12-2018

Genetic and Epigenetic Investigations on Pulmonary Hypertension Syndrome in Meat Type- Chickens

Khaloud Alzahrani
University of Arkansas, Fayetteville

Genetic and Epigenetic Investigations on Pulmonary Hypertension Syndrome in Meat Type-Chickens

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

by

Khaloud Alzahrani
Princess Nora University
Bachelor of Science in Animal Science, 2004
University of Arkansas
Master of Science in Cell and Molecular Biology, 2013

December 2018
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

_____
Douglas D Rhoads, Ph.D.
Dissertation Director

_____
Nicholas Anthony, Ph.D.
Committee Member

_____
Charles Rosenkrans, Ph.D.
Committee Member

_____
David S McNabb, Ph.D.
Committee Member

ABSTRACT

This dissertation presents a collection of studies that investigate the genetic and epigenetic associations to ascites phenotype in broiler chickens. Ascites is a significant metabolic disease associated with fast-growing meat-type chickens (broilers) and is a terminal result of pulmonary hypertension syndrome PHS. It is a multi-factorial syndrome caused by interactions between genetic, physiological, environmental, and managemental factors. It was estimated that ascites accounts for losses of about US$1 billion annually worldwide and for over 25% of broilers mortality. Although traditional and molecular genetic methods in the selection and in performance improvements, has greatly reduced ascites frequency, yet it has not eliminated its occurrence. Therefore, this dissertation aimed to 1) develop SNP assays for the gene region of HTR2B to examine the possible association with ascites phenotype and measure gene and allele specific expression in different tissues at different developmental age stages under hypoxic conditions, 2) investigate the association of mitochondrial prevalence in multiple tissues with ascites susceptibility and resistance in broilers, and genes known to regulate mitochondrial biogenesis were assessed, and 3) mapping genome-wide changes in chromatin accessibility for pulmonary artery tissue in ascites - susceptible and ascites- resistant lines under normal and hypoxic conditions using ATAC-seq technology (Assay for Transposase accessible Chromatin with high-throughput sequencing). Altogether, this collection of studies provides new insights into the genetic and epigenetic basis of the ascites syndrome in chicken.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and deep appreciation to my advisor, Dr. Douglas Rhoads, without him, I don't believe I would have reached where I am today. It is for his constant support, guidance, encouragement, and patience throughout my academic and research journey that I have reached this point in my studies. His ability to question my thoughts and reasoning helped me to grow as both a scientist and researcher. He has had a profound influence on the way that I approach scientific problems, find solutions and solve them. Dr. Rhoads has also helped me grow as a person, he has opened my eyes and helped me to see the bigger picture, I am a better person today from his lessons. Thank you for seeing my potential, for trusting in my abilities and most importantly thank you for always having my back when I needed support.

I would like to thank Dr. David S McNabb, Dr. Nicholas Anthony, and Dr. Charles Rosenkrans for their precious time, suggestions, and comments as members of my Graduate Student Advisory Committee.

To all my lab mates, both past and present, thank you for your help, encouragement, interesting discussions and enjoyment during my time here.

I must also thank the faculty, administration staff, and graduate students in the Cell and Molecular Biology program at the University of Arkansas.

Thank you to the Saudi Arabian Ministry of Education for funding throughout my academic time which allowed me to freely explore research directions.

A special thanks to my family especially my children Leyan, Rema, and Bayan for their love, patience, and understanding over the past years. I know it hasn't always been easy, but your

understanding of the importance of fulfilling my dream warmed my heart and made the guilt of being busy and unavailable sometimes more bearable.

Last of all, I would like to give my utmost sincerity to express my appreciation to my parents who taught me the importance of education, hard work, and everything they done for me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SUBMITTED ARTICLES

Chapter 2:

Polymorphisms Detection and Allele- Specific Expression of the HTR2B Gene for Pulmonary Hypertension Syndrome Association in Broilers. Khaloud Al-Zahrani*[,1,2], Sriram Krishna[1,3], Patrick Greenburg[2], Nicholas B. Anthony[1,3], and Douglas D. Rhoads[1,2] (*Submitted to Poultry Science*).

Chapter 3:

Further Investigation of Mitochondrial Biogenesis and Gene Expression of Key Regulators in Ascites- Susceptible and Ascites-Resistance Research Lines. Khaloud Al-Zahrani*[,1,2], Timothy Licknack[1,3], Destiny L. Watson[2], Nicholas B. Anthony[1,3], and Douglas D. Rhoads[1,2] (*Submitted to PLOS ONE*).

CHAPTER 1

Literature Review

Domestication History of Chicken

Chickens represent by far the most important poultry species that provides humans with a stable source of protein, including both meat and eggs (Eltanany & Distl 2010). Based on several molecular, historical and archeological studies, chicken was domesticated in Southeast Asia in 5400 BC and then spread around the world (West & zhou, 1988; Crawford, 1990; Tixier-Boichard *et al*., 2011). Other studies suggest that northern China represents one region of the earliest chicken domestication, possibly dating as early as 10,000 y before present (Sawai *et al*., 2010). Combined phylogenetic analyses showed that red jungle fowl (RJF, *Gallus gallus*) is considered the main ancestor of modern-day chicken breeds (Fumihito *et al*., 1994; Hillel *et al*., 2003; Twito *et al*., 2007). Nevertheless, based on several mitochondrial genome studies (categorized by common haplogroups), the matrilineal history of chickens should be extended to include more than one ancestor (Nishibori *et al*., 2005; Liu *et al*., 2006; Oka *et al*., 2007; Miao *et al*., 2013). Therefore, there is a controversial discussion whether domesticated chickens descend from a single ancestor; (RJF, *Gallus gallus*) in Southeast Asia or that multiple origins have contributed to the current chicken. Early domestication of chickens primarily focused on fulfilling various roles ranging from food and entertainment to religion and ornamentation. Various cultural practices spread them around the world over the course of thousands of years through trade, migration, and territorial conquests. In fact, the sport of cockfighting had tremendous influence not only in the domestication of the chicken but also on the distribution of fowl throughout the world. By progress of human culture and activities, chicken varieties have been developed, mostly for food consumption.

Domestication and Industrial Commercialization of Chicken

By the beginning of the 20th century, and continuing today, chickens were selected for specific

traits such as meat, and egg production especially in the United States and Europe. The breakthroughs that made today's massive, industrial bird farms possible were improved management techniques, nutritional evaluation (antibiotics and vitamins), and implementing breeding schemes. Intensive selection programs started approximately 60 years ago in highly controlled environment (1 generation/year) for meat (broiler lines) and egg production (layer lines). Modern commercial broiler lines are highly specialized, produced by crossing elite pedigree (pure) lines over several generations for an intense selection of multiple beneficial traits associated with meat production (Griffin *et al*., 1994; Deeb *et al*., 2002; Paxton *et al*., 2010). Nowadays, a commercial broiler chicken reaches the weight of 2 kg in 35 days, an egg layer reaches 2 kg at adulthood and lays 300 eggs/year, whereas the RJF does not reach the weight of 2 kg and does not lay more than 50 eggs/year. As illustrated in Figure 1.2.B, between 1925 to 2012, the weight of an average broiler was more than doubled while the number of weeks needed to grow a marketable chicken fell by 56%, the pounds of feed required for each pound of chicken fell by 61%, and the percent mortality fell by 78% (Zhao *et al*, 2004). It takes less than two pounds of feed to produce one pound of chicken (live weight), less than half the feed/weight ratio in 1925. Additionally, selective breeding increased the yield of pectoralis major muscles (by 79% in males and 85% in females) and of pectoralis minor (by 30% in males and by 37% in females) (Zuidhof *et al*., 2014; Buzala & Janicki, 2016). Compared to layer hens, broilers have more muscle fibers of greater size and their breast muscles grow 8 times more than layers (Aberle & Stewart, 1983) whereas broilers growth rate is 2- to 3-fold greater than in layers (Zheng *et al*., 2009). This suggests that innovative technology and selective breeding programs have led to the production of bigger size chickens without compromising the amount of time and feed required to grow them. In 2014, it was estimated that Americans eat more than 80 pounds of chicken a year, more than pork or

beef (Figure 1.2.A). Domestication processes tend to change animals' phenotypes by releasing them from natural selection, and favoring traits desired by humans, such as food production. However, a side effect of such practices can cause changes in traits other than those targeted for selections due to genetic linkage with undesirable genes. The genome diversity of today's domestic chickens is a result of the long-term domestication process, subsequent breed differentiation and intense selection for production. Altogether, this has yielded a dramatic phenotypic diversification of the chicken, both at the level of physiology and morphology.

The Chicken Genome

The chicken was the first bird, as well as the first domestic animal to have its genome sequenced and analyzed (Ellegren, 2005). The chicken genome provides a resource for researchers seeking to enhance the nutritional value of poultry and egg products, to benefit biomedical research, and to provide a key anchor species in which to understand the evolution of vertebrates. In fact, the chicken genome fills an essential gap located somewhere between mammals and fish on the tree of life. This is because chicken shared a common ancestor with mammals millions of years ago not previously covered by other genome sequences in the phylogenetic tree (Burt, 2006). The initial draft of the chicken genome (Hillier *et al*., 2004) was based on DNA from a single inbred female RJF *Gallus gallus*. Although this draft covered almost 86% of the genome, yet sex chromosomes (Z and W chromosomes), as well as the major histocompatibility (MHC) region on chromosome 16 were poorly represented in the final assembly. In 2006, subsequent re-sequencing of the chicken genome improved the final assembly to approximately 95% of the 1050 Mb genome (Burt, 2006). However, there was an area of 90 Mb not covered by any reads, suggested to be repetitive sequences. Over the years, further improvements were made to the avian genome reference assembly and a new chicken genome assembly was released in 2015 (Gallus_gallus-5.0)

(Schmid *et al*., 2015). The total length of chicken DNA is 1230.26 Mb, which is about one-third of human genome size, but approximately the same number of genes (Hillier *et al*., 2004; Schmutz & Grimwood, 2004) whereas the size of mitochondrial DNA (mtDNA) is 16,775bp (Desjardins & Morais, 1990). The chicken karyotype comprises 39 chromosome pairs as follows: 10 pairs of large autosomes (chromosomes 1–10), 28 pairs of microchromosomes (chromosomes 11–38), and a pair of sex chromosomes (chromosomes Z and W). Chromosome Z is a large chromosome and present as a pair in homogametic (ZZ) males whereas chromosome W is a microchromosome and present only in the heterogametic (ZW) females (Masabanda *et al*., 2004).

