muscle tissue samples were trimmed, paraffin-embedded into blocks, sectioned 4-5μm thickness and stained with H/E at the University of Delaware comparative pathology laboratory (Newark, DE). Both transverse and longitudinal sections of the P. major muscles were prepared and subsequently examined using an Olympus BX40 light microscope. Microscopic analysis of the muscle samples entailed examination for presence of tissue pathology associated with WB. For tissues with WB pathology, the tissue slides were scored to indicate the extent of tissue damage using the parameters applied in a previous study (Papah et al., 2017). Therefore, a subset of samples comprising unaffected Ross birds (n=3), moderately affected chickens (n=3) and Legacy chickens (n=3) were identified and used for RNA i*n situ* hybridization. Since all Legacy chickens were scored as unaffected, 3 samples were selected randomly from the rest to match those of the Ross groups. The respective paraffin embedded tissue blocks of the selected samples were used for RNA *in situ* hybridization.

5.3.2 RNA *In Situ* Hybridization Protocol

RNA *in situ* hybridization was performed using the RNAscope 2.5 assay for formalin-fixed paraffin-embedded (FFPE) protocols (Advanced Cell Diagnostics (ACD) Inc., Gateway, CA) according to manufacturer's instructions. Firstly, the samples were subjected to pretreatment protocol based on document number 322452 USM (ACD), while hybridization, amplification and target detection protocols used were based on document 322500-USM for RNAscope 2.5 HD Duplex detection kit (ACD). Briefly, 4-5 μm formalin-fixed and paraffin-embedded pectoral muscle tissue

sections were obtained from the selected paraffin embedded tissue blocks, pretreated with heat at 60 ºC for 1 hour, cleared in xylene and dehydrated in 100% EtOH followed by target retrieval for 30 min and then protease treatment for 30 minutes prior to hybridization.

This protocol targeted four genes in the pectoral muscles whose probes were prior developed by ACD. The probes for the target genes included *lipoprotein lipase* (*LPL*), Entrez ID 396219 targeting region 199-1306 of NM_205282.1; *perilipin 1* (*PLIN1*) Entrez ID 415487, targeting region 174-1143 of NM_001199486.1; *myosin binding protein C1* (*MYBPC1*) Entrez ID 418099, targeting region 118-1147 of XM_025155759.1 and *cysteine and glycine rich protein 3* (*CSRP3*) Entrez ID 174-1143 of NM_001199486.1. To allow for examination of expression of two related genes concurrently, the four probes were hybridized in pairs. Hence, the two pairs of genes examined included lipid-related genes (*LPL* and *PLIN1*) and muscle-related genes (*MYBPC1* and *CSRP3*).

Running parallel to target probes were positive and negative control probes. The positive control probes used were Gg-*UBC*-C2, detected by the red signal, and Gg-*PPIB*, detected by the green signal all ran in duplex. RNAscope 2-plex negative control probe was used as the negative control. To examine all target and control probes in this study, 4 tissue sections were obtained from each of the selected samples followed by processing for lipid-related genes, muscle-related genes, positive control probes and negative control probes. Hence, all the probes were hybridized to all selected sample sections for 2 hours at 40°C in a HybEZ oven (ACD), washed with 1X wash buffer and stored in 5X SSC solution overnight until the following day. On the second day, all the samples were subjected to amplification steps as well as signal

detection as per the protocol. For signal detection, the red signal targeted the mRNAs of *PLIN1* and *CSRP3* genes while the green signal targeted the mRNAs of *LPL* and *MYBPC1* genes. The tissue slides were then counter-stained with Gill's Hematoxylin II stain and subsequently examined using an Olympus BX40 light microscope and relevant photomicrographs collected using a Nikon DS-Fi2 camera and NIS Elements D software.

5.3.3 Gene Expression Data for Muscle-Related and Lipid-Related Genes

Besides RNA *in situ* hybridization, this study also used RNA-seq data of specific genes related to lipid metabolism and slow-myofiber phenotype frequently associated with WB myopathy. The genes were identified and selected from RNA-seq data in our laboratory. The RNA-seq data were derived from the P. major muscles belonging to 2 distinct high-breast-muscle-yield, purebred chicken broiler lines at 3 weeks of age (Papah et al., 2018) and another one at 7 weeks of age (Mutryn et al., 2015).

The protocol used in processing of the P. major muscle samples for RNA-seq were previously documented for chickens at 3 weeks of age (Papah et al., 2018), and 7 weeks of age (Mutryn et al., 2015). Of note is that the two RNA-seq datasets (week 3 and week 7) were sequenced using Illumina sequencing platform for short reads (Mutryn et al., 2015; Papah et al., 2018). To use the newer version of the software packages and the most recent chicken reference genome (Ensemble Gallgall 6.0 and gene annotation file release 95), raw RNA-sequencing reads for both week 3 and week 7 muscle samples were reanalyzed using the following steps: 1) sequence reads were quality-checked and quality-trimmed using FastQC v0.11.7 and trimmomatic v0.38, respectively; 2) the trimmed sequence reads were mapped to the chicken reference

genome using Hisat2 v2.1.0; 3) SAM files were converted to BAM files using samtools v1.9; 4) geometric normalization of library sizes and identification of differentially expressed genes (FDR-adjusted p-value < 0.05) were determined using Cuffdiff v2.2.1, a software tool within Cufflinks package (Trapnell et al., 2012, 2013).

The Cuffdiff software estimates gene expression abundance from aligned reads in two or more samples and tests the statistical significance of each observed change in expression between the conditions, by application of a beta negative binomial distribution model at gene- and transcript-level resolution (Trapnell et al., 2012). In the present study, the two conditions were unaffected and WB-affected chicken samples with several biological replicates in each case. The Cuffdiff software also uses statistical modeling to account for variability in gene and transcript measurements across biological replicates (Trapnell et al., 2012, 2013). We used the default pooled dispersion method to model for cross-replicate dispersion estimation in Cuffdiff. The Cuffdiff software identifies a differentially expressed gene or transcript by testing the observed log fold change in its expression against the null hypothesis of no change and assesses the significance of each comparison at (FDR-adjusted p-value < 0.05) (Trapnell et al., 2013). While the Cuffdiff software produces several output result-files, the focus of the present study was on the gene-level differential expression file, which is generated by examining the test differences in the summed FPKM values of transcripts sharing each gene identity between unaffected and affected conditions.

In addition, new adjustments incorporated during the processing of sample data at week 7 of age included use of 5 unaffected and 5 affected samples instead of 6 unaffected and 5 affected samples used in the previous study (Mutryn et al., 2015). This was because one unaffected sample (sample 51 unaffected) displayed molecular

characteristic consistent to those of affected muscle samples and was believed to be misclassified, as suggested by the authors (Mutryn et al., 2015), hence was excluded from the current analysis.

From the RNA-seq data of the two chicken lines, belonging to week 3 and week 7, the expression profile involving Log2 fold-change (Log2FC) of the selected genes between WB-affected and unaffected birds at each of the 2-time points was extracted. A time comparison of the lipid-related genes and muscle-related genes between week 3 and week 7 of age for each gene set was made. The lipid-related genes examined included *ATP-binding cassette sub-family A1* (*ABC1*), *angiopoietin-like factor 4* (*ANGPTL4*), *fatty acid translocase* (*CD36*), *fatty acid binding protein 4* (*FABP4*), *lipase G* (*LIPG*), *LPL*, *PLIN1*, *phospholipid transfer protein* (*PLTP*), *retinol binding protein 7* (*RBP7*) and *thyroid hormone responsive* (*THRSP*). Muscle-related genes examined included *ankyrin repeat domain 2* (*ANKRD2*), (*CSRP3*), *leiomodin 2* (*LMOD2*), (*MYBPC1*), *myomesin 3* (*MYOM3*), *myozenin 2* (*MYOZ2*), and *troponin I1, slow skeletal type* (*TNNI1*).

