FUNCTIONAL AND GENOMIC DRIVERS OF WOODEN BREAST IN COMMERCIAL BROILERS

A Dissertation

by

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ABSTRACT

Commercial meat-chickens (broilers) have been heavily selected for performance traits including high body weights, large breast muscle size, and improved feed efficiency. The wooden breast condition (WB) is a consequence of this selection along with several other physical and metabolic disorders. WB is currently considered a myopathy unique to broilers, causing decreased meat quality. It is not considered a threat to bird or consumer health, but inflammatory tissue due to WB is required to be excised during processing. The objective of this investigation was to detect molecular characteristics of WB through the use of global transcriptome and mitochondrial genome based comparative analyses between slow-growth chickens and commercial fast-growth broiler breeds. Our first investigation revealed that WB is genetic in origin and likely involves multiple organ systems. These findings are based on identification of an age-dependent transcriptional profile demonstrating altered regulation of cell proliferation and glycolysis, and markers of oxidative stress. Through our second investigation we observed the impact of dietary omega-6:3 ratio on age and breed related gene expression patterns and pinpointed genes specific to broilers and the characteristics of WB. Among the top pathways influenced were glycolysis, oxidative phosphorylation, and mitochondrial dysfunction, signifying the regulation of energy metabolism as central to the pathophysiology of WB. The third study illustrated a nuclear interaction with mitochondrial oxidative phosphorylation gene expression and deficiency in 42-day old broilers. This deficiency indicates an inability to produce adequate energy to support the

high metabolic rate of broilers and provides illumination to the role of the mitochondria in WB. Overall, these findings indicate that genes important to energy metabolism, cell proliferation and survival, and inflammation have been altered by selective pressure for broiler performance traits and their dysregulation has multi-system consequences which manifest macroscopically as WB. Functional classification of these genes showed commonalities between the genes in these lists suggesting that broilers share the biomedical profiles of obesity, diabetes, and cardiovascular disease.

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Professor Giridhar Athrey assisted in the data analysis for “Chapter 2”. The analyses depicted in “Chapter 3” were conducted in part (section 3.3.4.3) by Travis Williams of the Department of Poultry Science. All Athrey Lab members assisted in project management and dissections.

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NOMENCLATURE

ALA	alpha-Linolenic Acid
CBRO	Commercial Broiler Chickens
DPM	Deep Pectoral Myopathy
FA	Fatty Acid
FGCB	Fast Growth Commercial Broilers
LA	Linoleic Acid
LAY	Layer Chickens
MT	Mitochondria
ω -3	Omega-3
ω -6	Omega-6
R708	Ross 708 Commercial Broiler
RIN	RNA Integrity Number
RJF	Red Jungle Fowl
ROS	Reactive Oxygen Species
SGHB	Slow Growth Heritage Broilers
WB	Wooden Breast
WC	Wenchang Breed
WPR	White Plymouth Rock Heritage Broiler
WRR	White Recessive Rock Breed
WS	White Striping

XHH	Xingua Hybrid 1
XHI	Xinghua Hybrid 1

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1. INTRODUCTION TO THE MOLECULAR RELATIONSHIPS OF OXIDATIVE STRESS AND ENERGY METABOLISM IN WOODEN BREAST

1.1. Abridged History of Myopathies in the Poultry Industry

For the last 60 years the poultry industry has worked to increase growth rate, muscle development, and maturation of broilers in order to maximize production efficiency and reduce production costs (Bohren, 1953; Merritt et al., 1962; Jaap, 1963; Skoglund et al., 1966; Chambers et al., 1981; Fairfull and Chambers, 1984; Remignon et al., 1994; Paxton et al., 2010; Elwinger et al., 2016; Tallentire et al., 2018). These improvements have been accomplished through selective breeding and carefully maintaining multiple generations of broiler breeder flocks. However this has resulted in the genetic diversity of current commercial flocks becoming limited, promoting numerous health problems (Julian, 2005; Paxton et al., 2010; Tallentire et al., 2018). These problems include compromised immunity, vascular insufficiency, skeletal impairment, heat intolerance, and a class of disorders termed myopathies, which are generally considered meat quality issues rather than bird health concerns (Siller, 1985; Scheele, 1997; Malan et al., 2003; MacRae et al., 2006; Kuttappan et al., 2012, 2017a; b; Wideman et al., 2013; Sihvo et al., 2014; Mazzoni et al., 2015; Trocino et al., 2015; Tijare et al., 2016; Clark and Velleman, 2016; Cruz et al., 2017; Tarrant et al., 2017; Tallentire et al., 2018; Velleman et al., 2018; Hubert et al., 2018; Chen et al., 2019).

Generally, a myopathy constitutes a disorder of the skeletal muscle, often localized to a specific muscle tissue, in which cell structure and metabolism is impaired to the

level of macroscopic symptoms and muscle dysfunction. Myopathies can occur due to inheritance, chronic immune and endocrine disruptions, and environmental stimuli. For example, deep pectoral myopathy (DPM) is induced by wing flapping (Siller, 1985). Also, antibiotics such as Monensin (Umemura et al., 1984), hormone dysregulation such as the inhibition of prostaglandin (McLennan, 1985), and degeneration of mitochondria due to feed ingredients such as *Senna* seeds (Cavaliere et al., 1997) cause myopathies in chickens. Inherited (Asmundson and Julian, 1956; Asmundson et al., 1966) and nutritional muscular dystrophy (Weinstock et al., 1955) were the first myopathies to be investigated and are still seen in chickens. Due to the different modalities of inducing myopathies, it is difficult to pinpoint their pathophysiology. However, meat quality issues due to myopathies have become a prevalent consumer and economic concern in today's fast growth commercial broiler lines, necessitating intense investigation. Recent research has focused on the three most commonly observed broiler breast myopathies, DPM, white striping (WS), and wooden breast (WB), and a newly documented myopathy termed “spaghetti meat” (SM).

1.1.1. Overview of Current Breast Muscle Myopathies

Although they all are classified as myopathies of the breast, DPM affects the pectoralis minor while WS, WB, and SM affect the pectoralis major. DPM was first reported in broilers in 1980 (Richardson et al., 1980) and is commonly referred to as green muscle disease due to a characteristic green coloring of the pectoralis minor in affected birds. Numerous investigators have concluded that the very large pectoralis major of commercial broiler decreases blood flow to the pectoralis minor, resulting in

hemorrhage and necrosis of the muscle (Wight and Siller, 1980; Grunder et al., 1984; Siller, 1985; Lien et al., 2012; Bailey et al., 2015; Velleman, 2015; Kuttappan et al., 2016). The green coloring is caused by the release of iron from the hemorrhaged erythrocytes as they break down. Wight and Siller (1980) showed anucleated myofibers surrounded by fibrous and adipose tissues and disintegration of the sarcoplasmic reticulum, mitochondria, and Z-lines. This study suggested that DPM is a result of artificial selection for high growth rate and increased breast yield.

WS is so named based on the appearance of the affected pectoralis major, marked by white striations which run parallel to the muscle fibers. The manifestation of WS is not only altered appearance but also a decrease in mineral and protein content, water holding capacity, and an increase in fat and collagen content (Kuttappan, 2012; Kuttappan et al., 2012, 2013b; c; a; Petracci et al., 2013, 2014; Sihvo et al., 2014; Owens, 2014; Mazzoni et al., 2015; Mudalal et al., 2015; Bailey et al., 2015; Trocino et al., 2015; Vignale et al., 2017). Histologically it is characterized by variability in fiber size, necrosis of fibers, loss of cross striations, multinucleated cells, interstitial inflammation and fibrosis, lipidosis, and mononuclear cell infiltration (Kuttappan et al., 2013c; a; Mazzoni et al., 2015; Trocino et al., 2015). Few studies have investigated the molecular mechanisms of WS. Vignale et al. (2017) elucidated the activity of genes related to protein synthesis and degradation such as IGF-1, atrogin-1, insulin receptor, and MuRF1. MuRF1 and atrogin-1, which regulate ubiquitin mediated protein degradation in skeletal muscle, were up-regulated in WS birds, while IGF-1, an activator of muscle hypertrophy, was down-regulated. Vignale et al., (2017) suggested that these changes in gene expression

indicated that fast growth commercial broilers have a higher rate of muscle degradation, which leads to the development of WS. Pampouille et al. (2018) utilized a genome-wide association study of quantitative trait loci which identified possible genetic and molecular markers for WS and provided evidence for polygenic inheritance of the condition. These include several genes involved in muscle structure and metabolism, some known to be involved in neuromuscular disorders (Pampouille et al., 2018).

WB is a significant concern for the poultry industry due to its worldwide occurrence and escalating incidence (Mutryn et al., 2015; Bailey et al., 2015; Clark and Velleman, 2016; Sihvo et al., 2017; Cruz et al., 2017; Petracci et al., 2019). WB is characterized by an abnormally hard or "wooden" breast, with hardness originating at the cranial region in the least severe cases and extending the full length of the breast, from the cranial to the caudal region in the most severe cases. Breasts affected by WB demonstrate altered meat quality factors such as texture, shear force, pH and water holding capacity (Sihvo et al., 2014, 2017; Coble et al., 2014; Mazzoni et al., 2015; Velleman and Clark, 2015; Bailey et al., 2015; Trocino et al., 2015; Soglia et al., 2016; Tijare et al., 2016; Kuttappan et al., 2016, 2017b; Clark and Velleman, 2016; Cruz et al., 2017; Velleman et al., 2018).

SM, the most recently described myopathy, also primarily affects the pectoralis major. SM manifests as impaired muscle structure resulting in the separation of muscle fiber bundles and generally affects the cranial portion of the breast fillet (Baldi et al., 2018). WS and SM co-occur and share some histological features, but SM has increased perimysial and endomysial degradation of connective tissues, abundant infiltration of

inflammatory cells, and thin and split fibers surrounded by loose connective tissue (Baldi et al., 2018). Collagen of SM affected breasts is less mature compared to normal breast fillets, with fewer cross-links (Baldi et al., 2019). Furthermore, SM is higher in moisture content, but lower in protein and fat content than WS breasts (Baldi et al., 2018, 2019). SM breasts are downgraded for quality and used in further-processed products, resulting in economic losses (Baldi et al., 2018, 2019). Nutritional interventions for SM through altered arginine:lysine ratios were investigated by Zampiga et al. (2019), resulting in reduced incidence of SM ($P < 0.01$) at the highest arginine:lysine ratio. This reduction in SM incidence was hypothesized to be due to enhanced vasodilation and better blood flow to the muscle through increased production of nitric oxide through the arginine-nitric oxide pathway (Zampiga et al., 2019). However, the authors noted that the reduction in dietary lysine could also be responsible for the decreased incidence of SM as other researchers have shown increases in breast muscle myopathies due to increased dietary lysine concentrations (Cruz et al., 2017; Zampiga et al., 2019).

1.1.2. Significance of Myopathies to Food Production, Quality, and Safety

Chicken breast is the most consumed meat in the US, and the quality and safety of this food commodity is important both from an economic and a food security standpoint. Broiler breast myopathies decrease meat quality by altering the muscle ultrastructure, which in turn impacts texture, tenderness, and in some cases flavor. With high incidence rates, the annual economic losses due to broiler breast myopathies in the U.S. is roughly $200 million and increasing (Kuttappan et al., 2016). The pectoralis major is termed the breast when discussed as a meat product and is the most purchased cut, while the

pectoralis minor is termed the tender. The breast is popular for being high in protein content and low in fat. However, due to these myopathies, this desirable protein and fat ratio is altered, with increasing concentrations of fat and decreasing concentrations of protein (Petracci et al., 2014; Owens, 2014; Mazzoni et al., 2015; Mudalal et al., 2015; Soglia et al., 2016; Tijare et al., 2016; Kuttappan et al., 2017a; Tasoniero et al., 2017). The percentage of fat increase in breast meat affected by WB is 1.2-1.3% (Soglia et al., 2016; Tasoniero et al., 2016). Furthermore, as consumers have become aware of these myopathies, there is an increasing rejection of WB-affected meat, and a greater presence on social media (Gee, 2016; Rainey, 2016; Picchi, 2016; Jones, 2016; Prescott, 2017; Elder, 2017; Ngo, 2017; Pellegrini, 2017; Walansky, 2017; Keiger, 2017; Crews, 2017; Johnson, 2018; Petreycik, 2019; Versace, 2019; Burginger, 2019). Often, the public is misinformed on the source of these undesirable characteristics and misattribute them to questionable production practices or confuse them with genetically modified organisms. These views contribute to misinformation about animal agriculture and exacerbate food waste. Thus, it is imperative to improve public education on these crucial issues, while solutions are found and implemented.

1.2. Chicken Breast Muscle Characteristics

Although selective breeding of production animals developed around specific phenotypes, scientific advances in genetics and nutrition have created the ability to maximize the efficiency of selection using genotype data. Utilization of genomic-based approaches and the extent of multigenic traits may still be in its infancy, but trait heritability has been the basis of selection since roughly the 1980s (Chambers et al.,

1981; Fairfull and Chambers, 1984). Le Bihan-Duval et al. (2008) reported that growth and body composition traits such as body weight and abdominal fat were heritable (h^2 = 0.49 and 0.48 respectively), as were muscle characteristics such as glycolytic potential and muscle fiber cross-sectional area (h^2 = 0.43 and 0.41 respectively) in broilers. This study also reports that glycolytic potential was negatively correlated to the meat quality traits pf color, drip loss and shear force, and overall breast muscle weight (Le Bihan-Duval et al., 2008).

Chicken breast muscle physiology is well studied as it is the most valuable portion of the carcass and has been for more than 30 years. In 1994, Remignon et al. evaluated the muscle characteristics of slow-growth and fast-growth chicken lines to isolate muscle characteristics specific to growth-rate. This study found no differences in muscle fiber type at 55 weeks of age (Remignon et al., 1994). The breast was composed only of Type IIB fast-twitch fibers, cross-sectional areas were larger, and more numerous in the fast-growth line, compared to the slow growth line. However, some studies have identified very small percentages (0.5-10%) of Type IIA fibers in slow-growth and laying type chickens, especially those which are given room for movement (free-range or cage-free) (Remignon et al., 1995; Dransfield and Sosnicki, 1999; Scheuermann et al., 2004; MacRae et al., 2006; Velleman, 2007; Branciari et al., 2009; Clark and Velleman, 2016; Velleman et al., 2018).

The characteristics of the chicken breast muscle are primarily responsible for its popularity with consumers. It is high in protein and low in fat, while typically low in cost. Its stereotypical "taste of chicken" allows it to pair well with most recipes and it is

functional as a comminuted product, allowing it to have a variety of further processed food products, and is amenable to easy preparation. These desirable attributes are based not only on its Type IIB fibers and low-fat content but also the size and density of fibers, postmortem pH, and water holding capacity (WHC) (Rosser and George, 1986; Smith and Fletcher, 1988; Papa and Lyon, 1989; Dransfield and Sosnicki, 1999; Scheuermann et al., 2003; Lonergan et al., 2003; Berri et al., 2007; Fanatico et al., 2007; Le Bihan-Duval et al., 2008; Petracci and Cavani, 2012; Mazzoni et al., 2015; Petracci et al., 2015; Trocino et al., 2015; Baldi et al., 2018; Maxwell et al., 2018; Mueller et al., 2018; Golzar Adabi and Demirok Soncu, 2019).

Specifically, WHC affects the flavor and toughness of the breast through the amount of water retained or lost during cooking as well as the ability of the meat to absorb marinades and flavorings. WHC is directly affected by postmortem pH, with a low pH causing reduced WHC and a high pH causing increased WHC. However, pH only accounts for about one-third of WHC, and the other two-thirds is generally considered due to steric effects of muscle proteins. During rigor, the myofibrillar proteins form irreversible bonds, and muscle contraction occurs, reducing space for water storage. As rigor resolves, the contraction reduces, and several other muscle proteins such as z-lines have degraded, allowing the charged actomyosin complex to recruit water molecules, thus contributing to WHC. The specific combination of fat, functional protein, pH, and WHC is mainly responsible for the competence of chicken in comminuted products such as chicken nuggets. Deviations of these attributes result in downgrades in product

quality, reduced consumer acceptance, and economic losses (Betti et al., 2009; Kuttappan, 2012; Pellegrini, 2017; Kato et al., 2018; Petracci et al., 2019).

1.3. Known Mechanisms of Wooden Breast

First identified roughly 15 years ago and aggressively investigated during the last five years, many etiologies are proposed for WB. The most commonly investigated etiologies include nutritional deficiencies and toxicities, exercise induction, and hypoxia of the pectoralis major (Guetchom et al., 2012; Velleman and Clark, 2015; Bailey et al., 2015; Trocino et al., 2015; Soglia et al., 2016; Clark and Velleman, 2016; Cruz et al., 2017; Papah et al., 2017; Velleman et al., 2018; Sihvo et al., 2018; Bodle et al., 2018; Lilburn et al., 2018; Meloche et al., 2018; Livingston et al., 2019a; Chen et al., 2019). Despite this intense study, the molecular mechanisms and associated disease pathways driving WB remain unknown. There is, however, a consensus that WB is associated with the selection for fast growth-rate, occurring at highest frequency in the largest birds; slow growth varieties are not impacted (Mudalal et al., 2015; Mutryn et al., 2015; Velleman and Clark, 2015; Kong et al., 2017; Hubert et al., 2018; Meloche et al., 2018). Although similarities between WB and well-known myopathies such as white striping (WS) have been recognized, the histological characteristics of WB are well described, and include muscle fiber damage, interstitial fibrosis, infiltration of macrophages and an increase in fat and collagen content (Sihvo et al., 2014, 2017, 2018; Mazzoni et al., 2015; Velleman and Clark, 2015; Soglia et al., 2016; Clark and Velleman, 2016; Velleman et al., 2018; Lilburn et al., 2018; Meloche et al., 2018; Bowker et al., 2019).

At the molecular level, gene expression and metabolomics studies have shown that oxidative stress is one of the main features of WB tissue (Mutryn et al., 2015; Abasht et al., 2016; Clark and Velleman, 2016; Sihvo et al., 2017; Kong et al., 2017; Hubert et al., 2018; Papah et al., 2018). Reactive oxygen species (ROS), the free radicals produced in vivo by specialized enzymes such as NADPH-oxidase, nitric oxide synthase and myeloperoxidase, mitochondrial respiration and the monooxygenase activity of cytochrome p450, act as modulators of gene expression, increased cell proliferation, prevention of cell division, apoptosis, necrosis and cell death, and are responsible for the damage occurring (Weidinger and Kozlov, 2015) in oxidative stress (Halliwell, 2007; Bonnard et al., 2008; Mutryn et al., 2015; Netzer et al., 2015). In healthy tissues, ROS production is combated by antioxidants, preventing cellular damage. However, in tissues under oxidative stress, the ratio of ROS to antioxidants is highly imbalanced, and long-term cellular damage can occur. An example of this damage is direct modulation of the sodium/potassium-ATPase channels, resulting in changes in ion balance, which trigger changes in cellular calcium metabolism, causing increased concentrations of intracellular free calcium and subsequent cellular impairment (Sims and Muyderman, 2010). This characterization provides tremendous insight into the physiological occurrences of WB and provides an excellent foundation for the development of further investigations.

1.3.1. The Role of Selection for Performance in the Development of Wooden Breast

The poultry industry has made immense progress at increasing growth rate, breast yield, and feed efficiency of commercial broilers through careful selective breeding regimens and maintenance of pedigree flocks (Siller, 1985; Dransfield and Sosnicki,

1999; MacRae et al., 2006, 2007; Fanatico et al., 2007; Petracci and Cavani, 2012; Collins et al., 2014; Mazzoni et al., 2015; Velleman and Clark, 2015; Clark and Velleman, 2016). However, the quality of the meat produced is directly related to the morphological structure of the muscle, and growth-related selection has changed this structure. Intense selection has resulted in decreased capillary blood supply to the pectoralis major and minor, reduced connective tissue spacing between myofibers and muscle fiber bundles, and increased degeneration of myofibers (Scheele, 1997; Mahon, 1999; Mitchell, 1999; Dransfield and Sosnicki, 1999; MacRae et al., 2006, 2007; Berri et al., 2007; Fanatico et al., 2007; Petracci and Cavani, 2012; Kuttappan et al., 2012; Sihvo et al., 2014, 2018; Mazzoni et al., 2015; Trocino et al., 2015; Baldi et al., 2018; Lilburn et al., 2018; Soglia et al., 2019). Furthermore, myofibers of fast growth commercial birds are three to five times larger than those of slower growing birds (Dransfield and Sosnicki, 1999; Velleman, 2015). Larger fiber size combined with reduced capillary density is likely to impede nutrient/waste transfer to/from the cell affecting cellular health and longevity.

The muscular changes associated with selection for early rapid growth are exacerbated in the WB condition (MacRae et al., 2006, 2007; Petracci and Cavani, 2012; Mazzoni et al., 2015; Velleman and Clark, 2015; Trocino et al., 2015; Soglia et al., 2016; Clark and Velleman, 2016; Velleman et al., 2018). Some researchers hypothesized as early as 1999 (Mahon, 1999) that poultry growth rates might be hitting a maximum threshold at which myofiber metabolism would be compromised by its increased size and the inability of oxygen, nutrients, and waste to diffuse across the fiber. This idea has

been supported through the demonstration of increased numbers of necrotic, basophilic and hyaline fibers, and fibers with NADH rich rims or negative cores, which is indicative of mitochondrial dysfunction and altered oxidative metabolism in fast growth rate commercial broilers when compared to laying hens (MacRae et al., 2006, 2007). Multiple recent histological studies of WB have demonstrated that the same lesions are observed in both affected and unaffected birds, but affected birds show significantly more lesions (Mazzoni et al., 2015; Velleman and Clark, 2015; Trocino et al., 2015; Clark and Velleman, 2016; Sihvo et al., 2017, 2018; Velleman et al., 2018). Three of these investigations have surveyed and found an absence of the histological characteristics of WB in slow growth broiler or layer lines (Velleman and Clark, 2015; Clark and Velleman, 2016; Velleman et al., 2018).

Others have delved into the pathophysiology of WB through the use of transcriptomics, metabolomics, and proteomics. When investigating affected and unaffected birds of the same lines, these studies have identified pathways showing differential regulation to include those associated with cellular movement, proliferation, assembly, function and maintenance, protein synthesis, post-translational modification, protein folding and carbohydrate metabolism (Mutryn et al., 2015; Abasht et al., 2016; Kong et al., 2017; Kuttappan et al., 2017a; b; Schilling et al., 2017; Cai et al., 2018; Hubert et al., 2018; Papah et al., 2018). When comparing both affected and unaffected birds of the same fast growth commercial broiler line to a slow-growth variety they have observed differential regulation of the same pathways as discussed above, however they also observed differential regulation of disease pathways involved in organismal injury

or abnormalities and abnormal development and morphology of muscle (Velleman and Clark, 2015; Kong et al., 2017; Hubert et al., 2018). Comparison of fast-growth and slow-growth varieties indicated that mitochondrial activities such as oxidative phosphorylation and the tricarboxylic acid cycle are downregulated in breast muscles of fast growth broilers (Kong et al., 2017). These observations support the histological characteristics of WB. Furthermore, the molecular signatures of WB are suggestive of several diseases and disorders. The most significant included gastrointestinal disease, cardiovascular disease, hepatic system disease, neurological disease and cancer (Mutryn et al., 2015; Abasht et al., 2016; Kong et al., 2017; Kuttappan et al., 2017a; Hubert et al., 2018). This indicates that the underlying physiological and cellular processes impacted in these diseases are also being impacted in WB.

Although differences in gene expression and the subsequent alterations to physiological pathways can be detected when comparing unaffected and affected WB samples, the resolution is weak and cannot provide a definitive pathology. Unaffected samples may not have developed the condition at the time of collection or are asymptomatic (Abasht et al., 2016; Sihvo et al., 2017; Kuttappan et al., 2017a; b; Hubert et al., 2018). Data from these investigations suggest that asymptomatic WB tissue is not an adequate negative control for the determination of the molecular characteristics and pathophysiology of WB. Utilization of slow growth varieties as a negative control for WB investigations will not only provide a clear representation of the associated changes but will also allow for an increase in the understanding of genes and pathways which are

concurrently important for WB and production traits such as growth rate and feed efficiency.

1.3.2. The Role of Mitochondria and Oxidative Metabolism in Wooden Breast

The mitochondrion has crucial roles in cellular respiration, as well as several other factors specific to WB. As determined by Mutryn et al. (2015), gene expression studies of WB affected muscle samples indicate that intracellular calcium accumulation has the potential to impair cell membrane integrity. Interestingly, the mitochondria regulate cytosolic calcium concentrations, which in turn regulate the cellular reduction-oxidation reactions of a variety of transcription factors and cellular enzymatic reactions, control cellular, and mitochondrial metabolic pathways, and manage mitochondrial ROS production (Wallace, 2013; de Oliveira et al., 2017). Furthermore, calcium overload has the potential to activate proteases and lipases within the cell resulting in myofiber degeneration, a commonly observed histological feature of WB tissue (Halliwell, 2007; Mutryn et al., 2015). To this end, the role of mitochondria in WB occurrence or severity remains unclear. This gap in knowledge is surprising due to the mitochondrion's significance for muscle energetics, cell signaling, and death, which are all known features in WB progression.

It is well known that mitochondria are commonly called the powerhouse of the cell and that they function in oxidative metabolism to provide energy in the form of ATP to the body as well as facilitate the removal of wastes and act in cell signaling, differentiation and programmed cell death (Bonnard et al., 2008; Irwin et al., 2008; Angelini et al., 2009; Sims and Muyderman, 2010; Tuppen et al., 2010; Moreno-

Loshuertos et al., 2011; Velarde, 2013; Wallace and Chalkia, 2013; Netzer et al., 2015; Liao et al., 2015; Muir et al., 2016; Koch, 2016; Latorre-Pellicer et al., 2016; de Oliveira et al., 2017). However, the mitochondrion is the only organelle in the cell with a genome (Tuppen et al., 2010; Yarham et al., 2010; Wallace and Chalkia, 2013; Koch, 2016; Latorre-Pellicer et al., 2016). Mitochondrial DNA (mtDNA) is small, circular, and clonally inherited from the mother. It consists of two regions, the non-coding region, which controls mtDNA and the coding region, which codes for tRNAs, rRNAs, and 13 cellular energy production genes, and contains no introns (Takemoto et al., 1999; Wai et al., 2008; Tuppen et al., 2010; Moreno-Loshuertos et al., 2011; Wallace and Chalkia, 2013; Muir et al., 2016; Latorre-Pellicer et al., 2016). Mitochondrial DNA is haploid and does not recombine; it is typically stable over time, but due to its few repair mechanisms it tends to have a much higher mutation rate than nuclear DNA and mutations are commonly deleterious.

The number of mitochondria in a cell varies significantly by type and tissue, and they often have more than one genome sequence, a common condition known as heteroplasmy (DiMauro and Schon, 2001; Wai et al., 2008; Tuppen et al., 2010; Payne et al., 2013; Wallace and Chalkia, 2013; Abbott et al., 2014; Carelli et al., 2015; Lehmann et al., 2015; Luo et al., 2018). This heteroplasmy often acts as a sort of shield for an individual carrying mutant mtDNA mutations. Over a lifetime, the percentage of mutant mtDNAs increases, resulting in a decline in the individuals bioenergetic capacity below the minimum threshold and symptoms of the pathogenic mtDNA mutation ensue (Wallace and Chalkia, 2013; Abbott et al., 2014; Carelli et al., 2015; Lehmann et al.,

2015; Latorre-Pellicer et al., 2016). Due to this unique nature, pathogenic mtDNA mutations and the conditions caused by them are widespread, continually arising, can be localized to a specific tissue, are familial, and often age-related (Zeviani et al., 1991; Weber et al., 1997; Wai et al., 2008; Tuppen et al., 2010; Moreno-Loshuertos et al., 2011; Lehmann et al., 2015). This description fits the developmental stages observed in WB, as it arises at different ages, affects a high percentage, and occurs in varying severities throughout a broiler flock.

A critical trait of WB posing a dilemma for determining its pathology is its apparent localization to the breast muscle only. However, diseases caused by mitochondrial mutations typically localize to a tissue or region of the body and the observation of both histological and molecular markers of oxidative stress and myocyte degeneration, reveals their role in the pathogenesis of WB. We can determine the role of mitochondrial activities in WB through the use of Next Generation Sequencing (NGS) technologies and bioinformatics. However, investigations into human diseases in which mitochondria have recently been implicated such as diabetes, obesity, metabolic syndrome, stroke, Alzheimer's, and cancer have demonstrated that an understanding of the molecular characteristics of the disease is instrumental in determining the involvement of mitochondrial-related bioenergetic alterations (Barja and Herrero, 2000; Bonnard et al., 2008; Angelini et al., 2009; Sims and Muyderman, 2010; Yarham et al., 2010; Moreno-Loshuertos et al., 2011; D'Souza et al., 2011; Long et al., 2012; Velarde, 2013; Wallace and Chalkia, 2013; Muir et al., 2016; Latorre-Pellicer et al., 2016; Glancy et al., 2017; Maurya et al., 2018). It is through these unique characteristics of the mitochondria that

mitochondrial mutations can have significant physiological impacts; therefore, it is necessary to characterize the mitochondrial structure and gene expression related to WB.

1.4. Genetic and Nutritional Interactions as Potential Drivers of Wooden Breast

In contrast, based on the evidence for oxidative stress and hypoxia in WB, some hypotheses have suggested a genomic and nutritional interaction based on the high energy, soy-based diet typical of the commercial broiler (Haug et al., 2007; Koppenol et al., 2015; Cruz et al., 2017; Livingston et al., 2019c; Li et al., 2019). Although many nutritional interventions were assessed, none have managed to decrease the incidence of WB (Guetchom et al., 2012; Trocino et al., 2015; Cruz et al., 2017; Sobotik et al., 2018; Bodle et al., 2018; Lilburn et al., 2018; Livingston et al., 2019b; c; a). Still, other hypotheses have gone further to implicate endocrine disruptors such as phytoestrogens from soybean meal in poultry feed, as a potential driver of genomic/nutritional interaction. Phytoestrogens are naturally occurring, plant-derived, biologically active compounds which both structurally and functionally mimic estrogens when in humans and animals (Bacciottini et al., 2007; Cederroth and Nef, 2009; Patisaul and Jefferson, 2010; Stevenson et al., 2014).

Soybean meal, one of the main ingredients in industrial poultry feed, has one of the highest concentrations of phytoestrogens among plant-based feed sources (National Research Council et al., 1994; Payne et al., 2001; Haug et al., 2007; Stein et al., 2008; Cederroth and Nef, 2009; Patisaul and Jefferson, 2010; Gjorgovska et al., 2014; USDA ARS, 2016). Due to their similarity to estrogen, and ability to bind to both estrogen receptor α and ß, phytoestrogens act as endocrine disrupting compounds (Cederroth and

Nef, 2009; Patisaul and Jefferson, 2010; Gjorgovska et al., 2014). As estrogen regulates various vital physiological processes such as lipid and glucose metabolism, bone development, sexual maturation, and reproduction, perturbations of this suite of endocrine functions can potentially alter critical system-wide processes. Therefore, the nature and extent to which phytoestrogens drive occurrence and severity of WB deserve further attention.

The implications of high dietary concentrations of phytoestrogens are also relevant to mitochondrial cell signaling, and oxidative metabolism (Irwin et al., 2008; Sims and Muyderman, 2010; Mauvais-Jarvis, 2011; Velarde, 2013; Sarkar et al., 2015; Liao et al., 2015). One pathway involves alteration of mitochondrial cell signaling and mitochondrial hormonal regulation due to increased exposure to phytoestrogens. Recent work has shown the role of mitochondrial estrogen receptor-ß and its implications for mitochondrial bioenergetics and tumorigenesis (Velarde, 2013; Liao et al., 2015). Additionally, human studies have determined that oxidative stress in skeletal muscle is often induced by dysregulation of the metabolism of energy fuel substrates such as lipids and glucose, resulting in damage to the mitochondria and an increase in the buildup of ROS and fuel substrates (Marco et al., 1961; Bonnard et al., 2008; Irwin et al., 2008; Velarde, 2013; Netzer et al., 2015; Liao et al., 2015; de Oliveira et al., 2017). The endocrine disrupting actions of phytoestrogens are capable of inducing such a metabolic dysregulation, and this is a possible element in the pathogenesis of WB and warrants further investigation.

1.4.1. Impacts of Dietary Omega-6 and Omega-3 Supplementation to Oxidative Stress and Inflammation on Wooden Breast Gene Expression

Due to the many vital physiological activities of estrogens, the regulators of estrogen metabolism have become a popular topic in human and animal health. Currently, the most studied are the omega-3 (ω-3) and omega-6 (ω-6) polyunsaturated fatty acids. They boast a myriad of physiological effects including reduction of cholesterol, reduction of circulating levels of 17ß-estradiol, reduced binding of estradiol to the estrogen receptor, stabilization of electrical activity of cardiac myocytes, increased apoptosis of cancerous cells, increased 2-hydroxylation of estradiol, and protection for mitochondria (Marco et al., 1961; Lord et al., 2002; Gómez Candela et al., 2011; Cao et al., 2012; Dikshit et al., 2015; Jeromson et al., 2015; Behling et al., 2015; de Oliveira et al., 2017). These effects provide benefits such as reduced risk of cardiovascular and metabolic diseases, and reduced risk of multiple cancer types (O'Keefe et al., 1995; Lord et al., 2002; Simopoulos, 2002; Betti et al., 2009; Gómez Candela et al., 2011; Cao et al., 2012; Dikshit et al., 2015; Lorente-Cebrián et al., 2015; Jeromson et al., 2015; Behling et al., 2015).

Multiple studies have investigated increasing ω-3 fatty acid concentrations in chicken feed, as an indirect way to increase human ω-3 consumption (Ratnayake et al., 1989; O'Keefe et al., 1995; Newkirk and Classen, 2002; Rymer and Givens, 2005; Haug et al., 2007; Betti et al., 2009; Zuidhof et al., 2009; Koppenol et al., 2015; Carragher et al., 2016; Konieczka et al., 2017; Moghadam et al., 2017). A majority of these studies utilized fish-based additives as the source of ω-3s. The fish-based ω supplement did

increase the ω-3 content of the muscle, but also caused a fishy taste and decreased the shelf life of the product (Ratnayake et al., 1989; O'Keefe et al., 1995; Rymer and Givens, 2005; Koppenol et al., 2015). More recent studies investigated plant-based sources of ω-3s such as flax and canola, however most of these investigations demonstrated reduced growth rates and final carcass weights (Newkirk and Classen, 2002; Haug et al., 2007; Betti et al., 2009; Parveen et al., 2013; Carragher et al., 2016; Konieczka et al., 2017). Currently, the soy and corn-rich standard poultry industry diet is very high in ω-6s but very low in ω-3s (O'Keefe et al., 1995; Rymer and Givens, 2005; Haug et al., 2007; Dikshit et al., 2015). For humans, the recommended ratio of ω-6:3 is roughly 5:1. Ratios of 2-3:1 have demonstrated improvements in patients with cardiovascular disease, rheumatoid arthritis, asthma and multiple types of cancers while ratios of 10:1 have shown adverse effects (Simopoulos, 2002, 2010, 2016; Rymer and Givens, 2005; Gómez Candela et al., 2011; Jeromson et al., 2015).