Along with the chicken genome sequencing project, a consortium (Wong *et al*., 2004) generated 2.8 million SNPs from a comparison of the RJF reference sequence and partial genome scans of three different breeds: silkie, broiler, and layer lines. The chicken genome exhibits a high rate of polymorphism. In fact, the small chicken genome produced six times more single nucleotide polymorphisms SNP (>7,000,000 SNPs) than human and mammalian genomes, as well as considerable microsatellite content (375,000) (Eltanany & Distl, 2010). Single nucleotide polymorphisms (SNPs) make up the most abundant source of genetic variation in the chicken genome followed by short-length insertions and deletions (Indels). Populations of domestic chickens differ from the RJF by a large phenotypic variability due to the accumulation of mutations. High density SNP genotyping indicate that about 50% or more of ancestral genetic diversity is lost in today's commercial lines (Muir *et al*., 2008). Furthermore, it is estimated that there is about one SNP every 200 bp (Wong *et al*., 2004), where 70% of these SNPs are stable and common to all three breed lines (silkie, broiler, and layer). These genetic variants play an important role in driving genomic evolution. The estimated percentage of amino-acid altering SNPs ranged only between 0.3 and 1.2%. One of the major outcomes of the availability of a large database of

polymorphisms has been the development of SNP genotyping arrays to simplify screening of many individuals. Another major benefit of molecular markers has been in the detection of Quantitative Trait Loci (QTLs) associated with various phenotypes or diseases. Furthermore, genome wide association studies (GWAS) are important tools for identifying chromosomal regions of the genome associated with specific phenotype or trait. The combined analysis of SNP genotypes and performance data produces a list of QTLs. Till now, over 4,300 QTLs have been identified for chicken (http://www.animalgenome.org/cgi-bin/QTLdb/GG/index). However, GWAS often fall short in identify important genes or causal SNPs. This is because the genotyping arrays only search for association between pre-defined panels of markers, which mostly includes common SNPs leaving out the low-frequency causative alleles or mutations (Gheyas & Burt, 2013). Therefore, generally most regions detected through GWAS often explain only a small part of the genetic variance of a phenotype or trait (Maher, 2008). With the current development of next generation sequencing (NGS) technologies and data processing capabilities it is now possible to perform association analysis on all the variants detected from the whole-genome as it is also becoming relatively simple, fast and inexpensive procedure.

Genomic Selection in Poultry Industry

Since 1950, genetic selection in poultry has shown significant improvements, resulting in specialized egg laying breeds and fast-growing meat yield breeds. This extreme success has been achieved by selecting and breeding above-average birds using estimated breeding values without much knowledge about the number and nature of genes involved (Dekkers, 2005). This results in increasing the frequency of superior alleles in the next generation. After releasing the sequencing of the chicken genome in 2004, and as the technology and computing capacity developed, major

poultry breeding companies were actively interesting in investigating the use of genomic information to improve their breeding programs and selection strategies. This relationship between genotype and phenotype is of fundamental biological interest since Mendel postulated the existence of 'internal factors' that are passed on to the next generation. In the last decade, tremendous improvements have occurred in the methods available for the identification of DNA variation and in our understanding of how this DNA variation can influence phenotypes or traits. This has led to several technologies successively improved methodologies and strategies to enhance breeding programs. In this dissertation we will review some of the technologies and methods in genomic selection and their use in commercial breeding programs.

Restriction Fragment Length Polymorphisms

Restriction fragment length polymorphisms, or RFLPs, were the first molecular method of genetic-mapping that allows individuals to be identified based on unique patterns of restriction enzyme-digested DNA and probes for either specific genes or genomic dispersed repetitive element (Fulton, 2012). It was used to identify large insertions, deletions, and single base changes that occurred within the restriction enzyme digestion site that was found to influence traits of commercial importance. However, the main drawbacks of RFLPs were the relatively tedious and expense to develop and use, thus limiting the number of individuals that could be genotyped (Siegel, 2006).

Microsatellites

Microsatellites (MS), or Simple Sequence Repeats (SSRs) are short tandem repetitive sequences of short length (1 to 10 nucleotides) detected using the polymerase chain reaction (PCR) procedure (Vieira *et al*., 2016). Amplified PCR products from different individuals are then resolved on agarose gel electrophoresis (AGE) or polyacrylamide gel electrophoresis (PAGE), to reveal length variations (Powell *et al*., 1996). Microsatellites are presumably considered to be selectively neutral (not influenced by natural selection), and well-dispersed throughout the genome, and they are instead influenced by gene flow, genetic drift, and mutation. Therefore, these features are useful for estimating population differences and selection programs (Fulton, 2012). Although this method enhanced the identification of genomic regions that influenced traits, yet these regions were very large, containing potentially hundreds of genes.

Quantitative Trait Loci

Quantitative trait locus (QTL) analysis is a statistical method that links together phenotypic (trait measurements) and genotypic data (usually molecular markers) to explain the genetic basis of variation in multifactorial or quantitative traits (Miles & Wayne, 2008). QTL mapping is a powerful method to identify regions of the genome that co-segregate with a given phenotype in F2 populations. Generally, quantitative traits are complex and multifactorial influenced by many genes and environmental conditions (i.e., one or many QTLs can influence a trait or a phenotype). The animal genome QTL database (http://www.animalgenome.org) reported 125 publications identifying more than 2,400 QTL for 248 traits. Those traits include many aspects such as traits that influence egg quality, growth rate, behavior, specific disease resistance, and numerous metabolic disorders in chickens (Fulton, 2012). Mostly, these QTL searches were done using MS

markers and crosses between very diverse breeds, such as lines created by divergent artificial selection. Although QTL mapping provides valuable contributions, bridging the gap between genes and the phenotypic, yet QTL analysis is not without limitations. First, QTL studies require very large sample sizes and can only map allelic differences that segregates between the parents of the F2 cross. Another limitation of QTL mapping is that the DNA regions identified as QTLs are still so large that they likely contain hundreds of genes (Miles & Wayne, 2008; Majumder & Ghosh, 2005). There are other issues that can limit success of trait values determination in QTL analysis, such as the extent of gene-gene interaction (epistasis) and genotype-environment interaction (Majumder & Ghosh, 2005). However, there are many refinements and novel statistical approaches that help in overcoming these limitations enabling greater successes with QTL mapping and provides a significant advance in elucidating the genetic bases of economic importance traits.

Marker-Assisted Selection

In poultry, multiple QTL regions associated with specific phenotypic traits of economic importance were discovered using markers randomly distributed in the genome (microsatellites) (Wolc, 2015; Pértille *et al*., 2017). The goal was that, as important genomic regions associated with traits of commercial importance were identified, this information could be then used for marker-assisted selection (MAS). Selection could be done at an early age using DNA markers to identify those individuals that had superior performance. Currently, the industry is already using MAS to some extent, in their breeding programs. This can be used to increase the frequency of alleles of economic interest or to eliminate unfavorable alleles (Siegel, 2006). This is a successful practice but only to a limited extent. This is because most quantitative traits such as body weight

are influenced by many chromosomal regions in the genome, each with a small effect to the variation. Additionally, If the DNA marker was not close enough to the region to be selected for breeding, after a few next generations, recombination events would occur such that the positive association between a specific marker allele and commercially important trait is no longer present. Another major problem with MAS is the existence of negative associations with other traits of economic importance. For instance, selection for a specific marker that results in rapid growth rate could have an undesirable impact on other traits, such as egg size, shell strength, or feed efficiency (Fulton, 2012).

Genome-Wide Association Studies

Genome-wide association studies (GWAS) are an excellent and powerful complement tool to QTL mapping. GWAS compare common genetic variants in large numbers of affected subjects to those in unaffected controls (case-control design) to determine whether an association with a trait or phenotype exists. GWAS have been made possible by the identification of millions of genetic variations across the genome and the realization that a subset of these variations can capture common alleles via linkage disequilibrium (Witte, 2010). GWAS overcome the main limitations of QTL analysis but introduce several other drawbacks, but when conducted together, they compensate each other's limitations. Since QTLs contain up to hundreds of linked genes scattered thorough out the genome, which are then considered challenging to separate, GWAS produce many unlinked individual genes or even nucleotides. Although GWAS remain limited to organisms with genomic resources, combining the two techniques can increase the statistical power with fine mapping of associated genes. However, for many of the GWAS results, the findings explain only a limited amount of heritability which may reflects the small effect for most genetic variations identified by GWAS (Korte & Farlow, 2013). These issues can be overcome by

more detailed examinations that include analyses of less common variants with small effect size, use extremely large sample sizes, and well-characterized environmental exposures. In fact, sample sizes in the thousands and hundreds of thousands are typically required for GWAS to have sufficient statistical power to be able to detect the expected modest associations while examining hundreds of thousands of SNPs.