5.4 Results

5.4.1 RNA *In Situ* Hybridization of Lipid-Related Genes

To localize the expression of *LPL* in the P. major muscles in affected and unaffected chickens, we utilized RNA *in situ* hybridization technique. In our study, mRNA signal of *LPL* appeared to be localized in the endothelial layer of capillaries and small-caliber vasculature within the P. major muscles of both affected and unaffected chickens. In unaffected chickens, *LPL* mRNA signal was observed in the endothelium of capillaries and venules running between contiguous myofibers in both

Legacy (Figure 5.1a & b) and Ross birds (Figure 5.1c & d). *LPL* was expressed intermittently along the length of these blood vessels.

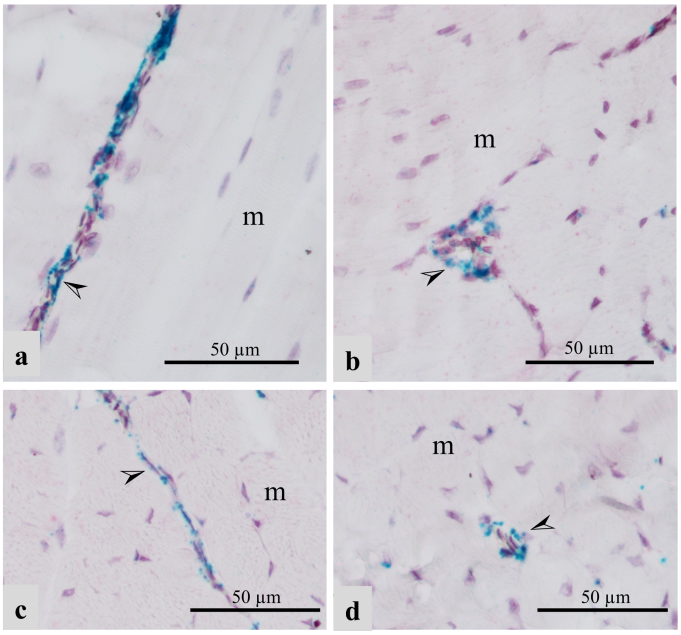

Figure 5.1: Expression of *LPL* mRNA in the vascular endothelium within the pectoralis major muscles of healthy chickens.

The *LPL* mRNA (green signal) is indicated by (arrowhead) in Legacy (**a** and **b**) and Ross (**c** and **d**) chicken lines. Blood vessels shown are (**a**: capillaries; **b**: venule) in Legacy chicken line, and (**c**: capillary; **d**: venule) in Ross chicken line. m; myofibers.

Similar presentation was also observed in the endothelium lining other small-caliber veins in Legacy chickens (Figure 5.2a**)** and unaffected Ross chickens (Figure 5.2b). Arteries in unaffected Legacy chickens (Figure 5.2a**)** and unaffected Ross chickens (Figure 5.2b) did not exhibit *LPL* mRNA signal. Conversely, affected chickens showed increased signal of *LPL* mRNA in the endothelial lining of small-

caliber veins compared to small-caliber arteries which exhibited lower *LPL* mRNA signal in their endothelial linings (Figure 5.2c & d).

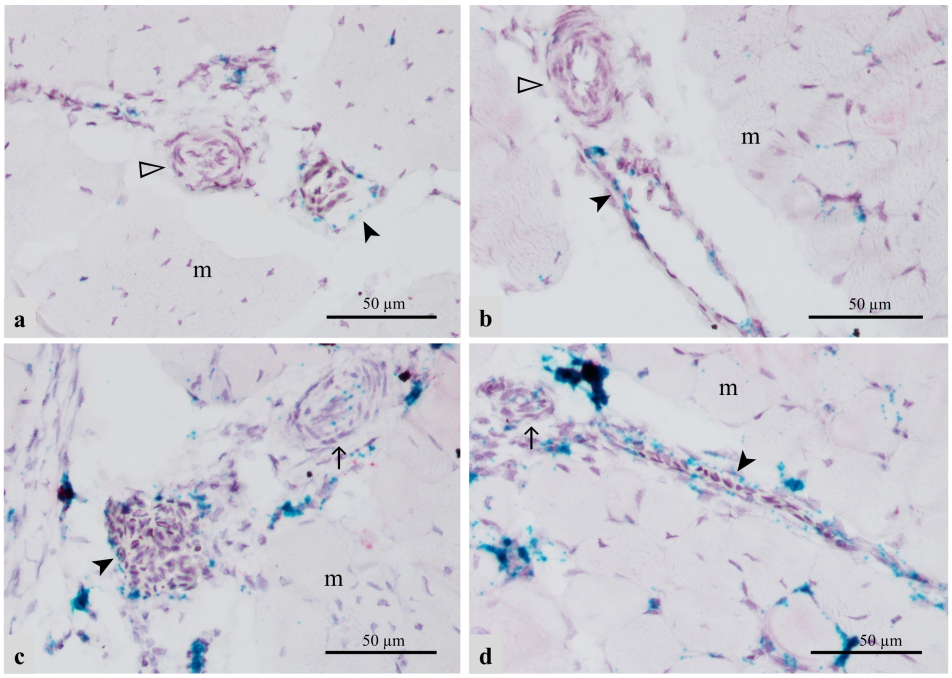

Figure 5.2: Expression of *LPL* mRNA in the endothelium of veins in chickens.

The *LPL* mRNA (green signal) in veins (closed arrowhead) in Legacy chicken (**a**) and Ross unaffected chicken (**b**), while arteries (open arrowhead) in respective chicken lines do not show LPL mRNA signal. Affected Ross chickens (**c** and **d**) with enhanced *LPL* mRNA signal in veins (closed arrowhead), while arteries show subtle *LPL* mRNA signal (arrow). Expression of *PLIN1* mRNA signal (red signal) in extracellular matrix possibly in developing adipocytes (**c** and **d**) can be seen. m; myofibers.

The expression of *LPL* in large arteries in affected chickens was almost nondetectable (Figure 5.3a). In addition, the *LPL* mRNA signal in affected chickens was enhanced in the endothelium of veins undergoing phlebitis, often characterized by intramural infiltrates as well as perivascular cuffing comprising primarily lymphocytic

cells and to a lesser extent, macrophages (Figure 5.3b). The macrophages found in affected tissues also expressed some *LPL* as evidenced by the *LPL* mRNA signal in the said cells (Figure 5.3b). Further, there appeared to be increased *LPL* mRNA signal in developing adipose tissue found in the extracellular matrix (ECM) between muscle bundles of affected chickens, (Figure 5.3a & c), capillaries between myofibers, as well as in some myofibers of affected birds (Figure 5.3c & d).