Balancing the ω-6:3 content of industry broiler feed could have a positive impact on several conditions which are currently causing tremendous losses to the poultry industry including WB, WS, green muscle disease, and sudden death syndrome (SDS) to name only a few. Furthermore, none of these studies have attempted to explain the physiological processes which result in the observed changes due to supplemented ω-3s, and few have considered differences in gene expression. Investigation of these processes utilizing multi-disciplinary approaches will help determine the extent to which nutritional modulation of ω-6:3 fatty acid ratios can influence oxidative stress in the muscle.

1.5. Motivation for the Current Research and Outcomes

As detailed previously, the most commonly observed theme in WB is a state of oxidative stress in the pectoralis major; the origins and implications of the offending ROS remain unknown to date. Furthermore, the intracellular calcium buildup and NADH displacement indicate dysfunction of the oxidative metabolism machinery and a severe threat to cellular health. This work proposes to utilize current "omics" technologies to determine both the origins and implications of this oxidative stress and its actions in the pathogenesis of WB. In this thesis, I achieved this objective using a combination of live-animal experiments, generation of molecular data, as well as bioinformatics approaches in a comparative framework. First, I completed a comparative analysis including chicken from multiple varieties (differing growth profiles) to explore transcriptional and genomic changes due to WB in both nuclear and mtDNA. Second, I used live animal experiments to determine the effects of omega ratios on the molecular signatures of wooden breast. Through these observations, a clear view of nutritional, age, and genetic interactions was developed. Altogether, the new knowledge generated here will help in the discovery of solutions for WB.

1.6. References

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2. INSIGHTS INTO THE MOLECULAR BASIS OF WOODEN BREAST BASED ON COMPARATIVE ANALYSIS OF FAST- AND SLOW-GROWTH BROILERS

2.1. Background

The domestic chicken (*Gallus gallus domesticus*) is a major agricultural species and a popular source of animal protein around the world. In the United States, chicken breast is the most consumed meat – per capita consumption surpassed 41kgs in 2015 (source: US Poultry) – and the broiler industry has a substantial economic footprint ($30 billion/year). Consumption of poultry has increased in step with human population growth as well as changes in consumption habits (OECD and FAO, 2017). While demand continues to grow, production is under enormous stress due to a variety of disorders (ascites, fatty liver disease) and meat quality issues such as green muscle disease and wooden breast (Siller, 1985; Scheele, 1997; Kuttappan, 2012; Kuttappan et al., 2012, 2013). Of these, wooden breast (WB) is the most recent problem that is negatively impacting breast meat quality. WB is a muscle condition which has been categorized as a breast myopathy from the well documented history of myopathies in broilers. The frequency of WB has risen steadily over the last five years, being reported globally with reduced consumer acceptability (Tasoniero et al., 2016) linked to economic losses (Tijare et al., 2016; Zambonelli et al., 2016). WB has become prominent within the last decade and has been reported to affect over 50% of commercial flocks (Abasht et al., 2016; Sihvo et al., 2017), but accurate estimates of global incidence are not known.

Pectoral myopathies are not new in broiler poultry species, and broiler chicken particularly have a well-documented history of dystrophies and myopathies, including pectoral myopathies induced by physical or nutritional stress (Wight and Siller, 1980; Randall, 1982; Van Vleet and Valentine, 2007a). For example, Siller *et al.* (1978) reported deep pectoral myopathy in both turkeys and broiler chicken induced by exercise. However, WB is different from previously studied pectoral myopathies in some important ways; the hallmarks of WB appear to be moderate to severe degenerative necrosis, with varying degrees of interstitial fibrosis. While some of these features have been previously reported in other myopathies, the co-occurrence of localized pectoral myopathy with fibrosis and striations has not been observed previously in broilers. WB is often observed in conjunction with white striping (WS), which is characterized by white striations that run parallel to the muscle fibers in the breast (Sihvo et al., 2017). These white striations can resemble marbling and are associated with increased fat and collagen content (Kuttappan et al., 2016). In the past, some pectoral myopathies have been assigned etiologies ranging from nutritional deficiency (e.g. selenium), to hypoxia or ionophore toxicity (Van Vleet and Valentine, 2007b; a). However, these etiologies have not been validated in WB. Despite intensive studies of WB, including histopathological analyses, serological studies, dietary interventions, gene expression, and metabolomics studies (Mutryn et al., 2015; Abasht et al., 2016; Cruz et al., 2017; Radaelli et al., 2017; Papah et al., 2017; Griffin et al., 2018), the causative factors of WB remain unknown.

Both WB and WS demonstrate varying degrees of severity and have been identified in multiple commercial varieties. Recent studies have described WB as a polyphasic myodegeneration (Sihvo et al., 2017), presenting lymphocytic phlebitis (Sihvo et al., 2017; Papah et al., 2017). The only common explanatory factor appears to be the growth rate of commercial broilers, with the most severe cases found in the heaviest male birds (Velleman, 2015; Griffin et al., 2018). Kuttappan *et al* (2012) reported that the rapid growth rate and high-energy diets both increase incidence of WS. A similar trend is observed with WB. Since 2000, average broiler weights have increased by 3 kg (6.5 lbs), representing a 55% increase (Source: U.S. Poultry). Due to the high value of breast meat in proportion to total carcass, increasing incidence and severity of WB translates into greater economic losses (Bailey et al., 2015).

While selective breeding for performance traits (e.g. growth rate, feed efficiency) and advances in nutrition, are largely responsible for growth rate improvements in broilers, it is not known whether WB, which is associated with growth rate, has a genetic basis. Recent studies have used gene expression (RNAseq) and metabolomics analyses to characterize WB (Mutryn et al., 2015; Abasht et al., 2016; Zambonelli et al., 2016), but these investigations have not been informative about the underlying cause(s) of WB. Whereas a previous report suggested low heritability for WB (Bailey et al., 2015), a recent report by Pampouille *et al.* (2018) describes the identification of quantitative traits loci (QTL) for WS in high-yield broilers, and further concludes that WS is a polygenic condition, supporting the hypothesis for a genetic basis for WB and WS.

In this study, the objective was to utilize comparative analyses to illuminate the basis of WB and to characterize its similarity to other known conditions. This study also evaluated the evidence for supposition that WB is a muscle myopathy. We addressed this objective by comparative transcriptomic analyses of WB samples against various genotypes/phenotypes, followed by pathway analyses and tests for enrichment of canonical pathways. This study did not specifically focus on molecular features of WB/WS co-occurrence, and hence we do not draw inferences regarding WS. Altogether, our study indicates that a) WB is an age-dependent disorder driven by transcriptional dysregulation in fast-growth broilers, and b) that WB molecular profiles suggest a complex syndrome potentially involving multiple organ systems. These results suggest a genetic basis to WB and emphasize the importance of deeper studies of the mechanistic basis of WB. These findings also suggest that WB is a condition with potential consequences for whole organism health.

2.2. Materials and Methods

2.2.1. Study Design and Source of Data

In this study, transcriptome data was generated from birds exhibiting WB, which was then analyzed comparatively with data generated for previous slow- and fast-growth broiler gene expression studies and published in the peer reviewed literature. The details of these samples, with links to original studies, are provided in Table 2.1. It is important to note that these studies focused on breast tissue specific gene expression and to our knowledge, WB was not the explicitly stated subject of investigation. However, a subset of these studies used modern commercial broilers, therefore we cannot be certain that

they were unaffected by WB; and based on the high frequency of WB in commercial broiler flocks (Cruz et al., 2017) it is likely that these samples include WB affected individuals.

To determine how the genetic background for growth profile drives the molecular signatures of WB, it is necessary to compare expression profiles across ages and genetic strains. WB has been reported in fast-growth broilers as early as two weeks of age, but with the most dramatic changes in severity occurring in the final three weeks before slaughter (Kuttappan et al., 2016; Radaelli et al., 2017). Therefore, we compared gene expression of pectoralis major muscle tissue from fast- and slow-growth broilers. While WB has been reported from all major commercial broiler strains, WB has not been reported in slow-growth and heritage broilers to date.

Secondly, as WB severity has been reported to increase with age and weight, we compared expression profiles among pectoralis muscle tissues from different age categories of both fast- and slow-growth. To answer these questions, we used a combination of data generated in house (reported above) in addition to reusing publicly available sequence data (NCBI Short Read Archive) that matched our analysis criteria. In total, nine publicly available datasets from six previously published studies were included in these comparisons (Table 2.1). In each instance, we selected studies that generated RNAseq data from the breast tissue, were sequenced on the Illumina platform, and were not from a pathogen challenge experiment. Three datasets were from an environmental ammonia challenge study, using the 42-day old broilers of the Arbor Acres strain (Aviagen, sample prefix ARAC). While these treatments influence their

gene expression profiles, our analyses showed that these three sample groups cluster together with other 42-day old fast-growth broilers. The inclusion of data from this experiment did not change the hierarchical clustering and separation of sample transcriptome profiles by performance and age profile; hence we retained all three datasets in further analyses.

Table 2.1 Summary of data used in comparative analysis, including information of chicken breed, tissue type, age of birds at sampling, SRA accession info, and authors of original study.

SRA accession ID	Chicken Breed/variety	Breed Type	Tissue Type	Age of bird at sampling	Authors
PRJNA339392	Arbor Acres	Commercial Broiler	Pectoralis major	42 days	Yi et al, 2016 [77]
PRJNA342997	WC and WRR	Heritage Broilers	Pectoralis major	120 and 180 days	Qui et al, 2017 [78]
PRJNA294010	Recessive White Rock (WRR) & Xinhua	Heritage & Indigenous	Pectoralis major	42 days	Chen et al. [79]
PRJNA273416	Ross 708 & Illinois Chickens	Commercial Broiler & Legacy Breed	Pectoralis major	6 and 21 days	Davis et al, 2015 [80]
PRJNA266323	WRR & XH	Heritage & Indigenous	Pectoralis major	42 days	Ouyang et al, 2015 [81]

WRR: White Recessive Rock, WC: Wenchang Chicken, XH: Xinghua Chicken

2.2.2. Sample Collection and RNA Extraction

Live animal studies and euthanasia procedures performed in-house were approved by the Texas A&M University's Animal Care and Use Committee (assurance number 2016-0065). Breast tissue samples were collected from eight, 42-day old chickens of a high-yielding commercial broiler strain. Animals for this study were obtained from a commercial broiler hatchery. The birds in the study were from an all-male flock and

raised on a three-phase industry standard diet. Two birds were randomly sampled from each of four replicate pens, containing 40 birds each. Birds were examined by palpation of the pectoralis major muscle prior to euthanasia. This approach has been used as a diagnostic method in several recent published studies (Mutryn, 2015; Abasht et al., 2016; Clark and Velleman, 2016). Birds were then euthanized by CO_2 exposure followed by cervical dislocation and dissected for collection of tissues for genetic analysis. The pectoralis major and pectoralis minor muscles were then examined for gross lesions and hardness of the muscle. Individual samples were classified as either WB+ or WB- based on observed hardness of breast tissue and the absence of other visible abnormalities.

While histological analyses have also been used for WB classification, they are perhaps more applicable for resolution of severity, rather than a diagnostic for presence-absence of WB. Furthermore, histological classification of breast tissue as 'normal' has not been found to be diagnostic of WB at the molecular level (Sihvo et al., 2017; Kuttappan et al., 2017). While it has been noted that WB and WS co-occur frequently, this study was focused on WB, and therefore did not specifically classify tissue for WS presence or severity. Of the eight individual birds sampled this way, six birds were classified as severe (WB+) based on palpation and gross lesions, whereas two other samples were less severe cases (WB-). Owing to the poor correlation between physical/histological and molecular markers of WB, all birds sampled in this study were classified into the WB group. Moreover, as the goal was to compare expression patterns

across genotypic backgrounds, this grouping allowed better resolution through increased biological replication of the WB group.

Tissue of size approximately $1cm^3$ was excised from the distal portion of the pectoralis major with a scalpel and immediately stored in RNAlater (Ambion Inc). After 24 hours of incubation at 4^0C, the excess RNAlater was removed and samples were stored at -80^0C until further processing. Total RNA was extracted from about 30mg of the tissue using the RNEasy Mini Kit (Qiagen Inc). Samples were checked for RNA quality and concentration on a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific).

2.2.3. RNA Sequencing and Transcriptome Analysis

Total RNA isolates were submitted for library preparation and RNA-sequencing at the AgriLife Genomics and Bioinformatics Center (Texas A&M University). Sample libraries were prepared by performing DNAse digest, followed by poly-A selection for mRNA molecules. Individual mRNA isolates were then pooled into three sample libraries – namely one library comprising two WB- samples, and two libraries each comprising three WB+ samples. These three sets of pooled samples were used for strand specific library preparation and the libraries were sequenced with 125bp single end sequencing.

The 11 datasets, including the in-house generated and downloaded datasets, were then processed identically. In brief, the raw RNAseq data was filtered for adapter contamination and quality trimmed using the program Trimmomatic (Bolger et al., 2014). Reads with average quality scores less than Q30 and/or shorter than 20bp in

length were discarded. High-quality reads were mapped to the *Gallus gallus* genome (Version 4.8, Ensembl Release 85, July 2016) using the short-read de-novo splice mapper STAR (Dobin and Gingeras, 2015, 2016), followed by counting of transcripts mapped to the 'exon' features using the tool HTSeq (Anders et al., 2015). The counts data for each sample were then compared for statistical significance using the EdgeR package on the R statistical platform (Robinson et al., 2010). First, low expressed genes across all libraries (CPM <2) were filtered out. Next, normalization factors were calculated for differences in library sizes, followed by estimation of common and then tagwise dispersion (GLM). We used the package COMBAT to check and correct for batch effects (Johnson et al., 2007; Nygaard et al., 2016). The quasi-likelihood based 'glmQLFTest' function was used to perform tests for significance between expression values among treatments. The QLF approach is known to provide greater protection against Type I error and can handle unbalanced designs better than exact tests.

A total of 10 pairwise contrasts were performed between WB data (generated in house) and downloaded broiler transcriptome data. Following the analyses in EdgeR, topTag tables were used interpretation and pathway analyses. For each of the 10 differential expression results, pathway analyses were performed using the Ingenuity Pathway Analysis platform (Qiagen Inc.). Only genes significant at FDR <0.05, and with Log2FoldChange smaller than -0.5 or greater than 0.5 were included in pathway analyses. Finally, the results from pathway core analyses (10 datasets) were all included in a 'Comparison Analyses' on the IPA platform, to characterize similarity of expression, shared canonical pathways, upstream regulators, and diseases.

2.2.4. Differential Expression Between Fast-Growth versus Slow-Growth Broilers

A secondary differential gene expression analysis was performed by grouping all the modern commercial broilers as the Fast-Growth Commercial Broiler (FGCB), and by grouping the Weichang (WC) and White Recessive Rock (WRR) samples as the Slow-Growth Heritage Broiler (SGHB). The Illinois strain and the hybrid WRR-XH crosses were left out of this comparison as they are neither a heritage breed nor a commercial variety. Differential expression analysis and pathway analysis was performed in the same way as described above.

2.2.5. Variant Analysis with RNAseq Data

A total of 54 sequence libraries (.fastq), including the 8 generated for this study, were used to generate variant calls and to identify shared and unique SNP variants among the breeds included. The Genome Analysis Toolkit (Van der Auwera et al., 2013) best practices pipeline for variant calling from RNAseq data was used to generate a set of high-quality variants for each sample using hard filtering. Briefly, STAR aligned reads (same used for differential expression analysis) were first processed to add read information and to mark duplicates using the tool Picard (Horner et al., 2010). Binary alignment files were then fed into HaplotypeCaller, followed by selection of SNP variants, and variant filtration. SNPs occurring in clusters within 35bp were filtered out, as were variant calls with Qual By Depth (QD) score <5, and Fisher strand bias > 35. The resultant set of high quality variants obtained this way were passed into the variant effect prediction software SnpEff (Cingolani et al., 2012). Variants annotated as having a 'High' impact modifier by SnpEff were used for comparison among samples. Due to the

variability in sequencing library size and depth of coverage, differences in number of variants were expected. Therefore, to account for potential bias arising from differences in sequencing depth, high-impact variants were compared only between the FGCB group, and the SGHB group. The combined high-impact variant list was generated by pooling all variants by ENSEMBL gene ID and removing duplicates.

2.3. Results

2.3.1. Global Gene Expression Patterns Are Explained by Growth Rate and Age

Across the 10 datasets, a total of 12,202 genes were expressed above threshold (CPM=>2) and were included in both the differential expression and pathway analyses. An ordination analysis using Non-metric Multidimensional Scaling (NMDS, Figure 2.1) showed that fast-growth breeds (ARAC, COB, ROSS and WR-XH Cross) overlapped each other, with younger (6 and 21 day old) fast-growth broilers being less proximate to WB samples, compared to 42 day old broilers (ARAC), indicating a clear age based expression similarity. The Illinois and Ross breeds (6 and 21 day old) both clustered by age and also by breed, showing a clear age-based segregation from 42-day old commercial broilers. The slow-growth breeds (WC and WRR, 120 day and older) formed clusters distinct from the fast-growth broilers. Furthermore, all WB samples formed a tight cluster, validating the observation that birds without obviously tangible WB symptoms are, nonetheless, not different at the molecular level. Therefore, birds from the same genetic background may not be suitable as a negative control (Figure 2.2).

Figure 2.1 Non-Metric Multidimensional Scaling plot of the RNAseq datasets in the multi-sample comparison study. The samples from each age and growth-rate group cluster clearly within groups. Commercial fast-growth broilers also appear more proximate to each other and are arranged from top to bottom in order of increasing age, while youngest and slowest growth breeds are furthest away from older, fast-growth breeds.

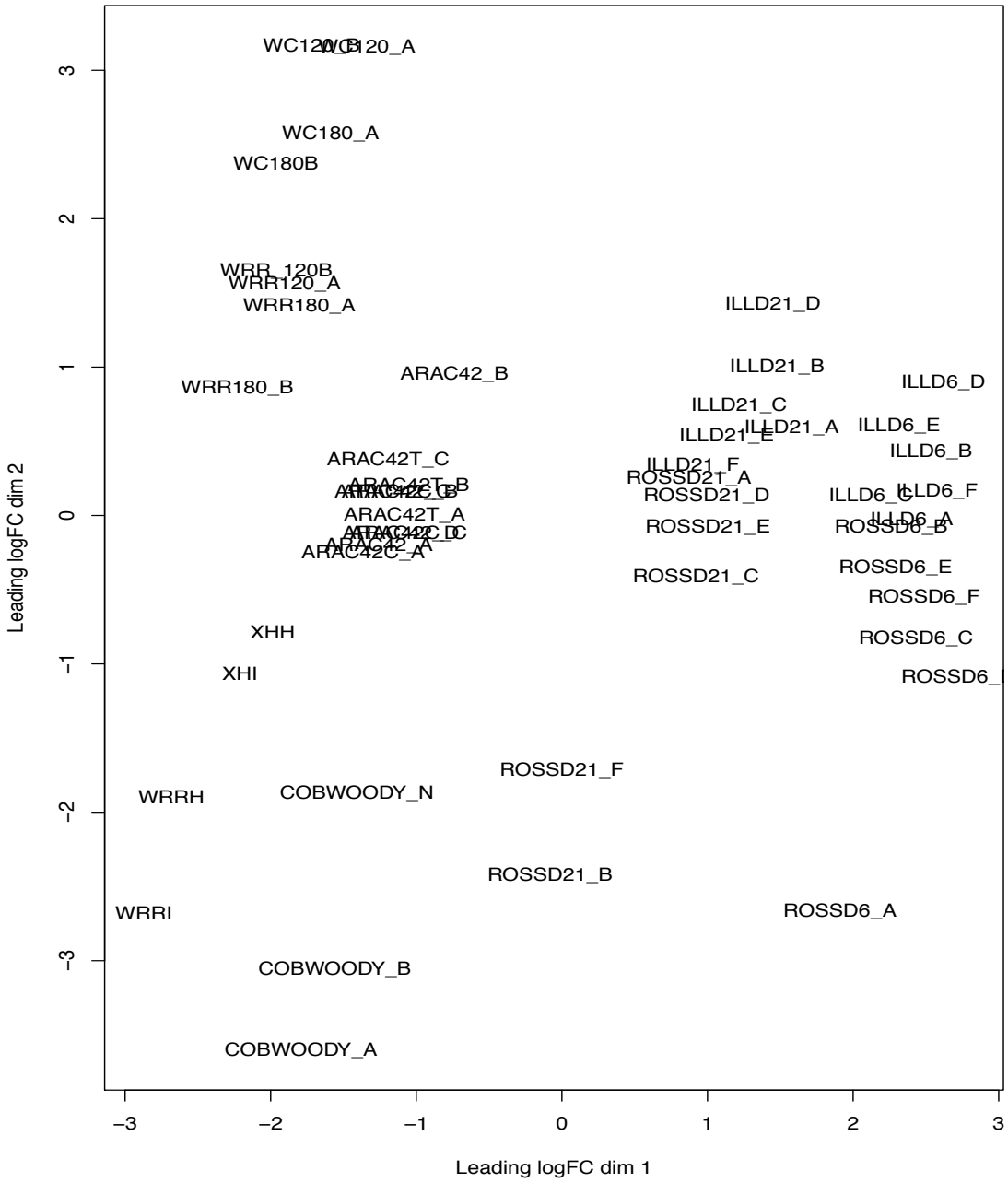
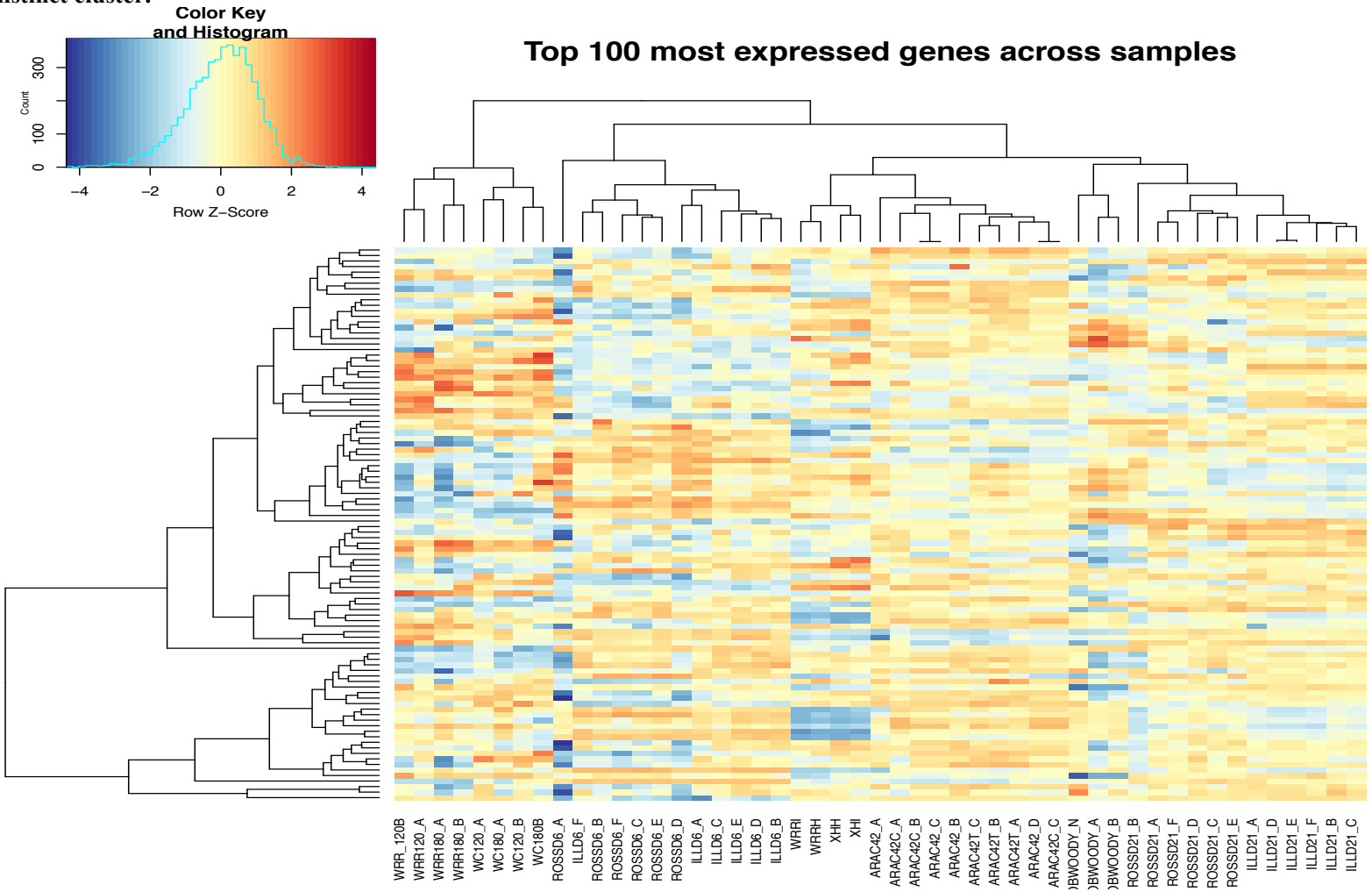


Figure 2.2 Hierarchical clustering heatmap of the top 100 most expressed genes, based on log CPM values, across samples. Clustering shows that wooden breast samples fall among Ross 21 day old and ARAC 42 old birds, with slow-growth heritage birds (WC and WRR) forming a distinct cluster.



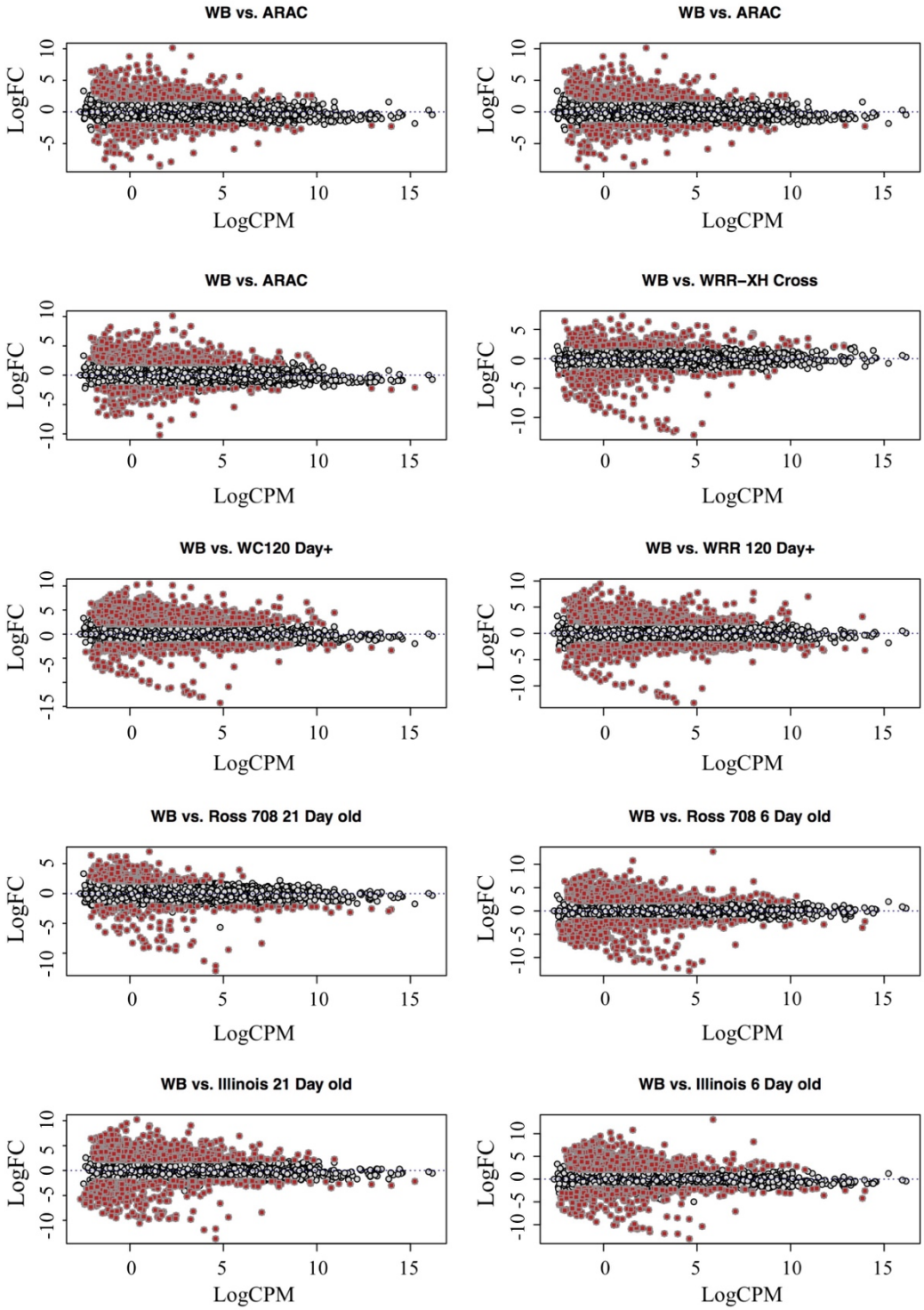
2.3.2. Comparison of Pairwise Differential Expression Analyses

Results from edgeR for each pairwise comparison against WB samples are summarized in Table 2.2 and mean-average plots for each comparison is shown in Figure 2.3. Complete list of differentially expressed genes with P-values for all pairwise comparisons are available in supplementary materials. Overall, the WC120+ and WRR120+ slow-growth varieties were most different from WB, based on the total number of differentially expressed genes (~2200). This number was approximately twice as high as any of the other comparisons. After the WC and WRR breeds, the Illinois 21D was most different (1702 genes differentially expressed), and Ross 21D being most similar (693 genes differentially expressed), with other comparisons falling in between. The three different datasets of 42-day ARAC (3 treatments in original study), were very similar to each other in their differences to WB (total of 1243, 1167, and 1330 DEG respectively). The top canonical pathways identified were also highly similar, with T-cell receptor signaling in all three comparisons and IL-8 signaling in comparison to both A and C groups of ARAC. Canonical pathways explained by these DE genes showed that IL-8 signaling and T-cell receptor signaling were recurrent terms, but all three comparisons shared TGFB1 and TNF as the upstream regulators.

Table 2.2 Summary of results from pairwise contrasts performed among RNAseq data using edgeR. Table shows the information on differentially expressed genes, summary of pathway analysis using IPA, upstream regulators for each dataset, and top disease terms.

Against	Arbor acres 42Day	Arbor acres 42Day	Arbor acres 42Day	WRR-XHCross	WC120D+	WRR120D+	ROSS-21D	ROSS-6D	ILL-21D	ILL-6D
Group	1	2	3	4	5	6	7	8	9	10
Up in WB	912	855	1001	372	1435	1227	438	686	1131	473
Down in WB	331	312	329	249	849	894	255	482	571	789
Total DE	1243	1167	1330	621	2284	2121	693	1168	1702	1262
Top Canonical PW	T Cell Receptor Signaling	CD28 Signaling in T Helper Cells	Axonal Guidance Signaling	eNOS signaling	Axonal Guidance Signaling	NRF2 Mediated Oxidative Stress	T-Cell Receptor Signaling	CD28 Signaling in T Helper Cells	Signaling by Rho Family of GTPases	CD28 Signaling in T Helper Cells
	IL-8 Signaling	T Cell Receptor Signaling	IL-8 Signaling	Calcium Signaling	Molecular Mechanisms of Cancer	IL-8 Signaling	Production of Nitric Oxide	IL-12 Signaling and Production of Macrophages	CD28 Signaling in T Helper Cells	T-cell Receptor Signaling
	Signaling by Rho Family of GTPases	Phospholipase C Signaling	T-cell Receptor Signaling	Hepatic Fibrosis/ Hepatic Stellate Cell Activation	T -cell Receptor Signaling	ERK/MAPK Signaling	CD28 Signaling in T Helper Cells	T-Cell Receptor Signaling	Tec Kinase Signaling	PI3K Signaling in B Lympocytes
Upstream Regs	TGFB1	TGFB1	TGFB1	TNF	TGFB1	TGFB1	Lipopolysaccharide	TP53	TNF	Lipopolysaccharide
	TNF	TNF	TNF	beta-estradiol	beta-estradiol	beta-estradiol	beta-estradiol	Lipopolysaccharide	beta-estradiol	beta-estradiol
	beta-estradiol	beta-estradiol	Lipopolysaccharide	TGFB1	TP53	TP53	TGFB1	beta-estradiol	TGFB1	TNF
Diseases	Cancer Organismal Injury	Cancer Organismal Injury	Cancer Organismal Injury	Cancer Organismal Injury	Cancer Organismal Injury	Cancer Organismal Injury	Cancer Organismal Injury	Cancer Organismal Injury	Cancer Organismal Injury	Cancer Organismal Injury
	Gastrointestinal Disease	Gastrointestinal Disease	Gastrointestinal Disease	Neurological Disease	Gastrointestinal Disease	Gastrointestinal Disease	Gastrointestinal Disease	Gastrointestinal Disease	Gastrointestinal Disease	Gastrointestinal Disease

Figure 2.3 Mean-Average plots for analysis of differentially expressed genes for each pairwise comparison performed against the wooden breast sample set. Points in red show the genes that were expressed at <-2 logFC or > 2 logFC, with an FDR <0.05



Comparing WB to Ross 708 (6 and 21 day old) showed greater differences at 6 days than at 21 days, with main pathway terms being CD28 Signaling in T-helper Cells and T-cell receptor signaling. Lipopolysaccharide and beta-estradiol were shared upstream regulators. Unlike the Ross strain, the Illinois 6- and 21-day old birds were distinct in their global expression profile, with greater differences to WB compared to the younger Ross strain birds. This observation would fit the known biology of the Illinois strain, which is a broiler line with a performance profile from the 1950's. However, pathway terms for these datasets still included CD28 signaling in T-helper Cells and T-cell receptor signaling, which were shared with the Ross strain. Both WC120+ and WRR120+ slow-growth breeds were most different from WB samples in the extent of differential expression, and with no overlap in the top three pathway terms.