Single Nucleotide Polymorphisms and SNP Chips

Simultaneously with the release of the chicken genome sequence in 2004, The International Chicken Polymorphism Map Consortium detected and released about 2.8 million single nucleotide polymorphisms, or SNPs (~1 every 400 base pairs) to the public domain (Wong *et al*., 2004). They were identified by comparing the RJF genome sequence with partial sequence information from three different domestic chicken strains: one Silkie (Chinese breed), two commercial broilers (meat type- broilers), and one inbred laboratory White Leghorn (egg-layer type). The mean rate was about five SNPs per kilobase (Wong *et al*., 2004). These identified SNPs have established the basis for all the large SNP genotyping platforms developed to date. SNPs are single nucleotide variants within the DNA sequence. They can be homozygous, heterozygous, or insertions/deletions (indels), and can occur within coding genes, non-coding regions of genes or in intergenic regions. Coding SNPs can exist in three main types 1) synonymous as (does not change amino acid) within coding regions, 2) nonsynonymous (changes amino-acid sequence), and 3) stop-gain/loss (causing the gain or loss of stop codons). Most identified SNPs convey no biological consequence when they occur within the non-coding regions of the DNA or occur as synonymous SNPs that cause no change in the amino acid sequence of a protein. In humans, 99.9% of the DNA sequence is identical between individuals within the same population. The remaining 0.1% is significant because it contains approximately 80-90% of all genetic variations as SNPs. SNP mutation mechanisms

include either transitions: purine-purine (A<>G) or pyrimidine-pyrimidine (C<>T) exchange, or transversions: purine-pyrimidine/pyrimidine-purine exchanges (A<>C, A<>T, G<>C, G<>T). Investigators can link a SNP with a phenotype by using GWAS where hundreds of thousands of SNPs can be scanned per sample.

Genomic selection in poultry would not be possible without development of high-density SNP chips. They provide genome-wide coverage and high throughput nature for rapid, massive, and relatively inexpensive genotyping. The first chicken SNP chip had only 3,000 (3K) SNPs (Muir *et al*., 2008), and was soon considered insufficient. After that medium-sized chips were developed that made a tremendous impact on genomic selection research and its implementation: a 60K chip developed with USDA funding and used by the Cobb and Hendrix groups (Groenen *et al*., 2011). In 2013, a high-density genotyping array (600 K) for chicken was commercially released for public use that allows for more complete identification of variation across the genome. (Kranis *et al*., 2013). Current methods are based on SNP variation and its influence on traits of interest. However, some studies suggest that other types of genetic variation such as copy number variation CNV and RNA splicing variants are reported in multiple species and may be important for trait determination and are worth exploring for selection (Zhang *et al*., 2014).

Next Generation Sequencing

Next Generation Sequencing (NGS) technology is a DNA sequencing technology which has revolutionized genomic research (Behjati & Tarpey, 2013). It is a cost-effective and viable alternative or replacement to current SNP genotyping methods, which has the potential to improve whole-genome selection (WGS), and GWAS in chicken meat and egg production. NGS can be used to sequence whole genome or targeted-region genomic sequence, gene expression

profiles (e.g. RNA-seq), and protein-DNA/RNA interactions (e.g. ChIP-seq) (Levy & Myers, 2016). Whole genome sequencing is by far the most all-encompassing method for identifying elements causing developmental defects in any organism. This method can detect any possible genetic variation in a genome related to functional alterations such as: SNPs, CNVs, large and small Indels, and chromosome rearrangements (Yan *et al*., 2014). Once the data is sorted and possible causative elements are identified, various techniques can be used to confirm or eliminate variants such as: qRT-PCR, in situ hybridization, microarrays, RNA-seq, siRNA, protein analysis, ChIP-seq etc., (Schmid e*t al*., 2015). The continued development of low-cost, high performance sequencing will keep expanding the diversity of genomics applications.

Epigenetics in Poultry

Most economically important traits in poultry production exhibit sustained phenotypic variability due to polygenic and environmental factors. Many GWAS have been conducted, and many QTL have been identified to be associated with commercially important traits, yet the variability of complex traits is only partially explained by genetic variation. It is now clear that the epigenome plays a significant role in directing and regulating gene expression due to environmental factors, and therefore, resulting in genetic variations across individuals (Frésard *et al*., 2013) The epigenome is considered as the second dimension to the genome, and it provides crucial insights to gene regulation besides the genomic sequence (Rivera & Ren, 2013). Epigenetics can explain how gene expression might be changed and stably maintained without affecting the genome sequence. Moreover, epigenetic marks could be transmitted to subsequent generations and influence offspring traits that occur either via epigenetic changes acquired during embryonic development, or through the inheritance of epigenetic marks via the gametes (Jablonka & Raz, 2009; Daxinger & Whitelaw, 2012). The epigenetic machinery includes chromatin accessibility,

packaging of DNA in nucleosomes, covalent modifications of histone proteins, DNA methylation, and regulatory non-coding RNA (such as: lncRNA, miRNA, and snoRNA), and higher-order chromatin architecture (such as: nucleosome positioning and occupancy, 3D chromatin structure). (Bernstein *et al*., 2007; Berger *et al*., 2009; Bonasio *et al*., 2010). Moreover, several studies revealed that epigenetic marks can actively contribute to the determination of patterns of gene activation or silencing, and influence lineage development and tissue-specific expression of genes (Youngson, & Whitelaw 2008; Mazzio, & Soliman, 2012; Feil & Fraga, 2012). With the current rapid development of NGS technologies and the significant drop of costs, the rate of growth in studies and publications of epigenomics have increased dramatically in several model organisms. Unfortunately, little is known about epigenetic mechanisms in chickens, although such mechanisms could contribute significantly to trait or phenotype variability. Understanding the epigenetic regulation of gene expression (when, where, and how much) in chickens remains to be elucidated. However, it is expected that in the next few years we will see an increase in epigenome data release and publications that will greatly contribute to our understanding of the genome-phenome relationship.

Genetic Selection Effects on Broiler Welfare

Intensive genetic selection of broiler breeders for commercial traits along with management, and nutritional improvements conducted by the poultry breeding programs have been successfully employed at an unprecedented magnitude over the recent decades. Consequently, several changes in production efficiency have greatly increased such as: growth rate while reducing the age to slaughter and feed conversion of the commercial birds. (Hunton, 1990). In fact, broiler body weights increased by over 400% where feed conversion improved by 50% when compared to chickens 60 years ago. (Zuidhof *et al*., 2014; Buzala & Janicki, 2016). Despite the success in the

beneficial effects of selection for economic important traits, it has been accompanied by undesirable side effects that include several behavioral, endocrinological, physiological, and immunological disorders. Long- term genetic selection for fast growth and high production efficiency often results in correlated responses in other traits where the selection response varies with the environment in which the selection takes place. In this review, some examples of undesirable side effects of intensive genetic selection for high production efficiency in poultry will be described. For instance, intense selection for rapid growth rate in broiler breeders has led to hyperphagic behavior or overconsumption due to aberrant hypothalamic satiety mechanisms (Rauw *et al*., 1998). Moreover, Broilers under intense selection pressure for increased body weight have increased deposition of excessive fat because of decreased lipolysis rates (Calabotta *et al*., 1985), and increased glucagon and insulin concentrations in the plasma (Sinsigalli *et al.,* 1987). Fast growth and heavy body weight have been implicated in musculoskeletal disorders in meat-type poultry (Julian, 1998). Sanotra *et al*. (2001) estimated that about 30% of broilers have high to moderate leg problems. This results from heavy body weights producing stress on the bones, tendons and ligaments of poor structural quality (Whitehead *et al*., 2003). Fast growth rate selection has also resulted in negative correlation for several reproduction traits. Fertility is a trait of major interest in the broiler industry, and selection for growth alone for multiple generations is likely to result in a decline in fertility or in males natural mating ability (Wolc *et al*., 2009). In the event of uncontrolled growth, male's ability to successively mate with the hens efficiently can be affected by their high body weight or leg problems. Both male and female influence egg fertilization and embryo development, and the contribution of each sex is influenced by genetic and non-genetic factors (Rauw *et al*., 1998). Factors that affect male fertility are: sperm motility, quality, metabolism, concentration, and percentage of abnormal or dead sperm cells (Wilson *et al*.,

1979). On the other hand, factors originating from broiler females include egg quality, and reproductive behavioral and physiological factors (Brillard, 2003). Subsequently, decreases in fertility cause reduction in the hatchability percentage, which indirectly limits the overall success of the poultry industry. It is estimated that economic losses are about 1 billion egg/year due to infertile eggs (Wolc *et al*., 2009). Anthony *et al*. (1989) showed that number of eggs produced from broiler breeders selected for high body weight was higher than broilers selected for low body weight, but there was a higher percentage of defective eggs. In addition to fertility problems, selection for growth rate also has a correlated negative effect on several health traits and immune performance. Broilers selected for high body weight showed lower antibody responses than a low body weight line when challenged with sheep erythrocytes (Miller *et al*., 1992). Havenstein et al. (1994) reported higher mortality rates at 42 days of age in a commercial broiler strain (9.7%) compared with random bred population (2.2%), primarily attributed to sudden death syndrome, ascites, and leg problems. Broilers selected for high body weight showed various leg- related problems such as: femoral head necrosis, valgus-varus deformities, rickets, and tibial dyschondroplasia; estimated to cause losses of about $120 million annually in poultry industry (Cook, 2000). In general, skeletal system integrity is affected by many factors including: managemental, environmental, and nutritional factors, along with aging, toxins, and, infectious diseases (Rath *et al*., 2000). Moreover, intense selection for rapid growth rate results in increased workload on the cardiovascular system predisposing birds to metabolic disorders such as right ventricular failure, cardiac arrythmias, ascites syndrome, and sudden death (Julian, 2005; Cherian, 2007). One of these important metabolic problems is ascites syndrome or pulmonary hypertension syndrome (PHS). Ascites is characterized by the accumulation of edematous fluid in one or more

of the peritoneal cavities of broilers, a condition known as "water belly" (Figure 1.2). The current dissertation aims to investigate some of the genetic and epigenetic aspects of PHS in broilers.