Localization of expression of *PLIN1* in the P. major muscles in affected and unaffected chickens was conducted using *in situ* RNA hybridization. The muscles in unaffected chickens (Legacy and Ross) did not show *PLIN1* expression. On the contrary, in the muscles of affected chickens, the signal of *PLIN1* mRNA was largely observed within the ECM and especially in developing adipose tissue that infiltrated muscle fascicles as well as in proximity to the vasculature (Figure 5.2c & d) and (Figure 5.3a, b & d). It should be noted that in all the tissues, the expression of *PLIN1* was frequently accompanied by the expression of *LPL*. Besides adipocytes which expressed *PLIN1*, it was not possible to resolve the identity of other cells expressing *PLIN1* gene.

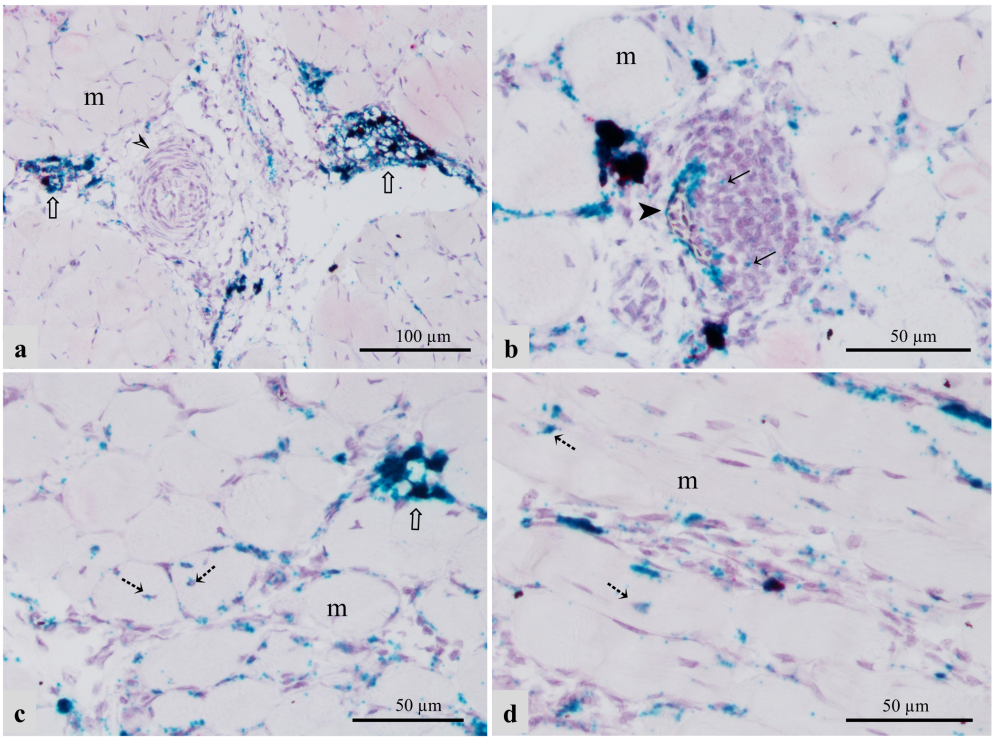

Figure 5.3: Expression of *LPL* mRNA (green signal) in the P. major muscle of affected chickens.

LPL mRNA signal is enhanced in several sites including developing adipocytes (⇧) in **a** and **c**; vein undergoing inflammation (closed arrowhead); capillaries (between myofibers), and some myofibers (dotted arrow) in **c** and **d**. A few macrophages also expressed *LPL* (solid arrow) in **b**. *PLIN1* mRNA signal (red signal) in developing adipocytes in **a** (open arrow), and generally localized in the extracellular matrix in **b** and **d**. Notice that a large artery in **a** (open arrowhead) in affected chicken does not express *LPL*. m; myofibers.

5.4.2 RNA-seq Gene Expression of Lipid-Related Genes

Gene expression data of DE lipid-related genes based on log2 fold-change between affected and unaffected chickens from week 3 of age was compared with those of chickens at week 7 of age. All the lipid-related genes except *ANGPTL4*, were

significant and upregulated in affected chickens compared to unaffected group at week 3 of age (Figure 5.4). At week 7 of age, 3 genes namely *ABCA1*, *ANGPTL4* and *PLTP* were significantly upregulated while *CD36*, *PLIN1*, and *THRSP* were downregulated (Figure 5.4). Other genes namely, *FABP4*, *LIPG*, *LPL* and *RBP7* were not differentially expressed between affected and unaffected chickens at week 7 of age (Figure 5.4).

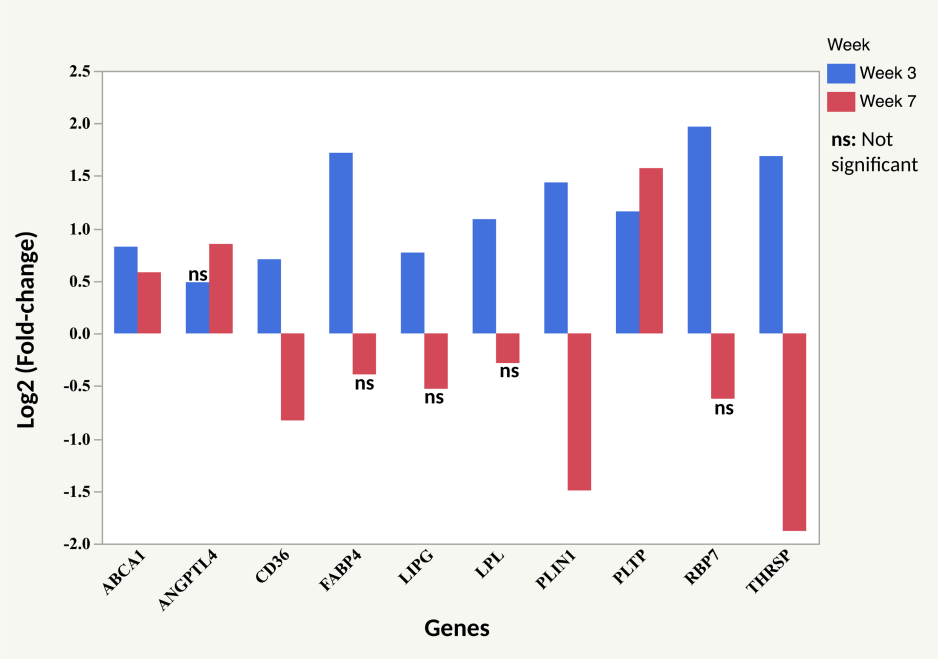

Figure 5.4: Log2 Fold-change of differentially expressed genes in WB associated with lipid metabolism between affected and unaffected broiler chickens at week 3 and week 7 of age.

Positive log2 Fold-change indicate upregulation in affected chickens while negative log2 Fold-change indicate down regulation in affected chickens. Notice that the majority of the genes are upregulated at week 3 and downregulated at week 7 of age. However, the expression of some genes were not statistically different between affected and unaffected chickens and are denoted as **ns** (not significant).

5.4.3 RNA *In Situ* Expression of Muscle-Related Genes

RNA *in situ* hybridization of *MYBPC1* in the P. major muscles of chickens in the present study demonstrated a stage wise expression pattern with respect to WB disease status. In Legacy chickens which are not affected by WB, the signal of *MYBPC1* mRNA exhibited intermittent focal distribution along the length of individual myofibers. The areas along the length of myofibers upon which *MYBPC1* was expressed appeared to vary widely with no predictable pattern. Similarly, the intensity of the *MYBPC1* mRNA signal varied from one region to another along myofibers (Figure 5.5a). An almost similar presentation of *MYBPC1* mRNA signal was seen in the P. major muscles of unaffected Ross chickens (Figure 5.5b). In affected Ross chickens, *MYBPC1* mRNA signal was greatly enhanced and was distributed homogenously within the myofibers, covering longer stretches of myofiber lengths (Figure 5.5c & d).