Pathway analysis of genes differentially expressed in slow growth varieties also yielded pathway terms that were not shared with other comparisons. Pathway analyses yielded hepatic fibrosis/hepatic satellite cell activation, calcium signaling, eNOS signaling, molecular mechanisms of cancer, NRF2 mediated oxidative stress, ERK/MAPK signaling, signaling by Rho family GTPases, Tec kinase signaling, and PI3K signaling in B lymphocytes as the top terms in ARAC (3 libraries), WRR-XH cross, WC120D+, WRR120D+, Ross 6D and 21D, and Illinois 6D and 21D respectively. Interestingly, despite the differences in the top canonical pathways identified when comparing WB gene expression to that of slow growth varieties rather than fast-growth varieties, the upstream regulators suggested by this differential gene expression include the same terms. Diseases identified by the differential gene expression of each

comparison evaluated included the same terms: Cancer, organismal injury, and gastrointestinal disease, with only one comparison indicating neurological disease. Finally, the common occurrence of T-cell receptor signaling and IL-8 Signaling suggest that molecules activated in these pathways may be suitable as biomarkers for detecting WB.

2.3.3. Multi-sample Comparison Analysis

The multi-sample comparison analysis allows identification of similarities and trends occurring across multiple datasets, specifically, identification of functions overrepresented across datasets. Pathways are considered significant if a greater number of molecules associated with a pathway are expressed than expected by chance. Based on the pathways with highest -log(P-values) and activation Z-scores, the top shared canonical pathways were T-cell receptor signaling, CD28 signaling in T-helper cells, and signaling by Rho family of GTPases. The top 50 pathway and disease terms are shown in Table 2.3. The top upstream regulators were TGFb1, TNF and TP53 and beta-estradiol. The comparison feature also generated a list of the top diseases and disorders; the top three disease terms that emerged from the consensus of the multi-sample comparison were Cancer, Abdominal Neoplasm and Solid Malignant Tumor. None of these top 100 disease terms pointed to muscle myopathies or other musculoskeletal disorders. Finally, the top disease signaling pathways were Cancer, MAPK and the P53 pathways.

Table 2.3 List of top 50 canonical pathway terms, and diseases and disorders identified from comparison analysis. In this analysis, multiple DEG datasets are compared to identify common pathway terms that are found more often than expected by chance.

Ranked by -log(P)	Diseases and Disorders	Canonnical Pathways
1	T Cell Receptor Signaling	Cancer
2	CD28 Signaling in T Helper Cells	abdominal neoplasm
3	Signaling by Rho Family GTPases	cancer
4	Phospholipase C Signaling	solid tumor
5	Axonal Guidance Signaling	malignant solid tumor
6	Tec Kinase Signaling	adenocarcinoma
7	FcÎ³ Receptor-mediated Phagocytosis in Macrophages and Monocytes	digestive system cancer
8	FXR/RXR Activation	digestive organ tumor
9	Germ Cell-Sertoli Cell Junction Signaling	abdominal cancer
10	CTLA4 Signaling in Cytotoxic T Lymphocytes	non-melanoma solid tumor
11	Semaphorin Signaling in Neurons	tumorigenesis of tissue
12	Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	neoplasia of epithelial tissue
13	Phagosome Formation	carcinoma
14	GP6 Signaling Pathway	malignant neoplasm of large intestine
15	Role of Tissue Factor in Cancer	intestinal cancer
16	B Cell Receptor Signaling	large intestine neoplasm
17	Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	intestinal tumor
18	Calcium-induced T Lymphocyte Apoptosis	large intestine adenocarcinoma
19	Primary Immunodeficiency Signaling	large intestine carcinoma
20	Colorectal Cancer Metastasis Signaling	intestinal carcinoma
21	Leukocyte Extravasation Signaling	gastrointestinal carcinoma
22	PI3K Signaling in B Lymphocytes	gastrointestinal tract cancer
23	LXR/RXR Activation	gastrointestinal neoplasia
24	Ephrin B Signaling	necrosis
25	Cardiac Î²-adrenergic Signaling	cell death
26	RhoGDI Signaling	apoptosis
27	CXCR4 Signaling	organization of cytoskeleton
28	Protein Kinase A Signaling	organization of cytoplasm
29	Î±-Adrenergic Signaling	organismal death
30	Opioid Signaling Pathway	morbidity or mortality
31	Breast Cancer Regulation by Stathmin1	liver lesion
32	Integrin Signaling	abdominal carcinoma
33	Acute Phase Response Signaling	tumorigenesis of epithelial neoplasm
34	iCOS-iCOSL Signaling in T Helper Cells	abdominal adenocarcinoma
35	PKCÎ¸ Signaling in T Lymphocytes	tumorigenesis of malignant tumor
36	Glioma Invasiveness Signaling	tumorigenesis of carcinoma
37	Paxillin Signaling	morphology of cells
38	ILK Signaling	morphology of body cavity
39	Th1 and Th2 Activation Pathway	migration of cells
40	Role of JAK1 and JAK3 in Î³c Cytokine Signaling	cell movement
41	G-Protein Coupled Receptor Signaling	liver carcinoma

Table 2.3 Continued

Ranked by -log(P)	Diseases and Disorders	Canonnical Pathways
42	Th2 Pathway	liver tumor
43	Hepatic Fibrosis / Hepatic Stellate Cell Activation	liver cancer
44	Sirtuin Signaling Pathway	hepatobiliary system cancer
45	Hereditary Breast Cancer Signaling	breast or colorectal cancer
46	Superoxide Radicals Degradation	genital tract cancer
47	Fc Epsilon RI Signaling	pelvic tumor
48	GÎ±i Signaling	pelvic cancer
49	Complement System	genitourinary tumor
50	Virus Entry via Endocytic Pathways	urogenital cancer

2.3.4. Fast-Growth versus Slow-growth Differential Expression

For this analysis, commercial broiler strains (ARAC, ROSS, WB) were included in the fast-growth commercial broiler (FGCB) group and the WC120+ and WRR120+ strains were grouped into the slow-growth heritage broilers (SGHB) group. This particular analysis was designed to separate out those genes that are upregulated or downregulated in FGCB irrespective of age, with the supposition that genes associated with the onset and progression of WB in fast-growth strains would be found across age categories (6 days to 42 days). A total of 11,766 genes were expressed above a threshold (CPM=>2), of which 6406 were differentially expressed (FDR<0.05, LogFC < -0.05 and >0.05). Of the total differentially expressed genes, 6168 genes were significantly downregulated in FGCB. These differentially expressed genes were then analyzed in IPA to identify canonical pathways and diseases/disorders. The top three canonical pathways based on -log(P) values were Mitotic Roles of Polo-like Kinase, ILK Signaling, and ERK/MAPK signaling. As observed with other pairwise comparisons, the main disease terms were Cancer, Solid Malignant Tumors, and Gastrointestinal Disease.

2.3.5. Variant Discovery from RNAseq Data

Variant calling and filtering of the datasets yielded average SNP calls of 79,539, and 83,902 for the FGCB and SGHB groups respectively. The individual variant calls were merged using GATK CombineVariants to generate a consolidated (merged) set of 709,959 and 279,339 high quality SNPs in FGCB and SGHB respectively. This difference in total variants was driven mainly by differences in the number of samples included in each group - namely 25 and eight for FGCB and SGHB respectively. The multi-sample VCFs annotated with snpEff to identify effects of the variants and to categorize impacts yielded 395 and 158 high-impact variants in FGCB and SGHB respectively. Overall, the proportions of effected features, and functional impacts, were evenly matched (Table 2.4) except where noted. Modifier effects (changes outside coding regions) was the most frequent effect, which was higher in FGCB compared to SGHB. High-impact variants which signify impacts within the coding sequences (e.g. frameshift, stop gained, stop lost), were of equal proportion in both groups, whereas both moderate, and low-impact variants were more frequent in SGHB.

Of the total high-impact variants, 37 were shared among all three fast-growth breeds (ARAC, Ross, WB), whereas 35 were shared among the two slow-growth breeds (WC120+ and WRR120+). Of these 72-total high-impact variants, 14 were found in both the slow and fast-growth breeds, leaving 23 unique high-impact variants in FGCB, and 21 unique high-impact variants in SGHB (Table 2.5). The membership of both lists are rich in genes involved in cell signaling, cell proliferation, and cellular response to stress (including hypoxia). Particularly notable genes with high-impact variants unique to the

FGCB group are SPEG, NPEPPS, and THYN1. These genes are involved in myocyte cytoskeletal development, linked to the cellular response to hypoxia, and associated with the induction of apoptosis, respectively. Notable genes with high-impact variants in the SGHB group were two myosin heavy chain genes (MYH1A, and MYH1B) and dystonin (DST). These genes are involved in motor activity and actin filament binding, and the assembly of collagen fibrils, respectively.

Table 2.4 Comparison of variant effect prediction between the slow-growth heritage broilers (SGHB), and the fast-growth commercial broilers (FGCB). Results from snpEff based on high-quality SNP variants are shown. Colored boxes highlight notable differences in predicted effects for any category. Green colored boxes show elevated frequency of effects that are less likely to be cause negative impacts, whereas red shaded boxes show elevated frequency of phenotype-changing effects.

Group		SGHB		FGCB	
	Type	Count	Percent	Count	Percent
Effects by impact	HIGH	158	0.03%	395	0.03%
	LOW	60566	11.67%	94998	8.01%
	MODERATE	17001	3.28%	30856	2.60%
	MODIFIER	441262	85.02%	1059364	89.35%
Effects by functional class	MISSENSE	17021	22.22%	30907	25.17%
	NONSENSE	50	0.07%	97	0.08%
	SILENT	59514	77.71%	91795	74.75%
	Missense - Silent ratio	0.286		0.336696	
Count by effects	3 prime UTR variant	53496	10.29%	82491	6.94%
	5 prime UTR premature start codon gain variant	544	0.10%	1302	0.11%
	5 prime UTR variant	3529	0.68%	8050	0.68%
	downstream gene variant	143715	27.65%	292411	24.60%
	initiator codon variant	1	0.00%	6	0.00%
	intergenic region	109334	21.03%	255700	21.51%
	intron variant	70624	13.59%	268782	22.61%
	missense variant	17001	3.27%	30856	2.60%

Table 2.4 Continued

Group		SGHB		FGCB	
	Type	**Count**	**Percent**	**Count**	**Percent**
Count by effects	non canonical start codon	0	0.00%	1	0.00%
	non coding exon variant	169	0.03%	369	0.03%
	splice acceptor variant	36	0.01%	107	0.01%
	splice donor variant	57	0.01%	152	0.01%
	splice region variant	837	0.16%	3099	0.26%
	start lost	9	0.00%	21	0.00%
	stop gained	50	0.01%	97	0.01%
	stop lost	10	0.00%	25	0.00%
	stop retained variant	24	0.00%	36	0.00%
	synonymous variant	59490	11.44%	91758	7.72%
	upstream gene variant	60901	11.72%	153487	12.91%
Count by genomic region	DOWNSTREAM	143715	27.69%	292411	24.66%
	EXON	76508	14.74%	122280	10.31%
	INTERGENIC	109334	21.07%	255700	21.57%
	INTRON	70124	13.51%	266869	22.51%
	SPLICE SITE ACCEPTOR	36	0.01%	107	0.01%
	SPLICE SITE DONOR	53	0.01%	145	0.01%
	SPLICE SITE REGION	747	0.14%	2771	0.23%
	UPSTREAM	60901	11.73%	153487	12.95%
	UTR 3 PRIME	53496	10.31%	82491	6.96%
	UTR 5 PRIME	4073	0.78%	9352	0.79%

2.3.6. Overlap of High Expression and High-Impact Variants

Genes with high-impact variants in either FGCB or SGHB were cross referenced against the list of significantly differentially expressed genes between the two groups. Twenty-three of the total 45 genes were also found to be significantly differentially expressed (Table 2.5). Genes that were up- or down-regulated in FGCB were found in

both high-impact variant lists. Interestingly, 17 of the 21 genes with high-impact variants in SGHB were also significantly differentially expressed, suggesting both a mechanistic and functional role for these genes.

Table 2.5 Lists of top high-impact variants in both the fast-growth commercial broilers, and slow-growth heritage broiler group. Highlighted genes are those that were also significantly differentially expressed in comparison of Fast versus Slow groups. The directionality of regulation is also shown highlighted for differentially expressed genes.

HI Variants Unique to FGCB	DGE	Direction	Description	Function
ATAD5	Not DE		ATPase family AAA domain-containing protein 5	Immunoglobulin prouction
C10orf71	Not DE		chromosome 10 open reading frame 71	Unknown
CELSR1	Not DE		Cadherin EGF LAG seven-pass G-type receptor 1	Calcium Ion Binding
CUEDC1	Not DE		CUE domain containing protein	Downregulation of Estrogen Receptor 1
DECR1	Not DE		2,4-dienoyl-CoA reductase 1	fatty acid beta-oxidation
ENSGALG00000004746	Not DE		Uncharacterized	Unknown
ENSGALG00000022316	Sig DE	Down in FGCB	C-type lectin family member	Fucose/Mannose Binding
ENSGALG00000023351	Sig DE	Down in FGCB	Small Integral Membrane Protein	Unknown
ENSGALG00000023846	Not DE		Uncharacterized	Unknown
ENSGALG00000026688	Not DE		Uncharacterized	Unknown
KIFC1	Not DE		Kinesin-like protein KIFC1	ATPase activity
MYCBPAP	Not DE		MYCPG-Associated Protein	Cell differentiation
NES	Sig DE		NEST Protein	intermediate filament binding
NPEPPS	Not DE		Puromycin-sensitive aminopeptidase	Cellular response to hypoxia
OGFR	Not DE		Opioid growth factor receptor	Regulation of cell growth
PKD1	Not DE		polycystin 1, transient receptor potential channel interacting	Regulation of calcium channels
RFC4	Not DE		replication factor C subunit 4	DNA-dependent ATP-ase activity
SPEG	Sig DE	Up in FGCB	SPEG Complex locus	Myocyte cytoskeletal development
STAT1	Sig DE	Up in FGCB	Signal transducer and transcription activator	Mediates cell viability in response to stress
THYN1	Sig DE	Up in FGCB	Thymocyte Nuclear Protein	Induction of apoptosis
TMEM108	Sig DE	Down in FGCB	Transmembrane Protein	cellular response to brain-derived neurotrophic factor stimulus
tvb	Not DE		TNF-related apoptosis inducing ligand	Tumor necrosis factor-activated receptor activity
WIPI1	Not DE			

Table 2.5 Continued

HI Variants Unique to SGHB	DGE	Direction	Description	Function
BFIV21	Not DE		MHC Class 1 component	Metal ion binding
Blec2	Sig De	Up in FGCB	C-type Lectin Like Receptor 2	Inhibition of natural killer cytotoxicity
C2CD5	Sig DE	Up in FGCB	C2 Calcium Dependent Domaning Containg 5	Calcium bion binding and calcium-dependent phospholipid binding
CASP10	Sig DE	Up in FGCB	Cystein-Aspartic Acid protease family	Regulation of apoptosis
CCNL1	Sig DE	Up in FGCB	Cyclin gene family member	pre-mRNA splicing and regulation of RNA polymerase II
CIRH1A	Not DE		Ribosome biogenesis factor	nucleolar processing of pre-18S rRNA
DST	Sig DE	Up in FGCB	Dystonin	Cytoskeletal linker protein
ENSGALG00000002067	Sig DE	Down in FGCB	Mannosyltranferase activity	Mannosylation of lipid-linked oligosaccharides
ENSGALG00000021835	Sig DE	Up in FGCB	Uncharacterized	Unknown
ENSGALG00000028551	Sig DE	Up in FGCB	Glutathione transferase	Cellular defense against toxic compounds
Lpin1	Not DE		Lipin 1 phospatidate phosphatase	Fatty acide metabolism, transcription regulation
MYH1A	Sig DE	Down in FGCB	myosin, heavy chain 1A, skeletal muscle	Microtubule motor activity, actin filament binding
MYH1B	Sig DE	Down in FGCB	myosin, heavy chain 1B, skeletal muscle	Microtubule motor activity, actin filament binding
RPL12	Not DE		ribosomal protein L12	Ribosomal large subunit assembly
SLC9A2	Sig DE	Up in FGCB	solute carrier family 9 member A2	Proton extrusion, regulation of pH, sodium absorption
STAMBP	Sig DE	Down in FGCB	STAM binding protein	Zinc metalloprotease activtity
STRN3	Sig DE	Up in FGCB	striatin 3	Calcium dependent calmodulin binding
SULT1E1	Not DE		Sulfotransferase	Sulfate conjugation of estradiol and estrone
TLN1	Sig DE	Down in FGCB	talin-1	actin filament binding
WRAP73	Sig DE	Down in FGCB	WD repeat containing, antisense to TP73	Regulation of mitotic spindle assembly
ZDHHC5	Not DE		palmitoyltransferase ZDHHC5	Palmitoyltransferase activity

2.4. Discussion

The hierarchical clustering of the pairwise differential expression analysis, and the 100 top pathway terms shared across the 10 comparisons showed that there is a clear age based clustering pattern; all 42 day old broilers (ARAC) cluster together, and based on the Z-score and P-values, are more similar to WB+ tissue in their gene expression

profiles and the pathways they activate. On the other hand, younger birds (6 and 21 day old) of slow and fast-growth breeds, and older birds of slow-growth breeds cluster together and separately from the 42-day old broilers. Interestingly, these results show that young broilers (Ross and Illinois 6 and 21 day old) and the slow-growth varieties (WC, WRR strains) appear to have a similar gene expression pattern, in contrast to 42-day old broilers. In summary, the comparison of pathways from multiple pairwise comparisons show that age, first, and then growth rate (broiler strain) are the main functional differentiators of WB tissue. The differentiation of the 120+ day old slow-growth broilers and 6 and 21 day old fast-growth broilers is especially notable, as they show that a) molecular signatures associated with WB are unique to older, fast-growth broilers, and b) that 21 day old modern broilers (Ross 708) are less similar to 21 day old Illinois breed than to 42 day old commercial broilers. These two points suggest an age-dependent transcriptome dysregulation in WB, which progresses with age, somewhere between the first and third week of life. This conclusion is similar to that reached by Griffin *et al.* (2018).

While gene expression and ontology analyses show which specific genes and molecular functions are involved in WB, design of appropriate remediation strategies requires a better resolution of the similarity of WB to known diseases. A clear understanding of diseases and conditions explained by gene expression patterns is necessary to narrow down specific endogenous as well as environmental factors driving WB. Specifically, we wanted to answer whether WB tissue expression patterns point to muscle myopathies, or whether such patterns are indicative of other conditions. While

genes important in muscle growth and cell differentiation are up-regulated in WB tissue, the totality of expressed genes and pathways show little support for myopathy as the underlying condition.

One notable cause for concern is the repeated occurrence of regulators and pathways that suggest neoplastic disorders. Upregulation of glycolysis, which was observed as the main pathway classifier in the same-background comparison is considered a "near-universal property" of primary and metastatic cancers (Gatenby and Gillies, 2004; Ganapathy-Kanniappan and Geschwind, 2013; Sajnani et al., 2017; Han et al., 2017). Oxidative stress and impaired glycolysis can both arise due to mitochondrial dysfunction. Multiple studies have confirmed the transcriptomic (Mutryn et al., 2015; Zambonelli et al., 2016) and metabolomic signatures of oxidative stress in WB (Abasht et al., 2016), and have also suggested mitochondrial dysfunction (Kong et al., 2017). Furthermore, genes important in glycolysis, angiogenesis, and apoptosis (up-regulated in WB) are transcriptionally regulated in hypoxic conditions (Brown and Wilson, 2004; Pouysségur et al., 2006), that are typical of tissue under oxidative stress.

The individual and comparison pathway analyses provided many of the same terms as being important among comparisons. The top pathways based on activation Z-score were T-cell receptor signaling, CD28 signaling in T-helper cells, and signaling by Rho family of GTPases. CD28 is involved in stimulation of T-cell activation and 23 molecules involved in this pathway were identified in the transcriptome data. CD28 signaling is involved in glucose metabolism, activation of T-cells, and costimulation of immune responses (Boomer and Green, 2010). T-cell receptors bind to antigenic

peptides presented by antigen presenting cells and are known to respond to various signal transduction pathways. They may be invoked to regulate cell proliferation, apoptosis, and cytotoxic killing (Mitsiades et al., 2002; Zhou et al., 2007). These processes may be activated in woody breast in response to apoptosis and necrosis occurring in breast tissue. Finally, the Rho family of GTPases are involved in regulating various processes, including reorganization of the actin cytoskeleton in response to growth factors and cytokines (Moorman et al., 1999; Kjoller and Hall, 1999; Schmitz et al., 2000). These pathway terms indicating abnormal expression patterns that together affect cell signaling, cytoskeletal organization, and inflammation have all been identified as features of WB. As it has been previously shown through histological and enzymatic assay that WS/WB does not have an infectious origin (Kuttappan et al., 2013), these immune responses are likely directed against endogenous cell proliferation and apoptotic processes (resembling neoplasms).

The disease terms from the multi-group comparison analysis in IPA also found the same top conditions as those found in pairwise comparisons. Many of these terms also invoke the digestive system (intestine, colon, liver, abdomen etc.), which is surprising considering all the analyses were based on differentially expressed genes in the pectoralis major tissue. While pathway analysis relies on over-representation or functional class scoring, they still rely on accurate annotations, cell specific information, and well described pathways for the accuracy of results (Khatri et al., 2012; García-Campos et al., 2015). Therefore, while it is possible that other organs may be involved, validation of that question will depend on additional sampling and wet-lab based

approaches. Other locations of organismal injury notwithstanding, these terms still suggest some important syndromes that can be considered very concerning. The major diseases and disorders explained by expression patterns also suggest abnormal cell proliferation and cell signaling mechanisms. The comparative analysis showed that over 80% of the top diseases identified by the pathway analyses indicate tumors, cancers and neoplastic conditions. Even as some caution is necessary in interpreting pathway analysis for chicken datasets, due to the majority of pathways described being from mammalian models, it has been shown repeatedly that pathway signatures do predict disease outcomes accurately based on shared molecular features (Yu et al., 2007; Ma and Kosorok, 2010; Aran et al., 2015; Shchetynsky et al., 2017). The reasons and basis for this similarity of WB to neoplastic disorders deserves further investigation.

Top canonical pathways emerging from the comparison between FGCB and SGHB groups indicated altered activity of multiple serine/threonine kinases including polo-like kinase (Plk), integrin-linked kinase (Ilk), and extracellular signal regulated kinase/mitogen-activated protein kinase (Erk/Mapk). All three pathways are implicated in the regulation of the cell cycle and cell survival. Specifically, Plk is induced by mitogens and is most abundant during metaphase of mitosis with activities including chromosome segregation, centrosome maturation and spindle assembly (Nigg, 1998; Liu and Erikson, 2003). Plk also functions in other stages of mitosis including inactivation of the anaphase-promoting complex and regulation of nuclear envelope breakdown during prophase (Nigg, 1998; Liu and Erikson, 2003; Strebhardt and Ullrich, 2006). It has also been shown that depletion of Plk in cancer cells induces apoptosis and it is now

considered a high potential target for intervention (Liu and Erikson, 2003; Strebhardt and Ullrich, 2006). Ilk impacts the cell cycle and survival through activation of key signaling pathways and stimulation of downstream effector proteins, while Erk/Mapk is similarly involved by activating a number of growth factors, cytokines and transcription factors (Bonni et al., 1999; Wu and Dedhar, 2001; Zhang and Liu, 2002; Qian et al., 2005). Erk/Mapk further promotes cell survival by phosphorylating and thus inhibiting the pro-apoptotic protein BAD (Bcl2 associated agonist of cell death) while also inducing the expression of cell survival genes (Bonni et al., 1999). Finally, of considerable interest is the ability of Ilk to anchor actin filaments to cell matrix contact sites, regulating changes in cell shape, cell migration, cell adhesion, as well as the ability to phosphorylate myosin in smooth muscle cells (Wu and Dedhar, 2001; Qian et al., 2005). Each of these pathways plays an essential role in the regulation of cell proliferation, maintenance, and death which are all physiological activities that have been identified as perturbed in the WB condition and thus this analysis provides a focused framework for further investigating the underlying mechanisms of the condition.

2.4.1. Molecular Basis of Wooden Breast

In studies of WB, it has been observed that few live bird or carcass quality variables are accurately predictive of the presence of WB (Athrey et al, unpublished). For example, in replicate flocks of broilers of the same breed raised under identical conditions, no combination of rearing or dietary variables has been found to prevent WB occurrence (Trocino et al., 2015; Radaelli et al., 2017). Furthermore, the severity of WB varies within the same flock under identical conditions (Kuttappan et al., 2013). Based

on these observations, it appears likely that nutritional interventions, while perhaps useful for ameliorating severity, may be of limited utility in eliminating wooden breast. On the other hand, these data suggest a genetic basis underlying WB. This hypothesis has received recent support with the identification of QTL for WS (Pampouille et al., 2018). While the relationships between WB and WS are not fully resolved, the co-occurrence of these two conditions make it more likely that WB has a genetic basis.

In this study we identified highly expressed genes that contained high-impact variants. This small subset of genes is involved in important cell proliferation and signaling functions. The identification of genes with high-impact variants that are also differentially expressed point towards a genetic basis that links the changes at the DNA level to functional expression. Such DNA variants, that are associated with expression differences are called Cis-acting regulatory variants, and are known to explain a substantial amount of phenotypic variation, as well as having a role in disease etiology (Pastinen and Hudson, 2004; Ongen et al., 2014; Brandler et al., 2018). In this study we identified a total of 44 high-impact variants, of which 21 were also significantly differentially expressed genes. While not all of these 21 genes may be directly affecting WB occurrence or severity, their expression patterns and the type of SNP modification make their involvement in WB highly probable. Particularly noteworthy genes identified in this analysis were MYH1A, MYH1B (high-impact variants in SGHB), the pair of which are myosin heavy chain genes. Both of these genes had ‘splice acceptor variants’ that may result in a different mature mRNA product and protein. These genes are members of a larger group of myosin genes which regulate development and function of

avian skeletal muscle (Camoretti-Mercado et al., 1993). Interestingly, MYH1B was also identified as a candidate gene for white striping in a QTL mapping study by Pampouille *et al.* (2018).

Another pair of notable genes with high-impact variants were DST (dystonin) and TMEM108 (Transmembrane protein). DST was modified in SGHB, whereas TMEM108 was modified in FGCB. DST is a cytoskeletal linker protein, which is involved in collagen trimerization and formation of scar tissue following injury (Dalpé et al., 1998). DST is known to interact with TMEM108, a gene that regulates the stability of microtubules, by recruiting TMEM108 for the transport of endosomal vesicles (Liu et al., 2007). Mutations in the DST gene have been identified as being responsible for hereditary neuropathy and dystonia (abnormal muscle tone) in mouse models (Ferrier et al., 2015). The high-impact variants and differential expression of these genes may be associated with the rigidity, collagen content, and microscopic features observed in WB.

While the short list of genes identified in this study have a high likelihood of being explanatory of WB, and even as potential diagnostic biomarkers for the condition, we stop short of calling these candidate genes; population level analyses such as association testing (GWAS) would be necessary to confirm if variants in these genes are causative of WB. However, these findings do lend support for a polygenic basis for WB, but perhaps occurring in conjunction with regulatory mechanisms that are yet to be identified and confirmed. Whether a putative genetic basis for wooden breast can be traced to linked loci under selection for growth traits or is a result of *de novo* mutations and structural variants, remains to be established.

The high frequency of WB within flocks is suggestive of underlying causes that are not highly variable among individuals of a flock. Any variation in severity could, therefore, be a result of particular genotypes, and the resulting allele-specific growth traits and nutritional interactions. Such a pattern is consistent with multi-genic traits, alleles for which may segregate in populations as heterozygotes, and the occurrence and severity of the condition may be driven by allele specific expression patterns (Dermitzakis, 2008; Lappalainen, 2015). A similar pattern would also be explained by *de novo* mutations.

The results from our multi-sample comparison analysis show that WB has an age-dependent expression pattern, with molecular signatures and phenotypic markers becoming more obvious in older birds. Such a phenomenon, called age-dependent penetrance, has been frequently observed in various heritable diseases. Recent studies have shown that some features of WB are observable as early as 2 weeks of age (Papah et al., 2017; Griffin et al., 2018), and therefore would explain our observation of 21-day old Ross birds being more similar to the older WB affected group. The inherited human neurodegenerative disorder, Huntington's Disease, is known to manifest in middle to later life, and gene expression studies show age-dependent expression and dysregulation of various signaling genes (Nguyen et al., 2008). Age-dependent disorders may also include heritable genetic mechanisms such *de novo* mutations, or as somatic mutations (Veltman and Brunner, 2012; Goldmann et al., 2016; Acuna-Hidalgo et al., 2016) that may affect genome organization, or repair mechanisms and increase penetrance of diseases in later life (Zane et al., 2014; Win et al., 2017). Further investigations of

genome organization, frequency of *de novo* mutations, or breakdown of repair mechanisms in fast-growth broilers is necessary to illuminate whether and how these processes may be important in WB.

2.5. Conclusions

Our study used transcriptomic datasets to compare pectoralis tissue from commercial broilers with wooden breast against multiple genotypic backgrounds and confirmed the previously reported molecular signatures in addition to previously unreported molecules and pathways. The comparison of tissue from fast-growth genetic backgrounds to those from slow-growth genetic backgrounds and different age classes suggests a genetic basis for WB that elicits age-dependent expression patterns in fast-growth broiler strains. The functional analyses of pathways from comparative data suggest that WB is a potentially polygenic, complex syndrome, with molecular similarities to neoplastic disorders. Through analysis of high-impact variants among the studied breeds, we identified a short list of genes with high-impact variants that are also significantly differentially expressed, suggesting Cis-Regulatory processes involving important developmental and cytoskeletal genes. This result underscores the need for deeper analyses to investigate the role of these genes, basis of these disease pathways, and similarities to complex disorders.

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3. TRANSCRIPTOMIC SIGNATURES OF WOODEN BREAST AND PERFORMANCE TRAITS OF BROILERS IN RESPONSE TO DIETARY OMEGA-6:3 RATIOS

3.1. Background

Poultry plays a major role in reducing global hunger through both meat and egg production, due to the ease of production and smaller environmental footprint compared to other livestock species. Over roughly the last 60 years, selection for body weight, feed efficiency and carcass traits, and improvements in nutrition and management strategies have greatly increased its production efficiency, expanding its availability and reducing cost. With these improvements, however, a variety of growth-rate related health and welfare issues have arisen. Specifically, breast muscle myopathies, a collection of conditions which decrease meat quality of the highest value portion of the carcass, have risen to an annual economic loss of roughly $200 million in the U.S. alone, with incidence rates continuing to increase (Kuttappan et al., 2016; Cruz et al., 2017). Currently, several breast muscle myopathies are affecting the poultry industry including wooden breast, white striping, and spaghetti meat (Baldi et al., 2018; Soglia et al., 2019; Petracci et al., 2019).

Myopathies are generally classified as localized disorders of the skeletal muscles, demonstrating cell structure and metabolic impairment, resulting in macroscopic symptoms and muscle dysfunction. In the case of a recent breast muscle myopathy called wooden breast (WB), these macroscopic symptoms include abnormal hardness, petechiae, and a viscous exudate resulting in increased shear force, reduced water

holding capacity, grainy texture (Sihvo et al., 2014; Velleman and Clark, 2015; Soglia et al., 2016; Clark and Velleman, 2016; Kuttappan et al., 2017b; Tasoniero et al., 2017; Meloche et al., 2018a). Molecular aspects most observed include inflammation, oxidative stress, and dysregulated glucose metabolism (Mutryn et al., 2015; Abasht et al., 2016; Sihvo et al., 2017; Kong et al., 2017; Kuttappan et al., 2017a; Papah et al., 2017, 2018; Cai et al., 2018; Hubert et al., 2018). These changes have led to a reduction in consumer acceptance as well as USDA FSIS requirements for removal of inflammatory tissue associated with these conditions (Brambila et al., 2017; Velleman et al., 2018; Aguirre et al., 2018; Soglia et al., 2018; Maxwell et al., 2018; USDA FSIS, 2018). WB is now being observed worldwide in commercial broiler flocks at high incidence - from 85% incidence in the U.S., 15% in Brazil and 60% in Italy (Mutryn et al., 2015; Abasht et al., 2016; Clark and Velleman, 2016; Sihvo et al., 2017; Cruz et al., 2017; Kuttappan et al., 2017b; Petracci et al., 2019). Understanding the molecular development of WB is necessary in order to determine methods of reducing the incidence and decreasing the economic impacts of this condition. Numerous investigations have observed deficiencies, toxicities, exercise induction and hypoxia as a means to identify the underlying pathology of WB, but definitive solutions remain elusive.

Transcriptome, proteome, and metabolome studies have demonstrated that WB is ubiquitous in fast-growth commercial lines (Mutryn et al., 2015; Velleman and Clark, 2015; Trocino et al., 2015; Abasht et al., 2016; Clark and Velleman, 2016; Kong et al., 2017; Kuttappan et al., 2017a; Schilling et al., 2017; Cai et al., 2018; Hubert et al., 2018;

Livingston et al., 2019b) (Hubert et al., unpublished data). Specifically, comparisons between fast- and slow-growth genetic backgrounds, and of several age categories, have demonstrated an age-dependent gene expression pattern unique to commercial fast-growth lines (Kong et al., 2017; Hubert et al., 2018). The history of broiler myopathies has shown that, in addition to the decrease in time to slaughter age and increased breast muscle size, muscle fibers of fast-growth commercial birds are three to five times larger than that of slower growing birds, have reduced connective tissue spacing between myofiber bundles, increased degeneration of myofibers, and decreased capillary blood supply to the pectoralis major and minor (Remignon et al., 1995; Mahon, 1999; Mitchell, 1999; Dransfield and Sosnicki, 1999; MacRae et al., 2006; Fanatico et al., 2007; Petracci and Cavani, 2012; Velleman and Clark, 2015). These features are exacerbated in WB affected birds (WB+), but have been observed in unaffected birds, and also in those given a WB score of zero (WB-) (Kuttappan et al., 2013b; Mazzoni et al., 2015; Velleman and Clark, 2015; Trocino et al., 2015; Clark and Velleman, 2016; Sihvo et al., 2017; Velleman et al., 2018). Furthermore, these studies have all identified molecular indicators of inflammation and immune response.

To date, the features of WB has not been found in slow-growth broiler and layer lines (Velleman and Clark, 2015; Clark and Velleman, 2016; Kong et al., 2017; Velleman et al., 2018; Hubert et al., 2018). The high frequencies (>85%) of WB in commercial broiler genotypes under standard rearing conditions mean that the same genotypes are not suitable as a control group (Velleman and Clark, 2015; Kong et al., 2017; Papah et al., 2017; Hubert et al., 2018)(Hubert et al., unpublished data). Various

lines of evidence show that WB- are asymptomatic rather than devoid of the condition. The data from these previous studies indicate that WB is genetic in origin and little impact to incidence can be made through altered management practices without a reduction in growth rate. Therefore, broiler genotypes that are not selected for high feed conversion ratio (FCR) are likely to offer insights into the causative mechanisms. Based on the observations of oxidative stress and muscle fiber degradation in WB, many proposed nutritional interventions include altered amino acid levels and antioxidant compounds like vitamin C (Cruz et al., 2017; Bodle et al., 2018; Meloche et al., 2018c; Livingston et al., 2019a). Inflammation and oxidative stress have been the focus of recent investigations of mechanism, as well as the search for solutions. As these features of WB can be regulated both by the genotype, as well as dietary factors, in this study, we investigated the relative contribution of diet and genotype. In case of the diet, we focused on fatty acids that have a role in ameliorating inflammation, while at the same time comparing fast and slow growth genotypes.