PHS in Chicken

PHS or ascites syndrome in broiler chickens is a negative result of selection for rapid growth in modern poultry. In the 1950s, PHS was first recognized as a problematic disease at high altitudes, under hypoxic conditions and colder weather (Smith, *et al*.,1954). However, since approximately 1980, PHS in broilers was also observed at lower altitudes even at sea level, and its incidence paralleled rapid growth rate, increased metabolic rate, and improved feed conversion (Scheele, 1996; Julian, 1998). PHS is caused by several processes all related to the need to ensure a high level of oxygen in the tissues. Nevertheless, the primary cause of PHS in broilers is the rapid growth rate along with insufficient pulmonary vascular capacity (Julian, 1998). Modern broilers can achieve market weight in 60% less time than broilers of 40 years ago (Baghbanzadeh & Decuypere, 2008). Selection for fast growth and increased muscle mass in broilers has not resulted in a proportionate increase in cardiopulmonary systems (Julian, 1989; 2007; Decuypere *et al*., 2000). In fact, the cardiopulmonary capacity of modern broilers is very similar to the old broiler strains (Lubritz *et al*., 1995). As a result, the heart and lungs are required to work very close to their physiological limit to meet the high oxygen demands required for metabolic processes. However, the cardiovascular and the respiratory systems capacity does not always meet the oxygen demands necessary for rapid growth, thus, leading to the development of PHS in broilers. Schmidt et al. (2009) have demonstrated that growth rate of the heart has decreased in modern broilers when compared to a heritage unselected line since 1950s. It is probable that this decrease in relative heart size contributes to the decrease in the cardiac capacity of modern broilers, and therefore results in higher incidence in heart related problems, such as PHS. There are a variety of additional

factors or secondary causes that can increase the incidence of PHS (Julian, 1993; Maxwell *et al*, 1997). Several environmental factors and management practices can prompt the development of PHS in broilers such as: poor ventilation, high altitude, cool temperatures, high feed intake, dietary energy content, continuous lighting, or poor air quality (Julian, 2000). Furthermore, some studies have shown that some microorganisms can cause respiratory damage or obstruct the pulmonary airways leading to greater resistance to blood flow and thus, PHS development. For instance, gram negative bacteria such as: *Escherichia, Salmonella,* and *Campylobacter*, produce a lipopolysaccharide (LPS) which can trigger pulmonary vasoconstriction leading to pulmonary hypertension in broiler chickens (Chapman *et al*., 2005). In the 1990s, PHS had become a leading cause of illness, death and carcass condemnation in the broiler industry and It has been estimated that ascites accounts for losses of about US$1 billion annually around the world (Maxwell & Robertson, 1997; Navarro et al., 2002). Mortality due to ascites could reach 25% in broiler flocks, and it was estimated to cost about 26 million dollars/year (Anthony & Balog, 2003). Nowadays, PHS is less an issue because of genetic research, improved management strategies, and intense selection against this problem by commercial breeder companies (Druyan & Cahaner, 2007; Pavilidis *et al*., 2007; Druyan *et al*., 2008).

PHS Physiology & Etiology

PHS is a multifactorial disease mediated by environmental, nutritional, genetic, physiological, and management factors that all seem to interact together to produce a series of events that culminate in ascites syndrome. (Decuypere *et al*., 2000; Balog, 2003; Julian, 2005). The general pathogenesis for this syndrome (Figure 1) has been extensively studied (Julian, 1993, 2000; Scheele, 1996; Hassanzadeh *et al*., 1997; Decuypere *et al*., 2000; Wideman, 2000; Druyan, 2012). The etiology of PHS in broilers is initiated with the increased metabolic rate induced by several factors such as

cold, moderate heat, air quality, elevated muscle mass, overeating, etc. This leads to an increased requirement for oxygen to meet the body's rapid growth. The architecture of modern broilers such as: small, rigid, and fixed lungs in the thoracic cavity, and heavy breast muscle can also increase the incidence of PHS. Since broilers have more genetic potential for rapid growth than for oxygen supply for that growth, the resultant physiological hypoxia causes an increased cardiac output and consequently elevated pressure in the pulmonary arteries. This increase in work load results in an enhanced pressure load on the right ventricle, to pump more deoxygenated blood to the lungs against the increased pressure. However, the increased blood flow rate through the lungs may not allow the red blood cells (RBC) to pick up a full load of oxygen. Thus, the hemoglobin oxygen saturation is not complete, leading to lower partial pressure of O_2 (hypoxemia) and higher partial pressure of CO_2 (hypercapnia) (Wideman & Kirby, 1995). Hypoxemia signals the body to produce more RBCs to elevate the blood's oxygen carrying capacity to the tissue, which increases blood viscosity and volume. This in turn raises the pressure required to move blood through the lung, and therefore worsens the pulmonary arteries pressure overload (Diaz *et al*., 1994; Julian, 2007). Cardiac muscle cells respond to chronic hypoxia by causing dilation and thickening (hypertrophy) of the right ventricular wall (Figure 1.3). Meanwhile, the valves between the right ventricle and the right atrium thicken and start to leak blood back into the atrium as the right ventricle gets more enlarged and dilated. Once valvular insufficiency occurs, a drop in cardiac output and pulmonary hypertension results, but at the same time marked pressure increases in the right atrium, sinus venosus, vena cava and portal vein, and eventually leads to right ventricle failure (RVF). The increased blood pressure in the veins, liver, and abdominal vessels forces the leakage plasma fluid (edema) out of the vessels, particularly the sinusoids of the liver, into the peritoneal spaces, which

is the most apparent sign of ascites. (Figure 1.2). (Wilson *et al*., 1988; Julian, 1993; Balog, 2003; Druyan, 2012; Wideman *et al*., 2013).

Experimental Methods of Inducing PHS In Broilers

Chickens are not only major livestock animals but also excellent model organisms for studying the genetic basis of phenotypic traits, like PHS. There are several methods that have been used for inducing PHS in broilers that include both invasive and noninvasive techniques for research purposes. One of the surgical techniques includes clamping the left pulmonary artery that results in immediate increase in cardiac output and pulmonary pressure leading to PHS development. Birds that underwent the surgery showed 90% ascites incidence compared to 8 % non-surgery control birds (Wideman & Kirby, 1995a). Although this method was highly effective in inducing PHS, yet it requires time and skill to perform, which restricted application to large scale commercial selection. A less invasive method was developed that involves intravenous injection of micro-particles into the systemic circulatory system (Wideman *et al*., 2002; Wideman & Erf, 2002). Micro-particles are then carried to the lungs where they become trapped in the pulmonary arterioles and block subsequent blood flow leading to the development of PHS. Non-invasive methods to induce PHS in broilers include cold stress (Lubritz & McPherson, 1994; Wideman *et al*., 1998; Sato *et al*., 2002), long photoperiods (Hassanzadeh *et al*., 2000; Julian, 1990), high elevation (Balog *et al*., 2000a), and dietary supplementation (Decuypere *et al*., 1994). Out of these listed techniques, high elevation was found to be superior to other methods in term of inducing PHS and has been used to develop excellent resource populations for many generations for several PHS related studies (Owen, *et al*., 1990; Balog *et al*., 2000a; Balog *et al*., 2000b; Anthony *et al*., 2001; los Santos *et al*., 2005; Pavlidis *et al*., 2007). This dissertation utilized a hypobaric model to

simulate a set elevation above sea level creating a hypoxic environment for PHS induction in broilers (Figure 1.5).

Divergent Selection for PHS

Since ascites was first observed in birds raised at high altitude, the use of natural or simulated high-altitude conditions was one of the first and simplest experimental techniques to be used (Hall & Machicao, 1968; Balog, 2003). Dr. Nicholas Anthony, (University of Arkansas, Poultry Science) has successfully developed divergently selected PHS-susceptible (SUS) and PHS-resistant (RES) lines of broiler chickens reared in a hypobaric chamber (2,900 m above sea level) to induce the disease (Anthony, 1998; Anthony and Balog, 2003; 2013; Pavlidis *et al*., 2007, Wideman *et al*., 2013). The hypobaric chamber simulated high altitude conditions via a partial vacuum. With the increase in altitude, the partial pressure of O_2 drops significantly resulting in hypoxia (Figure 1.5). Three separate groups of chicks from a commercial pedigree elite line which had experienced one generation of relaxed selection were transported to the poultry research facility at University of Arkansas in 1995, to serve as the base population. Those birds were placed in the hypobaric chamber and for the next six weeks information was collected such as: mortality, probable cause of death, ascites symptoms, total body weight, heart shape, right and total ventricle weight, and gender. At the end of the six-week trial, all remaining birds were euthanized by cervical dislocation and phenotyped as PHS-resistant or PHS-susceptible based on apparent symptoms. Subsequent breeding was performed using siblings of the birds challenged in the hypobaric chambers based on their ascites mortality records. Therefore, susceptible and resistant lines were generated based on long–term divergent selection in the hypobaric chamber. A separate group was randomly mated and maintained across all generations of selection under typical management and environmental conditions to serve as the control population, known as the relaxed

line (REL line). The line selection showed an average incidence of ascites of 98% in the SUS line, and 7% in the RES line by the 14th generation, while the REL line was approximately 66% (Figure 1.6) (Anthony *et al*., 2001; Balog *et al*., 2003; Pavlidis *et al*., 2007, Wideman *et al*., 2013).