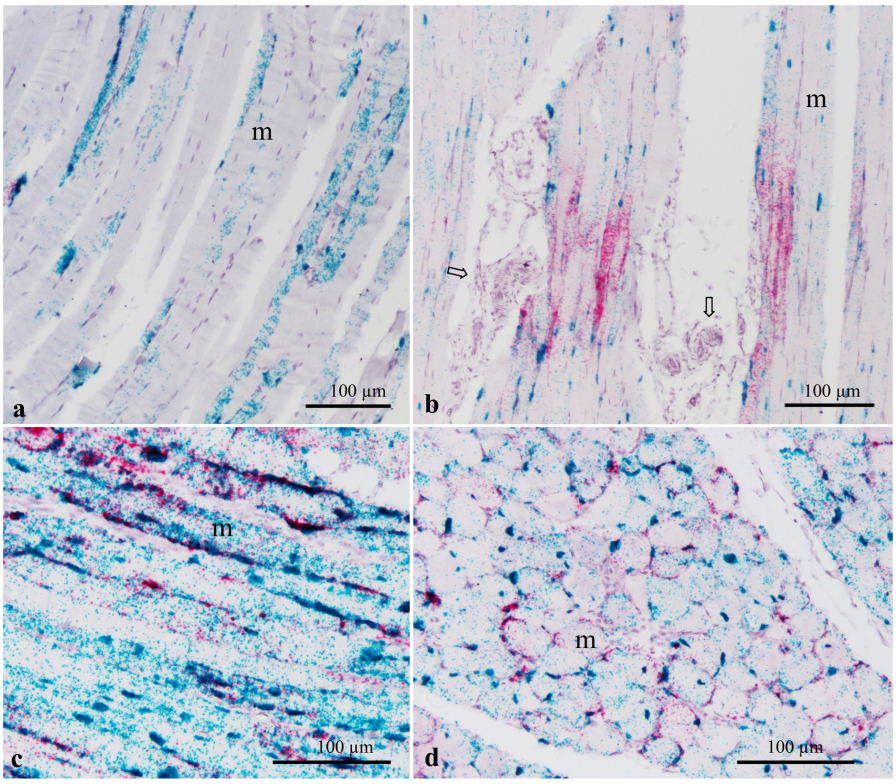

Figure 5.5: RNA *in situ* expression of *MYBPC1* and *CSRP3* genes in the P. major muscles of unaffected and WB-affected chickens.

Expression of *MYBPCI* (green signal) and *CSRP3* (red signal) mRNA in the in the P. major muscle of Legacy (**a**), Ross unaffected (**b**) and Ross WB-affected chickens (**c** and **d**). Notice the focal to multifocal expression of *MYBPC1* in Legacy and Ross unaffected, and increased *MYBPC1* mRNA signal assuming a homogenous distribution in myofibers of Ross affected chickens. *CSRP3* expression in Legacy chickens is largely absent, the expression in Ross unaffected chickens occur frequently in myofiber portions that are contiguous to tissues in the extracellular matrix as vasculature (open arrow), while in affected chickens, *CSRP3* mRNA signal is increased and largely distributed towards the sarcolemma of myofibers (**c** and **d**). Notice that *CSRP3* is consistently co-expressed with *MYBPC*1, but not the other way. m; myofibers.

Localization of *CSRP3* gene was examined using RNA *in situ* hybridization in Legacy, Ross unaffected and Ross affected chickens. The mRNA signal of *CSRP3* was almost non-existent in the P. major muscles of Legacy chickens (Figure 5.5a). On the other hand, *CSRP3* mRNA signal was observed in unaffected Ross chickens in a focal manner and in very limited portions of individual myofibers. In particular, the *CSRP3* mRNA signal in unaffected Ross chickens was present in portions of myofibers that were adjacent to the vasculature and/or other structures localized within the extracellular matrix (Figure 5.5b). In some instances, the signal was stronger in areas where myofibers appeared to be impinged by contiguous vascular tissues (Figure 5.5b). In these regions, the expression of *MYBPC1* was not altered. In affected chickens, *CSRP3* mRNA signal was enhanced assuming a multifocal distribution among fibers (Figure 5.5c). Additionally, the *CSRP3* mRNA signal in myofibers of affected samples appeared to be highly concentrated along the periphery towards the sarcolemma. In such cases, *CSRP3* was always co-expressed with *MYBPC1* in myofibers (Figure 5.5c & d).

5.4.4 RNA-seq Gene Expression of Muscle-Related Genes

Examination of differential expression of all 7 muscle-related genes between WB-affected and unaffected chickens revealed upregulation in the P. major muscle of affected chickens at week 3 and week 7 of age (Figure 5.6). However, while all the 7 genes were significantly expressed higher in affected chickens at week 7, only 3 genes were significant at week 3, namely *CSRP3*, *MYBPC1* and *LMOD2* (Figure 5.6). Further, *CSRP3* had the highest fold-change at both week 3 (log2FC 2) and week 7 of age (log2FC 6.2) (Figure 5.6).

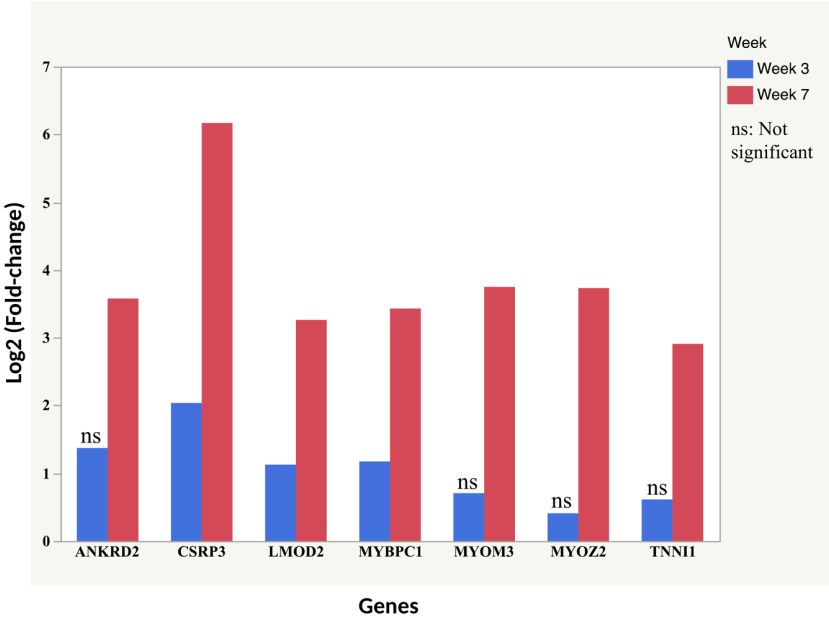

Figure 5.6: Log2 Fold-change of differentially expressed genes in WB associated with slow myofiber type transition between affected and unaffected broiler chickens at week 3 and week 7 of age.

Positive log2 Fold-change indicate upregulation in affected chickens while negative log2 Fold-change indicate down regulation in affected chickens. Notice that while all genes are upregulated in affected chickens at week 3 and week 7 of age, the genes in the later age group have appreciably higher fold change. However, the expression of some genes between affected and unaffected group were not statistically different and are denoted as **ns** (not significant).