The omega-6 (ω-6) and omega-3 (ω-3) polyunsaturated fatty acids (PUFAs) are well known essential fatty acids (EFAs) and their physiological impacts are well studied in both human and animal health. The ω-6 and ω-3 PUFAs are best known for their modulation of inflammation, with ω-6 PUFAs being pro-inflammatory while ω-3 PUFAs are anti-inflammatory. These impacts on inflammation are elicited through the production of prostaglandins and leukotrienes and the regulation of gene expression either by indirectly altering signaling pathways which initiate at the plasma membrane or by directly interacting with nuclear receptors (Calder, 1998, 2001, 2003, 2006; Miles

and Calder, 1998; Simopoulos, 2002; Stulnig, 2003; Gómez Candela et al., 2011; Komprda, 2012; Wiktorowska-Owczarek et al., 2015). The standard high-energy corn-soy based broiler diets are high in ω-6 PUFAs but low in ω-3 PUFAs (National Research Council et al., 1994; Aviagen, 2014a; b; Cobb, 2015, 2018). The NRC Nutrient Requirements of Poultry (1994) recommends the ω-6 Linoleic acid (LA) as 1% of the diet but has no recommendation for the ω-3 œ-Linolenic acid (ALA) (National Research Council et al., 1994). These two EFAs often have antagonistic physiological activities and are metabolically competitive, but numerous studies have demonstrated that they are both necessary for normal growth and development and that the ratio in which they are found in the diet plays an important role (Simopoulos, 2002, 2010, 2016; Burdge and Calder, 2005; McNamara et al., 2007; Gómez Candela et al., 2011; Cherian, 2015; Koppenol et al., 2015; Pusceddu et al., 2015; Jeromson et al., 2015; Dias et al., 2015; Wiktorowska-Owczarek et al., 2015; Nobili et al., 2016; Baker et al., 2016; Moatt et al., 2017).

To our knowledge no studies have addressed the impact of the ratio of ω-6:3 on inflammation in relationship to WB. However, several have investigated the physiological functions of PUFAs in chicken, mainly to increase the EFA composition of poultry products for human consumption (Ratnayake et al., 1989; Lin et al., 1989; Fritsche et al., 1991; O'Keefe et al., 1995; Rymer and Givens, 2005; Haug et al., 2007; Betti et al., 2009; Zuidhof et al., 2009; Dikshit et al., 2015; Cherian, 2015; Koppenol et al., 2015; Carragher et al., 2016; Ravindran et al., 2016; Willson et al., 2017).

Furthermore, comparative analyses of the impact of dietary ω-6:3 ratios on the body weight and gene expression of both a fast-growth commercial broiler and a slow-growth heritage broiler would provide useful insights into the genetic impacts of selection for broiler performance traits. For this aspect of the study, we compared the fast-growing commercial broiler (Ross 708 breed) against the White Plymouth Rock (WPR) breed. The WPR is a slow-growth heritage broiler is a foundational breed for the modern commercial broiler, picked for its large final body size and white feathering and crossed with the Cornish for its large muscles and wide-set legs. Although the growth-rate of WPR is very slow compared to that of the modern broiler, which reaches slaughter weight around 6-8 weeks, its final body size is similar at 3-4 kg. The WPR have not been selectively bred in recent years and are typically seen only in backyard flocks, keeping their genetic variation similar to those used in the original cross. Therefore, we expect the WPR to be a genetic contrast where there is a divergent selection for fast growth (feed conversion ratio), but not on final body weight. This study aimed to utilize comparative analyses between fast-growth commercial broilers and their progenitor, the WPR, to filter out body weight specific gene expression, measure the impact of nutrition, and narrow down on genes associated with WB.

3.2. Materials and Methods

3.2.1. Animals

In this study, we used Ross 708 broilers to represent the fast-growth commercial broiler chickens and White Plymouth Rock heritage broiler chickens. A total of one hundred and twenty straight run birds of each breed were divided randomly into two

treatment groups - a high ω-3 diet, a low ω-3 diet - and a control group. All birds were raised in a single open-sided barn at the Texas A&M University Poultry Center in floor pens. Pens were divided by breed and diet, in a randomized block design with duplicate pens for each group. Food and water were provided *ad libitum*, with birds reared under an industry standard lighting program as recommended by the Aviagen Broiler Handbook (Aviagen, 2014b).

3.2.2. Diet

Experimental diets were formulated based on the Aviagen Ross 708 nutritional guidelines (Aviagen, 2014a). Starter, grower, and finisher feeds were calculated to maximum energy and nutritional requirements. Treatment diet formulations were energetically equivalent to the control, with the replacement of soy oil with canola oil or a canola/soy blend in order to alter the ω-6:3 ratio of each diet. Nutrient composition and calculation of the ω-6:3 ratio of the diet was based on the USDA ARS National Nutrient Database for Standard Reference (USDA ARS, 2016a; b; c; d). A complete basal diet formulation is included in Table A-1. Specifically, the ω-6:3 ratio of the control diet (C) which contained only soy oil was calculated as 20:1, while treatment diet 1 (T1) which contained only canola oil was calculated as 5:1 and treatment diet 2 (T2) which contained both soy and canola oil was calculated as 11:1 (Table A-2). Starter, grower, and finisher phase diets were made in individual batches roughly three days before the feed change date. Feed was delivered in hanging feeders, and birds were checked on twice daily.

3.2.3. Tissue Collection

We sampled birds for dissection and WB scoring at two-time points until the age of market weight - namely at day 11 and day 42. We chose these intervals to observe the effects of age and body weight on gene expression in addition to the impact of the treatment diets. WB was scored by a single trained individual on a scale of 0-3, with 0 having no macroscopic signs of WB and 3 demonstrating severe WB. At the time of euthanasia, ten birds were haphazardly selected from each breed and treatment group and CO_2 gas was administered according to AUP IACUC 2016-0065.

Immediately following euthanasia, individual body weights were recorded, and then each sampled bird was scored for WB through visual appraisal and physical palpation of the excised pectoralis major. These methods of examination are widely accepted and utilized as an adequate means of determining the presence of WB in a variety of investigations (Kuttappan et al., 2013a; b, 2016; Sihvo et al., 2014, 2017, 2018; Mutryn et al., 2015; Tijare et al., 2016; Cruz et al., 2017; Papah et al., 2017; Cai et al., 2018; Aguirre et al., 2018; Maxwell et al., 2018; Norring et al., 2018). Breast tissue samples were collected using sterile dissection procedures within 30 minutes of euthanasia and stored in RNALater following the manufacturer's protocol (Ambion Inc, ThermoFisher Scientific) at a 5:1 ratio. Tissue samples were then stored at 4°C for a minimum of 24 hours, removed from the RNALater, and stored at -80°C until RNA isolation.

3.2.4. RNA Extraction and Quality Control

Total RNA was extracted using TRIzol Reagent following the manufacturer's protocol (ThermoFisher Scientific, Waltham, MA). Quality was assayed with the Agilent

Bioanalyzer 2100 RNA 6000 Nano chip kit (Agilent Technologies, Santa Clara, CA). Only samples with RNA Integrity Number (RIN) $\geq$ 8 were used for library prep. RNA isolates of sufficient quality were quantified with the Qubit Fluorometer (ThermoFisher Scientific). Also, DNA contamination was estimated using the Qubit dsDNA Broad Range analysis kit before further processing.

3.2.5. RNA Sequencing

Library preparation for RNA sequencing (RNAseq) with Illumina was performed in-house with the Lexogen QuantSeq 3'mRNA Library Prep Kit (Lexogen, Vienna, Austria). For each library 2 μg of total RNA was used, and a total of 96 single-indexed libraries were prepared. The quality of library generation was checked with the Agilent TapeStation D1000 DNA ScreenTape and concentration was determined using the Qubit dsDNA High Sensitivity Kit (ThermoFisher Scientific). 72 libraries (n=6) were of sufficient quality and quantity for sequencing. Individually barcoded libraries were pooled in equimolar proportions and submitted to the Texas A&M University Institute for Genome Sciences and Society (TIGSS, College Station, TX) for sequencing on the Illumina NextSeq (Illumina, San Diego, CA) platform. Single end reads of 75 bp were generated with an average of 4.8 million reads. The R package ssizeRNA (1.3.1) was used to perform a Power Analysis for this study, which showed that the replication was sufficient to provide in 99% power at FDR $\leq$ 0.05 (Bi and Liu, 2016).

3.2.6. Data Analysis

Individual body weights for each breed and diet treatment from each dissection age were evaluated by one-way ANOVA (RStudio Team, 2015; R Core Team, 2019). We

performed a Wilcoxon signed-rank test to determine significant differences in WB severity and a chi-square test for WB incidence with the MASS package (version 7.3-51.3) in the R statistical platform (Venables and Ripley, 2002; RStudio Team, 2015). For both analyses, we considered differences to be statistically significant at p-value < 0.05.

RNAseq data obtained from TIGSS was quality checked with FastQC version 0.11.6 and MultiQC version 1.4.Lexogen specific adapter sequences were trimmed with Trim_Galore version 0.4.3 (Martin, 2011; Ewels et al., 2016; Babraham Institute, 2018a; b). Also, reads shorter than 35 bp or with an average quality score less than Q30 were removed. After quality check, four samples showing higher than 75% sequence duplication were removed from further analysis (WPR 11d: 13 T2 - 82%, 30 C - 75.4%; R708 11d 51 T1 - 82%; WPR 42d 208 C - 81%). The remaining samples were aligned to the *Gallus gallus* genome (Version 4.8, Ensembl Release 85, July 2016) with the short-read de-novo splice mapper STAR (version 020201) and reads mapping to exons were counted with HTseq-Count (version 0.9.1) (Dobin et al., 2013; Anders et al., 2015; Dobin and Gingeras, 2015). The EdgeR program (version 3.22.1) in the R statistical platform (3.5.2) was used for statistical analysis of differential gene expression (Robinson et al., 2010; McCarthy et al., 2012; RStudio Team, 2015; R Core Team, 2019). Normalization factors were calculated for differences in library sizes and common and tagwise dispersions were estimated (GLM). The likelihood ratio test 'glmLRT' function was used to test for significant differential expression between

groups at an FDR < 0.05. Genes identified as significantly differentially expressed were further investigated through gene ontology and pathway analysis using the Ingenuity Pathway Analysis (IPA) program (Qiagen, Hilden, Germany). Differentially expressed (DE) genes for each comparison were also filtered into unique and common genes between comparisons using Venny 2.1.0 in order to identify breed, age, and WB specific gene expression (Oliveros, 2007). These data sets were then annotated with IPA.

3.3. Results

3.3.1. Body Weight and Wooden Breast Incidence and Severity

The ω-6:3 ratio (represented by C, T1, T2 diets) did not have a significant impact on body weights at sampling time points for either broiler breed based on a one-way ANOVA (Figure 3.1). The body weights showed high consistency within the control and T2 treatments for the R708 broilers, whereas the T1 treatment showed more variability. Body weights were similar for all diets in the WPR broilers. The R708 broiler growth-rate matched the performance expected in Ross 708 Broiler: Performance Objectives which is indicated by the red line in Figure 3.1 ($\chi^2 = 0.064$, $P = 0.8$) (Aviagen, 2014c). The most recent performance objective manual for WPR found was published in 1926 by University of Illinois, and the average body weight at that time for similar ages is shown by the blue line in Figure 3.1 (Mitchell et al., 1926). The WPR broilers in this study averaged 107g at 11 days of age and 480 g at 42 days of age, indicating that the growth-rate of WPRs has increased since the 1920s ($\chi^2 = 11.004$, $P= 0.0009$).

WB incidence was significantly different at 21 and 31 days of age between the three diets, based on a χ^2 test ($P < 0.01$), but not at 42 days of age, as all diets were at 100%

incidence in the Ross 708 breed. At no time point did the WPR breed demonstrate any of the diagnostic criteria for WB, whereas the R708 broilers had a WB incidence of zero for all diets only at 11 days of age. WB severity varied between both ages and diets, but at no time point was there a significant difference in severity of WB between the diets based on Wilcoxon signed-rank tests for each age (Figure 3.2). WB percent incidence by age and diet for each breed is displayed in Table 3.1.

Figure 3.1 Box plots demonstrating the body weights in grams for WPR and R708 at 11, 21, 31, and 42 days of age for each diet. Growth-rate for R708 matched that specified in the Aviagen management guide (red line) and WPR exceeded its published growth-rate from 1926 (blue line).

Figure 3.2 Violin plot of WB severity (scored 0-3) by diet at 21, 31, and 42 days of age. At no point was severity significantly different between diets. Only R708 are represented as WB did not occur in WPR.

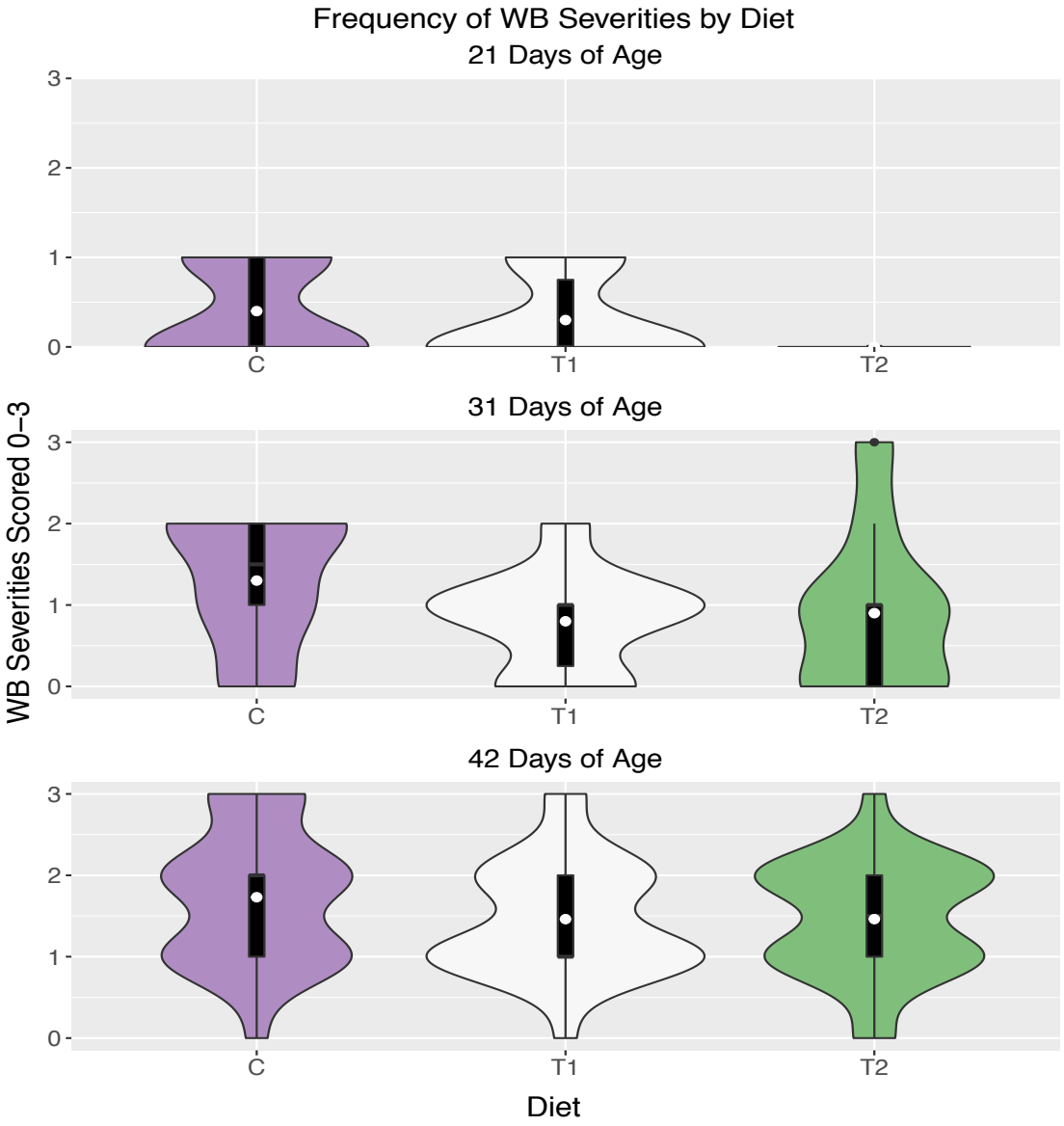


Table 3.1 Summary table of percent incidence of WB for each breed and diet at 11, 21, 31, and 42 days of age.

WB Incidence (%)				
Breed	Age (days)	T1	T2	C
WPR	11d	0	0	0
	21d	0	0	0
	31d	0	0	0
	42d	0	0	0
R708	11d	0	0	0
	21d	30	0	40
	31d	70	60	80
	42d	100	100	100

3.3.2. Pairwise Comparisons of Differential Gene Expression

Global analysis of expression counts data for the entire transcriptome dataset (all breeds, diets, and time points) had a low common dispersion estimate of 0.15 (Robinson et al., 2010; McCarthy et al., 2012; Anders et al., 2013). Estimates of tagwise dispersion showed that 75% of the data had a biological coefficient of variation (BCV) under 0.76 (Figure B-1), with lowly expressed genes showing higher dispersion. A total of 12,135 total genes were detected (at CPM >1), and across our comparisons we saw from 1 - 29% of DE genes. All the comparisons between ages, or groups showed hundreds of differentially expressed genes, whereas limited or no differences in gene expression (FDR < 0.05) were seen when comparing between diets within each age and within each breed (i.e. WPR 11d T1 v C, and etc.) (Figure B-2). For this reason, these results are not discussed further.

A summary of differential gene expression for all other comparisons made is presented in Table 3.2. Figure 3.3 shows the comparison of gene expression between breast tissue sampled from birds 11 days of age and 42 days of age for each breed and each diet, e.g. R708 C 11d v 42 (n=~6). For the R708 breed on the control diet, 267 genes were significantly differentially expressed at FDR $\leq$ 0.05 between 11 days of age and 42 days of age (Figure 3.3A). In contrast, the R708 T1 comparison found 618 DE genes between 11 and 42 days of age (Figure 3.3C), which is about 2.5x greater than the DE genes observed for the control diet comparison between ages, and interestingly the R708 T2 comparisons showed 850 DE genes for this comparison.

Similarly, for the WPR, 115 genes and 172 genes were differentially expressed in the C and T1 diets between 11 and 42 days of age respectively (Figures 3.3B&D). Although differential gene expression was expected to be observed in these comparisons due to growth, these results shows a diet x age interaction on gene expression. We found this interactive effect (diet x age) on gene expression to be statistically significant, based on a χ^2 test in both varieties ($P < 0.0001$). In summary, our results show that diet had a differential effect on age-based differential gene expression.

When comparing gene expression between varieties for each diet (n=~12) (Figure 3.4) ignoring age, we found 1,674, 1,337, and 1,218 genes were differentially expressed for T1, T2 and C diets respectively, between WPR and R708. The T1 and T2 diet (Figure 3.4A&B) elicit greater differential gene expression between the two varieties than the control diet (Figure 3.4C), indicating that diet may be uniquely altering gene

expression in each breed. The diet x breed interactive effect was statistically significantly based on χ^2 test (P = 3.16 E -20).

Finally, we compared gene expression between ages within and between broiler varieties (n=~18) (Figure 3.5) - ignoring the diets. This post-hoc comparison was based on the observation that dietary treatments did not elicit dramatic differences in gene expression profiles within ages. This analysis showed 1,169 and 2,425 DE genes between 11 and 42 days of age for WPR and R708 varieties respectively (Figures 3.5A&C) and 2,496 and 3,504 DE genes between WPR and R708 varieties at 11 days of age and 42 days of age respectively (Figures 3.5 B&D).

Table 3.2 Summary table of differentially expressed genes from pairwise contrasts performed among RNAseq data using edgeR.

Contrast	Control	DE Up	DE Down	Total DE	Percent DE
R708 42d C	**R708 11d C**	137	130	267	2
R708 42d T1	**R708 11d T1**	358	260	618	5
R708 42d T2	**R708 11d T2**	537	313	850	7
WPR 42d C	**WPR 11d C**	35	80	115	1
WPR 42d T1	**WPR 11d T1**	61	111	172	1
WPR 42d T2	**WPR 11d T2**	78	219	297	2
R708 C	**WPR C**	740	478	1218	10
R708 T1	**WPR T1**	960	714	1674	14
R708 T2	**WPR T2**	686	651	1337	11
R708 11d	**WPR 11d**	1214	1282	2496	21
R708 42d	**WPR 42d**	2183	1321	3504	29
WPR 42d	**WPR 11d**	488	681	1169	10
R708 42d	**R708 11d**	1450	975	2425	20

Figure 3.3 Mean abundance plots (logFC by logCPM) of differential gene expression by diet for both breeds between 11 and 42 days of age. Purple stars indicate genes FDR < 0.05 while blue lines indicate logFC 2 and -2. Plots A, C, and E represent R708 gene expression, while plots B, D, and F represent WPR gene expression between ages for the C, T1, and T2 diets respectively. R708 showed more differentially expressed genes than WPR, as did T1 and T2 diets.

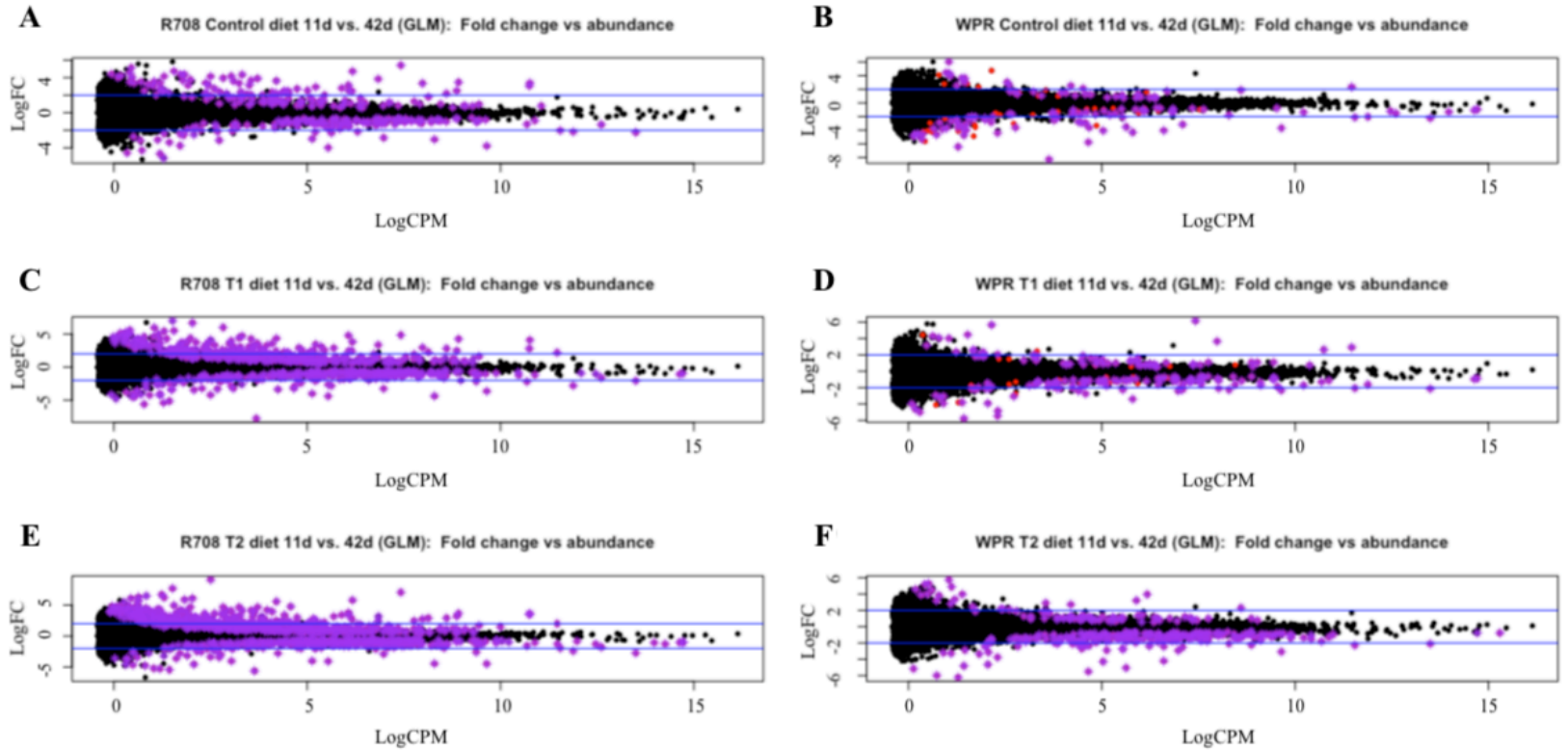


Figure 3.4 Mean abundance plots (logFC by logCPM) of differential gene expression by diet between breeds ignoring age. Purple stars indicate genes FDR < 0.05 while blue lines indicate logFC 2 and -2. Plots A, B, and C represent gene expression due to diet between breeds for the T1, T2 and C diets respectively. The T1 and T2 diets showed more differentially expressed genes than the C diet.

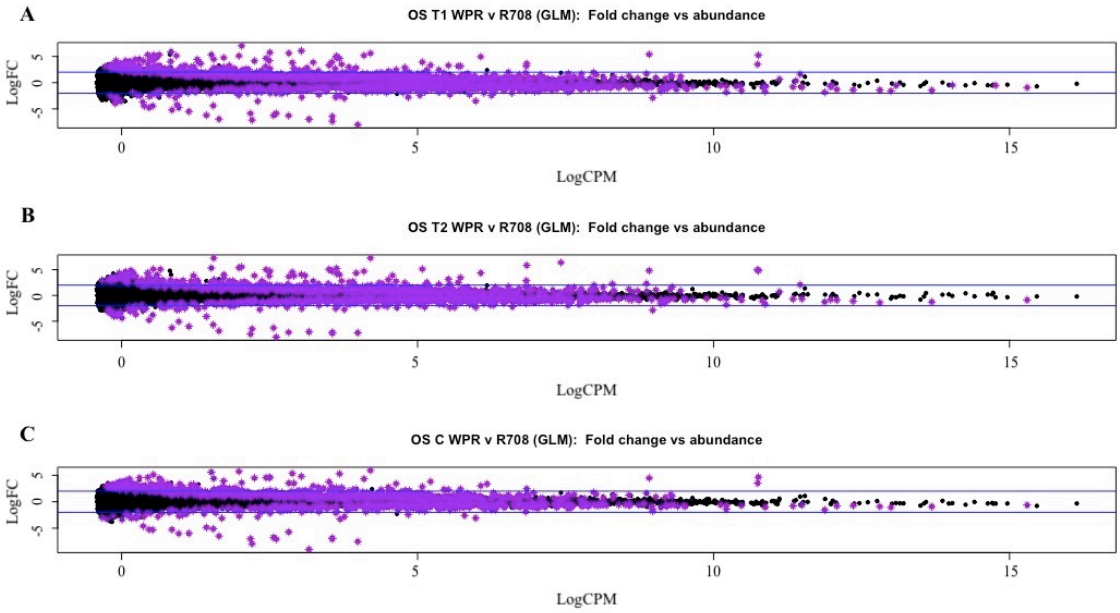
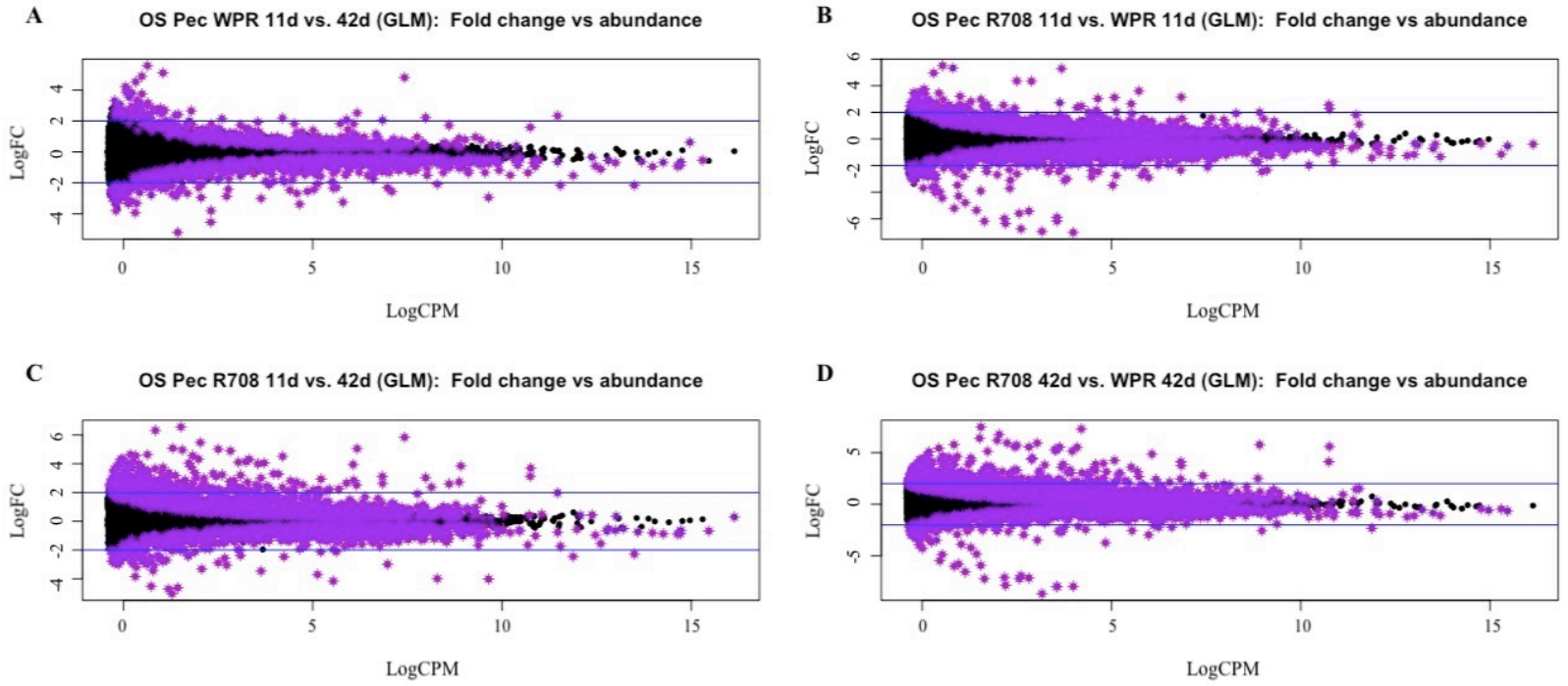


Figure 3.5 Mean abundance plots (logFC by logCPM) of differential gene expression. Purple stars indicate genes FDR < 0.05 while blue lines indicate logFC 2 and -2. Plots A and C represent age-based gene expression by breed, while plots B and D represent gene expression between breeds at 11 and 42 days of age respectively. R708 showed more differentially expressed genes than WPR between 11 and 42 days of age. Also, the 42d of age comparison showed more differentially expressed genes than the 11d comparison.



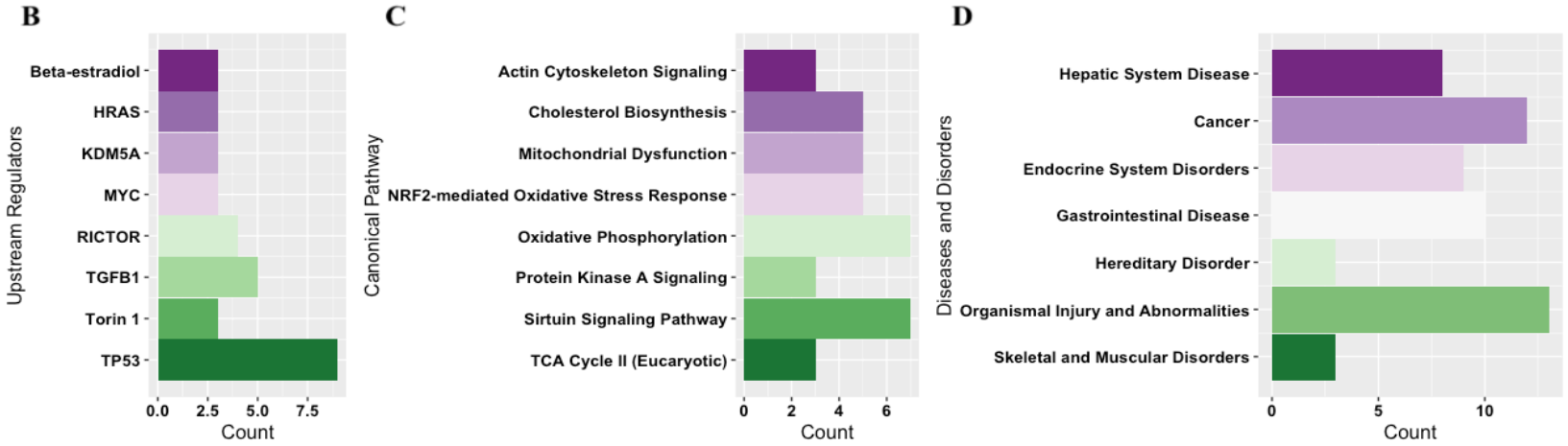
3.3.3. Pathways Activated by Differentially Expressed Genes

The results of differential gene expression analysis (from edgeR) were used in Core Pathway analysis in IPA considering only the DE genes (FDR < 0.05). The Core Pathway analysis returned Top Canonical pathways demonstrating oxidative stress and dysregulated energy metabolism, cell cycle regulation, and immune response. Several of the specific canonical pathways identified were repeatedly observed over all 13 pairwise comparisons. This was also true of the upstream regulators and diseases and disorders identified by IPA (Table A-3). The identified terms from the 13 pairwise comparisons are reported as a histogram (Figure 3.6). The most observed upstream regulators include TP53 and TGFB1 (Figure 3.6B), while the most observed Canonical Pathways are oxidative phosphorylation and sirtuin signaling (Figure 3.6C). Finally, organismal injury and abnormalities, cancer, gastrointestinal disease, and endocrine system disorders topped the disease and disorders list (Figure 3.6D).

Figure 3.6 Summary of the number of differentially expressed genes and core pathway analyses by IPA for each pairwise comparison. A table (A) is used to represent the up, down, and total differentially expressed genes (FDR < 0.05). Histograms (B, C, and D) represent the frequency of results observed from the IPA core pathway analyses as many terms for Upstream Regulators, Canonical Pathways, and Diseases and Disorders, respectively, were repetitive between the pairwise comparisons.