Genetics of PHS

PHS has both genetic and environmental components with estimates of heritability ranging from 0.1 to 0.7 (Lubritz *et al.,* 1995; de Greef *et al*., 2001; Moghadam *et al*., 2001). A cross of the SUS and RES lines was used to generate an F2 population. GWAS of the F2 population used a 3,072 SNP panel and identified several chromosomal regions that were associated with ascites phenotype (Krishnamoorthy *et al*., 2014). Further genetic and statistical analysis identified a total of 7 regions on 4 chromosomes that might contain potential candidate genes for resistance/susceptibility to PHS: Three regions on Gga1: 0.6-1.1, 18.3-21.5, and 127.0-128.3 Mbp; two regions on Gga9: 13.5-14.8, and 15.5-16.3 Mbp; one region on Gga27: 2.0-2.3 Mbp; and three regions on GgaZ:31.2-34, 47.1-48.9, and 65.0-66.0 Mbp on GgaZ (according to Galgal 4 assembly, 2011). The most statistically significant regions were the two on Gga9 that included three potential candidate genes: AGTR1 (Angiotensin II Type 1 Receptor), UTS2D, (Urotensin 2 Domain Containing protein located on Gga9, and HTR2B, (serotonin receptor/transporter type 2B) (Wideman *et al*., 2013; Krishnamoorthy *et al*., 2014, Dey *et al*., 2016). These genes have all been shown to be involved in the development of PAH in humans and mice PAH (Cuffe *et al*., 2014; Palatini *et al*., 2009, Chassagne *et al*., 2000; Pousada *et al*., 2015 Ong *et al*., 2008, Ullmer *et al*., 1995, Launay *et al.,* 2002, West *et al*., 2016). This dissertation is the result of extensive analysis of the region encompassing the HTR2B gene. Separately, others have investigated the region encompassing AGTR1 and UTS2D. Dey et al (2016) showed that AGTR1 and UTS2D genes are only marginally associated with ascites when extended to additional experimental and commercial

populations. Subsequent multi-generational GWAS using a 60k SNP panel in the REL line identified regions on Gga2 around 70 Mbp, and on Gga Z around 60 Mbp as candidates for association with PHS. The region on Gga 2 was found to be associated with resistance in male broilers and contained the genes for melanocortin-4 receptor (MC4R) and cadherin 6 (CDH6). Within the Gga Z region was the gene for myocyte enhancer factor 2C (MEF2c). This region was putatively associated with PHS resistance in males and females (Tarrant *et al*., 2017). However, attempts to use these two regions for Marker-Assisted-Selection (MAS) in breeding showed that it was not reliable for selection for PHS. Recently, our research group has used whole genome resequencing (WGR) in the REL line to identify 31 candidate QTLs for ascites phenotype. These regions are under further investigation. One of these regions was validated by Dey et al (2018) and identified the carboxypeptidase Q (CPQ) gene on chromosome 2 (near 127 Mbp) to be associated with PHS resistance in male birds (Dey *et al.,* 2018).

SYNOPSIS

The current dissertation deals primarily with the genetic and epigenetic investigations of PHS in broiler chickens. The first study (chapter 2) was designed to investigate the association of HTR2B with PHS in broilers. HTR2B gene located on chromosome 9 (Gga9:15 Mbp) was one of the candidate genes that showed association to PHS phenotype based on the F2 cross of the RES and SUS lines. SNP assays were developed for the gene region of HTR2B to examine the possible association with ascites phenotype. Moreover, expression of HTR2B gene, and allele specific expression (ASE) using SNP located in exon 3 of the HTR2B gene (T>C rs315854205) were determined in different tissues at different developmental age stages under normal and hypoxic conditions. The second study (chapter 3) was an extended investigation to our previous survey of the association of mitochondrial prevalence in multiple tissues with ascites susceptibility and

resistance in broilers. Previously we reported that for a small sample set of breast muscle at 22 weeks of age for RES and SUS males, the samples from SUS males had approximately twice the ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nucDNA), and that this difference correlated with a difference in the level of expression of *PPARGC1A*. This dissertation has further investigated this apparent difference and extended the analyses to both genders, multiple tissues, and different developmental stages. Furthermore, the relative expression of five genes known to regulate mitochondrial biogenesis were assessed. The aim of third study (chapter 4) was to map genome-wide changes in chromatin accessibility resulting from hypoxic challenge. The analysis used ATAC-seq technology (Assay for Transposase accessible Chromatin with high-throughput sequencing) to identify changes in promoter accessibility for pulmonary artery tissue in PHS-susceptible and PHS- resistant lines. This is the first attempt to examine epigenetic modifications associated with hypoxic relative to ambient conditions in broilers. Finally, chapter 5 discusses the results and highlights concluding remarks of this dissertation and its importance to poultry industry.

REFERENCES

Aberle, E. D., & Stewart, T. S. (1983). Growth of fiber types and apparent fiber number in skeletal muscle of broiler- and layertype chickens. *Growth*. 47:135–144.

Anthony, N. B., Dunnington, E. A., & Siegel, P. B. (1989). Egg production and egg composition of parental lines and F1 and F2 crosses of White Rock chickens selected for 56-day body weight. *Poultry Science*, 68(1), 27-36.

Anthony, N. B., Balog, J. M., Hughes, J. D., Stamps, L., Cooper, M. A., Kidd, B. D., Liu, X., Huff, G. R., & Rath, N. C. (2001). Genetic selection of broiler lines that differ in their ascites susceptibility 1. Selection under hypobaric conditions. *Proceedings 13th European Symposium on Poultry Nutrition, Blankenberge, Belgium,* pp 327-328.

Anthony, N. B. & Balog, J. M. (2003). Divergent selection for ascites: Development of susceptible and resistant lines. Proc. 52nd Ann. *National Breeders Roundtable,* 39-58.

Baghbanzadeh, A., & Decuypere, E. (2008). Ascites syndrome in broilers: Physiological and nutritional perspectives. *Avian Pathology*, *37*(2), 117–126.

Balog, J. M., Anthony, N. B., Cooper, M. A., Kidd, B. D., Huff, G. R., Huff, W. E., & Rath, N. C. (2000a). Ascites syndrome and related pathologies in feed restricted broilers raised in a hypobaric chamber. *Poultry Science*, 79:318-323.

Balog, J. M., Huff, G. R., Rath, N. C., & Huff, W. E. (2000b). Effect of dietary aspirin on ascites in broilers raised in a hypobaric chamber. *Poultry Science,* 79:1101-1105.

Balog, J.M. (2003). Ascites syndrome (pulmonary hypertension syndrome) in broiler chickens: are we seeing the light at the end of the tunnel? *Avian & Poultry Biology Reviews*, 14(3), 99126.

Behjati, S., & Tarpey, P. S. (2013). What is next generation sequencing? *Archives of Disease in Childhood. Education & Practice Edition*, *98*(6), 236–238.

Berger, S. L., Kouzarides, T., Shiekhattar, R., & Shilatifard, A. (2009). An operational definition of epigenetics. *Genes & Development,* 23:781–783.

Bernstein, B. E., Meissner, A., & Lander, E.S. (2007). The mammalian epigenome. *Cell,* 128:669–681.

Bonasio, R., Tu, S., & Reinberg, D. (2010). Molecular signals of epigenetic states. *Science,* 330:612–616.

Brillard, J. (2003). Practical aspects of fertility in poultry. *World's Poultry Science Journal, 59*(4), 441-446.

Burt, D.W. (2006) Chicken genome: Current status and future opportunities. *Genome Research,* 15: 1692- 1698.

Buzala, M., & Janicki, B. (2016). Review: Effects of different growth rates in broiler breeder and layer hens on some productive traits. *Poultry Science*, *95*(9), 2151–2159.

Calabotta, D. F., Cherry, J. A., Siegel, P. B., & Jones, D. E. (1985). Lipogenesis and lipolysis in fed and fasted chicks from high and low body weight lines. *Poultry Science*, 64(4), 700-704.

Chassagne, C., Eddahibi, S., Adamy, C., Rideau, D., Marotte, F., Dubois-Randé, J. L., Adnot, S., Samuel, J.L., & Teiger, E. (2000). Modulation of angiotensin II receptor expression during development and regression of hypoxic pulmonary hypertension. *American Journal of Respiratory Cell & Molecular Biology*, 22(3), 323-332.

Chapman, M. E., W., Erf, G. F., and Wideman, R.F. (2005) Pulmonary Hypertensive responses of broilers to bacterial lipopolysaccharide (LPS): Evaluation of LPS source and dose, and impact of pre- existing pulmonary hypertension and cellulose micro-particle selection. *Poultry Science,* 84:432-441

Cherian, G. (2007). Metabolic and Cardiovascular Diseases in Poultry: Role of Dietary Lipids1. *Poultry Science*, *86*(5), 1012–1016.

Cook, M. E. (2000). Skeletal deformities and their causes: introduction. *Poultry Science, 79*(7), 982-984.

Crawford, R.D. (1990) Origin and History of Poultry Species. In: Crawford. R.D., Ed., Poultry Breeding & Genetics. *Elsevier*, Amsterdam.

Cuffe, J. S. M., Walton, S. L., Steane, S. E., Singh, R. R., Simmons, D. G., & Moritz, K. M. (2014). The effects of gestational age and maternal hypoxia on the placental renin angiotensin system in the mouse. *Placenta*, 35(11), 953-961.

Daxinger, L., & Whitelaw, E. (2012). Understanding transgenerational epigenetic inheritance via the gametes in mammals. *Nature Reviews Genetics*, *13*, 153.