5.5 Discussion

The present study aimed at demonstrating the evidence as well as mechanistic relationship of lipid dysregulation and occurrence of slow myofiber-phenotype in a typically glycolytic P. major muscle during the development of WB in modern broiler chickens. By using RNA *in situ* hybridization and RNA-seq, it was possible to examine the expression of specific genes as they relate to WB disease process in the

early phase (week 3 of age) and late phase (week 7 of age). Some of the genes related to lipid metabolism and myofiber remodeling, and their respective implications in muscle metabolism are discussed.

LPL is the rate-limiting enzyme involved in the hydrolysis of circulating triglycerides (TGs) in chylomicrons, or portomicrons in chickens (Fraser et al., 1986), and in very low-density lipoproteins (VLDL) resulting in release of free fatty acids, monoglycerides and other chylomicron/portomicron remnants for use by subjacent tissue or for storage (He et al., 2018). While *LPL* is known to be expressed by parenchymal cells of a number of tissues including both white and brown adipose tissue, skeletal and cardiac muscles, brain, macrophages as well as some smooth muscles of large blood vessels in mammals (He et al., 2018; Preiss-Landl et al., 2002), knowledge about its cellular expression in chickens remains scarce. In the present study, we have demonstrated for the first time, that *LPL* is expressed by the vascular endothelium, especially in capillaries and small-caliber veins within the P. major muscles of chicken in both health (Legacy chickens and unaffected Ross birds) and disease (WB-affected Ross chickens) states.

The low *LPL* mRNA signal in the arterial end accompanying a higher one in the capillary and venous endothelia in affected chickens may explain the occurrence of the artery-sparing lymphocytic phlebitis lesion in WB (Papah et al., 2017; Sihvo et al., 2017). We speculate that the enhanced *LPL* expression in veins compared to arteries indicates elevated lipoprotein hydrolysis activity in the venous end of affected chickens. This in turn causes increased permeability of the venous endothelium not only to free fatty acids (FFA) and lipoprotein remnants such as LDL following hydrolysis of VLDL (Curtiss et al., 2012) and possibly portomicrons (Sato et al.,

2009) in the blood stream, but also to inflammatory cells including monocytes, lymphocytes and heterophils. The escaping immune cells contribute to the development of the lymphocytic phlebitis lesion. Indeed, this observation is in line with a previous study which showed a role of LPL in development of vascular diseases such as in atherogenesis (Goldberg, 1996).

Evaluation of expression of lipid-related genes based on RNA-seq profile suggests an increase of lipid metabolism in affected chickens at week 3 age. This is demonstrated by the upregulated genes whose functions include the following processes: uptake of lipids from circulating lipoprotein facilitated by *LPL* (He et al., 2018), and to some extent, *LIPG* (Jaye et al., 1999; Kratky et al., 2005); transportation of FFA across the plasma membrane into the cytoplasm of cells by *CD36* for β-oxidation or re-esterification into triglycerides (Nickerson et al., 2009); intracellular binding and shuttling of lipids and other hydrophobic molecules across different organelles within cells by *FABP4* (Zimmerman and Veerkamp, 2002) and *RBP7* (Ross, 2016), and storage of lipids by *PLNI1* (Hansen et al., 2017) and *THRSP* (Schering et al., 2017). Other lipid-related biological functions that are likely occurring in affected chickens include elevated cholesterol metabolism as evidenced by the upregulation of *ABCA1* which is involved in reverse cholesterol transport (Oram and Lawn, 2001), together with *PLTP* (Wolfbauer et al., 1999) and *LIPG*, which encodes endothelial lipase (Jaye et al., 1999). It should be noted that both *ABCA1* and *PLTP* were significantly upregulated in affected chickens at week 3 and week 7 of age, indicating that active cholesterol biosynthesis and transport accompanies all stages of WB disorder. The current presentation agrees with recent

studies in our laboratory that suggested increased cholesterol biosynthesis in the P. major muscles of feed-efficient chickens, which also show high susceptibility to WB (Abasht et al., 2019; Zhou et al., 2015).

The RNA-seq expression profile of lipid-related genes in affected chickens at week 7 reveals statistically non-significant (*FABP4, LIPG, LPL* and *RBP7*) and significant downregulation (*CD36, PLNI1* and *THRSP*) downregulation, suggesting dysregulation of lipid metabolism between week 3 and week 7. While direct biological changes leading to this dysregulation are not apparent in the present study, other indirect causes such as hypoxia, which has been reported previously in WB-affected chickens (Mutryn et al., 2015; Sihvo et al., 2018), could be playing a significant role in the aforementioned metabolic disruption. It has been widely established that hypoxia increases the expression of *ANGPTL4*, a potent inhibitor of LPL activity (Drager et al., 2013; Yao et al., 2013). Another study showed that acute hypoxia decreases transcription of *PPARγ*, *LPL* and *CD36* in white and brown adipose tissue (Jun et al., 2012). Based on these observations, it is likely that hypoxia, starts earlier in life and increases gradually to reach a threshold when it affects other functional processes such as lipid metabolism. The upregulation of *ANGPTL4* in affected chickens at week 7 in this study supports heightened hypoxia at market age. Additionally, hypoxia is implicated in increased cholesterol metabolism especially involving *ABCA1* in macrophages (Crucet et al., 2013), which compares favorably with our findings at both week 3 and week 7 of age. Besides its effects on metabolism, hypoxia is also suspected to augment development of lymphocytic phlebitis that characterizes WB. This follows the role of hypoxia in activating vascular endothelial

cells to increase their adhesiveness for leucocytes (Michiels et al., 2000), thereby allowing immune cells to cross the endothelial-cell barrier contributing to phlebitis.

The present study has also demonstrated presence of a consistent overexpression of slow-twitch myofiber-related genes in the P. major muscles of WB-affected chickens at week 3 and week 7 of age (market age). Even though the expression of *ANKRD2, MYOM3, MYOZ2* and *TNNI1* were not statistically significant in affected compared to unaffected chickens at week 3, they showed similar directionality as those genes whose expression were significantly upregulated in affected chickens at both age groups. This observation suggests that the onset and progression of WB in chickens is accompanied by expression of slow-twitch skeletal muscle genes, and that the expression increases with the severity of the disease as evidenced at week 7 of age. Corroborating the present findings, a study conducted by Kong et al. (2017) between unselected and genetically selected modern broiler chickens also showed upregulation of *TNNI1, MYOZ2, MYBPC1*and *LMOD2* in the latter group (Kong et al., 2017), indicating a consistent molecular signature in the P. major muscles of modern broiler chickens. Given that P. major muscles in chickens are almost purely type II muscle (glycolytic) fibers (Barnard et al., 1982), it follows, therefore, that the metabolism governing type II muscle fibers is different from that of type I (oxidative fibers) (Schiaffino and Reggiani, 2011). Hence, the appearance of slow-type proteins in a primarily glycolytic muscles such as P. major muscle would inevitably bring alterations in the overall muscle metabolism, especially relating to muscle bioenergetics. With the knowledge that the P. major muscles of modern broiler chickens have lower mitochondrial content with respect to their weight (Hakamata et al., 2018; Reverter et al., 2017), it is reasonable to suggest that the emergence of the

slow-twitch muscle fibers would place undue bioenergetic demands to the muscle, especially in affected chickens. Indeed, skeletal muscles have been known to communicate their energy demands to other organs through paracrine and endocrine signaling mechanisms, effected by myokine secretions (Giudice and Taylor, 2017). With this in mind, the emergence of slow-type muscle proteins in the P. major in the present study, would likely result in a shift or dysregulation of muscle metabolism, and therefore, adaptive responses with respect to muscle bioenergetics and myofibrillar structure taking effect. The upregulation of genes associated with increased lipid uptake and transport at week 3 of age in affected chickens, which coincide with the time when the expression of slow muscle genes begin to rise, serves to support changes in muscle metabolism towards oxidative phosphorylation. More specifically, *MYBPCI*, whose expression was upregulated in affected chickens at all time points in the current study, has been associated with intramuscular lipid deposition in beef cattle (Tong et al., 2015). This observation also corroborates the findings of previous studies on WB which showed alterations of carbohydrate and lipid metabolism (Abasht et al., 2016; Zambonelli et al., 2016). Additionally, our findings agree with a recent study which showed that increased expression of lipid metabolism genes in the P. major muscles of male broiler chickens as well as the cranial ends of the same muscle group was suggestive of metabolic switch and increased susceptibility to WB (Brothers et al., 2019).