A

Contrast	R708 42d C	R708 42d T1	R708 42d T2	WPR 42d C	WPR 42d T1	WPR 42d T2	R708 C	R708 T1	R708 T2	R708 11d	R708 42d	WPR 42d	R708 42d
Control	R708 11d C	R708 11d T1	R708 11d T2	WPR 11d C	WPR 11d T1	WPR 11d T2	WPR C	WPR T1	WPR T2	WPR 11d	WPR 42d	WPR 11d	R708 11d
Total Obs.							12135						
DE Up	137	358	537	35	61	78	740	960	686	1214	2183	488	1450
DE Down	130	260	313	80	111	219	478	714	651	1282	1321	681	975
Total DE	267	618	850	115	172	297	1218	1674	1337	2496	3504	1169	2425



3.3.4. Unique and Shared Gene Lists for Diet, Age, Breed, Inflammation, and WB

3.3.4.1. ***Comparison of Differential Gene Expression Due to Diet between Broiler Breeds***

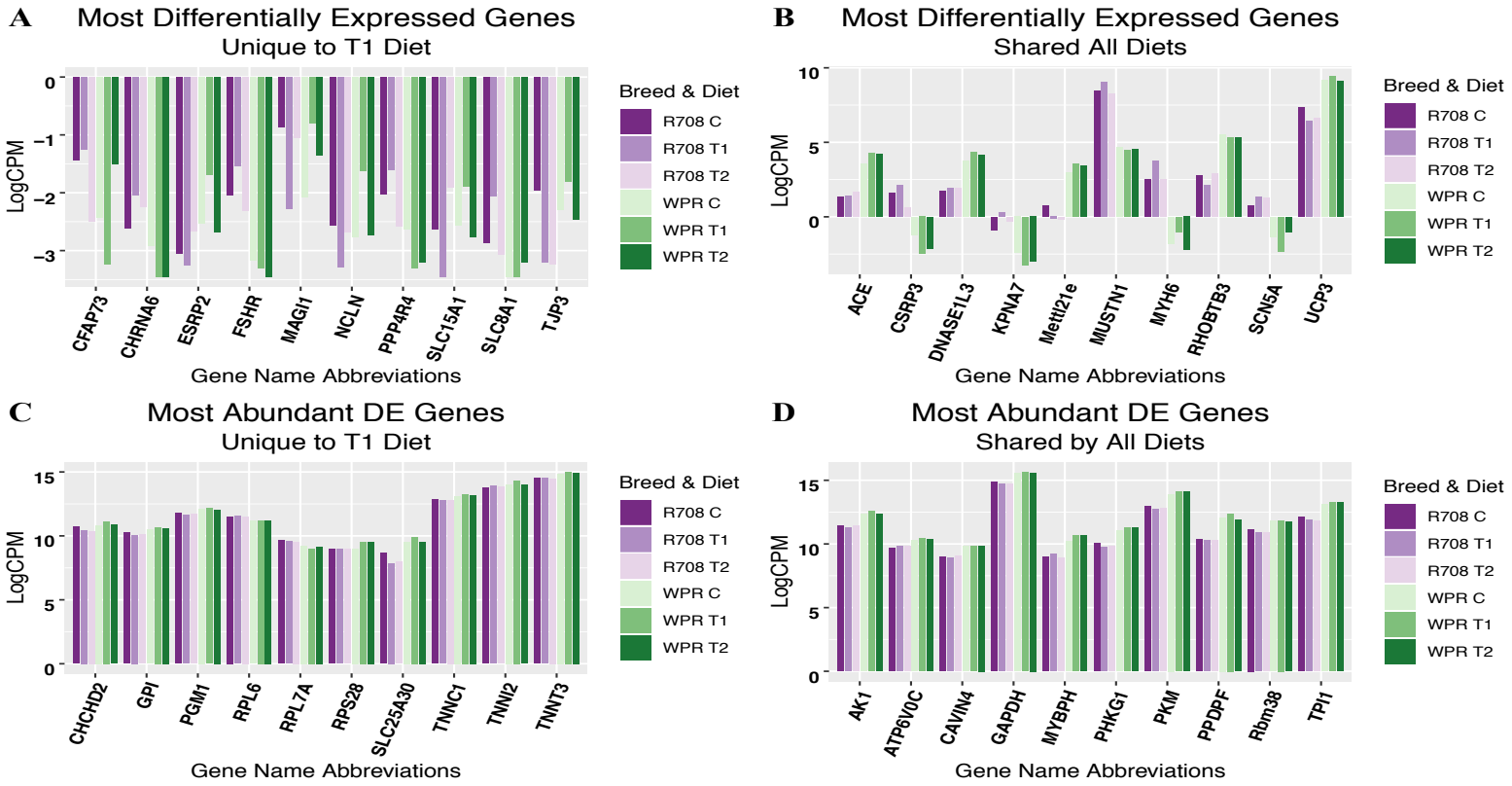
To narrow down the list of genes associated with age, diet, and WB status differences, we generated lists of shared and unique genes from pairwise comparisons. First, we focused on filtering out the genes associated with the impact of diet on gene expression between broiler breed. A three-way comparison (Figure 3.4, WPR v R708 by diet) was used to generate unique and shared gene lists to identify these differences. The 10 most DE (by logFC) and 10 most abundant genes unique to the T1 diet regardless of breed and shared among all diets are shown in Figure 3.7. The shared list helped identify breed-specific gene expression (Figure 3.7B&D).

Of interest in the shared list are multiple genes associated with cellular development and function of skeletal and cardiac muscle (ACE, CSRP3, MUSTN1, MYH6, SCN5A, UCP3, AK1, CAVIN4, MYBPH, PHKG1, RHOBTB1, METTL21E), glucose metabolism (PKM, TPI1, GAPDH, PHKG1), energy metabolism (AK1, UCP3), regulation of the cell cycle and gene expression (RBM38, METTL21E, CSRP3), and immune function (KPNA7, ATP6V0C). Among the members of the shared list, the DE genes were uniformly higher in WPR than in R708 (Figure 3.7D). We also observed that expression (logCPM) in WPR was uniformly higher than R708 breed in members of the shared list (Figure 3.7C), except for RPL7A.

The list unique to the T1 diet (Figure 3.7A&C), regardless of breed, included genes associated with neurotransmission (CHRNA6, FSHR, GPI), cellular structure (CFAP73,

MAGI1, NCLN, TJP3), hypoxia (CHCHD2), glucose metabolism (GPI, PGM1), mitochondrial transport and signaling (SLC25A30, CHCHD2), muscle function (TNNC1, TNNI2, TNNT3), regulation of gene expression (ESRP2, FSHR, PPP4R4, RPL6, RPL7A, RPS28) and cytoplasmic calcium concentrations (SLC8A1). Interestingly, the members of the unique list all showed decreased expression in the T1 diet, regardless of broiler breed (Figure 3.7A).

Figure 3.7 Bar plots showing the (logCPM) ten most differentially expressed and the ten most abundant differentially expressed genes (FDR < 0.05) unique to the T1 diet or shared by all diets between breeds. Plots A and C represent genes which were only differentially expressed in the T1 diet between WPR and R708, while plots B and D represent gene which were differentially expressed in all diets between WPR and R708.



3.3.4.2. ***Differential Expression Patterns in Diet x Age Combinations***

To identify shared and unique DE gene lists based on diet and age combinations, we performed a six-way intersection analysis of the DGE results (Figure 3.3). Seven genes were associated with age in WPR, 124 in R708, whereas 11 were shared in both breeds. These results are summarized in Figure 3.8. Of the 11 DE genes identified as shared between all comparisons, only five were annotated in IPA (Figure 3.8A) and these include genes involved in cytokine control of granulocytes and macrophages and inflammation (CSF2RA), intracellular transport and motility (BC048507), mitochondrial creatine biosynthesis (GATM), regulation of translation (IGF2BP3) and metabolic and growth processes and cardiac function (NRP3). Regardless of broiler breed, expression of BC048507, GATM, and IGF2BP3 decreased with age, while expression of CSF2RA and NPR3 increased with age.

The 10 most DE genes unique to R708 (Figure 3.8B) include activities such as glycosaminoglycan metabolism (CRYL1), histamine metabolism (HNMT) and regulation of inflammation and immune response (PTX3, RGS1), metalloaminopeptidase activity (LVRN), modulation of Rho family GTPases (MCF2), the TCA cycle and the malate-aspartate shuttle (MDH1), muscle structure and function (MYH6, MYOM3, RGS1), and neuronal development (TTLL7). Here too, regardless of broiler breed, the expression of CRYL1, HNMT, MCF2, MDH1, and TTL7 decreased with age, although the decrease of expression is much less pronounced in CRYL1, MCF2, MDH1 and TTL7 for the WPR than the R708. LVRN increased with age regardless of broiler breed, however its increase is more pronounced in R708 than WPR.

MYH6, MYOM3, PTX3, and RGS1 increased with age in R708 broilers but decrease with age in WPR. Of the seven genes identified as unique to the WPR breed, six were annotated (Figure 3.8C) and they all function in cell and muscle development (ACE, FRAS1, MYH2, NRG4, TNMD, TUBA1B). The expression of all these genes (ACE, FRAS, MYH2, TNMD, and TUBA1B) decreased with age regardless of broiler breed, except NRG4 which increased with age in WPR but remains constitutively expressed in R708.

Figure 3.8 Bar plots showing the (logCPM) most differentially expressed genes (FDR < 0.05) between ages unique to each breed and shared between breeds. Plot A represents the five genes which were differentially expressed between 11 and 42 days of age in both breeds, while plots B and C represent the top ten, and six total genes only expressed in the R708 and WPR respectively.

3.3.4.3. ***Differential Gene Expression Related to Performance Traits***

To narrow down on bodyweight and performance related gene expression, the top 100 most variable genes for each breed by age, was merged into a list of the average abundance in logCPM. The absolute value of the difference between day 11 and day 42 was sorted to identify the top 25 highly variable genes for each breed. These 25 candidate genes for each breed were then compared using the merge function from the reshape2 package in R (Wickham, 2007; RStudio Team, 2015; R Core Team, 2019) and confirmed using the Venn diagram tool Venny (Oliveros, 2007) to generate three lists of candidate performance-associated genes. In total, 18 genes were identified for each breed as "unique" or not being shared, while they shared seven genes (shared). Interestingly 13 of the 18 genes unique to R708 are related to cell growth and differentiation, muscle function and development, or intracellular ion concentration while only 8 out of the 18 unique WPR genes are related to the same biological functions. It is also to be noted that 6 of the 7 shared top 25 most highly variable genes between the two breeds are related to these biological functions as well (Table A-4).

The boxplots of gene abundance (Figure 3.9) show that the 7 shared genes exhibit a decreasing trend between day 11 and day 42 while the median of the same genes in the WPR remains approximately the same but has a smaller range at day 42 than on day 11 (Figure 3.9A). It is also of interest to note that he unique R708 genes demonstrate an overall increasing abundance trend from day 11 to day 42 (Figure 3.9B), while the unique WPR genes exhibit an overall slightly decreasing abundance trend. The boxplots of the absolute value of the difference between days 11 and 42 for breed (Figure

3.10A&B) show that for both the shared and unique genes the median logCPM for the R708 is at least 0.75 logCPM greater than that of the WPR.

Figure 3.9 Box plots of shared and unique broiler performance-associated gene abundance (logCPM) in both breeds at 11 and 42 days of age. Plot A shows the expression variance of the genes which were differentially expressed in both breeds while plot B shows the expression variance of genes which were differentially expressed unique to each breed.

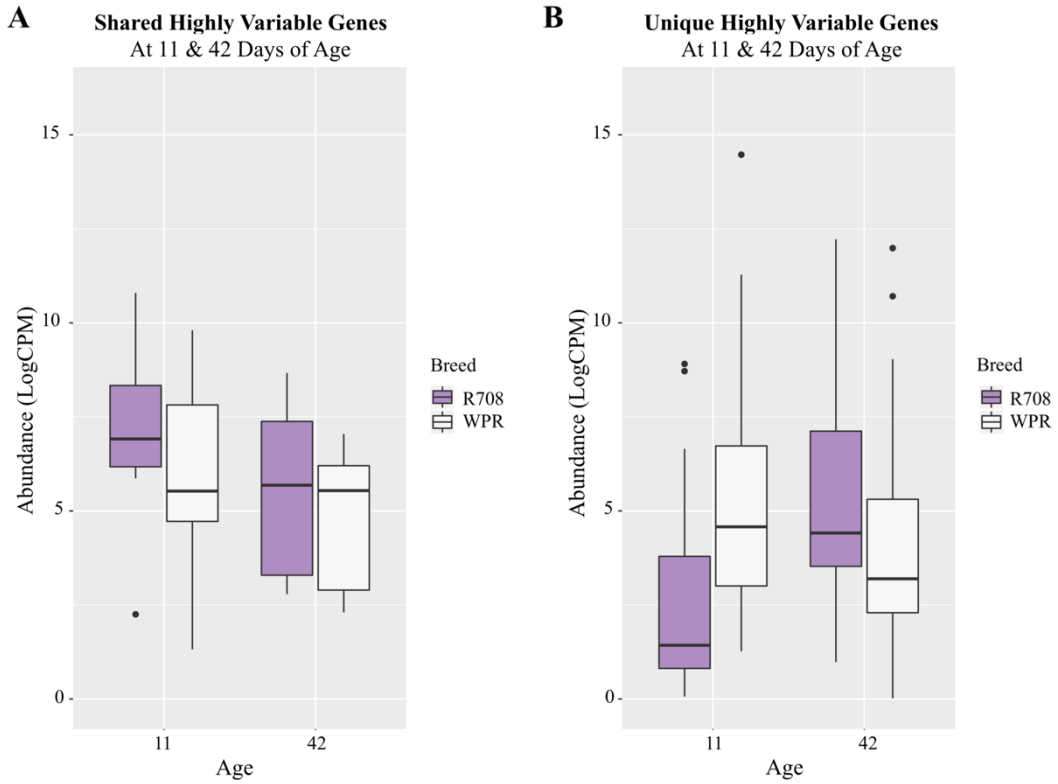
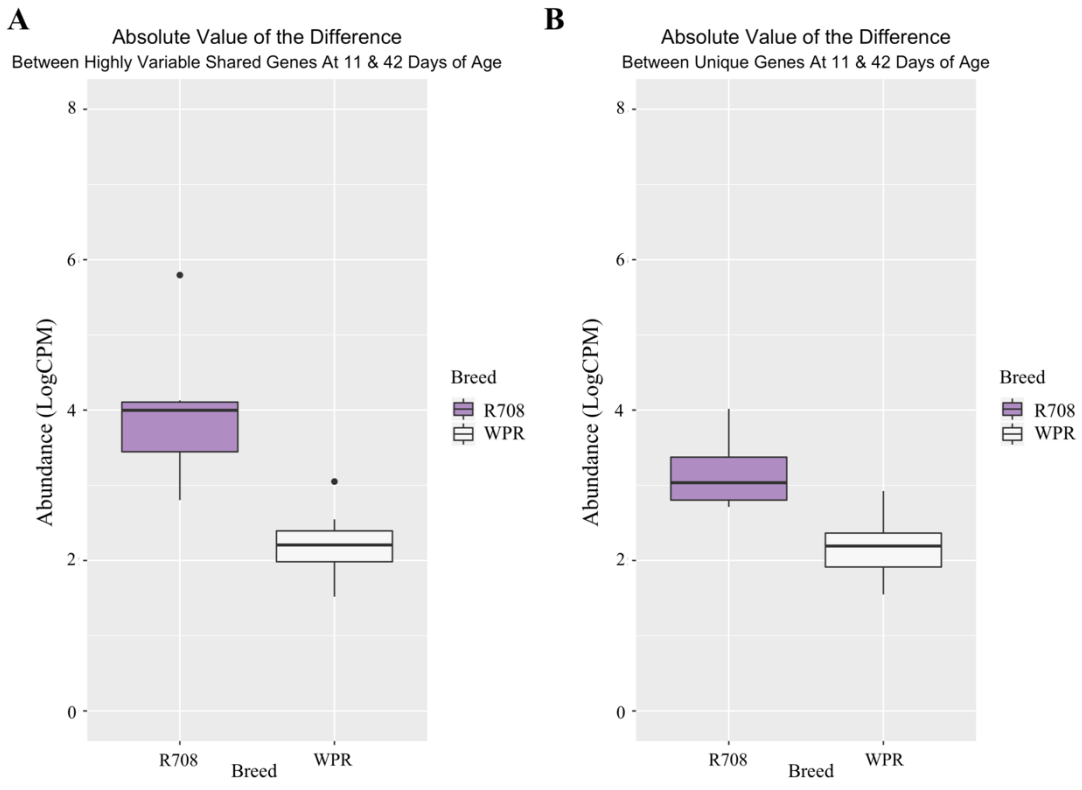


Figure 3.10 Box plots of the absolute value of the difference between highly variable shared genes and unique genes for each breed. Plot A shows the variance of the expression of the shared genes between breeds, while plot B shows the variance of the expression of the genes unique to each breed.



3.3.4.4. ***Wooden Breast Specific Differential Expression***

To filter out genes specific to WB, we compared the DGE lists of four pairwise comparisons, namely the 11d WPR v R708, 42d WPR v R708, WPR 11d v 42d and R708 11d v 42d DE genes (Figure 3.5). This yielded a list of 262 DE genes shared by the three data sets used in this comparison containing R708 samples (11d WPR v R708, 42d WPR v R708 and R708 11d v 42d). As 100% of 42d R708 birds were affected by WB, we expect all samples in this category to share WB associated expression. WB has been observed in commercial fast-growth broilers as early as 14 days of age; therefore, we expect 11 day old R708 broilers to also be displaying expression profiles indicative of WB (Papah et al., 2017, 2018; Chen et al., 2019).

The resulting lists of shared and unique genes were annotated for function with IPA (Figure 3.11). Also, the frequencies of gene product types (Figure 3.11C) and the location in the cell (Figure 3.11D) for the entire WB DE gene list were identified. Functions of these genes include cellular development and differentiation (CSRP3, IQCA1), nuclear protein import (IPO7), calcium and pH-dependent gap junction channels (GJA8), modulation of inflammation (HPGD), muscle structure and function (LMOD2, MYH6, MYOM3, UCP3, AK1, CAMK2G, LIMCH1, MUSTN1), energy metabolism (NDUFB10, PGK1), iron storage and transport (FTH1), amino acid synthesis (PHGDH), and regulation of the cell cycle and gene expression (METTL21E, KLHL31).

3.3.4.5. ***Influence of Diets on Inflammation Observed in Wooden Breast***

We performed intersection analysis to identify the effects of the diets on inflammation associated gene expression patterns. The DE results generated from pairwise comparisons of broiler breed x diet (Figure 3.4) were utilized with the Bioprofiler function in IPA to identify chemokines, cytokines, leukotrienes, interleukins, prostaglandins, and glucocorticoids, as these genes are known to play a role in immune and inflammatory processes. Only four genes were found to be DE between breeds for this analysis (Figure 3.12). Of these four genes, all were DE in T1 and C, while only CXCL14 was DE for all diets. Furthermore, expression for three of the four inflammation-associated genes demonstrated increased expression in R708 compared to WPR. HPGD was the only gene from this list with elevated expression in WPR.

Figure 3.11 Bar plots showing the (logCPM) ten most differentially expressed and the most abundant differentially expressed genes (FDR < 0.05) (plots A and C) and histograms of their type and location frequency (plots B and D) between ages associated with WB.

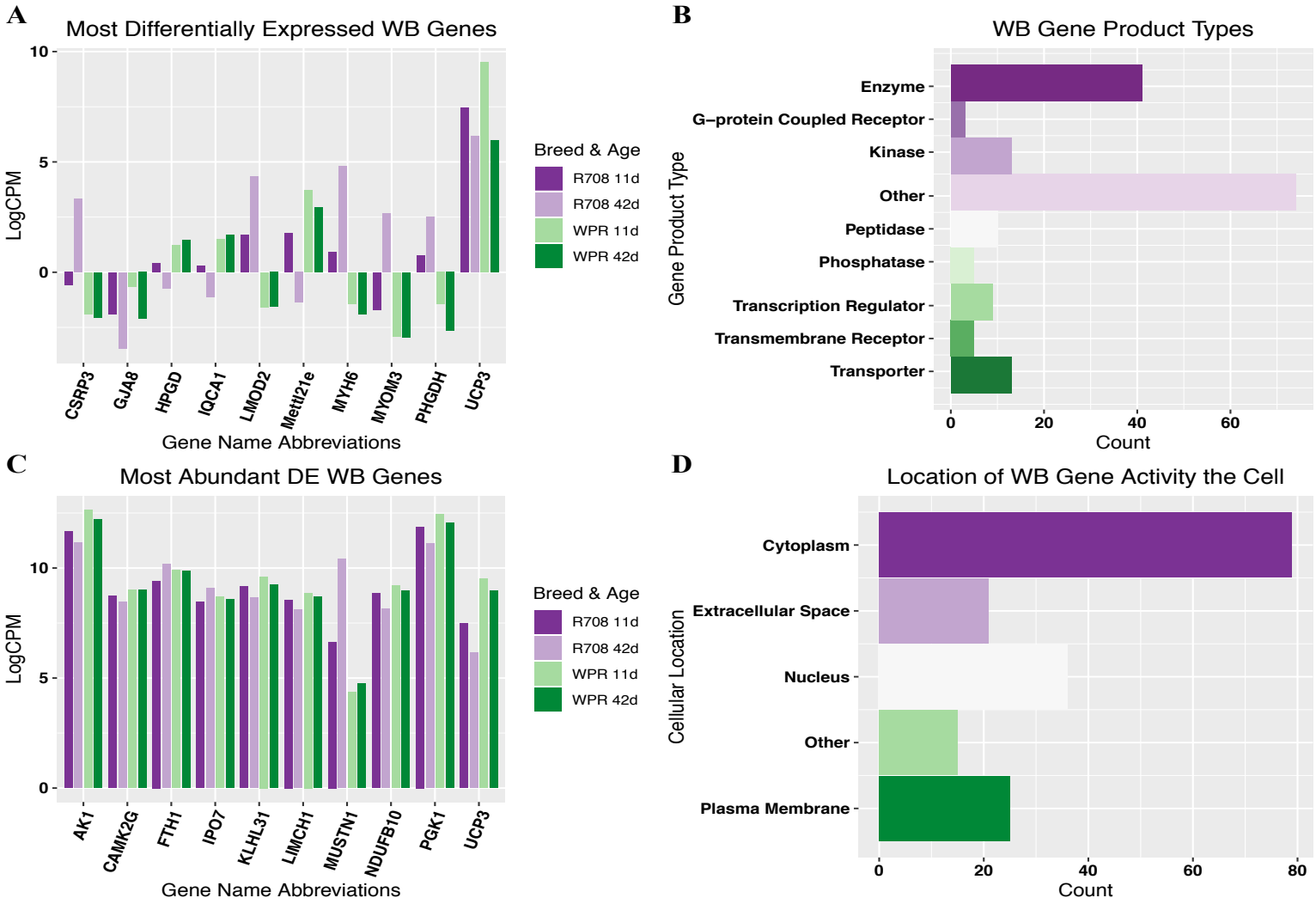
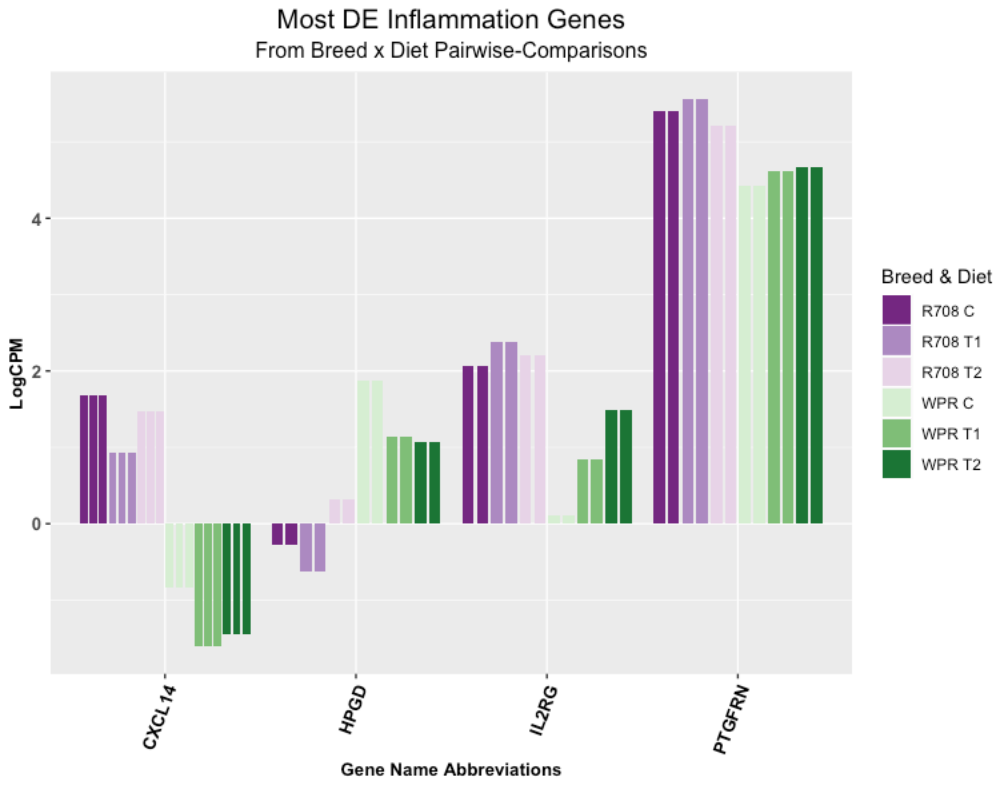


Figure 3.12 Bar plot showing the (logCPM) four differentially expressed genes (FDR < 0.05) from breed x diet pairwise comparisons associated with inflammation. The number of sections per bar indicates that the gene was differentially expressed in multiple diets (e.g. three sections in a bar means the gene was differentially expressed in all three diets, while two sections in a bar means the gene was differentially expressed in only two diets). Three of the four genes were differentially expressed in the C and T1 diet, while only one gene was differentially expressed in all three diets.



Inflammation-associated genes that were significantly DE were all of high relevance to the symptoms of WB. Interestingly, CXCL14 is known to have antimicrobial activity and chemotactic activity for monocytes but not for dendritic cells, macrophages, neutrophils, or lymphocytes. IL2RG, is required for execution of IL9 activities, which

stimulate cell proliferation and prevents apoptosis. HPGD is a dehydrogenase which acts in prostaglandin metabolism and its pathways also include transcriptional mis-regulation in cancer, while PTGFRN is a receptor inhibitor for prostaglandin F2.

3.4. Discussion

Historically, several breast muscle myopathies have been nutrition/management induced (Weinstock et al., 1955; Umemura et al., 1984; Siller, 1985; McLennan, 1985; Cavaliere et al., 1997). Also, considering the specificity of the current nutritional guidelines for broiler chickens and as WB is most commonly observed in the largest and fastest growing birds, a nutritional etiology has been a very popular area for investigation (Guetchom et al., 2012; Trocino et al., 2015; Cruz et al., 2017; Meloche et al., 2018b; c; Bodle et al., 2018; Zampiga et al., 2018; Livingston et al., 2019a). Although the diets did not impact WB severity or incidence at slaughter age in this investigation, we found that specific dietary oil sources with varying ω-6:3 ratios impacted gene expression. Furthermore, through comparative analyses of breeds and diets, we narrowed down the genes impacted by selection for performance traits in modern fast-growth commercial broilers, and specifically those that stand out in WB.

3.4.1. Pathways Perturbed by Diet and Age

The pairwise comparisons of age and diet by breed (Figure 3.3) provided insights into the differences in growth and metabolism due to broiler breed. Regardless of diet, R708 11d v 42d canonical pathways were largely shared, whereas the WPR 11d v 42d canonical pathways varied highly by diet and were dissimilar to the those for the R708 comparisons (Table A-3). Mitochondrial dysfunction, oxidative phosphorylation and

sirtuin signaling were the only three pathways shared across all 6 pairwise comparisons. As these comparisons were between 11 and 42 days of age those genes indicating alteration of these pathways are most likely due to the natural aging process and not necessarily indicative of disease. All of these pathways are involved in energy metabolism, cell cycle progression and aging and are interrelated.

The Sirtuins are a seven member family of enzymes described as histone deacetylases and its members inhabit different locations of the cell and perform several important activities for normal development and the processes of aging and disease (Zee et al., 2010; Verdin et al., 2010; Bonda et al., 2011; Schug and Li, 2011; Jing et al., 2011; Corbi et al., 2013; Gonzalez Herrera et al., 2015; Ren et al., 2017; Mendes et al., 2017). Sirtuin 3 is localized to the mitochondrion, regulates skeletal muscle metabolism and insulin sensitivity through deacetylation of acetyl-CoA synthase 2 and glutamate dehydrogenase, and plays a role in hepatic lipid metabolism (Zee et al., 2010; Verdin et al., 2010; Jing et al., 2011; Mouchiroud et al., 2013). Sirtuin 1, although localized to the nucleus, controls mitochondrial function through the deacetylation of FOXO and PGC-1**α** (Zee et al., 2010; Verdin et al., 2010; Jing et al., 2011; Mouchiroud et al., 2013). A recent study on chicken sirtuins showed that Sirtuin 3 was predicted to be located in the nucleus, whereas Sirtuin 1 was predicted to be both nuclear and cytoplasmic (Ren et al., 2017).

In Ren et al.'s study (2017), predicted activities were also different with sirtuin 3 acting in amino acid biosynthesis. While sirtuins 1, 2, 5, and 6 were predicted to act in central intermediary metabolism, and sirtuin 4 is predicted to function in transcription

regulation (Ren et al., 2017). Sirtuin 7 is predicted to have no enzymatic activities in chicken (Ren et al., 2017). Ren et al. (2017) investigated the expression of the sirtuins in ten chicken tissues, finding that all seven sirtuins were expressed in all ten tissues, with sirtuin 2 and 7 being the most highly expressed in pectoralis muscle. Also, age related changes in sirtuin expression of liver tissue demonstrated that sirtuin 1, 3, 4, 6, and 7 increase in expression with age while sirtuin 5 remains constitutively expressed (Ren et al., 2017). These findings require deeper investigation as they indicate that activities of individual sirtuins in chicken may not be analogous to the known activities in humans.

Pairwise comparisons of the impact of the diet on gene expression between the two broiler breeds provided insights regarding overall metabolic differences between WPR and R708 (Figure 3.4). No pathways were shared between all comparisons, but C and T1 diets both impacted protein kinase A signaling and the NRF2-mediated oxidative stress response, while C and T2 diets impacted glycolysis I. T1 and T2 diets impacted Actin Cytoskeletal signaling (Table A-3). As the T1 diet and the control diet were the most different, the sharing of activated pathways in these two treatments are most likely demonstrative of gene expression differences due to broiler breed rather than an impact of diet. The alteration of Glycolysis I pathway in both the C and T2 may be indicative of the higher ω-6:3 ratio in these two diets, as increasing ω-3 concentrations has been shown to decrease glucose metabolism (Jump et al., 1994; Andrade-Vieira et al., 2013; Flachs et al., 2014). Many studies have demonstrated that higher ω-6:3 ratios lead to insulin resistance and dysregulation of food intake (Simopoulos, 2002, 2010, 2016; Sartorelli et al., 2010; Gómez Candela et al., 2011; Li et al., 2014; Jeromson et al., 2015;

Wanders et al., 2019). The alteration of Actin Cytoskeleton signaling in T1 and T2 may be due to the higher inclusion of ω-3s in these diets. ω-3s are recognized for their modulation of the Actin Cytoskeleton due to their roles in the normal development of cell structures and survival, the brain, retina, as well as T-cell activation (Plowman et al., 2005; Mazelova et al., 2009; Hou et al., 2012, 2016; Schmidt et al., 2015). These results indicate that the diets modulated gene expression differences in limited but crucial ways.

The pairwise comparisons focusing on gene expression due to age (Figure 3.5) further provided insights into metabolic and growth-related differences between the two breeds. Pathways identified between 11d and 42d regardless of breed again included mitochondrial dysfunction, oxidative phosphorylation and sirtuin signaling, further supporting suggesting that these pathways may have notable roles in normal growth and development (Table A-3). Interestingly, this age comparison for the WPR showed alteration of cholesterol biosynthesis pathways, while it also showed alteration of gluconeogenesis I and NRF2-mediated oxidative stress responses for the R708. The comparisons of gene expression differences at 11d and 42d between the two breeds shared only one pathway, namely the NRF2-mediated oxidative stress response. Myocyte degradation observed as a feature of WB is predicted to be a response to high levels of oxidative stress and NRF-2 may be a potential target for amelioration. The other pathways observed (Table A-3) perturbed between 11d WPR v R708 and 42d WPR v R708 are all associated with cell proliferation and are most likely representative of the drastic differences in growth-rate and body size between the two breeds (Figure 3.1) at these two ages.

These breed/age comparisons also provided insights into the pathogenesis of WB. Oxidative stress is a defining molecular feature of WB and in this study, the NRF2-mediated oxidative stress response pathway showed up in four of five total WPR v R708 comparisons, as well as the R708 11d v 42d comparison. NRF2 is a cytosolic transcription factor for phase II detoxifying genes, which is activated in response to high levels of oxidative stress (Itoh et al., 1999; Ishii et al., 2000; Kang et al., 2005; Nguyen et al., 2009; Singh et al., 2010; Ungvari et al., 2011; Lee et al., 2018). During redox homeostasis, NRF2 is tethered to KEAP1, a cytoskeleton anchoring protein, and actin filaments which assist KEAP1 in retaining NRF2 in the cytosol (Itoh et al., 1999; Kang et al., 2005; Nguyen et al., 2009; Singh et al., 2010).

Several genes are upregulated in response to NRF2-antioxidant responsive element activation and are categorized based on their functions in glutathione homeostasis, drug metabolism, excretion/transporter, and iron metabolism or stress response protein (Kang et al., 2005; Singh et al., 2010). These genes include Glutathione-S-Transferase, **γ**-glutamyl-cysteinyl-glycine (aka GSH), NADPH quinone oxidoreductase-1, UDP-glucuronosyltransferases, Microsomal epoxide hydrolase, Ferritin, and Heme oxygenase-1, to name only a few (Kang et al., 2005; Singh et al., 2010). Furthermore, as inflammation is a natural immune response, NRF2 plays a role in the regulation of inflammation by inhibiting the NF-kB-dependent proinflammatory genes (Kang et al., 2005; Singh et al., 2010; Ungvari et al., 2011; Lee et al., 2018). Based on these activities and NRF2 deficient animal studies, NRF2 has been linked to several diseases including Alzheimer's, Parkinson's, Huntington's, cardiovascular disease, pulmonary disease, and

cancer (Chan et al., 2001; Cho et al., 2006; Liu et al., 2010; Singh et al., 2010; Ungvari et al., 2011). NRF2-antioxidant response element mechanisms have been proposed as therapeutic intervention candidates for these conditions based on the ability to directly or indirectly alter the NRF2/KEAP1 pathway (Nguyen et al., 2009; Singh et al., 2010). Better understanding of the structure of the NRF2-mediated pathway and their regulation in broilers will likely be informative for remedying the cascade of inflammatory processes observed in WB.