Decuypere, E.C., Vega, T., Bartha, J., Buyse, J., Zoons, & Albers, G.A. (1994). Increased sensitivity to triiodothyronine (T3) of broiler lines with a high susceptibility for ascites. *Poultry Science*, 35:287-297.

Decuypere, E., Buyse, J., & Buys, N. (2000). Ascites in broiler chickens: exogenous and endogenous structural and functional causal factors. *World's Poultry Science Journal*, 56(04), 367-377.

Deeb, N., & Lamont, S. J. (2002). Genetic architecture of growth and body composition in unique chicken populations. *Journal of Heredity*, 93(2), 107-118.

De Greef, K. H., Janss, L. L., Vereijken, A. L., Pit, R., & Gerritsen, C. L. (2001). Diseaseinduced variability of genetic correlations: ascites in broilers as a case study. *Journal of Animal Science,* 79(7), 1723-1733.

Dekkers, C. M. (2005). Implementation of marker assisted selection into breeding programs. *4th European poultry genetics symposium,* Croatia.

Desjardins, P., & Morais, R. (1990). Sequence and gene organization of the chicken mitochondrial genome: A novel gene order in higher vertebrates. *Journal of Molecular Biology*, *212*(4), 599–634.

Dey, S., Krishna, S., Anthony, N.B., and Rhoads, D.D. (2016). Further investigation of a quantitative trait locus for ascites on chromosome 9 in broiler chicken lines. *Poultry Science,* 96(4), 788- 797.

Dey, S., Parveen, A., Tarrant, K. J., Licknack, T., Kong, B. C., Anthony, N. B., & Rhoads, D. D. (2018). Whole genome resequencing identifies the CPQ gene as a determinant of ascites syndrome in broilers. *PLoS ONE*, *13*(1), 1–17.

Diaz, G. J., Julian, R. J., & Squires, E. J. (1994). Cobalt-induced polycythaemia causing right ventricular hypertrophy and ascites in meat-type chickens. *Avian Pathology,* 23:91–104.

Dodgson, J. B. (2007). The Chicken Genome: Some Good News and Some Bad News. 1 THE GOOD NEWS: SYMPOSIUM Transcriptional Profiling: Array of Hope. *Poultry Science*, 1453–1459.

Druyan, S. (2012). Ascites Syndrome in Broiler Chickens - A Physiological Syndrome Affected by Red Blood Cell. *Blood Cell - An Overview of Studies in Hematology*.

Ellegren, H. (2005) The avian genome uncovered. *Trends in ecology and evolution,* 20: 180-186.

Eltanany, M., & Distl, O. (2010). Genetic diversity and genealogical origins of domestic chicken. *World's Poultry Science Journal*, *66*(4), 715–726.

Falconer, D.S., & Mackay, T.F. (1996) Introduction to Quantitative Genetics. 4th edn. (Harlow, Pearson Prentice Hall).

Feil, R., & Fraga, M. F. (2012). Epigenetics and the environment: Emerging patterns and implications. *Nature Reviews Genetics*, *13*(2), 97–109.

Frésard, L., Morisson, M., Brun, J. M., Collin, A., Pain, B., Minvielle, F., & Pitel, F. (2013). Epigenetics and phenotypic variability: Some interesting insights from birds. *Genetics Selection Evolution*, *45*(1), 1–12.

Fulton, J. E. (2012). Genomic selection for poultry breeding. *Animal Frontiers*, *2*(1), 30–36.

Fumihito, A., Miyake, T., Sumi, S., Takada, M., Ohno, S. & Kondo, N. (1994) One subspecies of the red jungle-fowl (G.g. gallus) suffices as the matriarchic ancestor of all domestic breeds. *Proceedings of National Academy of Sciences,* 91: 12505-12509.

Griffin, H. D., & Goddard, C. (1994). Rapidly growing broiler (meat-type) chickens. Their origin and use for comparative studies of the regulation of growth. *International Journal of Biochemistry*, 26(1), 19-28.

Grisel, J. E., & Ph, D. (1998). Quantitative Trait Locus Analysis. *Alcohol Research & Health,* 169–174.

Groenen, M. A., Megens, H.-J., Zare, Y., Warren, W. C., Hillier, L. W., Crooijmans, R. P., … Cheng, H. H. (2011). The development and characterization of a 60K SNP chip for chicken. *BMC Genomics*, 12, 274.

Hall, S. A., & Machicao, N. (1968) Myocarditis in broiler chickens reared at high altitude. *Avian Diseases*, 12: 75-84.

Hassanzadeh, M., Buys, N., Dewil, E., Rahimi, G. & Decuypere, E. (1997). The prophylactic effect of vitamin C supplementation on broiler ascites incidence and plasma thyroid hormone concentration. *Avian Pathology*, 26, 3344.

Hassanzadeh, M., Bozorgmerifard, M. H., Akbari, A. R., Buyse, J., & Decuypere, E. (2000). Effect of intermittent lighting schedules during the natural scotoperiod on T3-induced ascites in broiler chickens. *Avian Pathology,* 29:433-439.

Havenstein, G. B., Ferket, P. R., Scheideler, S. E., & Larson, B. T. (1994). Growth, livability, and feed conversion of 1957 vs 1991 broilers when fed "typical" 1957 and 1991 broiler diets. *Poultry Science*, 73(12), 1785-1794.

Hillel, J., Groenen, M.A., Tixier-Biochard, M., Korold, B., David, L., Kirzhner, M. V., Burke, T., Barre-Dirief, A., Crooijmans, M.A., Elo, K., Feldman, M.W., Freidlin, P.J., Mäki-Tanila, A., Oortwijn, M., Thomson, P., Vignal, A., Wimmers, K. & Weigend, S. (2003) Biodiversity of 52 chicken populations assessed by microsatellite typing of DNA pools. *Genetics Selection Evolution,* 35: 533-557.

Hunton, M. (1990). Industrial breeding and selection [in poultry]. *Developments in Animal & Veterinary Sciences (Netherlands)*. Elsevier.

Hillier, L. W., W. Miller, E. Birney, W. Warren, R. C. Hardison, C. P. Ponting, P. Bork, D. W. Burt, M. A. M. Groenen, M. E. Delany, J. B. Dodgson, A. T. Chinwalla, P. F. Cliften, S. W. Clifton, K. D. Delehaunty, C. Fronick, R. S. Fulton, T. A. Graves, C. Kremitzki, D. Layman, V. Magrini, J. D. McPherson, T. L. Miner, P. Minx, W. E. Nash, M. N. Nhan, J. O. Nelson, L. G. Oddy, C. S. Pohl, J. Randall-Maher, S. M. Smith, J. W. Wallis, S. P. Yang, M. N. Romanov, C. M. Rondelli, B. Paton, J. Smith, D. Morrice, L. Daniels, H. G. Tempest, L. Robertson, J. S. Masabanda, D. K. Griffin, A. Vignal, V. Fillon, L. Jacobbson, S. Kerje, L.

Andersson, R. P. M. Crooijmans, J. Aerts, J. J. van der Poel, H. Ellegren, R. B. Caldwell, S. J. Hubbard, D. V. Grafham, A. M. Kierzek, S. R. McLaren, I. M. Overton, H. Arakawa, K. J. Beattie, Y. Bezzubov, P. E. Boardman, J. K. Bonfield, M. D. R. Croning, R. M. Davies, M. D. Francis, S. J. Humphray, C. E. Scott, R. G. Taylor, C. Tickle, W. R. A. Brown, J. Rogers, J. M. Buerstedde, S. A. Wilson, L. Stubbs, I. Ovcharenko, L. Gordon, S. Lucas, M. M. Miller, H. Inoko, T. Shiina, J. Kaufman, J. Salomonsen, K. Skjoedt, G. K. S. Wong, J. Wang, B. Liu, J. Yu, H. M. Yang, M. Nefedov, M. Koriabine, P. J. deJong, L. Goodstadt, C. Webber, N. J. Dickens, I. Letunic, M. Suyama, D. Torrents, C. von Mering, E. M. Zdobnov, K. Makova, A. Nekrutenko, L. Elnitski, P. Eswara, D. C. King, S. Yang, S. Tyekucheva, A. Radakrishnan, R. S. Harris, F. Chiaromonte, J. Taylor, J. B. He, M. Rijnkels, S. Griffiths-Jones, A. Ureta-Vidal, M. M. Hoffman, J. Severin, S. M. J. Searle, A. S. Law, D. Speed, D. Waddington, Z. Cheng, E. Tuzun, E. Eichler, Z. R. Bao, P. Flicek, D. D. Shteynberg, M. R. Brent, J. M. Bye, E. J. Huckle, S. Chatterji, C. Dewey, L. Pachter, A. Kouranov, Z. Mourelatos, A. G. Hatzigeorgiou, A. H. Paterson, R. Ivarie, M. Brandstrom, E. Axelsson, N. Backstrom, S. Berlin, M. T. Webster, O. Pourquie, A. Reymond, C. Ucla, S. E. Antonarakis, M. Y. Long, J. J. Emerson, E. Betran, I. Dupanloup, H. Kaessmann, A. S. Hinrichs, G. Bejerano, T. S. Furey, R. A. Harte, B. Raney, A. Siepel, W. J. Kent, D. Haussler, E. Eyras, R. Castelo, J. F. Abril, S. Castellano, F. Camara, G. Parra, R. Guigo, G. Bourque, G. Tesler, P. A. Pevzner, A. Smit, L. A. Fulton, E. R. Mardis, and R. K. Wilson. (2004). Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. *Nature*, 432:695-716.

Jablonka, E., & Raz, G. (2009). Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. The Quarterly Review of Biology, 84:131–176.