Increasing expression of slow myofiber-type genes in affected muscles with disease severity may also reflect an adaptive response of the P. major muscle to biomechanical or overload stress as a result of a rapid increase in muscle weight over a short period. Fast-to-slow muscle transitions have been previously observed to be

induced by stretch overload resulting in immobilization in a lengthened position (Kostek et al., 2007; Pette and Staron, 2000) and mechanical stress (Akimoto et al., 2013). Along these lines, affected broiler chickens, which frequently have high-breast muscle yield/weight, due to elevated myofiber hypertrophy (Velleman, 2015) may be considered to have the greatest impact of biomechanical stress due to stretch overload. The low activity characterizing the behavior of modern broiler chickens further serves to support this argument. We therefore hypothesize that the rapid increase of pectoral muscle weight would inevitably impart stretch overload stress on individual myofibers resulting in immobilization in a lengthened position, thereby inducing the expression of slow myofiber genes in the predominantly glycolytic muscle. This is especially true for myofibers located superficially on angulated or curved regions of the pectoral muscles (e.g. cranial region), which are frequently affected first in comparison to those located in deeper regions.

In the case of muscle overload stress, it would be expected that some of the "first responder genes" will be those associated with mechanosensing, whose role would be to bring about muscle adaptability and homeostasis. Accordingly, several genes that were upregulated in affected chickens in the current study have been linked with mechano-sensing and enhancement of structural support in muscles. *CSRP3* gene, which encodes muscle LIM protein (MLP) (Arber et al., 1994), and found in the sarcolemma, costameres and the sarcomere of muscles where it binds specific proteins of the myofiber in the aforementioned regions (Vafiadaki et al., 2015), has been shown to play a role in mechano-signaling processes (Chaillou et al., 2015; Gehmlich et al., 2008). CSRP3's role of mechanosensing and structural support in the current study is evidenced by the localization of the *CSRP3* mRNA near the sarcolemma and

probably costameres of myofibers where the protein product (MLP) is thought to confer its maximal mechanosensory effect in response to external mechanical stress. Further, the focal expression pattern of *CSRP3* in areas of myofibers that are contiguous with large blood vessels in unaffected chickens suggests a response to external mechanical force. It is, therefore, not surprising that *CSRP3* had the highest expression at both time points, especially at week 7 (Log2 FC 6.2) coinciding with the severe degree of pathology of the muscles possibly due to increased fibrosis from WB.

Another slow myofiber gene associated with myofibrillar support is *MYBPC1*, which encodes the myosin-binding protein C, slow skeletal isoform (MyBP-C) (Reinach et al., 1982) that binds to myosin and titin thereby conferring stability and maintenance of the sarcomeric A-band (Kontrogianni-Konstantopoulos and Ackermann, 2010). Therefore, increased expression of *MYBPC1* in affected chickens, coupled with homogenous distribution of its mRNA in myofibers further affirms its role in maintaining muscle integrity, especially in the face of WB. Indeed, the association of *MYBPC1* with progression of muscular dystrophy in chickens (Obinata and Shinbo, 1987) and mice (Kurasawa et al., 1999), and WB in the current study suggest the importance of this gene in development of myopathies. Taken together, these observations demonstrate the role of *MYBPC1* gene as a potential biomarker for WB progression in meat-type chickens.

ANKRD2, which belongs to the members of the ankyrin repeat protein, and known to interact with titin at the sarcomeric I-band, is highly expressed in slow-twitch compared with fast-twitch muscles (Chaillou et al., 2015; da Costa et al., 2007). Through its mechanosensory action, the expression of *ANKRD2* in oxidative striated

muscles has been associated with a response to increased mechanical stress thereby contributing to maintenance of structural stability of muscles (Chaillou et al., 2015). While the same function has not been reported for type II muscle fibers, it is possible that the upregulation of *ANKRD2* in the P. major muscles of affected chickens in the current study could be a response to increased mechanical stress emanating from individual myofibers that are under continuous hypertrophy, fibrosis or weight overload as the pathology of WB progresses.

Leiomodin 2 is a protein that belongs to a class of potent tandem-G-actin binding nucleators that promote actin polymerization in muscles (Chen et al., 2015). In promoting actin nucleation, leiomodin proteins aid in lengthening, assembly, maintenance of actin/thin filament lattice structure in muscles as well as muscle contraction (Szatmári et al., 2017). LMOD2 functions primarily as an actin nucleator in the cardiac muscles (Pappas et al., 2015), and to a lesser extent, in mammalian adult skeletal muscles (Conley et al., 2001; Szatmári et al., 2017). Therefore, the upregulation of *LMOD2* in the present study is in line with increased muscle fiber-type remodeling in WB-affected chickens specifically relating to actin filament functions. As an actin-binding protein, LMOD2 is thought to act in concert with other slow myofiber-type proteins in imparting structural support and functionality within the P. major muscles. It is worth noting that the expression of *LMOD2* in affected chickens increases from week 3 to week 7 suggesting an association with the progression of the WB myopathy.

Myomesin 3 protein encoded by the *MYOM3* gene, belongs to myomesin family that localize in the M-band of the sarcomere (Schiaffino and Reggiani, 2011;

Schoenauer et al., 2008). Myomesin proteins solely found in striated muscles, are involved in binding of myosin to other proteins such as titin thereby stabilizing the thick filament lattice and the sarcomere structure in general during periods of sustained mechanical loading (Obermann et al., 1997; Schoenauer et al., 2008). *MYOM3* was found to be highly expressed in slow muscles (Schoenauer et al., 2008). In the present study, the upregulation of *MYOM3* alongside other slow-type muscle genes in the P. major muscles of affected chickens at week 7 of age provides evidence of the association of slow myofiber-phenotype with WB disease process. Given that the severe form of the WB myopathy frequently occurs around market age (week 7 of age), it is likely that the upregulation of *MYOM3* in affected chickens at this stage, serves to augment the structural support of the remaining healthy myofibers in the face of the WB disease. This, therefore, is in line with the adaptive response of the P. major muscles to increased mechanical loading of the breast muscle that is complexed with WB condition.