3.4.2. Differential Gene Expression Specific to Breed and Age

Considering the previous growth performance curves (R708 v WPR growth, Figure 3.1) the genes identified here assist in illuminating the differences in growth-rates between the two breeds, and partially demonstrate at a molecular level how commercial broilers are able to increase their muscle mass at a faster rate when compared to the heritage breeds. Six genes, MUSTN1 (Musculoskeletal, Embryonic Nuclear Protein 1), MYH15 (Myosin Heavy Chain 15), MYH1A (Myosin Heavy Chain 1A), MYH1B (Myosin Heavy Chain 1B), MYH1D (Myosin Heavy Chain 1D), and ONCM2 (Oncomodulin) code proteins for musculoskeletal growth and homeostasis with the first four being highly variable only in R708's and the remaining two being highly variable only in WPR's. Additionally, Gene ontology of 5 out of the 7 shared genes, CA3 (Carbonic Anhydrase III), HBAD (HBM; Hemoglobin Subunit Mu), PROCA1 (Protein Interacting with Cyclin A1), HBE1 (Hemoglobin Subunit Epsilon 1), and HBAA (HBA1; Hemoglobin Subunit Alpha 1) reveals a shared function; acting through iron-

iron and oxygen binding. These functions play critical roles in transporting nutrients, such as iron and oxygen, in support of developing and maintaining skeletal muscle.

Mitochondrial proton leak through uncoupling proteins of the electron transport chain has associated with low feed efficiency. Uncoupling protein 3 (UCP3) expression is increased in WPR vs R708 and further increased by administration of the T1 diet (Figure 3.7B). This trend was also reported by Zhou et al. (2015) when comparing high feed efficiency broilers to low feed efficiency broilers. They reasoned that this, combined with other factors they observed may be explanatory of the increased levels of oxidative stress in the high feed efficiency broilers (Zhou et al., 2015). They also noted that lipid metabolism was decreased in high feed efficiency broilers. UCP3 is predominantly expressed in the skeletal muscle (Rousset et al., 2004; Bezaire et al., 2005; Fritz et al., 2006; De Marchi et al., 2011; Harmancey et al., 2015; Oliveira et al., 2016; Fan et al., 2016; Tang et al., 2017). Increased UCP3 expression has been correlated to increased fatty acid oxidation and increases in UCP3 expression reduces intramuscular fatty acid storage, increases fatty acid transport at the plasma and mitochondrial membranes (Bezaire et al., 2005; Nowinski et al., 2015; Oliveira et al., 2016; Fan et al., 2016; Tang et al., 2017). The reduced UCP3 expression in high feed efficiency broilers may be an indicator of decreased lipid metabolism.

Interestingly, UCP3 expression is requisite for proper mitochondrial function, and lowered UCP3 expression have been associated with reduced insulin sensitivity, lipotoxicity, glucotoxicity, and increased oxidative stress (Rousset et al., 2004; Fritz et al., 2006; Harmancey et al., 2015; Tang et al., 2017). Furthermore, studies in transgenic

mice have demonstrated that UCP3 does not function in cold-induced thermogenesis or uncoupling of mitochondrial respiration (Rousset et al., 2004). These findings warrant further investigation as they indicate that the higher expression of UCP3 may be exerting a protective effect on the WPR by shifting from glycolysis to lipid metabolism and decreasing oxidative stress. If this is the mode of action, then the finding that UCP3 expression was highest in birds fed the T1 diet indicates that increasing the concentration of ω-3's in the diet may also have a beneficial effect.

Finally, cardiovascular disease (sudden death syndrome, ascites, inadequate vessel density) is a common concern in modern commercial broiler chickens (Scheele, 1997; Olkowski et al., 1998, 1999; Olkowski and Classen, 1998; Malan et al., 2003; Pan et al., 2005; Olkowski, 2007; Wideman et al., 2013; Tarrant et al., 2017; Sihvo et al., 2018). The expression of angiotensin converting enzyme (ACE) is increased in WPR vs R708. ACE is responsible for the conversion of angiotensin I to angiotensin II a vasoconstrictor, inactivation of the vasodilator bradykinin, and is a member of the Renin-Angiotensin-Aldosterone System (also known as RAS) (Erdös, 1975; Niu et al., 2002; Bealer, 2002; Cabo et al., 2012).

ACE is most often associated with cardiovascular disease. The product of ACE, angiotensin II is highly pro-inflammatory, and a known vasoconstrictor, which increases basal heart rate, and activates free-radical generation and oxidative stress through upregulation of NADPH oxidases (Bealer, 2002; Das, 2004, 2016; Briones et al., 2012; LeMieux et al., 2016). Based on these activities, ACE has also been implicated in obesity, metabolic disorder, and diabetes. The decreased expression of ACE in

commercial broilers compared not only to the WPR, but also across ages, may explain their lower heart rate and higher occurrence of ascites (Scheele, 1997; Olkowski and Classen, 1998; Olkowski et al., 1999; Olkowski, 2007; Wideman et al., 2013).

3.4.3. Differential Gene Expression Specific to Wooden Breast

The WB specific gene list contained seven of the genes already discussed. Specifically, UCP3, MUSTN1, MYOM3, MYH6, METTL21E, CSRP3, and AK1. This overlap was expected as WB is highly correlated with growth-rate and breast muscle yield, but also numerous concurrent conditions found in modern commercial broilers. 15-Hydroxyprostaglandin dehydrogenase (HPGD) functions in the metabolism of prostaglandins and is involved in inflammation. Expression of HPGD was similar in WPR at 11 and 42 days of age but significantly lower in 11-day old R708, and extremely down-regulated in 42-day old R708 (Figure 3.10). Eleven genes were unique to the WB gene list (GJA8, IQCA1, LMOD2, PHGDH, CAMK2G, FTH1, IPO7, KLHL31, LIMCH1, NDUFB10, PGK1). Gene ontology for these indicates a strong association with the known molecular characteristics of WB - namely cell proliferation, altered energy metabolism, inflammation, calcium regulation, and degeneration and regeneration of myofibers.

Two genes in particular stand out for their potential significance in WB - GJA8 and PGK1. Intracellular calcium accumulation is a hallmark of WB tissue (Mutryn et al., 2015) and GJA8 is a component of calcium and pH-dependent gap junction channels, facilitates cell to cell communication, and allows passive diffusion of nutrients, metabolites and second messengers (Beyer and Willecke, 2000; Dobrowolski and

Willecke, 2009; Šeda et al., 2017). While most information about GJA8 in the literature pertains to the development of cataracts (Beyer and Willecke, 2000), recent research has linked reduced GJA8 expression to cardiac fibrosis, hypertrophy, decreased blood pressure, hypertension, and insensitivity of skeletal muscle to insulin (Šeda et al., 2017). As GJA8 is significantly downregulated in R708, this may also explain the elevated rate of cardiovascular disease in commercial broilers.

Dysregulation of glucose metabolism is also a frequently observed characteristic of WB. Furthermore, it has been shown that under hypoxic conditions like those of WB, glycolysis is enhanced in order to compensate for decreased OXPHOS and maintain energy production of the cell (Jackman and Willis, 1996; Guzy et al., 2005; Zheng, 2012; McGarry et al., 2018). Phosphoglycerate kinase 1 (PGK-1), a key enzyme in glycolysis from the WB gene list showed high transcript (logCPM) abundance levels in all breeds. However, compared to the WPR and R708 11d samples, expression of PGK-1 was lowest in 42-day old R708 broilers. Deficiency of PGK-1 has been associated with central nervous system dysfunction, hemolytic anemia and myopathies (Fujii et al., 1980; Tsujino et al., 1995; Sotiriou et al., 2010; Sakaue et al., 2017). Hereditary Parkinsonism is an age dependent neuromuscular disease which has been associated with PGK-1 deficiency in human males and heterozygotes are often considered asymptomatic (Sakaue et al., 2017). These deficiencies in PGK-1 are typically linked to missense mutations (Fujii et al., 1980; Tsujino et al., 1995; Sotiriou et al., 2010; Sakaue et al., 2017). Downregulation of PGK-1 in commercial broilers contrasts with their high energy requirements. Also, as the main energy source in breast muscle is glycolysis,

reduced expression of PGK-1 is likely correlated to the muscle fiber degradation observed in WB.

3.4.4. Inflammatory Gene Expression in Relation to Diets and Wooden Breast

It has been demonstrated that selection for today's fast-growth commercial broilers has enhanced inflammatory and cell-mediated immune responses (Cheema et al., 2003). Considering the metabolic costs of immune function and the selection for the high metabolic rate in fast-growth broilers, nutrition plays a major role in the supplying the energy demands of modern broilers. The ω-3 PUFA ALA and its metabolites eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are highly studied in human health and are credited widely as having beneficial effects on numerous diseases including cardiovascular disease, metabolic disease, multiple cancer types, neurological conditions, and inflammatory diseases (Simopoulos, 2002, 2010; Lorente-Cebrián et al., 2015; Patrick and Ames, 2015; Jeromson et al., 2015; Behling et al., 2015; Wiktorowska-Owczarek et al., 2015; de Oliveira et al., 2017). Several of these disorders are commonly observed by canonical pathways, regulators, or predicted diseases and disorders in molecular investigations into the pathogenesis of WB (Mutryn et al., 2015; Kong et al., 2017; Kuttappan et al., 2017a; Hubert et al., 2018; Papah et al., 2018).

Our work demonstrated DE inflammation-associated genes specific to all three diets (T1, T2, and C) between breeds (Figure 3.11). The only shared gene in the inflammation list was HPGD which was also observed in the WB specific gene list. This gene is significantly lower expressed in R708 than WPR, and from the comparison used for the WB gene list, we know its expression is lowest in 42-day old R708. As prostaglandins

are a metabolite of arachidonic acid (an ω-6) it is notable that HPGD was least expressed in the T1 diet for R708 while most expressed for R708 in the C diet, although these differences in expression were not significant. Interestingly, HPGD not only acts in inflammation, but has been identified as a tumor suppressor for breast, pancreatic, and gastrointestinal cancers (Yan et al., 2004; Wolf et al., 2006; Mehdawi et al., 2017; Arima et al., 2018). All three of these cancers appear commonly on the diseases and disorders list of WB pathway analyses.

Only one gene, C-X-C motif chemokine ligand 14 (CXCL14) was identified as DE between breeds in all diets and thus is of particular interest. CXCL14 acts as chemoattractant for immature dendritic cells and activated monocytes (Shellenberger et al., 2004; Starnes et al., 2006). It has also been identified in chemotaxis of natural killer cells to inflamed areas and to have possible roles in oncogenesis (Shellenberger et al., 2004; Starnes et al., 2006). Expression of CXCL14 has been shown to be ubiquitous in normal body tissues, but significantly decreased in many cancers (Shellenberger et al., 2004; Starnes et al., 2006; Augsten et al., 2009; Lu et al., 2016). However, up-regulation of CXCL14 has been shown in MCF7 breast cancer cells in response to mitochondrial induced oxidative stress (Pelicano et al., 2009; Lu et al., 2016). CXCL14 has also been shown to have significantly increased expression in inflammatory conditions such as arthritis, obesity, and atherosclerosis (Lu et al., 2016). Receptors for CXCL14 are still unidentified, resulting in a poor understanding of if its regulators (Lu et al., 2016). We chose to explore it further in our pairwise comparisons for age and strain ignoring diet. We observed that CXCL14 was highly DE between WPR and R708 at both 11 and 42

days of age (FDR < 0.001), but not different between ages within either breed (FDR = 0.85 and 0.9 WPR and R708 respectively). Based on these characterizations, the upregulation of CXCL14 in R708 broilers supports previous observations of inflammation symptoms and immune infiltration in WB and suggests a possible molecular marker for the condition.

3.5. Conclusions

Our treatment diets (ω-6:3 ratio) had no impact on WB incidence and severity at slaughter age and we did not observe DE genes between diets at a specific age for either breed. Although several previous investigations had success in reducing the severity of WB, none achieved a reduction in incidence without a corresponding reduction in growth-rate, indicating that WB is not susceptible to dietary intervention. We did observe an impact of diet on DE genes between breeds and ages, and inflammation related genes. This allowed for the refinement of a WB associated DE gene list, as well as provided insight into the impact of selection for body weight and feed conversion rate. Further research to expound upon the influence of the WB associated DE gene list on the condition is needed. Also, comparison of these genes to the proteome would provide essential information relating modulation of transcription and translation in modern fast-growth commercial broilers and important conditions like WB.

3.6. References

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4. MITOCHONDRIAL GENOME STRUCTURE AND FUNCTION, AND MITO-NUCLEAR INTERACTIONS IN WOODEN BREAST

4.1. Background

Wooden breast (WB) is a broiler chicken breast muscle disorder characterized by oxidative stress at the tissue and molecular levels (Mutryn et al., 2015; Abasht et al., 2016; Clark and Velleman, 2016; Sihvo et al., 2017; Cruz et al., 2017; Kuttappan et al., 2017b; Petracci et al., 2019). Since the 1950s, broiler breeds have been under heavy selection for breast muscle yield, feed efficiency, and body weight (Bohren, 1953; Warren, 1958; Merritt et al., 1962; Jaap, 1963; Chambers et al., 1981; Fairfull and Chambers, 1984; Paxton et al., 2010; Qanbari et al., 2019). The breast muscle is typically the highest value portion of the carcass and breast muscle myopathies like WB are a large economic concern for the poultry industry. A consensus has emerged that WB is associated with the fast growth-rate of commercial broilers (CBRO) and the main characteristics are muscle fiber degradation, fibrosis, infiltration of fat, collagen, macrophages and T-lymphocytes at the histological level (Trocino et al., 2015; Kuttappan et al., 2017b; Papah et al., 2017; Livingston et al., 2019b; Chen et al., 2019). At the molecular level, oxidative stress, inflammation, and altered energy metabolism are definitive of WB (Mutryn et al., 2015; Abasht et al., 2016, 2019; Kong et al., 2017; Kuttappan et al., 2017a; Hubert et al., 2018; Papah et al., 2018; Livingston et al., 2019a). The identification of inflammatory and immune activities in WB is also a consumer health concern, and inflammatory tissue is required to be removed from the carcass

(USDA FSIS, 2018), further downgrading product quality and increasing economic losses.

A notable feature of WB and several other related myopathies is that they are found in fast-growth, high-feed efficiency strains, and altered energy metabolism is a unifying theme. Metabolic rate is positively correlated with growth-rate and feed efficiency, or an organism's ability to produce and convert energy to body mass, specifically muscle mass. This energy, in the form of ATP, is necessary for cell maintenance and replication as well as whole organism health and survival. On the other hand, the metabolic rate required for rapid growth-rate can be inversely related to feed efficiency in that higher metabolic rates typically coincide with high higher levels of heat production and oxygen consumption, and thus decreased feed efficiency (Fairfull and Chambers, 1984; Jackson and Diamond, 1996; Ojano-Dirain et al., 2007; Bottje et al., 2017; Tallentire et al., 2018). Therefore, it is remarkable that selection in modern broilers has achieved the highly desirable trifecta of high feed efficiency, rapid growth rate, and high metabolic rate. Optimized nutrition and controlled environments have contributed to this success, but this economically desirable combination has come at the cost of various metabolic disorders.

Oxidative stress, a primary molecular feature of WB, is a disruption of the balance between reactive oxygen species (ROS), and antioxidant defense (Clanton, 2007; Halliwell, 2007; Weidinger and Kozlov, 2015; McGarry et al., 2018). ROS are produced normally in the cell and are essential for intracellular signaling, regulation of inflammatory responses, and gene expression (Halliwell, 2007; Sims and Muyderman,

2010; Weidinger and Kozlov, 2015). Antioxidants, either produced *in vivo* or consumed as part of the diet, combat excess reactive oxygen species (Clanton, 2007; Halliwell, 2007; Sims and Muyderman, 2010; Weidinger and Kozlov, 2015). Tissues experiencing oxidative stress can experience cellular damage and subsequent cell death if the oxidant/antioxidant balance is not restored. As oxidative stress proceeds, ion balance changes, modulating sodium/potassium-ATPase channels and cellular calcium metabolism, resulting in elevated intracellular free calcium and apoptosis (Clanton, 2007; Halliwell, 2007; Sims and Muyderman, 2010; Weidinger and Kozlov, 2015). One of the foci of ROS production is the mitochondria. The mitochondria regulate cytosolic calcium concentrations, acting in cell signaling, differentiation, and programmed cell death (Johnstone et al., 2002; Bonnard et al., 2008; Sims and Muyderman, 2010; Netzer et al., 2015; Liao et al., 2015). The mitochondrion, well known as the powerhouse of the cell, is responsible for the majority of the cell's ATP production. Thus, the mitochondrion also, in large part, drives feed efficiency in agricultural animals. This role has been studied in many species of production livestock (Kiessling, 1977; Pitchford, 2004; Schenkel et al., 2004; Bottje et al., 2006, 2009; Bottje and Carstens, 2009; Kelly et al., 2010, 2011; Tinsley et al., 2010; Toyomizu et al., 2011; Young and Dekkers, 2012; Sharifabadi et al., 2012; Weller et al., 2013; Fu et al., 2017).

While mitochondrial involvement in WB has been of interest recently in WB (Papah et al., 2017, 2018; Sihvo et al., 2018; Livingston et al., 2019a; Abasht et al., 2019), relatively little attention has been devoted to mitochondrial genetics in broiler myopathies. Part of this gap in the knowledge can be traced to the fact that the broiler

breast is “white meat”, comprised mostly of type IIB muscle fibers. Type IIB muscle fibers have very few mitochondria and rely on anaerobic methods of ATP production (Jackman and Willis, 1996; Hudson et al., 2017). Only two recent studies have included the mitochondria in their histologic investigations of WB in CBRO (Papah et al., 2017; Sihvo et al., 2018).

The mitochondria provide a unique opportunity for better understanding the impacts of selective breeding for performance traits as well as the selective forces of domestication (Kiessling, 1977; Desjardins and Morais, 1990; Liu et al., 2006; Guan et al., 2007). Few investigations have focused on the signatures of selection arising from domestication (comparing against ancestral Red Jungle Fowl) versus those arising through selection for production traits (comparing against slow growth chicken varieties) (Chambers et al., 1981; Remignon et al., 1994; Liu et al., 2006; Kanginakudru et al., 2008; Paxton et al., 2010; Miao et al., 2013; Collins et al., 2014; Tallentire et al., 2018; Qanbari et al., 2019). Selection for CBRO has resulted in changes to muscle morphology such as reduced spacing for connective tissue between fiber bundles, increased size of fibers, increased rates of degradation and regeneration, and decreased capillary blood supply (Mahon, 1999; Dransfield and Sosnicki, 1999; MacRae et al., 2006, 2007; Fanatico et al., 2007; Petracci and Cavani, 2012; Velleman and Clark, 2015; Velleman, 2015; Sihvo et al., 2018). Muscle morphological structure not only impacts function, but directly determines meat quality and the morphology observed in breast muscle myopathies like WB (Petracci and Cavani, 2012; Sihvo et al., 2014, 2017, 2018; Petracci et al., 2015; Velleman, 2015; Clark and Velleman, 2016; Velleman et al., 2018).

Furthermore, several investigations have indicated that “normal” or unaffected WB CBRO samples are asymptomatic rather than negative for the condition (Velleman and Clark, 2015; Kong et al., 2017; Papah et al., 2017; Hubert et al., 2018). As most nutritional and management approaches to eliminate WB have been unsuccessful, it is increasingly important to identify the impact of selective breeding for body size, breast muscle accretion and feed efficiency (Trocino et al., 2015; Cruz et al., 2017; Bodle et al., 2018; Livingston et al., 2019b; a). Thus, comparative analyses of chicken mitochondria varieties can be a valuable perspective for determining the pathophysiology of WB.

In this study, we utilized comparative genomics approaches to investigate the structure and function of the mitochondrial genome in relation to WB. First, we compared mitochondrial gene expression patterns from the breast tissue of fast- versus slow-growth broilers. Following this, we investigated if functionally important genes carried significant variants (mutations) that may be associated with breed and WB incidence. Differential gene expression of mitochondrial genes is likely to be directly informative about energy metabolism and aberrations in WB. Additionally, due to the primarily uniparental inheritance of the mitochondrial (MT) genome, the patterns of genetic variation at functionally active genes may reflect the selection history of commercial broilers. On the other hand, it is possible that the adoption of line breeding in broiler production is so recent (<70 years) that the broiler MT genome has not diverged sufficiently from foundational and ancestral strains, potentially constraining efficient energy metabolism

4.2. Materials and Methods

4.2.1. Sample Collection and Storage

Tissue samples for RNA sequencing were collected as a part of a study investigating WB molecular signatures in fast and slow growth broilers. Full details on methods for this study can be found in Hubert, 2019 (Dissertation Chapter 3). Birds utilized for tissue collection included 60 White Plymouth Rock (WPR) and 60 CBRO. Dissections were done at 11 and 42 days of age and euthanization was by CO_2 exposure, followed by cervical dislocation. Animal care and euthanasia procedures were performed according to protocols approved by Texas A&M's Institute for Animal Care and Use Committee (AUP IACUC 2016-0065). Approximately two grams of breast tissue was surgically dissected and stored in RNALater (Ambion Inc.) at a 5:1 ratio following the manufacturer guidelines. Breast tissue samples were stored at 4°C for a minimum of 24 hours and then removed from the RNALater and stored at -80°C until RNA isolation. Genomic data for comparative variant analysis were originally collected for previous studies at the Texas A&M University Poultry Center. Blood and tissue samples were utilized in order to generate data for this study. Blood was collected from four Ross broilers (CBRO), four Vietnam Red Jungle Fowl, two Richardson's Red Jungle Fowl (RJF), two Hy-line Browns (Hy-Line International, Dallas Center, IA), and two Rhode Island Reds (LAY). Blood samples was stored in Longmire buffer until DNA isolation and purified using a Qaigen DNEasy column-based kit (Qiagen).

4.2.2. Sample Processing

4.2.2.1. ***RNA Extraction and Sequencing***

Total RNA was extracted from 100 mg sections of *Pectoralis major* tissue samples through the TRIzol Reagent method following the manufacturer's protocol (ThermoFisher Scientific, Waltham, MA). All samples were quantified on a Nanodrop spectrophotometer and the quality of the RNA isolates was checked using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) with the RNA 6000 Nano Kit following the kit manual. Samples yielding an RNA integrity number above 8 were retained for further analyses and those which did not were re-extracted until a satisfactory RIN was met. Finally, the concentration of both RNA and DNA in these samples was checked using Qubit Fluorometric quantitation (ThermoFisher Scientific). The concentration of DNA measured in each sample was calculated as the percent of contaminant genomic DNA and was generally low with an average of about two percent.

RNA sequencing (RNAseq) libraries were prepared in-house with the Lexogen QuantSeq 3'mRNA Library Prep Kit (Lexogen, Vienna, Austria) for Illumina. A total of 96 single-indexed libraries were generated, checked for quality with the Agilent TapeStation D1000 DNA ScreenTape, and concentration was determined using the Qubit dsDNA High Sensitivity Kit (ThermoFisher Scientific). Of these, 72 libraries (n=18) were of sufficient quality and quantity. Libraries were diluted to 2 µg, pooled, and submitted for sequencing of 75 bp single-end reads on an Illumina NextSeq (Illumina, San Diego, CA). Sequencing of total RNA isolated was performed by the

Texas A&M University Institute for Genome Sciences and Society (College Station, TX).

4.2.2.2. ***DNA Extraction and Sequencing***

DNA was extracted from blood samples through the use of the DNeasy Blood and Tissue Kit (Qiagen, Inc., Hilden Germany) and quality control was carried out on a Nanodrop spectrophotometer. High quality samples were then used for whole genome sequencing which was performed by the Texas A&M University Institute for Genome Sciences and Society (College Station, TX). Paired-end, 150 base pair reads were sequenced on an Illumina NextSeq.

4.2.3. Data Analysis

4.2.3.1. ***RNA Sequencing Data***

Briefly, RNA sequence data was checked for quality with FastQC (version 0.11.6) and MultiQC (version 1.4), and adapters were trimmed with Trim_Galore version 0.4.3 (Martin, 2011; Ewels et al., 2016; Babraham Institute, 2018a; b). Reads with an average quality score lower than Q30 and length less than 35 bp were removed. RNA sequencing data were aligned to the *Gallus gallus* genome (Version 4.8, Ensembl Release 85, July 2016) with the short-read de-novo splice mapper STAR (version 020201) and reads mapping to exons were counted with HTseq-Count (version 0.9.1) (Anders et al., 2013; Dobin and Gingeras, 2015; Herrero et al., 2016; Aken et al., 2016; Ruffier et al., 2017). Normalized counts from each mRNA libraries were used to perform analysis of differential gene expression in the EdgeR package in R (Robinson et al., 2010; McCarthy et al., 2012; Anders et al., 2013; RStudio Team, 2015; R Core Team, 2019).

Genes were identified as significantly differentially expressed (FDR <0.05) using the likelihood ratio test 'glmLRT' function. Differentially expressed genes specific to the mitochondrial genome were then extracted from the data set and ten commonly observed genes were selected for comparison to SNP data. Expression data (logCPM) of genes were then used to create a heatmap with the gplots package in R in order to visualize differences in gene expression (RStudio Team, 2015; R Core Team, 2019; Warnes et al., 2019).

4.2.3.2. ***DNA Sequencing Data***

The whole genome sequence data were checked for quality, trimmed with Trimmomatic and aligned with BWA (Li and Durbin, 2009; Bolger et al., 2014). Variant calling was performed following the GATK best practices pipeline, followed by the annotation of variant effects using the SnpEff program for effect prediction (McKenna et al., 2010; Cingolani et al., 2012). Mitochondrial SNP data was separated and used in further analyses. Individual sample mitochondrial chromosome variant call files were combined by bird variety (CBRO, LAY, RJF) using the GATK CombineVariants tool, for the purpose of generating summary statistics by breed type. Tajima's D was then calculated for each of these files using VCFtools (Danecek et al., 2011) in order to observe the impact of selection on each variety and the results were graphed using the ggplot2 package in the R statistical platform (RStudio Team, 2015; Wickham, 2016; R Core Team, 2019). To investigate MT variants associated with differentially expressed MT genes, ten mitochondrial genes differentially expressed across all pairwise comparisons were identified, and SNP data for these genes were subsetted using

VCFtools. We then observed the compared SNPs and their effects at these loci across all varieties and individuals.

4.3. Results

4.3.1. RNA Sequencing Results

Thirty-six annotated mitochondrial genes obtained Ensembl BioMart (*Gallus gallus* genome, version 4.8, Ensembl Release 85, July 2016) specific to the chicken mitochondrial genome were included in the analysis (Herrero et al., 2016; Aken et al., 2016; Ruffier et al., 2017). Twenty-five of the 36 mitochondrial genes were differentially expressed in pairwise comparisons of fast- versus slow-growth broilers (FDR < 0.05). The number of observations across all pairwise comparisons for each of mitochondrial gene observed as differentially expressed is shown in Figure 4.1. We then utilized the SNPeff predicted impacts and the frequency of their observation to select ten genes for further analyses (Table 4.1). A heatmap of the logCPM for each of the ten mitochondrial genes comparing the WPR to the CBRO at 11 and 42 days of age is shown in Figure 4.2. In general, mitochondrial gene expression was similar between ages than variety, with a decrease in expression from 11 to 42 days of age for both strains. However, NADH Dehydrogenase 3 (ND3) and an Mt_tRNA (ENSGALG00000018366 and ENSGALG00000018372 respectively) were downregulated in CBRO compared to WPR regardless of age, with FDR < 0.02 and minimum logFC < -3. These were the only genes considered differentially expressed at 11 days of age between the two varieties. At 42 days of age ATP Synthase 6 (ATP6) and NADH Dehydrogenase (ND2) were downregulated in CBRO (ENSGALG00000018368,

FDR = 0.008, logFC = -1.86 and ENSGALG00000018378, FDR = 0.02, logFC = -1.78 respectively). Within variety, CBRO mitochondrial gene expression for the nine protein-coding genes significantly decreased from 11 to 42 days of age. In the WPR, only six of the protein-coding genes significantly decreased expression from 11 to 42 days of age, NADH Dehydrogenase 6 (ND6), NADH Dehydrogenase 1 (ND1), ND3, Cytochrome C Oxidase 3 (COX3), Cytochrome C Oxidase 2 (COX2), and NADH Dehydrogenase 4 (ND4) (ENSGALG00000018357, ENSGALG00000018382, ENSGALG00000018366, ENSGALG00000018367, ENSGALG00000018370, ENSGALG00000018364 respectively, FDR < 0.05, logFC < -1.8).

Figure 4.1 Histogram of differentially expressed mitochondrial genes across all RNAseq pairwise comparisons.

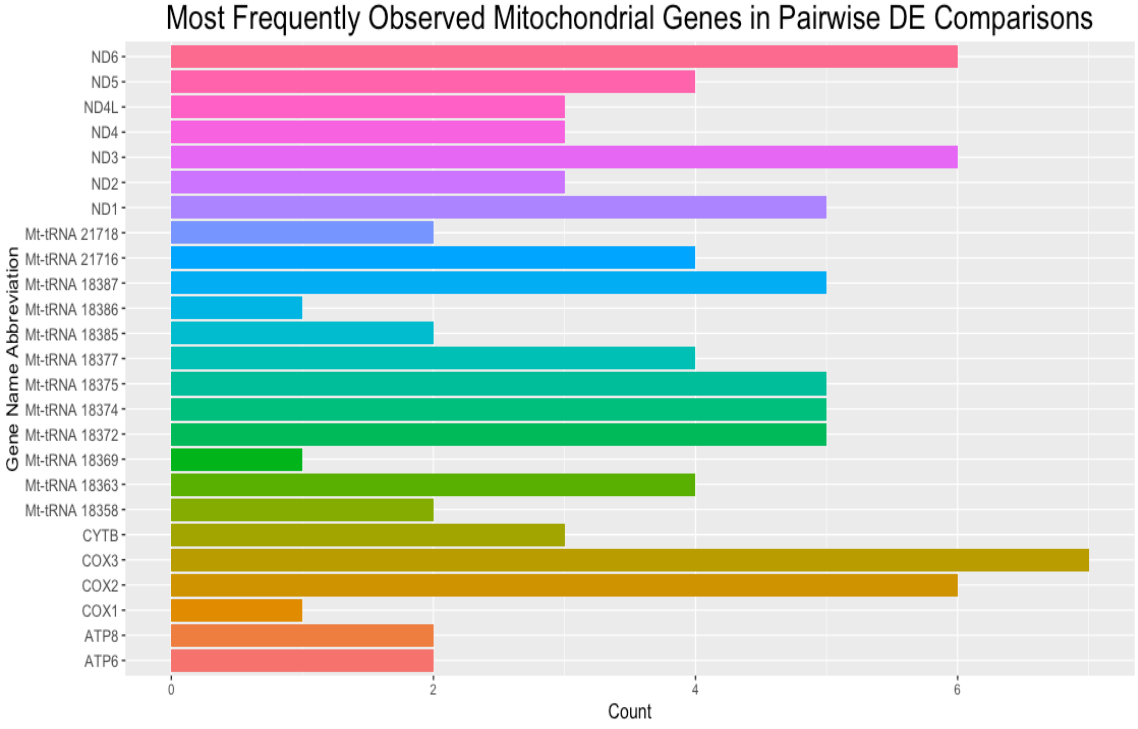
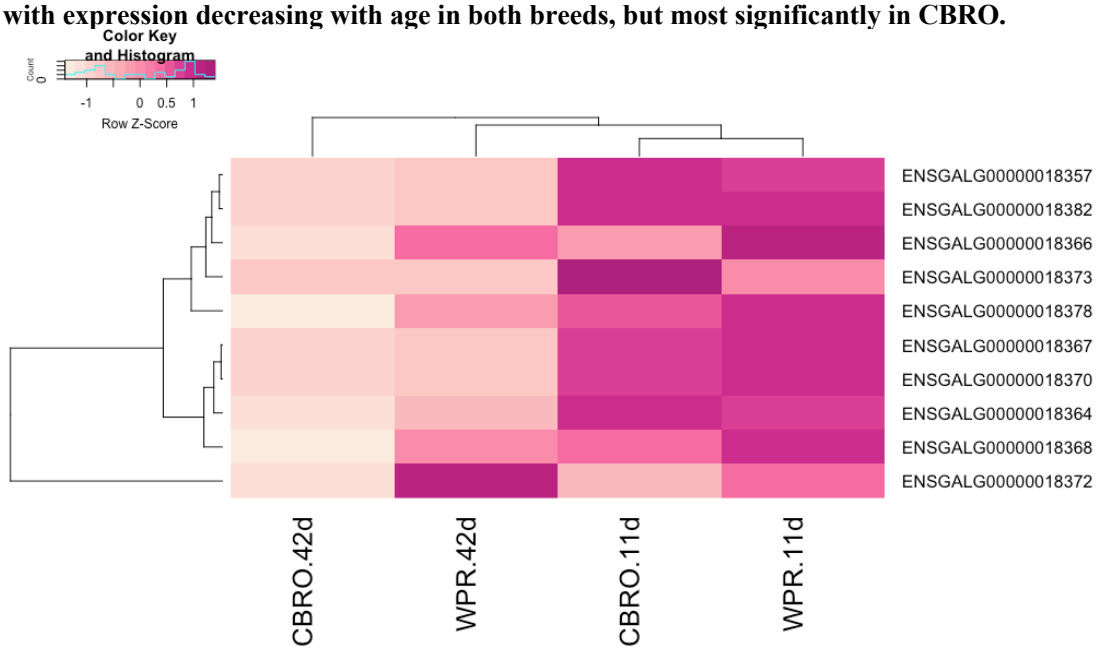


Table 4.1 Summary of the ten mitochondrial genes selected for further analyses. The table contains the gene name abbreviation, the Ensembl release 85 gene ID, the start and end coordinates for the coding sequence (bp), the gene type, and a short description.