Julian, R.J. (1989). Lung volume of meat-type chickens. *Avian Diseases*, 33, 174176

Julian, R. J. (1990). Pulmonary hypertension: a cause of right heart failure, ascites in meat-type chickens. *Feedstuffs,* 78:19-20.

Julian, R. J. (1993). Ascites in poultry. *Avian Pathology*. 22:419–454.

Julian, R.J. (1998). Rapid growth problems: ascites and skeletal deformities in broilers, *Poultry Science*, 77, 17731780.

Julian, R.J. (2005). Production and growth-related disorders and other metabolic diseases of poultry*A review. *The Veterinary Journal*, 169, 350369

Julian, R. J. (2007). The Response of the Heart and Pulmonary Arteries to Hypoxia, Pressure, and Volume. A Short Review1. *Poultry Science*, *86*(5), 1006–1011.

Korte, A., & Farlow, A. (2013). The advantages and limitations of trait analysis with GWAS: a review. *Plant Methods*, *9*, 29.

Kranis, A., Gheyas, A. A., Boschiero, C., Turner, F., Yu, L., Smith, S., Talbot, R., Pirani, A., Brew, F., Kaiser, P., Hocking, P.M., Fife, M., Salmon, N., Fulton, J., Strom, T.M., Haberer, G., Weigend, S., Preisinger, R., Gholami, M., Qanbari, S., Simianer, H., Watson, K.A., Woolliams, J.A., & Burt, D. W. (2013). Development of a high density 600K SNP genotyping array for chicken. *BMC Genomics*, *14*, 59.

Krishnamoorthy, S., Smith, C. D., Al-Rubaye, A. A., Erf, G. F., Wideman, R. F., Anthony, N. B., & Rhoads, D. D. (2014). A quantitative trait locus for ascites on chromosome 9 in broiler chicken lines. *Poultry science*, 93(2), 307-317.

Launay, J.-M., Hervé, P., Peoc'h, K., Tournois, C., Callebert, J., Nebigil, C. G., Etienne, N., Drouet, L., Humbert, M., Simonneau, G., & Maroteaux, L. (2002). Function of the serotonin 5-hydroxytryptamine 2B receptor in pulmonary hypertension. *Nature Medicine*, *8*, 1129.

Levy, S. E., & Myers, R. M. (2016). Advancements in Next-Generation Sequencing. *Annual Review of Genomics & Human Genetics*, *17*(1), 95–115.

Liu, Y.P., Wu, G.S., Yao, Y.G., Miao, Y.W., Luikart, G., Baig, M., Beja-Pereira, A., Ding, Z.L., Palanichamy, M.G. & Zhang, Y.P. (2006) Multiple maternal origins of chickens: Out of the Asian jungles. *Molecular Phylogenettics & Evolution,* 38: 12-19.

los Santos, F. S. D., Tellez, G., Farnell, M. B., Balog, J. M., Anthony, N. B., Pavlidis, H. O., & Donoghue, A. M. (2005). Hypobaric hypoxia in ascites resistant and susceptible broiler genetic lines influences gut morphology. *Poultry Science,* 84:1495-1498.

Lubritz, D. L., & McPherson, B. N. (1994). Effect of genotype and cold stress on incidence of ascites in cockerels. *Journal of Applied Poultry Research* 3:171-178.

Lubritz, D. L., Smith, J. L., & McPherson, B. N. (1995). Heritability of ascites and the ratio of right to total ventricle weight in broiler breeder male lines. *Poultry Science*, 74(7), 1237-1241.

Majumder, P. P., & Ghosh, S. (2005). Mapping quantitative trait loci in humans: achievements and limitations. *Journal of Clinical Investigation*, *115*(6), 1419–1424.

Masabanda, J., Burt, D.W., O'Brien, P.C.M, Vignal, A., Fillon, V., ... (2004) Molecular cytogenetic definition of the chicken genome: the first complete avian karyotype. *Genetics,* 166:1367–1373

Maxwell, M. H. & Robertson, G.W. (1997). World broiler ascites survey 1996. *Poultry Science Int*. 36:16-19

Mazzio, E. A., & Soliman, K. F. (2012). Basic concepts of epigenetics: Impact of environmental signals on gene expression. *Epigenetics*, *7*(2), 119–130.

Miao, Y. W., Peng, M. S., Wu, G. S., Ouyang, Y. N., Yang, Z. Y., Yu, N., Liang, J.P., Pianchou, G., Beja-Pereira, A., Mitra, B., Palanichamy, M.G., Baig, M., Chaudhuri, T.K., Shen, Y.Y., Kong, Q.P., Murphy, R.W., Yao, Y.G., & Zhang, Y. P. (2013). Chicken domestication: An updated perspective based on mitochondrial genomes. *Heredity*, *110*(3), 277–282.

Miles, C. & Wayne, M. (2008). Quantitative trait locus (QTL) analysis. *Nature Education,* 1(1):208

Miller, L.L., Siegel, P.B., & Dunnington, E.A. (1992). Inheritance of antibody response to sheep erythrocytes in lines of chickens divergently selected for 56-day body weight and their crosses. *Poultry Science,* 71, 47–52.

Moghadam, H. K., McMillan, I., Chambers, J. R., & Julian, R. J. (2001). Estimation of genetic parameters for ascites syndrome in broiler chickens. *Poultry Science,* 80(7), 844-848.

Muir, W. M., Wong, G. K.-S., Zhang, Y., Wang, J., Groenen, M. a M., Crooijmans, R. P. M., Huanmin, Z., Ron, O., Addie, V., Annemieke, J., Gerard A. A. Albers, Cindy, T. L., Mary, E., Delany, S. M., & Cheng, H. H. (2008). Genome-wide assessment of worldwide chicken SNP genetic diversity indicates significant absence of rare alleles in commercial breeds. *Proceedings of the National Academy of Sciences*, *105*(45), 17312–17317.

Navarro, P., Koerhuis, A.N.M., Chatziplis, D., Visscher, P.M. And Haley, C.S. (2002) Genetic studies of ascites in a broiler population. Proc. 7th World Congress on Genetics Applied to Livestock Production, *Montpellier, France, August* 19-23, 2002.

Nishibori, M., Shimogiri, T., Hayashi, T. & Yasue, H. (2005) Molecular evidence for hybridization of species in the genus Gallus except for Gallus varius. *Animal Genetics,* 36: 367-375.

Oka, T., Ino, Y., Nomura, K., Kawashima, S., Kuwayama, T., Hanada, H., Amano, T., Takada, M., Takahata, N., hayashi, Y. & Akishinonomiya, F. (2007) Analysis of mtDNA sequences shows Japanese native chickens have multiple origins. *Animal Genetics,* 38: 287-293

Ong, K. L., Wong, L. Y., & Cheung, B. M. (2008). The role of urotensin II in the metabolic syndrome. *Peptides*, 29(5), 859-867.

Owen, R. L., Wideman, R. F., Hattel, A. L., & Cowen, B. S. (1990). Use of a hypobaric chamber as a model system for investigating ascites in broilers. *Avian Diseases,* 34:754-758.

Pavlidis, H. O., Balog, J. M., Stamps, L. K., Hughes, J. D. J., Huff, W. E., & Anthony, N. B. (2007). Divergent selection for ascites incidence in chickens. *Poultry Science*, *86*(12), 2517–2529.

Paxton, H., Anthony, N. B., Corr, S. A., & Hutchinson, J. R. (2010). The effects of selective breeding on the architectural properties of the pelvic limb in broiler chickens: a comparative study across modern and ancestral populations. *Journal of Anatomy,* 217(2), 153-166.

Pértille, F., Moreira, G. C. M., Zanella, R., Nunes, J. D. R. D. S., Boschiero, C., Rovadoscki, G. A., Mourão, G.B., Ledur, M.C., & Coutinho, L. L. (2017). Genome-wide association study for performance traits in chickens using genotype by sequencing approach. *Scientific Reports*, *7*1–11.

Powell, W., Machray, G.C., & Provan, J. (1996). Polymorphism revealed by simple sequence repeats. *Trends in Plant Science*. 1:215-222.

Pousada, G., Baloira, A., & Valverde, D. (2015). Molecular and clinical analysis of TRPC6 and AGTR1 genes in patients with pulmonary arterial hypertension. *Orphanet Journal of Rare Diseases*, 10(1), 1.

Preisinger, R. (2012). Genome-wide selection in poultry. *Animal Production Science*, *52*(2–3), 121–125.

Rath, N. C., Huff, G. R., Huff, W. E., & Balog, J. M. (2000). Factors regulating bone maturity and strength in poultry. *Poultry Science, 79*(7), 1024-1032.

Rauw, W. M., Kanis, E., Noordhuizen-Stassen, E. N., & Grommers, F. J. (1998). Undesirable side effects of selection for high production efficiency in farm animals: a review. *Livestock Production Science,* 56(1), 15-33

Rivera, C.M., & Ren, B. (2013). Mapping human epigenomes. *Cell* 155:39–55.

Sanotra, G.S., Lund, J.D., Erksbøl, A.K., Petersen, J.S. & Vestergaard, K.S. (2001) Monitoring leg problems in broilers: a survey of commercial broiler production in Denmark. *World's Poultry Science Journal* 57, 55–69

Sato, T., K., Tezuka, H., Shibuya, T., Watanabe, H., Kamata, & Shirai, W. (2002). Cold-induced ascites in broiler chickens and its improvement by temperature-controlled rearing. *Avian Diseases,* 46:989-996.

Sawai, H., Kim, H. L., Kuno, K., Suzuki, S., Gotoh, H., Takada, M., Naoyuki, T., Yoko, S., & Akishinonomiya, F. (2010). The Origin and Genetic Variation of Domestic Chickens with Special Reference to Junglefowls *Gallus g. gallus* and *G. varius*. *PLoS ONE*, *5*(5), e10639.