Myozenin 2 protein, also called calsarcin 1 or FATZ 2 (filamin-, actinin-, and telethonin-binding protein of the Z-disc), and encoded by the *MYOZ2* gene, is localized exclusively in the Z-disc of muscles. *MYOZ2* gene is expressed in cardiac muscles and slow-twitch skeletal muscles of mammalian adults and developing embryos (Frey et al., 2000; Schiaffino and Reggiani, 2011). Myozenin primarily binds calcineurin/calmodulin and other proteins in the Z-disc such as alpha-actinin, telethonin and myotilin (Frey et al., 2000; Schiaffino and Reggiani, 2011), which in the present case, would bring about structural stability of the muscle sarcomere during the progression of WB in chickens. Lastly, *TNNI1*, which encodes inhibitory troponin I of the troponin protein subunits in slow-skeletal muscles (Sheng and Jin, 2016), has

been demonstrated to be involved in myofiber switches in response to changes in functional demands in muscle such as mechanical unloading (Stevens et al., 2002). This agrees with our current observation to a certain extent, where the late stage of WB is associated with increased myodegeneration and myonecrosis, and hence, the remaining functional myofibers would be subjected to increased workload.

Additionally, while it was previously thought that the expression of slow-type myofiber genes in P. major muscles of WB-affected chickens was due to myoregeneration (Mutryn et al., 2015), the current study clarifies that it is not the case. This is exemplified by the expression of *CSRP3* and *MYBPC1* genes in mature myofibers of affected chickens in the present study.

5.6 Conclusion

This is the first study to show the expression of *LPL* by the vasculature endothelium in chickens. This study also provides mechanistic insights into alteration of lipid metabolism in the course of WB disease process. Further, the present study confirms the occurrence of slow myofiber phenotype during the progression of WB in chickens and its role in skeletal muscle remodeling. The association of some slow myofiber genes such as *MYBPC1* with muscle dystrophies, which are known to occur due to genetic defects, also suggests a possibility of genetic alterations affecting gene expression or protein functions in WB.

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Chapter 6

CONCLUSION AND FUTURE RESEARCH DIRECTION

This dissertation has provided for the first time, a comprehensive evaluation of phenotypic, morphological and molecular changes associated with the pathology and pathogenesis of WB in commercial broiler chickens. By employing a time-series approach, as well as utilization of different chicken strains comprising the commercial genetically rapid growth-selected broiler lines and unselected slow growing multi-purpose chickens of the 1950s, we were able to provide new knowledge regarding the mechanistic insights into the development of WB in chickens. Firstly, we established that WB exhibits an earlier onset than when detected by clinical examination. This was demonstrated through microscopic analysis which showed that the disease assumes a progressive course with acute phase characterized by localized vasculitis limited to small-caliber veins perivascular lipid infiltration and deposition, and myodegeneration occurring in the earlier stages, followed by a chronic fibrotic phase at later stages.

Secondly, we reported that molecular changes associated with WB precede morphological and phenotypic alterations. In this case, it was demonstrated that the onset and early pathogenesis of WB is characterized by molecular alterations involving muscle bioenergetics, particularly those involving lipid and carbohydrate metabolism. Additionally, vascular pathology, remodeling of extracellular matrix, compromised muscle function as well as response to inflammation were reported as being pertinent to the onset and early pathogenesis of WB in commercial meat-type

chickens. In essence, the molecular study not only confirmed the earlier morphological study, but also helped in explaining the mechanistic developmental pattern of the same.

In the third study which focused on the elucidation of involvement of myofiber-type phenotype in WB at the molecular level, we demonstrated that the process indeed occurs in affected P. major muscles. On this, we determined that the expression of genes encoding slow myofiber proteins increased with the severity of pathology over time. Further, the expression of some of the slow-myofiber-type genes such as *CSRP3* and *MYBPC1* were expressed in mature fibers, contrary to the earlier knowledge that their expression was associated with myoregeneration. Lastly, we confirmed that alteration of lipid metabolism accompanied the development of WB between the early stage and the late stage of the disease. Additionally, we reported for the first time, the expression of *LPL* by the vascular endothelial cells of capillaries and small-caliber veins of all chickens, a feature that is in sharp contrast to that in mammals so far. We also showed that cellular expression of *LPL* from the vascular endothelial cells increased in affected cases compared to unaffected chickens, thereby providing a mechanistic insight into the increased lipid uptake, transport and storage that frequently characterizes WB. Overall, the findings from studies contained in this dissertation shows the complexity that exist in the pathogenesis of WB in commercial broiler chickens.

Based on the findings from these studies, future research on WB can be centered on stabilization of bioenergetic metabolism and enhancement of inherent and endogenous antioxidant systems to counter the effects of oxidative stress. This can be accomplished by use of exogenous agents as feed additives that can promote

metabolic homeostasis and augment the antioxidant system without compromising on the health of the chickens or muscle quality. Other areas of interest could be optimization of the vascular network of the P. major muscle in order to counter the development of hypoxia, and promotion of adequate distribution of oxygen throughout the muscle. The latter can be achieved through identification of chickens with sufficient small-caliber vasculature with high muscle yield and then selecting for the trait.

Appendix A

IDENTIFICATION OF GENES ASSOCIATIED WITH SKIN CONTAMINATION IN PECTORAL MUSCLES

A.1 Background

Differentially expressed (DE) genes from the current study (weeks 2, 3 and 4) were scrutinized for potential skin contamination inadvertently obtained following muscle biopsy sampling process prior to functional analysis. This scrutiny was important to ensure that all DE genes analyzed downstream were derived from Pectoralis major muscles (affected and unaffected) and that they were largely free from skin contamination, which could impact downstream analysis. It was therefore necessary that a list of skin-derived genes (potential contaminant genes) were known beforehand so that they could be filtered out from the overall list of DE genes across all 3 time points before downstream functional analysis.

A.2 Strategy

To accomplish the above objective, we scrutinized for candidate muscle biopsy samples from all 3 datasets (week 2, 3 and 4) that could potentially be used to generate skin-derived genes. The aim was to come up with 2 groups of samples (those without skin contamination vs those with skin contamination), all belonging to the same disease state (affected or unaffected) to avoid confounding effect of disease.

Identification of significant differentially expressed genes between the 2 groups using Cuffdiff v2.2.1 software would then provide an idea of genes that are highly enriched in the skin-contaminated samples vs those that are not contaminated with skin. It follows therefore that genes that are highly enriched in the skin contaminated samples become true skin contaminants if found in the DE gene sets of muscle samples analyzed for disease vs non-disease within each of the three time points. Consequently, such skin gene contaminants become candidates for removal before functional analysis of the DE genes from muscle.

To be able to identify samples from the current dataset that contained the skin tissue, (that would eventually form one group of samples with skin contamination), we checked for the expression levels (FPKM values) of a few genes known to be primarily expressed by the skin across all samples. The candidate skin genes included keratin-associated genes such as *keratin, type I cytoskeletal 9-like* (*KRT9L*), *keratin, type I cytoskeletal 10* (*KRT10*) and *keratin 15* (*KRT15*). The hypothesis was that a muscle biopsy sample with skin tissue would have the skin-derived gene exhibiting higher expression values (FPKM values) compared to the sample that did not have skin contamination. Additionally, the consistency of expression levels of the above listed skin- derived genes in the candidate samples was considered. The hypothesis was that the expression levels of the skin genes should be consistent in all the muscle samples that contained skin tissue compared to those that did not have skin contamination.