Gene Abbr.	Ensembl Gene ID	Gene Start (bp)	Gene End (bp)	Gene type	Description
ATP6	ENSGALG00000018368	9240	9923	protein_coding	ATP synthase subunit a [Source:UniProtKB/Swiss-Prot;Acc:P14092]
COX1	ENSGALG00000018373	6645	8192	protein_coding	Cytochrome c oxidase subunit 1 [Source:UniProtKB/Swiss-Prot;Acc:P18943]
COX2	ENSGALG00000018370	8331	9014	protein_coding	Cytochrome c oxidase subunit 2 [Source:UniProtKB/Swiss-Prot;Acc:P18944]
COX3	ENSGALG00000018367	9923	10706	protein_coding	Cytochrome c oxidase subunit 3 [Source:UniProtKB/Swiss-Prot;Acc:P18945]
Mt_tRNA	ENSGALG00000018372	8124	8258	Mt_tRNA	
ND1	ENSGALG00000018382	4050	5024	protein_coding	NADH-ubiquinone oxidoreductase chain 1 [Source:UniProtKB/Swiss-Prot;Acc:P18936]
ND2	ENSGALG00000018378	5241	6281	protein_coding	NADH-ubiquinone oxidoreductase chain 2 [Source:UniProtKB/Swiss-Prot;Acc:P18937]
ND3	ENSGALG00000018366	10776	11126	protein_coding	NADH-ubiquinone oxidoreductase chain 3 [Source:UniProtKB/Swiss-Prot;Acc:P18938]
ND4	ENSGALG00000018364	11486	12863	protein_coding	NADH-ubiquinone oxidoreductase chain 4 [Source:UniProtKB/Swiss-Prot;Acc:P18939]
ND6	ENSGALG00000018357	16184	16705	protein_coding	NADH-ubiquinone oxidoreductase chain 6 [Source:UniProtKB/Swiss-Prot;Acc:P18941]

Figure 4.2 Hierarchical clustering heatmap of the 10 mitochondrial genes from pairwise RNAseq comparisons, based on average logCPM values for each breed and age. Clustering shows that gene expression is most similar between the 11d samples and then the 42d samples, with expression decreasing with age in both breeds, but most significantly in CBRO.



4.3.2. Mitochondrial Variants in the Wooden Breast Phenotype

Forty-three SNPs were identified within the coding sequences of nine out of the ten MT genes investigated (Table 4.2). The exception (ENSGALG00000018372) was a Mt_tRNA gene, which demonstrated no SNPs within its coding sequence. However, 41 of the 43 SNP variants identified act as modifiers (upstream or downstream regulators) of the Mt_tRNA. SNP variants were found at the same loci and the frequency of the variant allele was similar between the three breeds (Table 4.3). Low impact synonymous variants accounted for 33 of the 43 SNPs, whereas nine were moderate impact missense

variants, and one was a high impact variant. High-impact variants are typically non-synonymous changes in the coding sequence. The single high-impact variant occurred in the Cytochrome C Oxidase 1 (COX1) gene in the form of a stop loss missense mutation. Also, all SNPs identified were either up- or downstream gene modifiers for Mt_tRNAs and other mitochondrial protein coding genes. Of the 43 SNPs identified, the majority were transitions (39) and the rest were transversions [9:1, TS:TV]. Transitions typically outnumber transversions, as transversions tend to be nonsynonymous. Furthermore, based on gene expression data, all these ten genes carrying the SNP variants are significantly down regulated in 42-day old CBRO.

Table 4.2 Summary of the SNP variants identified within the protein coding sequences of the mitochondrial genes. The table contains the gene name abbreviation and Ensembl release 85 gene IDs, the directional regulation of the gene in CBRO, the location of the SNP in the gene coding sequence (bp), possible alleles at the loci, and the predicted effect of the SNP.

Gene Abbr.	Ensembl ID	Regulation (up or down)	Location (bp)	Alleles (ref:alt)	Predicted Effect
ND1	ENSGALG00000018382	down	4580	G:A	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018381, ENSGALG00000018379, and ND2 ENSGALG00000018378; downstream gene variant and modifier of ENSGALG00000018372
ND2	ENSGALG00000018378	down	5369	C:T	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018380, ENSGALG00000021716, and COX1 ENSGALG00000018373; downstream gene variant and modifier of ENSGALG00000018372
			5718	T:G	Moderate impact missense variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018380, ENSGALG00000021716, and COX1 ENSGALG00000018373; downstream gene variant and modifier of ENSGALG00000018372
			5750	A:G	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018380, ENSGALG00000021716, and COX1 ENSGALG00000018373; downstream gene variant and modifier of ENSGALG00000018372
			5826	T:C	Moderate impact missense variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018380, ENSGALG00000021716, and COX1 ENSGALG00000018373; downstream gene variant and modifier of ENSGALG00000018372
			5928	C:A	Moderate impact missense variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018380, ENSGALG00000021716, and COX1 ENSGALG00000018373; downstream gene variant and modifier of ENSGALG00000018372

Table 4.2. Continued

Gene Abbr.	Ensembl ID	Regulation (up or down)	Location (bp)	Alleles (ref:alt)	Predicted Effect
COX2	ENSGALG00000018370	down	8420	C:T	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018380, ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018372
			8464	T:C	Moderate impact missense variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018380, ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018372
			8609	T:C	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018380, ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018372
			8774	C:T	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018380, ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018372
ATP6	ENSGALG00000018368	down	9375	A:G	Moderate impact missense variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018380, ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018372
			9533	A:G	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018380, ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018372
			9593	G:A	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018380, ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018372
			9650	A:G	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018380, ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018372
			9797	G:A	Moderate impact missense variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018380, ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018372

Table 4.2. Continued

Gene Abbr.	Ensembl ID	Regulation (up or down)	Location (bp)	Alleles (ref:alt)	Predicted Effect
COX3	ENSGALG00000018367	down	10072	A:G	Moderate impact missense variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018380, ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018372
			10249	A:G	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018375, ENSGALG00000018372
			10261	G:A	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018375, ENSGALG00000018372
			10303	T:C	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018375, ENSGALG00000018372
			10438	T:C	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018375, ENSGALG00000018372
			10660	C:T	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018375, ENSGALG00000018372
ND3	ENSGALG00000018366	down	10968	T:C	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018375, ENSGALG00000018372
			10997	T:C	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018377, ENSGALG00000018376, ENSGALG00000018375, ENSGALG00000018372

Table 4.2. Continued

Gene Abbr.	Ensembl ID	Regulation (up or down)	Location (bp)	Alleles (ref:alt)	Predicted Effect
ND4	ENSGALG00000018364	down	11683	A:G	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018372, ENSGALG00000021720, ENSGALG00000018363
			11963	C:T	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018372, ENSGALG00000021720, ENSGALG00000018363
			11998	T:C	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018372, ENSGALG00000021720, ENSGALG00000018363
			12094	T:C	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018372, ENSGALG00000021720, ENSGALG00000018363
			12268	C:T	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018372, ENSGALG00000021720, ENSGALG00000018363
			12454	T:C	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018372, ENSGALG00000021720, ENSGALG00000018363
			12481	A:G	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018372, ENSGALG00000021720, ENSGALG00000018363
			12547	G:A	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018372, ENSGALG00000021720, ENSGALG00000018363
			12679	T:C	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNAs ENSGALG00000018372, ENSGALG00000021720, ENSGALG00000018363
ND6	ENSGALG00000018357	down	16346	G:A	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNA ENSGALG00000018358; downstream gene variant and modifier ND4L ENSGALG00000021719, ND4 ENSGALG00000018364
			16586	A:G	Low impact synonymous variant; upstream gene variant and modifier of MT_tRNA ENSGALG00000018358; downstream gene variant and modifier ND4 ENSGALG00000018364, MT_tRNA ENSGALG00000021720

Table 4.3 Summary of the frequency of the reference and alternate alleles for each identified SNP loci for the nine mitochondrial protein-coding genes for each of the three breeds. The table contains the gene name abbreviation, SNP location (bp) within the gene coding sequence, and columns for each breed containing the reference and alternate alleles and their relative frequencies.

Gene Abbr.	Location	LAY		CBRO		RJF	
ND1	4580	G:0	A:1	G:0.375	A:0.625	G:0.667	A:0.333
ND2	5369	C:0.666667	T:0.333333	C:1	T:0	C:1	T:0
	5718	T:1	G:0	T:1	G:0	T:0.583333	G:0.416667
	5750	A:1	G:0	A:1	G:0	A:0.8	G:0.2
	5826	T:1	C:0	T:0.75	C:0.25	T:1	C:0
	5928	C:0	A:1	C:0	A:1	C:0	A:1
COX1	6758	T:0	C:1	T:0.25	C:0.75	T:0.666667	C:0.333333
	6800	T:0	C:1	T:0.25	C:0.75	T:0.666667	C:0.333333
	6899	A:0	G:1	A:0.25	G:0.75	A:0.571429	G:0.428571
	7166	C:1	T:0	C:1	T:0	C:0.8	T:0.2
	7361	T:1	C:0	T:1	C:0	T:0.833333	C:0.166667
	7530	C:0	G:1	C:0	G:1	C:0	G:1
	7644	T:1	G:0	T:0.875	G:0.125	T:1	G:0
	7691	T:1	C:0	T:1	C:0	T:0.666667	C:0.333333
	8070	T:0	C:1	T:0.25	C:0.75	T:0.571429	C:0.428571
COX2	8420	C:1	T:0	C:1	T:0	C:0.833333	T:0.166667
	8464	T:0	C:1	T:0.25	C:0.75	T:0.666667	C:0.333333
	8609	T:0	C:1	T:0.25	C:0.75	T:0.571429	C:0.428571
	8774	C:0.5	T:0.5	C:1	T:0	C:1	T:0
ATP6	9375	A:1	G:0	A:1	G:0	A:0.833333	G:0.166667
	9533	A:0	G:1	A:0.333333	G:0.666667	A:0.571429	G:0.428571
	9593	G:0	A:1	G:0.333333	A:0.666667	G:0	A:1
	9650	A:1	G:0	A:1	G:0	A:0.833333	G:0.166667
	9797	G:0	A:1	G:0	A:1	G:0	A:1
COX3	10072	A:0	G:1	A:0.25	G:0.75	A:0.666667	G:0.333333
	10249	A:1	G:0	A:1	G:0	A:0.666667	G:0.333333
	10261	G:1	A:0	G:0.75	A:0.25	G:1	A:0
	10303	T:1	C:0	T:1	C:0	T:0.666667	C:0.333333
	10438	T:0	C:1	T:0	C:1	T:0	C:1
	10660	C:1	T:0	C:0.75	T:0.25	C:1	T:0

Table 4.3. Continued

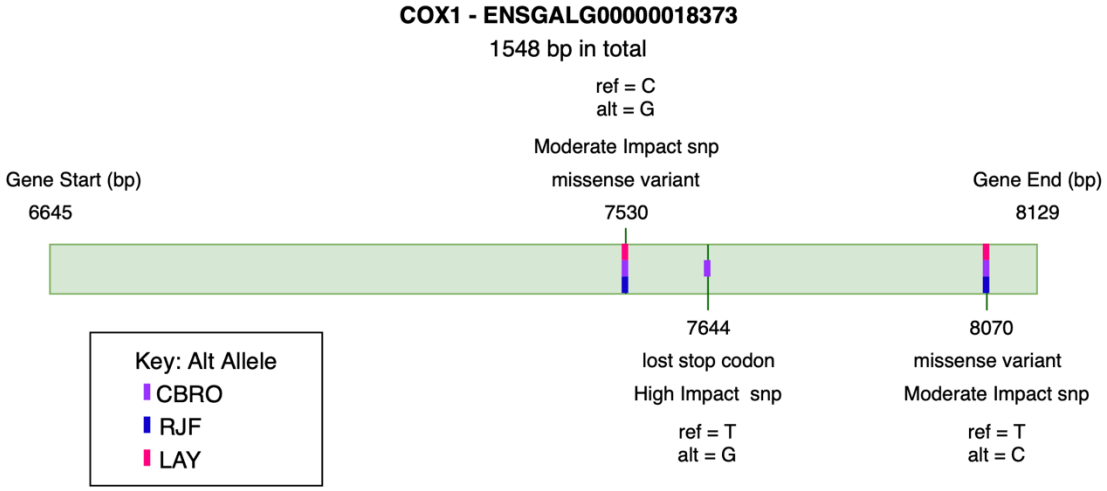
Gene Abbr.	Location	LAY		CBRO		RJF	
ND3	10968	T:0	C:1	T:0.25	C:0.75	T:0.571429	C:0.428571
	10997	T:0	C:1	T:0.25	C:0.75	T:0	C:1
ND4	11683	A:1	G:0	A:1	G:0	A:0.833333	G:0.166667
	11963	C:0	T:1	C:0.25	T:0.75	C:0.571429	T:0.428571
	11998	T:0.5	C:0.5	T:1	C:0	T:1	C:0
	12094	T:0	C:1	T:0	C:1	T:0	C:1
	12268	C:1	T:0	C:1	T:0	C:0.833333	T:0.166667
	12454	T:0	C:1	T:0.25	C:0.75	T:0.571429	C:0.428571
	12481	A:1	G:0	A:1	G:0	A:0.833333	G:0.166667
	12547	G:1	A:0	G:1	A:0	G:0.833333	A:0.166667
	12679	T:0	C:1	T:0	C:1	T:0	C:1
ND6	16346	G:1	A:0	G:1	A:0	G:0.875	A:0.125
	16586	A:0	G:1	A:0.333333	G:0.666667	A:0	G:1

Next, we estimated the Tajima's D statistic to detect signatures of selection along the mitochondrial genome, for each chicken breed (Figure 4.3). A negative value of Tajima's D is indicative of decreasing genetic diversity due to a directional selection, whereas positive values are indicative of balancing selection and increased genetic diversity. The location and directionality of peaks in Figure 4.3 are similar between LAY, CBRO and RJF at most locations across the mitochondrial genome. For RJF there are no negative Tajima's D values and several positive ones, indicating a high level of genetic diversity as we would expect from a population not undergoing any type of selection or bottleneck. The Tajima's D values for LAY are most similar to those for the

RJF except at one position around 13000 bp, indicating lower genetic diversity, and suggesting directional selection at this locus. The CBRO, although showing positive values in the same locations as the RJF and LAY, was less so, and had pronounced negative values for Tajima's D at around 7500 bp window. This peak sits atop the COX1 (ENSGALG00000018373, Table 4.1) gene, located between 6645 and 8192 bp, and is thus likely to be the gene experiencing directional selection in CBRO. COX1 contains nine known SNPs, one of which (7644 bp) is a high impact variant due to a lost stop codon, and two moderate impact missense SNP variants (7530 and 8070 bp) (Figure 4.4). COX1 is the main subunit of cytochrome c oxidase in respiratory complex IV, the final enzyme in oxidative phosphorylation (Debray et al., 2014; Dennerlein and Rehling, 2015). Mutations in COX1 have been linked to complex disorders such as primary ovarian insufficiency, Parkinson's disease, Alzheimer's disease, and encephalomyopathy in humans (Jiang et al., 2004; Frautschy, 2010; Hornig-Do et al., 2012; Arnold et al., 2013; Choi et al., 2013; Debray et al., 2014; Zhen et al., 2015; Shukuri et al., 2016; Hoffmann et al., 2018), suggesting the importance of this gene in several critical pathways. These disorders terms have been previously associated with WB by pathway analyses of the transcriptome from our lab.

Figure 4.3 Line graph of the values obtained by the calculation of the Tajima's D statistic to quantify the effect of selection on the mitochondrial genome across the three chicken breeds (CBRO = commercial broilers, LAY = laying chickens, RJF = ancestral red jungle fowl).

Figure 4.4 Diagram of the COX1 gene demonstrating the moderate-high impact SNPs within its coding sequence and the observation of these SNPs by breed. The location (bp) of the SNP is marked along the gene coding sequence and the reference and alternate alleles are noted, along with the predicted effect. Identification of the SNP within each breed is indicated by a colored marker at the location of the SNP. The two moderate impact SNP variants were observed in all three breeds so three colored markers are shown at these locations, while the high impact SNP variant was only observed in CBRO, as indicated by only one colored marker.

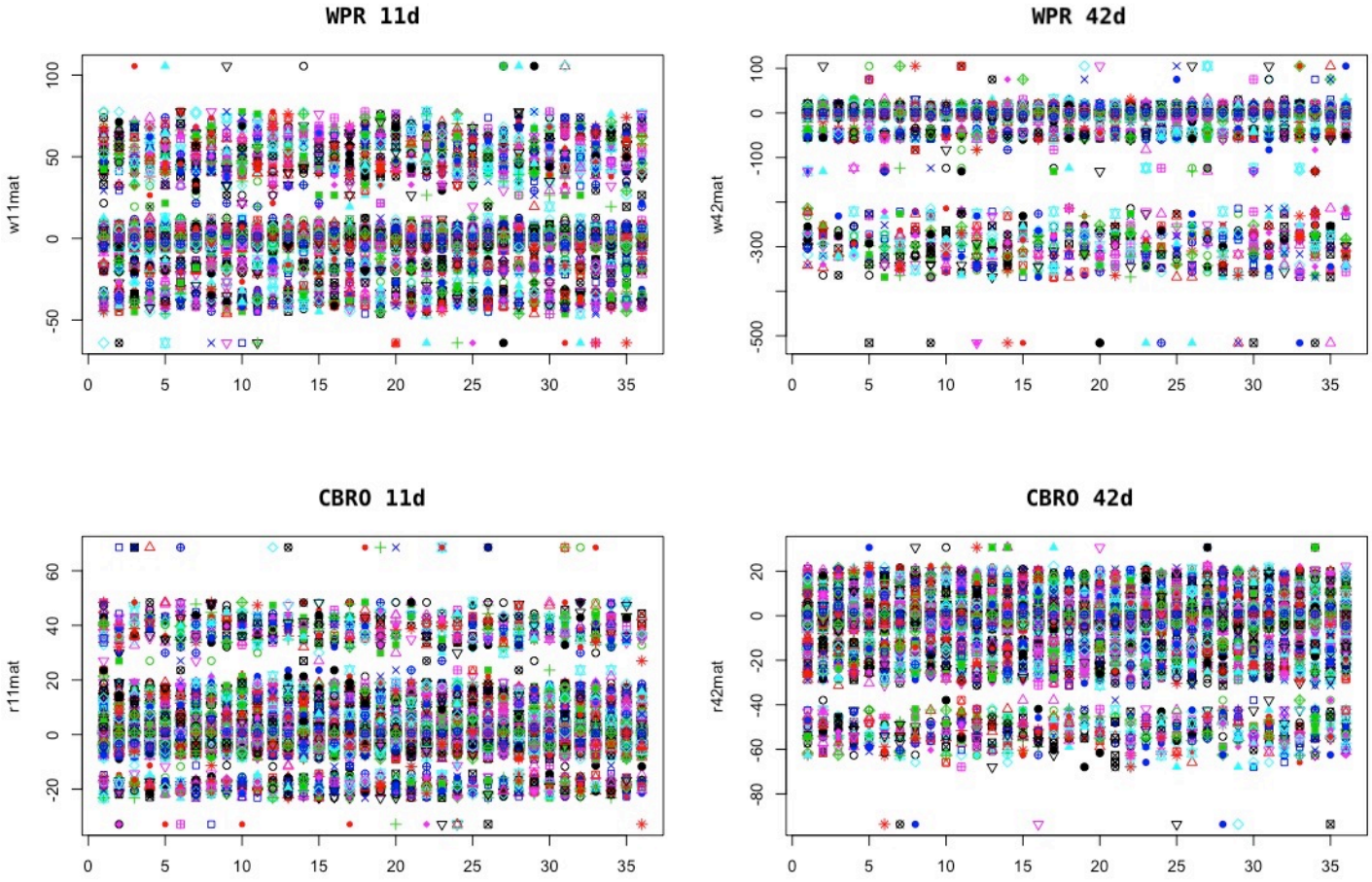


4.3.3. Divergent Expression Ratios of OXPHOS Counterparts

Considering the low impact of the majority of the SNPs identified in the mitochondrial genome, we investigated why the expression of these genes was different in the CBRO versus the WPR (slow-growth broiler). Particularly, we investigated the interaction of nuclear and mitochondrial genes, given that nuclear-encoded and MT-encoded genes work together to ensure normal cellular function. Given the importance of energy metabolism and oxidative stress in WB, we compared gene expression patterns in the nuclear and mitochondrial counterparts of the oxidative phosphorylation pathway

(OXPHOS). Also, of the polypeptides required for OXPHOS, only 13 are encoded by the mitochondrion, while roughly 70 are encoded by the nuclear (NUC) genome (Smeitink et al., 2001). To this end, the expression ratios for 51 NUC encoded MT associated genes (available from Ensebml BioMart, *Gallus gallus* genome, version 4.8, Ensembl Release 85, July 2016) involved in OXPHOS and the 36 MT encoded genes were compared between 11 and 42-day old broilers of either variety (Table A-5). Nuclear genes were subsampled 36 at a time to calculate the NUC:MT expression ratios. Each unique MT-NUC gene combination was resampled without replacement 1000 times for each of the four data sets (WPR and CBRO, 11 and 42 days of age), resulting in all 51 NUC OXPHOS genes being sampled approximately 20 times. Although gene expression was significantly down-regulated in both varieties between 11 and 42 days of age, this analysis demonstrated that in CBRO a high frequency of NUC:MT gene expression ratios fall close to zero, indicating that oxidative capacity is not different between the two ages (Figure 4.5). In WPR, we see a very surprising pattern where the 11- and 42-day old expression ratios were significantly different (Figure 4.5). Considering the immense change in body size seen in CBRO, the results suggest that the performance of the MT components of OXPHOS are constrained in CBRO, which in turn may cause or exacerbate hypoxia, oxidative stress, and altered energy metabolism observed in WB.

Figure 4.5 Frequency of NUC: MT OXPHOS gene expression ratios generated by resampling unique NUC: MT OXPHOS gene combinations without replacement 1000 times for each breed and age. For CBRO, NUC: MT OXPHOS gene expression ratios tend to fall near 0 indicating no change to OXPHOS capacity with an increase in age, while the NUC: MT OXPHOS gene expression ratios for the WPR show significant deviation from zero with increased age.



4.4. Discussion

Our investigation of mitochondrial gene expression confirmed the inhibition of OXPHOS observed by others (Kong et al., 2017; Papah et al., 2018). All of the gene products of the protein-coding MT genes observed here are involved in OXPHOS. The ND genes encode enzymes which act as NADH dehydrogenases, while the COX genes encode Cytochrome C Oxidase subunits, and ATP6 encodes the complex V ATP synthase which produces ATP from ADP (Smeitink et al., 2001; Sparks et al., 2005; Reinecke et al., 2009; Fuhrmann and Brüne, 2017; Pearce et al., 2017). Oxidative phosphorylation is an aerobic pathway in which energy molecules from beta-oxidation, glycolysis, and the TCA cycle are converted to ATP in the mitochondria and is required for proper cell function and maintenance (Kunz, 2001; Smeitink et al., 2001; Conley et al., 2001; Reinecke et al., 2009). Although muscles can utilize anaerobic metabolic pathways, OXPHOS is the most efficient method of ATP production for the cell, producing 38 molecules of ATP per glucose versus 2 (Conley et al., 2001; Reinecke et al., 2009; Hudson et al., 2017; Fuhrmann and Brüne, 2017). As OXPHOS is under both mitochondrial and nuclear genetic control, discerning phenotypic impacts is extremely complex. However, several conditions have been linked to OXPHOS disorders including diabetes, lactic acidosis, epilepsy, neuropathy, cardiomyopathy, and myopathy (Sparks et al., 2005; Misu et al., 2007; Olsson et al., 2011; Frazier et al., 2019).

Modern broiler chickens are particularly well known for their high growth rate and feed efficiency, especially with respect to breast muscle production. Breast muscle fiber type is consistent between varieties of chicken, regardless of growth-rate or genotype,

although fiber number, size, and density is variable (Kiessling, 1977; Remignon et al., 1994, 1995; Dransfield and Sosnicki, 1999; Scheuermann et al., 2003, 2004; MacRae et al., 2006; Velleman, 2007; Branciari et al., 2009; Clark and Velleman, 2016; Velleman et al., 2018). However, hypertrophy has been implicated as the main change responsible for breast size increase in modern CBRO (Roy et al., 2006; Velleman, 2007; Berri et al., 2007; Le Bihan-Duval et al., 2008; Trocino et al., 2015). The mechanisms behind this desirable performance phenotype have long been investigated, but recently Reverter et al., (2017) identified that a strong positive relationship exists between mitochondrial content of the breast and thigh ($P<0.0001$). They also observed a negative correlation of mitochondrial content with breast muscle yield ($P=0.037$) and high levels of individual variation (Reverter et al., 2017). However, increased breast muscle mass (low mitochondrial content) was also associated with an increased proportion of abdominal fat (Reverter et al., 2017). This phenotype coincides with biochemical characterizations of obesity and metabolic disorders, all of which have been associated with the aberrant molecular characteristics of WB.

Several studies have observed the transcriptome, metabolome, and proteome in relation to WB severity, finding impacts on glucose metabolism, OXPHOS, and oxidative stress (Mutryn et al., 2015; Abasht et al., 2016, 2019; Clark and Velleman, 2016; Kong et al., 2017; Kuttappan et al., 2017a; Cai et al., 2018; Hubert et al., 2018; Papah et al., 2018). Results of these investigations demonstrated increased MT beta-oxidation of fatty acids, while peroxisomal beta-oxidation of fatty acids was decreased in CBRO (Abasht et al., 2019). Also, the lipogenic enzyme ATP-citrate lyase was up-

regulated in CBRO indicating citrate export from the MT for cytosolic production of acetyl-CoA (Abasht et al., 2019). This further validates the correlation of increased body fat observed by Reverter et al. (2017) and the association with obesity models.

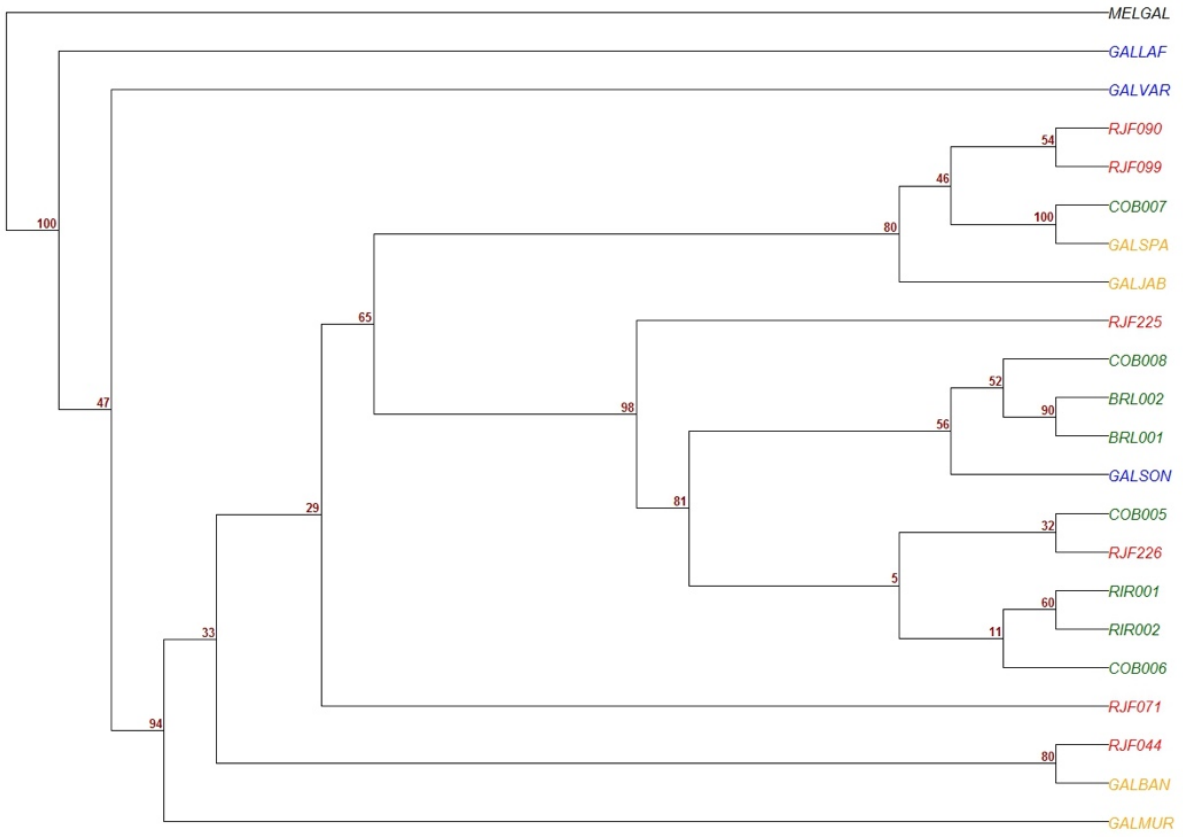
The extreme growth rate of CBRO has also been associated with an increase in cardiovascular disease and inflammatory states. These conditions are also associated with WB by diminished cardiovascular efficiency due to decreased blood vessel density, and resultant hypoxia. Recently, Sihvo et al. (2018) investigated this relationship finding that WB exhibits reduced vessel density which exacerbates hypoxic conditions. Mitochondria can adapt to hypoxic conditions through the activation of hypoxia-inducible factor-1α (HIF1α) which shuts down electron transport chain complex IV and up-regulating expression of pyruvate dehydrogenase kinase (decreasing pyruvate conversion to Acetyl CoA) to reduce oxygen consumption by the MT (Guzy et al., 2005; Solaini et al., 2010; Fuhrmann and Brüne, 2017; McGarry et al., 2018). HIF1α also stimulates glycolysis by up-regulating the expression of glucose transporters (Solaini et al., 2010; Fuhrmann and Brüne, 2017; McGarry et al., 2018). Hypoxic conditions increase the production of reactive oxygen species, furthering cellular damage and inflammation (Solaini et al., 2010; Fuhrmann and Brüne, 2017; McGarry et al., 2018) exacerbating WB. Concurrently, hypoxia downregulates OXPHOS, and stimulates anaerobic glycolysis, both of which increase inflammation. The downregulation of the mitochondrial counterparts of OXPHOS in WB found in this study supports the hypoxia-driven oxidative stress and inflammation mechanism in action. Additionally, the expression ratios of MT-nuclear OXPHOS counterparts suggests that diminished

mitochondrial gene expression may be a limiting factor in supplying the energy demands of fast growth, therefore instigating the symptoms of WB.

The causes of diminished mitochondrial function can be varied. While nonsynonymous mutations have been identified in some human disorders (e.g. See Warburg Phenomenon), we found only one nonsynonymous change in CBRO, but this variant was also shared among other chicken breeds. Furthermore, an MT phylogenetic tree including CBRO, RJF, and LAY breeds (based on MT genetic distances) shows remarkable conservation of the MT genome across members of *Gallus gallus* (Figure 4.6). When contrasted against the divergence of nuclear genomes across members of *Gallus gallus*, the similarity of MT genomes is not only surprising, but emerges as a potential factor limiting continued phenotypic improvements that depend on MT genome products.

A second, complementary factor affecting mitochondrial function is the number of mitochondria. Although few mitochondria are expected in muscles composed of type IIB fibers, those impacted by diffuse WB typically demonstrates aggregations of 10-25 mitochondria between the myofibrils, with the observation of more than 40 in those scored as severely affected (Sihvo et al., 2018). However, areas of unaffected tissue in both "normal" and affected WB samples demonstrated single mitochondria or rows of only two or three lined up along the myofibrils (Sihvo et al., 2018). Histologically a swollen matrix chamber, loss of cristae, and vacuolation were observed in the broiler breast muscles regardless of WB, but the degree of these changes was increased in WB muscles (Sihvo et al., 2018). Furthermore, normal mitochondria were also observed in all cases, just in varying degrees. These findings are representative of those previously observed by Papah et al. (2017), but with the addition of detail regarding increased mitochondrial content compared to non-WB CBRO samples.

Figure 4.6 Phylogenetic tree of mitochondrial genetic distances of CBRO, RJF, and LAY as well as other members of *Gallus gallus* demonstrating conservation of the mitochondrial genome.



4.5. Conclusions

Although several studies have considered the role of the mitochondrion in WB, to our knowledge this is the first study to directly investigate the gene expression profiles specific to the MT genome, MT genome variants in CBRO, as well as investigate the cooperative regulation of OXPHOS by nuclear genes in relationship to WB. Our data showed that MT OXPHOS genes were downregulated in WB affected CBRO, compared to WPR. The diminished function of MT genes was not a result of mutations affecting MT transcription, but our data is unable to rule out alteration of MT gene translation. Sequence conservation in the MT genome across *Gallus gallus*, combined with its uniparental inheritance of MT genomes, suggests that unlike the nuclear genome, the MT genome has remained relatively unaffected by directional selection for performance traits. These findings suggest that the MT genome structure and function may be the constraint in growth performance, and exacerbating WB.

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5. CONCLUSIONS

5.1. Overview of Our Work

The work completed here investigated the roles of genetic background, age, and diet in causing wooden breast (WB). A major takeaway from this work and emerging work from various researchers is that diet has no role in causing or ameliorating WB. Instead, we found strong support for a genetic basis of WB in broilers defined by a history of selection for feed conversion ratio (and consequently, growth rate). Although several studies have investigated the molecular architecture of WB and the role of its characteristics, this is the first body of work to investigate pathophysiological mechanisms through comparative analyses with fast- and slow-growth varieties as well as the ancestral RJF. WB is an inherited, age-dependent multi-system condition characterized by dysregulation of oxidative phosphorylation (OXPHOS).

Growth-rate, high body weight, breast muscle mass, and feed efficiency (performance traits) have long been linked to WB through observation of variation in incidence and severity. The molecular characteristics of hypoxia, oxidative stress, and dysregulated glucose metabolism have been observed by many. As the breast muscle is anaerobic due to its muscle fiber composition, few have considered the role of the mitochondria (MT), although its damage, degradation, and altered gene expression have been described. Here we validated previous observations of the transcriptional profile of WB and linked selection for broiler performance to aberrant OXPHOS. Furthermore, we

found an abnormal pattern of nuclear: MT OXPOS expression in 42-old birds affected by WB.

The MT is an intrinsic producer of reactive oxygen species (ROS) and oxidative stress. This is in large part due to the activities of the electron transport chain and OXPHOS. Furthermore, attenuated MT DNA repair mechanisms and a close relationship with the electron transport chain increase its susceptibility to damage by ROS during oxidative stress. Conversely, MT are also severely affected when oxygen is limiting, or under conditions of hypoxia. Although they seem antagonistic, hypoxia and ROS work in conjunction to produce oxidative stress, oxidative damage, and inflammation. As both, oxidative stress and hypoxia are hallmarks of WB tissue, observation of MT degradation is not surprising. However, it has not been observed if cellular and MT damage in WB is a consequence of OXPHOS ROS production or a symptom of the overall condition.

5.2. Relevance to Industry

WB presents not only substantial economic losses for the industry but also consumer related propagation of miss-information. Reduction of WB has so far, only been attained along with reduced growth-rate, typically due to decreased caloric intake during the growth period. All manner of antioxidants, vitamins, and minerals have been hypothesized as functional in combating oxidative stress or as possibly being limiting for normal cellular growth and development. Combined with these previous findings, this work substantiates that WB cannot be mitigated by nutritional/management practices. These findings indicate that selection methods besides phenotypes/genotypes may be

necessary in order to completely abrogate WB. However, this is a costly and inefficient process which will require years for the industry to implement. Furthermore, without the ability for informed selection of pedigree lines, this would be unreasonable. The work completed here offers some potential applications for future genomic selection programs.