A.3 Methodology

Upon application of the above strategy, we were able to determine that the affected samples (n =11) from week 3 dataset (4 unaffected vs 11 affected) provided a higher statistical power with sufficient and balanced biological replicates to run differential expression analysis using Cuffdiff v2.2.1, and identification of relevant DE genes. From the 11 affected samples, we identified 6 non-skin contaminated samples and 5 skin-contaminated samples (Appendix A.1). The two groups were run using Cuffdiff v2.2.1 software to yield differentially expressed genes between them. While muscle biopsy samples at week 2 and 4 showed promise for being candidates for identification of skin-derived genes, the groupings possessed a lower statistical power compared to the sample group at week 3. For example, at week 2, unaffected samples had (2 non-skin contaminated samples vs 4 skin contaminated samples) while affected group had (7 non-skin contaminated samples vs 3 skin contaminated samples). Similarly, week 4 samples had (5 non- skin contaminated samples vs 1 skin contaminated samples) for unaffected group, while affected group had (8 non-skin contaminated samples vs 2 skin contaminated samples).

Upon generation of DE genes following Cuffdiff analysis of non-skin contaminated vs skin- contaminated samples from week 3, we determined that any gene with log2 FC 31 from this geneset was a potential skin contaminant and was subsequently removed from DE genes of all 3 datasets of the 3 time points. Only those genes that did not have potential skin contaminants were used for downstream functional analysis.

Table 1 Appendix A: Week 3 affected samples used to identify skin-derived genes

Table A.1: Week 3 affected samples used to identify skin-derived genes

Non-skin contaminated samples	**Skin-contaminated samples**
1795_wk3_Mod2	446_wk3_Mod1
1737_wk3_Mod3	362_wk3_Mod4
1794_wk3_Mod6	339_wk3_Mod5
458_wk3_Sev3	1651_wk3_Sev1
457_wk3_Sev4	
390_wk3_Sev5	

Appendix B

PARAMETERS AND CRITERIA APPLIED FOR SELECTION OF BIOPSY SAMPLES USED IN RNA-SEQ ANALYSIS

Muscle Biopsy sample selection criteria for chickens at Week 2 and week 4 of age

Parameters	Unaffected	Affected
Gross changes	No lesions	Multifocal to diffuse pallor, petechial to ecchymotic hemorrhage, multifocal edema
Gross evaluations (gWBD score)	0 to 1	≥ 3
Microscopic WBD score	0 to 2	≥ 2
Body weight (g)	2396 to 3605	2730 to 3647

Muscle Biopsy sample selection criteria for chickens at week 3 of age

Parameters	Unaffected	Moderate	Severe
Gross changes	No lesions	Localized pallor, petechial hemorrhage, subcutaneous edema	Multifocal to diffuse pallor, petechial to ecchymotic hemorrhage, multifocal edema
Gross evaluations (gWBD score)	0	0 to 2	≥ 3
Microscopic WBD score	0 to 2	2 to 3	≥ 3
Body weight (g)	2747 to 2965	3431 to 3555	2965 to 3883

Appendix C

UPSTREAM REGULATORS PREDICTED TO BE ACTIVATED BY IPA IN THE PECTORAL MUSCLES OF CHICKENS AFFECTED WITH WBD AT WEEK 3 OF AGE

Upstream Regulator	Expression (Log2FC)	Molecule Type	Activation z-score	p-value of overlap
CEBPA	1.373	transcription regulator	3.83	8.96E-07
CD44	1.242	other	2.756	0.00314
C3	1.083	peptidase	2.414	0.000856
MAP3K1	0.875	kinase	2.599	0.000248
SPI1	0.871	transcription regulator	2.378	0.00369
CSF1	0.724	cytokine	2.567	0.000166
FN1	0.589	enzyme	2.965	0.00368
D-glucose		chemical - endogenous mammalian	3.179	2.19E-10
palmitic acid		chemical - endogenous mammalian	2.566	0.000119
D-fructose		chemical - endogenous mammalian	2.207	0.00283
nitric oxide		chemical - endogenous mammalian	2.06	0.00137
reactive oxygen species		chemical toxicant	2.173	0.176
NFkB (complex)		complex	2.878	0.00405
PI3K (complex)		complex	2.462	0.000205
Ap1		complex	2.189	0.0013
Pdgf (complex)		complex	2.187	0.0821
IL15		cytokine	2.599	0.205
TNFSF11		cytokine	2.572	0.0029
Jnk		group	2.674	0.00162
Vegf		group	2.655	0.00199
TGFB1		growth factor	3.81	4.75E-15
IGF1		growth factor	2.047	0.00000759
PPARG		ligand-dependent nuclear receptor	2.098	0.000134
HIF1A		transcription regulator	2.752	0.0000473
JUN		transcription regulator	2.312	7.14E-10

Appendix D

UPSTREAM REGULATORS PREDICTED TO BE INHIBITED BY IPA IN THE PECTORAL MUSCLES OF CHICKENS AFFECTED WITH WBD AT WEEK 3 OF AGE

Upstream Regulator	Expression Log2FC	Molecule Type	Activation z-score	p-value of overlap
vitamin E		chemical drug	-2.40	0.00374
Collagen(s)		complex	-2.22	0.00008
ITCH		enzyme	-2.00	0.00086
ACOX1		enzyme	-2.12	0.01990
CAT		enzyme	-2.22	0.00775
ALDH1A2		enzyme	-2.43	0.00007
Alpha catenin		group	-2.79	0.00716
JAG2		growth factor	-2.00	0.01260
INSR		kinase	-3.95	0.00000
CR1L		other	-2.61	0.00001
VCAN	0.798	other	-2.70	0.00000
MYC		transcription regulator	-2.05	0.00000
KLF3		transcription regulator	-2.11	0.16200
IRF4		transcription regulator	-2.19	0.15200
NCOR1		transcription regulator	-2.22	0.00027
NEUROG1		transcription regulator	-2.33	0.00002
TP73		transcription regulator	-2.57	0.00348
RB1		transcription regulator	-2.71	0.00089
MYCN		transcription regulator	-3.60	0.00000
TFRC		transporter	-2.00	0.00326
SFTPA1		transporter	-2.24	0.03740
HBA1/HBA2		transporter	-2.333	6.54E-07

Appendix E

REPRINT PERMISSION FOR CHAPTER 3

Our Ref: KB/CAVP/P19/1236 09 July 2019
Dear Michael B. Papah

Michael B. Papah, Erin M. Brannick, Carl J. Schmidt & Behnam Abasht (2017) Evidence and role of phlebitis and lipid infiltration in the onset and pathogenesis of Wooden Breast Disease in modern broiler chickens, Avian Pathology, 46:6, 623-643, DOI: 10.1080/03079457.2017.1339346

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Appendix F

APPROVAL FOR USE OF AGRICULTURAL ANIMALS IN RESEARCH

UNIVERSITY OF DELAWARE

COLLEGE OF AGRICULTURE AND NATURAL RESOURCES

AGRICULTURAL ANIMAL CARE AND USE COMMITTEE

Application for Use of Agricultural Animals

In Teaching or Research

AACUC Protocol Number:

TITLE OF PROJECT: (44) 07-08-14R
Identifying the Onset of a Novel Muscle Disorder in Chickens through Differential Gene Expression and Histologic Analyses

INSTRUCTOR/PRINCIPAL INVESTIGATOR

Behnam Abasht	B. Abasht	08/06/14
Printed Name	**Signature**	**Date**

(This section for Committee use only)

Application Approved (date) 7-29-14

Application Rejected (date) _____

Reason for Rejection _____

Robert L. Alphin	7-29-14
Signature, Animal Care and Use Committee	**Date**