The comparative analysis of the impact of diet and age between White Plymouth Rock chickens and commercial broilers was highly informative of the genetic changes associated with selection for performance traits and narrowing down on WB specific gene expression. It allowed for isolation of signatures of normal growth and development which have been selected upon in commercial broilers and identification of those which are now aberrantly regulated and likely involved in WB pathogenesis. Furthermore, although the diets did not demonstrate a significant impact on gene expression, an age and breed related interaction with the diet was identified. Genes differentially expressed due to age or breed and further impacted by diet yield insights into nutritional and genomic interplay which provides insights about the role of feed ingredients on normal growth and development.

5.3. Possible Applications and Future Directions

This work demonstrates identified genes which could be used as biomarkers for genetic testing of pedigree lines. Selecting based on OXPHOS function is one method which could be implemented with little effort. Another option would be creating a custom panel for divergently expressed genes involved in cellular functions like signaling, development, and differentiation, regulation of inflammation and metabolism,

and muscle structure and function such as CXCL14, GJA8, UCP3, LMOD2, CSRP3, HPGD, and IQCA1 which were identified as WB- and inflammation-associated in commercial broilers in this work. Poultry primary breeders are adopting genomic selection into their selection practices, and therefore selecting for beneficial genotypes is highly feasible. However, as the commercial broilers (which experience WB) are the product of complex AB♂ x CD♀ crosses, substantial work is needed to evaluate individual lines and the impact of selection for specific variants in the ultimate hybrid product.

The candidate biomarkers identified here could also provide a foundation on which further methods of ameliorating WB could be tested. Many of these genes are targets in ongoing research for disease therapies and there is a potential to model those investigations in broilers. However, this would be a completely new direction for WB research, requiring high technical skills, extensive funding, and tightly controlled rearing conditions. Finally, this too would most likely be inefficient and require not only years to complete and validate, but consumer education and acceptance could become an issue as well.

APPENDIX A

SUPPLEMENTARY TABLES

Table A-1. Diet formulation including basal diet ingredients (percent) for starter, grower, and finisher feeds.

			Percent of Diet for each ingredient		
		Name:	Starter	Grower	Finisher
IngrCode	Ingredient Name	Version:	17	18	19
2	TAMU CORN #21		0.512	0.5495	0.6045
21	TAMU SOYBEAN ML48%		0.365	0.335	0.2955
50	DL-MET98		0.003575	0.003	0.0029
53	LYSINE HCL		0.002425	0.0017	0.001775
54	L-THREONINE 98.5%		0.001325	0.000825	0.000725
61	Oil		0.05	0.05	0.05
70	LIMESTONE		0.01425	0.01305	0.01225
73	BIOFOS 16/21P		0.01565	0.0139	0.0127
75	SALT		0.00465	0.0046	0.00365
76	SODIUM BICARB		0	0.00005	0.00135
79	TAMU TRACE MINERALS		0.0005	0.0005	0.0005
80	TAMU VITAMINS		0.0015	0.0015	0.0015
SAND	sand		0.029105	0.02665	0.012745

Table A-2. Summary of canola oil concentration and estimated omega-6:3 ratio for T1, T2, and C for starter, grower, and finisher feeds.

Diet	Canola Oil % Inclusion	Omega 6-3 Ratio
T1 Starter	5%	4.4:1
T2 Starter	0.75	11.2:1
C Starter	0	20:1
T1 Grower	5	4.49:1
T2 Grower	0.75	11.6:1
C Grower	0	19.7:1
T1 Finisher	5	4.64:1
T2 Finisher	1	11:1
C Finisher	0	20:1

TableA-3. Summary of differential gene expression results from pairwise contrasts and results of the core pathway analysis in IPA. Table shows the specific information for each pairwise data set on differentially expressed genes associated IPA analysis of core pathways, upstream regulators for each dataset, and top disease and disorder terms.

Against	**R708 42d C**	**R708 42d T1**	**R708 42d T2**
Control	**R708 11d C**	**R708 11d T1**	**R708 11d T2**
DE Up	137	358	537
DE Down	130	260	313
Total DE	267	618	850
Top Canonical PW	Oxidative Phosphorylation	Oxidative Phosphorylation	Mitochondrial Dysfunction
	Mitochondrial Dysfunction	Mitochondrial Dysfunction	Oxidative Phosphorylation
	TCA Cycle II (Eukaryotic)	Sirtuin Signaling Pathway	Sirtuin Signaling Pathway
	Superpathway of Cholesterol Biosynthesis	TCA Cycle II (Eukaryotic)	Superpathway of Cholesterol Biosynthesis
	Sirtuin Signaling Pathway	Complement System	TCA Cycle II (Eukaryotic)
Upstream Regs	KDM5A	KDM5A	TP53
	INSR	TP53	KD5MA
	RICTOR	methypredisolone	torin 1
	RB1	MAPT	metribolone
	arsenic trioxide	dexamethasone	mono-(2-ethylhexyl)phthalate
Diseases	Hereditary Disorder	Cancer	Cancer
	Organismal Injury and Abnormalities	Organismal Injury and Abnormalities	Organismal Injury and Abnormalities
	Skeletal and Muscular Disorders	Hereditary Disorder	Gastrointestinal Disease
	Gastrointestinal Disease	Skeletal and Muscular Disorders	Hepatic System Disease
	Hepatic System Disease	Connective Tissue Dissorders	Endocrine System Disorders

TableA-3. Continued

Against	**WPR 42d C**	**WPR 42d T1**	**WPR 42d T2**
Control	**WPR 11d C**	**WPR 11d T1**	**WPR 11d T2**
DE Up	35	61	78
DE Down	80	111	219
Total DE	115	172	297
Top Canonical PW	NAD Phosphorylation and Dephosphorylation	Mitochondrial Dysfunction	Oxidative Phosphorylation
	Cell Cycle: G1/S Checkpoint Regulation	Epithelial Adherenes Junction Signaling	Mitochondrial Dysfunction
	Epithelial Adherens Junction Signaling	Oxidative Phosphorylation	Sirtuin Signaling Pathway
	Cyclins and Cell Cycle Regulation	Remodeling of Epithelial Adherens Junctions	Superpathway of Cholesterol Biosynthesis
	Hepatic Fibrosis/Hepatic Stellate Cell Activation	Sirtuin Signaling Pathway	CD28 Signaling in T Helper Cells
Upstream Regs	STAT5B	RICTOR	RICTOR
	FGF1	DMD	Esrra
	ADRB	AMOT	POR
	ESR1	AMOTL2	arsenic trioxide
	YY1	AMOTL1	INSR
Diseases	Cancer	Cancer	Cancer
	Organismal Injury and Abnormalities	Organismal Injury and Abnormalities	Organismal Injury and Abnormalities
	Reproductive System Disease	Endocrine System Disorders	Gastrointestinal Disease
	Respiratory Disease	Cardiovascular Disease	Hepatic System Disease
	Cardiovascular Disease	Skeletal and Muscular Disorders	Neurological Disease

TableA-3. Continued

Against	**R708 C**	**R708 T1**	**R708 T2**
Control	**WPR C**	**WPR T1**	**WPR T2**
DE Up	740	960	686
DE Down	478	714	651
Total DE	1218	1674	1337
Top Canonical PW	Protein Kinase A Signaling	Protein Kinase A Signaling	Glycolysis I
	NRF2-mediated oxidative stress response	NRF2-mediated oxidative stress response	Actin Cytoskeleton Signaling
	RhoGDI Signaling	Actin Cytoskeleton Signaling	Aldosterone Signaling in Epithelial Cells
	Complement System	IL-8 Signaling	Regulation of Actin-based Motility by Rho
	Glycolysis I	Renin-Angiotensisn Signaling	Glutamate Receptor Signaling
Upstream Regs	KRAS	TP53	TGFB1
	TGFB1	TGFB1	beta-estradiol
	TP53	beta-estradiol	TP53
	HRAS	dexamethasone	HNRNPA2B1
	methapyrilene	KRAS	HRAS
Diseases	Cancer	Cancer	Cancer
	Organismal Injury and Abnormalities	Organismal Injury and Abnormalities	Organismal Injury and Abnormalities
	Endocrine System Disorders	Endocrine System Disorders	Endocrine System Disorders
	Gastrointestinal Disease	Gastrointestinal Disease	Gastrointestinal Disease
	Hepatic System Disease	Hepatic System Disease	Hereditary Disorder

TableA-3. Continued

Against	**R708 11d**	**R708 42d**	**WPR 42d**	**R708 42d**
Control	**WPR 11d**	**WPR 42d**	**WPR 11d**	**R708 11d**
DE Up	1214	2183	488	1450
DE Down	1282	1321	681	975
Total DE	2496	3504	1169	2425
Top Canonical PW	EIF2 Signaling Protein Ubiquitination Stress Response NRF2-mediated Oxidative Stress Response Breast Cancer Regulation by Stathmin 1 Mechanisms of Viral Exit from Host Cells	NRF2-Mediated Oxidative Stress Response Actin Cytoskeleton Signaling Protein Kinase A Signaling Signaling by Rho Family GTPases Breast Cancer Regulation by Stathmin 1	Oxidative Phosphorylation Mitochondrial Dysfunction Sirtuin Signaling Pathway Cholesterol Biosynthesis I Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	Mitochondrial Dysfunction Oxidative Phosphorylation Sirtuin Signaling Pathway Gluconeogenesis I NRF2-mediated Oxidative Stress Response
Upstream Regs	TP53 RRP1B CDKN1A ESR1 MYC	TP53 TGF1B beta-estradiol MYC HRAS	RICTOR torin 1 TP53 mono-(2-ethylhexyl)phthalate methylprednisolone	TP53 MYC TGFB1 FOXO1 torin 1
Diseases	Cancer Organismal Injury and Abnormalities Endocrine System Disorders Gastrointestinal Disease Hepatic System Disease	Cancer Organismal Injury and Abnormalities Endocrine System Disorders Gastrointestinal Disease Hepatic System Disease	Cancer Organismal Injury and Abnormalities Endocrine System Disorders Reproductive System Disease Gastrointestinal Disease	Cancer Organismal Injury and Abnormalities Endocrine System Disorders Gastrointestinal Disease Hepatic System Disease

Table A-4. List of unique and shared most variable genes associated to broiler performance traits between breeds. Gene name abbreviations and Ensembl release 85 gene IDs are provided. Genes identified by bold font are related to cell growth and differentiation, muscle function and development, and intracellular ion concentration.

Unique To Ross 708	Gene ID	Unique to WPR	Gene ID	Shared Genes	Gene ID
TNNT2	ENSGALG00000000302	BF1	ENSGALG00000000178	**HBAD**	ENSGALG00000007463
TNNI1	ENSGALG00000000313	BTF3	ENSGALG00000000395	**HBAA**	ENSGALG00000007468
MUSTN1	ENSGALG00000001709	ACE	ENSGALG00000000498	**GATM**	**ENSGALG00000023435**
CSRP3	ENSGALG00000004044	**FKBP51**	ENSGALG00000000947	**PROCA1**	ENSGALG00000027107
MYOM3	ENSGALG00000004155	**ONCM2**	ENSGALG00000003465	**CA3**	**ENSGALG00000027638**
SCN5A	ENSGALG00000006112	ANKRD!	ENSGALG00000006491	**HBE1**	ENSGALG00000028273
GREM1	ENSGALG00000009724	**PDK4**	ENSGALG00000009700	Novel Gene	ENSGALG00000028612
JCHAIN	ENSGALG00000011551	**ACTC1**	ENSGALG00000009844		
DCDC2	ENSGALG00000012668	SUN Domain-containing protein3-like	ENSGALG00000013105		
PRUNE2	ENSGALG00000015167	HNRPK	ENSGALG00000014366		
MYH15	ENSGALG00000015358	Uncharacterized Protein	ENSGALG00000022685		
RGCC	ENSGALG00000016954	RTN4RL2	ENSGALG00000023441		
IGLL1	ENSGALG00000021139	**BFIV21**	ENSGALG00000024372		
EX-FABP	ENSGALG00000024011	SNORA2	ENSGALG00000027108		
MYH1A	ENSGALG00000026748	**MYH1D**	ENSGALG00000027323		
MYH1C	ENSGALG00000027177	**CHAC1**	ENSGALG00000027874		
Uncharacterized Protein	ENSGALG00000027412	Uncharacterized Protein	ENSGALG00000028102		
MYH1B	ENSGALG00000028134	**GADD45B**	ENSGALG00000028143		

Table A-5. List of nuclear and mitochondrial oxidative phosphorylation genes used for the nuclear: mitochondrial gene expression ratio calculation. The table contains the location (NUC or MT), the Ensembl release 85 gene and transcript IDs. description, associated chromosome, coding sequence start and end location (bp), gene name abbreviation, and the gene type.

Location	Ensembl Gene ID	Ensembl Transcript ID	Description	Chromosome Name	Gene Start (bp)	Gene End (bp)	Associated Gene Name	Gene type
MT	ENSGALG00000018356	ENSGALT00000029076		MT	16708	16775		Mt_tRNA
MT	ENSGALG00000018357	ENSGALT00000029077	NADH-ubiquinone oxidoreductase chain 6 [Source:UniProtKB/Swiss-Prot;Acc:P18941]	MT	16184	16705	ND6	protein_coding
MT	ENSGALG00000018358	ENSGALT00000029078		MT	16108	16177		Mt_tRNA
MT	ENSGALG00000018359	ENSGALT00000029079		MT	16039	16107		Mt_tRNA
MT	ENSGALG00000018360	ENSGALT00000029080	Cytochrome b [Source:UniProtKB/Swiss-Prot;Acc:P18946]	MT	14893	16035	CYTB	protein_coding
MT	ENSGALG00000018361	ENSGALT00000029081	NADH-ubiquinone oxidoreductase chain 5 [Source:UniProtKB/Swiss-Prot;Acc:P18940]	MT	13071	14888	ND5	protein_coding
MT	ENSGALG00000018364	ENSGALT00000029084	NADH-ubiquinone oxidoreductase chain 4 [Source:UniProtKB/Swiss-Prot;Acc:P18939]	MT	11486	12863	ND4	protein_coding
MT	ENSGALG00000018366	ENSGALT00000029086	NADH-ubiquinone oxidoreductase chain 3 [Source:UniProtKB/Swiss-Prot;Acc:P18938]	MT	10776	11126	ND3	protein_coding
MT	ENSGALG00000018367	ENSGALT00000029087	Cytochrome c oxidase subunit 3 [Source:UniProtKB/Swiss-Prot;Acc:P18945]	MT	9923	10706	COX3	protein_coding
MT	ENSGALG00000018370	ENSGALT00000029090	Cytochrome c oxidase subunit 2 [Source:UniProtKB/Swiss-Prot;Acc:P18944]	MT	8331	9014	COX2	protein_coding
MT	ENSGALG00000018373	ENSGALT00000029093	Cytochrome c oxidase subunit 1 [Source:UniProtKB/Swiss-Prot;Acc:P18943]	MT	6645	8192	COX1	protein_coding

Table A-5. Continued

Location	Ensembl Gene ID	Ensembl Transcript ID	Description	Chromosome Name	Gene Start (bp)	Gene End (bp)	Associated Gene Name	Gene type
MT	ENSGALG00000018378	ENSGALT00000029098	NADH-ubiquinone oxidoreductase chain 2 [Source:UniProtKB/Swiss-Prot;Acc:P18937]	MT	5241	6281	ND2	protein_coding
MT	ENSGALG00000018382	ENSGALT00000029102	NADH-ubiquinone oxidoreductase chain 1 [Source:UniProtKB/Swiss-Prot;Acc:P18936]	MT	4050	5024	ND1	protein_coding
MT	ENSGALG00000018370	ENSGALT00000029090	Cytochrome c oxidase subunit 2 [Source:UniProtKB/Swiss-Prot;Acc:P18944]	MT	8331	9014	COX2	protein_coding
MT	ENSGALG00000018371	ENSGALT00000029091		MT	8261	8329		Mt_tRNA
MT	ENSGALG00000018372	ENSGALT00000029092		MT	8124	8258		Mt_tRNA
MT	ENSGALG00000018373	ENSGALT00000029093	Cytochrome c oxidase subunit 1 [Source:UniProtKB/Swiss-Prot;Acc:P18943]	MT	6645	8192	COX1	protein_coding
MT	ENSGALG00000018374	ENSGALT00000029094		MT	6573	6643		Mt_tRNA
MT	ENSGALG00000018375	ENSGALT00000029095		MT	6508	6573		Mt_tRNA
MT	ENSGALG00000018376	ENSGALT00000029096		MT	6434	6506		Mt_tRNA
MT	ENSGALG00000018377	ENSGALT00000029097		MT	6362	6430		Mt_tRNA
MT	ENSGALG00000018378	ENSGALT00000029098	NADH-ubiquinone oxidoreductase chain 2 [Source:UniProtKB/Swiss-Prot;Acc:P18937]	MT	5241	6281	ND2	protein_coding
MT	ENSGALG00000018379	ENSGALT00000029099		MT	5172	5240		Mt_tRNA
MT	ENSGALG00000018380	ENSGALT00000029100		MT	5102	5172		Mt_tRNA
MT	ENSGALG00000018381	ENSGALT00000029101		MT	5025	5096		Mt_tRNA

Table A-5. Continued

Location	Ensembl Gene ID	Ensembl Transcript ID	Description	Chromosome Name	Gene Start (bp)	Gene End (bp)	Associated Gene Name	Gene type
MT	ENSGALG00000018382	ENSGALT00000029102	NADH-ubiquinone oxidoreductase chain 1 [Source:UniProtKB/Swiss-Prot;Acc:P18936]	MT	4050	5024	ND1	protein_coding
MT	ENSGALG00000018383	ENSGALT00000029103		MT	3967	4040		Mt_tRNA
MT	ENSGALG00000018384	ENSGALT00000029104		MT	2346	3966		Mt_rRNA
MT	ENSGALG00000018385	ENSGALT00000029105		MT	2273	2345		Mt_tRNA
MT	ENSGALG00000018386	ENSGALT00000029106		MT	1297	2272		Mt_rRNA
MT	ENSGALG00000018387	ENSGALT00000029107		MT	1228	1296		Mt_tRNA
MT	ENSGALG00000021716	ENSGALT00000035258		MT	6280	6355		Mt_tRNA
MT	ENSGALG00000021717	ENSGALT00000035259	ATP synthase protein 8 [Source:UniProtKB/Swiss-Prot;Acc:P14093]	MT	9085	9249	ATP8	protein_coding
MT	ENSGALG00000021718	ENSGALT00000035260		MT	10708	10775		Mt_tRNA
MT	ENSGALG00000021719	ENSGALT00000035261	NADH-ubiquinone oxidoreductase chain 4L [Source:UniProtKB/Swiss-Prot;Acc:P18942]	MT	11196	11492	ND4L	protein_coding
MT	ENSGALG00000021720	ENSGALT00000035262		MT	12864	12932		Mt_tRNA
NUC	ENSGALG00000026732	ENSGALT00000043689	NADH:ubiquinone oxidoreductase core subunit V1 [Source:CGNC Symbol;Acc:123]	5	89593	93802	NDUFV1	protein_coding
NUC	ENSGALG00000000508	ENSGALT00000000694	succinate dehydrogenase complex, subunit B, iron sulfur (Ip) [Source:CGNC Symbol;Acc:63429]	21	162038	170418	SDHB	protein_coding

Table A-5. Continued

Location	Ensembl Gene ID	Ensembl Transcript ID	Description	Chromosome Name	Gene Start (bp)	Gene End (bp)	Associated Gene Name	Gene type
NUC	ENSGALG00000000842	ENSGALT00000001235	Gallus gallus NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2, 8kDa (NDUFA2), mRNA. [Source:RefSeq mRNA;Acc:NM_001302137]	13	392987	394389	NDUFA2	protein_coding
NUC	ENSGALG00000001330	ENSGALT00000002026	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C1 (subunit 9) [Source:CGNC Symbol;Acc:911]	27	3493403	3495227	ATP5G1	protein_coding
NUC	ENSGALG00000001355	ENSGALT00000034898	NADH:ubiquinone oxidoreductase subunit A8 [Source:CGNC Symbol;Acc:929]	17	8704356	8706618	NDUFA8	protein_coding
NUC	ENSGALG00000001452	ENSGALT00000002207	ATP synthase, H+ transporting, mitochondrial Fo complex subunit B1 [Source:CGNC Symbol;Acc:1004]	26	3157146	3161840	ATP5F1	protein_coding
NUC	ENSGALG00000002033	ENSGALT00000003162	NADH:ubiquinone oxidoreductase subunit B6 [Source:CGNC Symbol;Acc:1444]	14	13393595	13395738	NDUFB6	protein_coding
NUC	ENSGALG00000002490	ENSGALT00000003923	ubiquinol-cytochrome c reductase core protein II [Source:CGNC Symbol;Acc:1783]	14	15085440	15096857	UQCRC2	protein_coding
NUC	ENSGALG00000003625	ENSGALT00000005739	NADH:ubiquinone oxidoreductase subunit S5 [Source:CGNC Symbol;Acc:2647]	23	5287781	5289451	NDUFS5	protein_coding
NUC	ENSGALG00000004302	ENSGALT00000033730	NADH:ubiquinone oxidoreductase subunit A10 [Source:CGNC Symbol;Acc:3165]	7	6552331	6592902	NDUFA10	protein_coding

Table A-5. Continued

Location	Ensembl Gene ID	Ensembl Transcript ID	Description	Chromosome Name	Gene Start (bp)	Gene End (bp)	Associated Gene Name	Gene type
NUC	ENSGALG00000005465	ENSGALT00000008778	NADH:ubiquinone oxidoreductase subunit B10 [Source:CGNC Symbol;Acc:4071]	14	6130748	6132664	NDUFB10	protein_coding
NUC	ENSGALG00000005749	ENSGALT00000009231	cytochrome c oxidase subunit IV isoform 1 [Source:CGNC Symbol;Acc:4285]	11	17077534	17081626	COX4I1	protein_coding
NUC	ENSGALG00000005789	ENSGALT00000009300	ubiquinol-cytochrome c reductase core protein I [Source:CGNC Symbol;Acc:4319]	12	9046001	9054965	UQCRC1	protein_coding
NUC	ENSGALG00000006073	ENSGALT00000009801	NADH:ubiquinone oxidoreductase subunit AB1 [Source:CGNC Symbol;Acc:4554]	14	6864896	6866786	NDUFAB1	protein_coding
NUC	ENSGALG00000006753	ENSGALT00000010913	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1 [Source:CGNC Symbol;Acc:5096]	1	4144466	4151271	ATP5C1	protein_coding
NUC	ENSGALG00000007096	ENSGALT00000011489	ubiquinol-cytochrome c reductase, complex III subunit VII, 9.5kDa [Source:CGNC Symbol;Acc:5357]	13	16453956	16455280	UQCRQ	protein_coding
NUC	ENSGALG00000007205	ENSGALT00000011661	cytochrome c oxidase subunit VIa polypeptide 1 [Source:CGNC Symbol;Acc:50396]	15	9297230	9298519	COX6A1	protein_coding
NUC	ENSGALG00000007926	ENSGALT00000012869	ATP synthase, H+ transporting, mitochondrial Fo complex subunit D [Source:CGNC Symbol;Acc:5993]	18	10897737	10900757	ATP5H	protein_coding
NUC	ENSGALG00000008066	ENSGALT00000013095	Gallus gallus ubiquinol-cytochrome c reductase, complex III subunit X (UQCR10), mRNA. [Source:RefSeq mRNA;Acc:NM_001302149]	15	11034185	11035607	UQCR10	protein_coding

Table A-5. Continued

Location	Ensembl Gene ID	Ensembl Transcript ID	Description	Chromosome Name	Gene Start (bp)	Gene End (bp)	Associated Gene Name	Gene type
NUC	ENSGALG00000008084	ENSGALT00000013121	NADH:ubiquinone oxidoreductase core subunit S3 [Source:CGNC Symbol;Acc:6122]	5	22088781	22093418	NDUFS3	protein_coding
NUC	ENSGALG00000008239	ENSGALT00000013407	Gallus gallus NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa (NDUFB3), mRNA. [Source:RefSeq mRNA;Acc:NM_001302167]	7	10893771	10897452	NDUFB3	protein_coding
NUC	ENSGALG00000008606	ENSGALT00000014015	NADH:ubiquinone oxidoreductase core subunit S1 [Source:CGNC Symbol;Acc:6532]	7	12077169	12090936	NDUFS1	protein_coding
NUC	ENSGALG00000008613	ENSGALT00000014034	NADH:ubiquinone oxidoreductase subunit A1 [Source:CGNC Symbol;Acc:6539]	4	16454195	16454917	NDUFA1	protein_coding
NUC	ENSGALG00000008821	ENSGALT00000014337	NADH:ubiquinone oxidoreductase subunit A5 [Source:CGNC Symbol;Acc:50588]	1	22078542	22084456	NDUFA5	protein_coding
NUC	ENSGALG00000009076	ENSGALT00000014768	NADH:ubiquinone oxidoreductase subunit B5 [Source:CGNC Symbol;Acc:6907]	9	16903953	16907474	NDUFB5	protein_coding
NUC	ENSGALG00000009286	ENSGALT00000015106	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C3 (subunit 9) [Source:CGNC Symbol;Acc:52137]	7	16150149	16153215	ATP5G3	protein_coding
NUC	ENSGALG00000009703	ENSGALT00000015797	NADH:ubiquinone oxidoreductase core subunit S7 [Source:CGNC Symbol;Acc:56235]	28	3064298	3067588	NDUFS7	protein_coding
NUC	ENSGALG00000011325	ENSGALT00000018479	NADH:ubiquinone oxidoreductase subunit A12 [Source:CGNC Symbol;Acc:8606]	1	45179843	45187320	NDUFA12	protein_coding

Table A-5. Continued

Location	Ensembl Gene ID	Ensembl Transcript ID	Description	Chromosome Name	Gene Start (bp)	Gene End (bp)	Associated Gene Name	Gene type
NUC	ENSGALG00000013167	ENSGALT00000021508	succinate dehydrogenase complex flavoprotein subunit A [Source:CGNC Symbol;Acc:49461]	2	85681939	85697842	SDHA	protein_coding
NUC	ENSGALG00000013191	ENSGALT00000021552	NADH:ubiquinone oxidoreductase subunit S6 [Source:CGNC Symbol;Acc:9959]	2	86002617	86007620	NDUFS6	protein_coding
NUC	ENSGALG00000014121	ENSGALT00000022855	cytochrome c oxidase subunit 5A [Source:CGNC Symbol;Acc:10534]	10	1891957	1896138	COX5A	protein_coding
NUC	ENSGALG00000014310	ENSGALT00000023134	NADH:ubiquinone oxidoreductase core subunit V2 [Source:CGNC Symbol;Acc:52803]	2	98526884	98541978	NDUFV2	protein_coding
NUC	ENSGALG00000014644	ENSGALT00000002698	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle [Source:CGNC Symbol;Acc:49066]	Z	2151456	2159196	ATP5A1	protein_coding
NUC	ENSGALG00000015372	ENSGALT00000024803	ATP synthase, H+ transporting, mitochondrial Fo complex subunit E [Source:CGNC Symbol;Acc:11448]	Z	53307243	53309110	ATP5I	protein_coding
NUC	ENSGALG00000015751	ENSGALT00000025402	ATP synthase, H+ transporting, mitochondrial Fo complex subunit F6 [Source:CGNC Symbol;Acc:11749]	1	101882851	101884143	ATP5J	protein_coding
NUC	ENSGALG00000015906	ENSGALT00000031654	cytochrome c oxidase subunit VIIa polypeptide 2 (liver) [Source:CGNC Symbol;Acc:11867]	3	80197965	80201301	COX7A2	protein_coding
NUC	ENSGALG00000016336	ENSGALT00000026351	NADH:ubiquinone oxidoreductase subunit B9 [Source:CGNC Symbol;Acc:12221]	2	138071064	138074938	NDUFB9	protein_coding

Table A-5. Continued

Location	Ensembl Gene ID	Ensembl Transcript ID	Description	Chromosome Name	Gene Start (bp)	Gene End (bp)	Associated Gene Name	Gene type
NUC	ENSGALG00000017380	ENSGALT00000028065	ubiquinol-cytochrome c reductase hinge protein [Source:CGNC Symbol;Acc:13054]	8	20498970	20500444	UQCRH	protein_coding
NUC	ENSGALG00000017675	ENSGALT00000032767	NADH:ubiquinone oxidoreductase core subunit S8 [Source:CGNC Symbol;Acc:13124]	5	199552	201194	NDUFS8	protein_coding
NUC	ENSGALG00000019445	ENSGALT00000030826	cytochrome c oxidase subunit 6C [Source:CGNC Symbol;Acc:13868]	2	128069939	128075639	COX6C	protein_coding
NUC	ENSGALG00000022539	ENSGALT00000036114	Gallus gallus ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (ATP5B), mRNA. [Source:RefSeq mRNA;Acc:NM_001031391]	LGE22C19W28_E50C23	954486	965125	ATP5B	protein_coding
NUC	ENSGALG00000022813	ENSGALT00000036704	NADH:ubiquinone oxidoreductase subunit V3 [Source:CGNC Symbol;Acc:15744]	1	108926008	108928938	NDUFV3	protein_coding
NUC	ENSGALG00000025819	ENSGALT00000044199	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 [Source:CGNC Symbol;Acc:3283]	11	7596758	7603866	UQCRFS1	protein_coding
NUC	ENSGALG00000025999	ENSGALT00000045941	NADH dehydrogenase [Source:RefSeq peptide;Acc:NP_001289036]	2	26144942	26148789	NDUFA4	protein_coding
NUC	ENSGALG00000026108	ENSGALT00000046192	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit [Source:CGNC Symbol;Acc:56808]	JH375570.1	172	2062	ATP5D	protein_coding

Table A-5. Continued

Location	Ensembl Gene ID	Ensembl Transcript ID	Description	Chromosome Name	Gene Start (bp)	Gene End (bp)	Associated Gene Name	Gene type
NUC	ENSGALG00000026530	ENSGALT00000046236	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C2 (subunit 9) [Source:CGNC Symbol;Acc:64862]	JH376268.1	49431	50544	ATP5G2	protein_coding
NUC	ENSGALG00000027277	ENSGALT00000045899	succinate dehydrogenase complex subunit C [Source:CGNC Symbol;Acc:63505]	JH376252.1	4289	9884	SDHC	protein_coding
NUC	ENSGALG00000027607	ENSGALT00000045518	NADH:ubiquinone oxidoreductase core subunit S2 [Source:CGNC Symbol;Acc:66024]	AADN03017199.1	21	1922	NDUFS2	protein_coding
NUC	ENSGALG00000027688	ENSGALT00000043614	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 4, 15kDa [Source:CGNC Symbol;Acc:55666]	1	79257200	79258833	NDUFB4	protein_coding
NUC	ENSGALG00000027963	ENSGALT00000045264	cytochrome c oxidase subunit 7C [Source:HGNC Symbol;Acc:HGNC:2292]	Z	61059202	61062815	COX7C	protein_coding
NUC	ENSGALG00000028026	ENSGALT00000045576	NADH:ubiquinone oxidoreductase subunit S4 [Source:HGNC Symbol;Acc:HGNC:7711]	Z	15962742	16010010	NDUFS4	protein_coding

APPENDIX B

SUPPLEMENTARY FIGURES

Figure B-1. Plot demonstrating the biological coefficient of variation (BCV) for all samples based on average logCPM for each sample. Tagwise dispersion is represented by the black dots and showed that 75% of the data had a BCV under 0.76. Common dispersion was estimated as 0.15 and is represented by the blue line. Trended dispersion is represented by the red line.

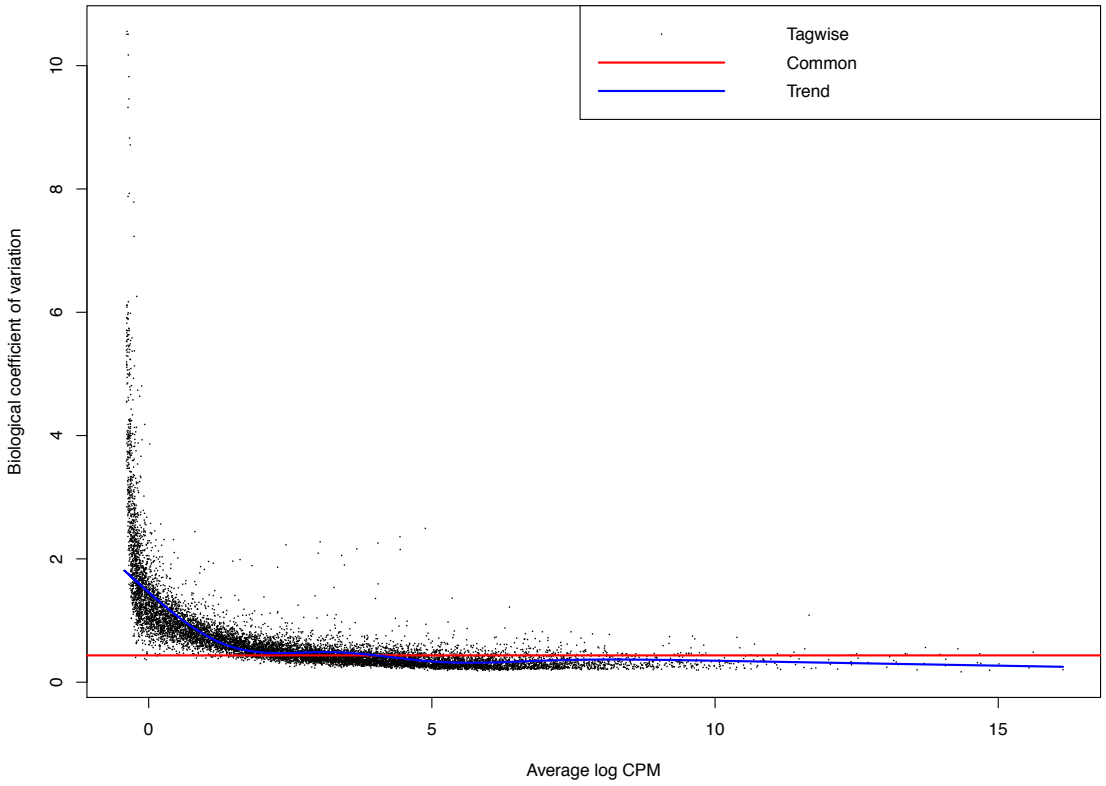

Figure B-2. Mean abundance plots (logFC by logCPM) of differential gene expression by age for both breeds between diets. Red dots indicate genes P < 0.05, while blue lines indicate logFC 2 and -2. An FDR < 0.05 was required for significance and no genes in these pairwise comparisons met that criteria. Plots A, B, E, F, I, and J represent WPR gene expression, while plots C, D, G, H, K, and L represent R708 gene expression between diets at 11 and 42 days of age respectively.