Scheele, C.W. (1996). Ascites in chickens, Thesis, Landbouwuniversiteit, Wageningen, The Netherlands.

Schmidt, C. J., Persia, M. E., Feierstein, E., Kingham, B., & Saylor, W.W. (2009). Comparison of a modern broiler line and a heritage line unselected since the 1950s. *Poultry Science,* 88:26102619.

Schmid, M., J. Smith, D. W. Burt, B. L. Aken, P. B. Antin, A. L. Archibald, C. Ashwell, P. J. Blackshear, C. Boschiero, C. T. Brown, S. C. Burgess, H. H. Cheng, W. Chow, D. J. Coble, A. Cooksey, R. P. M. A. Crooijmans, J. Damas, R. V. N. Davis, D.-J. de Koning, M. E.

Delany, T. Derrien, T. T. Desta, I. C Dunn, M. Dunn, H. Ellegren, L. Eöry, I. Erb, M. Farré, M. Fasold, D. Fleming, P. Flicek, K. E. Fowler, L. Frésard, D. P. Froman, V. Garceau, P. P. Gardner, A. A. Gheyas, D. K. Griffin, M. A. M. Groenen, T. Haaf, O. Hanotte, A. Hart, J. Häsler, S. B. Hedges, J. Hertel, K. Howe, A. Hubbard, D. A. Hume, P. Kaiser, D. Kedra, S. J. Kemp, C. Klopp, K. E. Kniel, R. Kuo, S. Lagarrigue, Susan J. Lamont, D. M. Larkin, R. A. Lawal, S. M. Markland, F. McCarthy, H. A. McCormack, M. C. McPherson, A. Motegi, S. A. Muljo, A. Münsterberg, R. Nag, I. Nanda, M. Neuberger, A. Nitsche, C. Notredame, H. Noyes, R. O'Connor, E. A. O'Hare, A. J. Oler, S. C. Ommeh, H. Pais, M. Persia, F. Pitel, L. Preeyanon, P. Prieto Barja, E. M. Pritchett, D. D. Rhoads, C. M. Robinson, M. N. Romanov, M. Rothschild, P.-F. Roux, C. J. Schmidt, A.-S. Schneider, M. G. Schwartz, S. M. Searle, M. A. Skinner, C. A. Smith, P. F. Stadler, T. E. Steeves, C. Steinlein, L. Sun, M. Takata, I. Ulitsky, Q. Wang, Y. Wang, W. C. Warren, J. M. D. Wood, D. Wragg, and H. Zhou (2015). Third report on chicken genes and chromosomes. *Cytogenic & Genome Research*. 145: 78–179

Sinsigalli, N. A., McMurtry, J. P., Cherry, J. A., & Siegel, P. B. (1987). Glucose tolerance, plasma insulin and immunoreactive glucagon in chickens selected for high and low body weight. *The Journal of Nutrition*, 117(5), 941-947.

Smith, A. H., Wilson, W. O., & Pace, N. (1954). The effect of high altitude on the growth of turkeys. *Growth*, 18:27-35.

Tarrant, K., Dey, S., Kinney, R., Anthony, N.B., and Rhoads, D.D. (2017). Multi-generational genome wide association studies identify chromosomal regions associated with ascites phenotype. *Poultry Science*, p. pew478.

Twito, T., Weigend, S., Blum, S., Granevitze, Z., Feldman, M.W., Perl-Treves, R., Lavi, U. & Hillel, J. (2007) Biodiversity of 20 chicken breeds assessed by SNPs located in gene regions. *Cytogenetic & Genome Research,* 117: 319-326.

Ullmer, C., Schmuck, K., Kalkman, H. O., & Lübbert, H. (1995). Expression of serotonin receptor mRNAs in blood vessels. *FEBS Letters*, *370*(3), 215–21.

Vieira, M. L. C., Santini, L., Diniz, A. L., & Munhoz, C. F. (2016). Microsatellite markers: What they mean and why they are so useful. *Genetics & Molecular Biology*, *39*(3), 312–328.

West, J. D., Carrier, E. J., Bloodworth, N. C., Schroer, A. K., Chen, P., Ryzhova, L. M., Gladson, S., Shay, S., Hutcheson, J.D., & Merryman, W. D. (2016). Serotonin 2B receptor antagonism prevents heritable pulmonary arterial hypertension. *PLoS ONE*, *11*(2), 1–18.

Whitehead, C.C., Fleming, R.H., Julian, R. J. & Sørensen, P. (2003) Skeletal problems associated with selection for increased production. In: Muir, W.M. and Aggrey, S.E. (eds) *Poultry Genetics, Breeding & Biotechnology*. CAB International, Wallingford, UK, pp. 29–52.

Wideman, R. F., & Kirby, Y. K. (1995a). A pulmonary artery clamp model for inducing pulmonary hypertension syndrome (ascites) in broilers. *Poultry Science*, 74:805-812.

Wideman, R. F., Wing, T., Kirby, Y. K., Forman, M. F., Marson, N., Tackett, C. D., & RuizFeria, C. A. (1998). Evaluation of minimally invasive indices for predicting ascites susceptibility in three successive hatches of broilers exposed to cool temperatures. *Poultry Science*, 77:1565-1573.

Wideman, R.F. (2000). Cardio-pulmonary hemodynamics and ascites in broiler chickens. *Avian & Poultry Biology Review*, 11, 2143

Wideman, R. F., Erf, G. F., Chapman, M. E., Wang, W., Anthony, N. B., & Xiaofang, L. (2002). Intravenous Micro-Particle Injections and Pulmonary Hypertension in Broiler Chickens: acute post-injection mortality and ascites susceptibility. *Poultry Science*, 81:1203-1217.

Wideman, R. F., & Erf, G. F. (2002). Intravenous micro-particle injection and pulmonary hypertension in broiler chickens: cardio-pulmonary hemodynamic responses. *Poultry Science*, 81:877-886.

Wideman, R. F., Rhoads, D. D., Erf, G. F., & Anthony, N. B. (2013). Pulmonary arterial hypertension (ascites syndrome) in broilers: a review. *Poultry Science*, 92(1), 64-83.

Wilson, H., Piesco, N., Miller, E., & Nesbeth, W. (1979). Prediction of the Fertility Potential of Broiler Breeder Males. *World's Poultry Science Journal, 35*(2), 95-118.

Wilson, J. B., Julian, R. J., & Barker, I. K. (1988). Lesions of right heart failure and ascites in broiler chickens. *Avian Diseases*, 32:246–261.

Witte, J. S. (2010). Genome-Wide Association Studies and Beyond. *Annual Review of Public Health*, *31*(1), 9–20.

West, B. & zhou, B. X. (1989) Did chickens go north? New evidence for domestication. *World's Poultry Science Journal,* 45: 205-218.

Wolc, A., White, I.M., Olori, V.E., & Hill, W.G. (2009). Inheritance of fertility in broiler chickens. *Genetics, Selection, Evolution: GSE*, *41*(1), 47.

Wolc, A. (2015). Genomic selection in layer and broiler breeding- LOHMANN TIERZUCHT GmbH, 4–11.

Wong, G.K., Liu, B., Wang, J., Zhang, Y., Yang, X., Zhang, Z., Meng, Q., Zhou, J., Li, D., Zhang, J., Ni, P., Li, S., Ran, L., Li, H., Zhang, J., Li, R., Li, S., Zheng, H., Lin, W., Li, G., Wang, X., Zhao, W., Li, J., Ye, C., Dai, M., Ruan, J., Zhou, Y., Li, Y., He, X., Zhang, Y,, Wang, J., Huang, X., Tong, W., Chen, J., Ye, J., Chen, C., Wei, N., Li, G., Dong, L., Lan, F., Sun, Y., Zhang, Z., Yang, Z., Yu, Y., Huang, Y., He, D., Xi, Y., Wei, D., Qi, Q., Li, W., Shi, J., Wang, M., Xie, F., Wang, J., Zhang, X., Wang, P., Zhao, Y., Li, N., Yang, N., Dong, W., Hu, S., Zeng, C., Zheng, W., Hao, B., Hillier, L.W., Yang, S.P., Warren, W.C., Wilson, R.K., Brandström, M., Ellegren, H., Crooijmans, R.P., vanderPoel, J.J., Bovenhuis, H., Groenen, M.A., Ovcharenko, I., Gordon, L., Stubbs, L., Lucas, S., Glavina, T., Aerts,

A., Kaiser, P., Rothwell, L., Young, J.R., Rogers, S., Walker, B.A., van Hateren, A., Kaufman, J., Bumstead, N., Lamont, S.J., Zhou, H., Hocking, P.M., Morrice, D., de Koning, D.J., Law, A., Bartley, N., Burt, D.W., Hunt, H., Cheng, H.H., Gunnarsson, U., Wahlberg, P., Andersson, L., Kindlund, E., Tammi, M.T., Andersson, B., Webber, C., Ponting, C.P., Overton, I.M., Boardman, P.E., Tang, H., Hubbard, S.J., Wilson, S.A., Yu, J., Wang, J., Yang, H. & International Chicken Polymorphism Map Consortium. (2004). A genetic variation map for chicken with 2.8 million single nucleotide polymorphisms. *Nature,* 432:717-722.

Yan, Y., Yi, G., Sun, C., Qu, L., & Yang, N. (2014). Genome-wide characterization of insertion and deletion variation in chicken using next generation sequencing. *PLoS ONE,* 9: e104652

Youngson, N. A., & Whitelaw, E. (2008). Transgenerational Epigenetic Effects. *Annual Review of Genomics & Human Genetics*, *9*(1), 233–257.

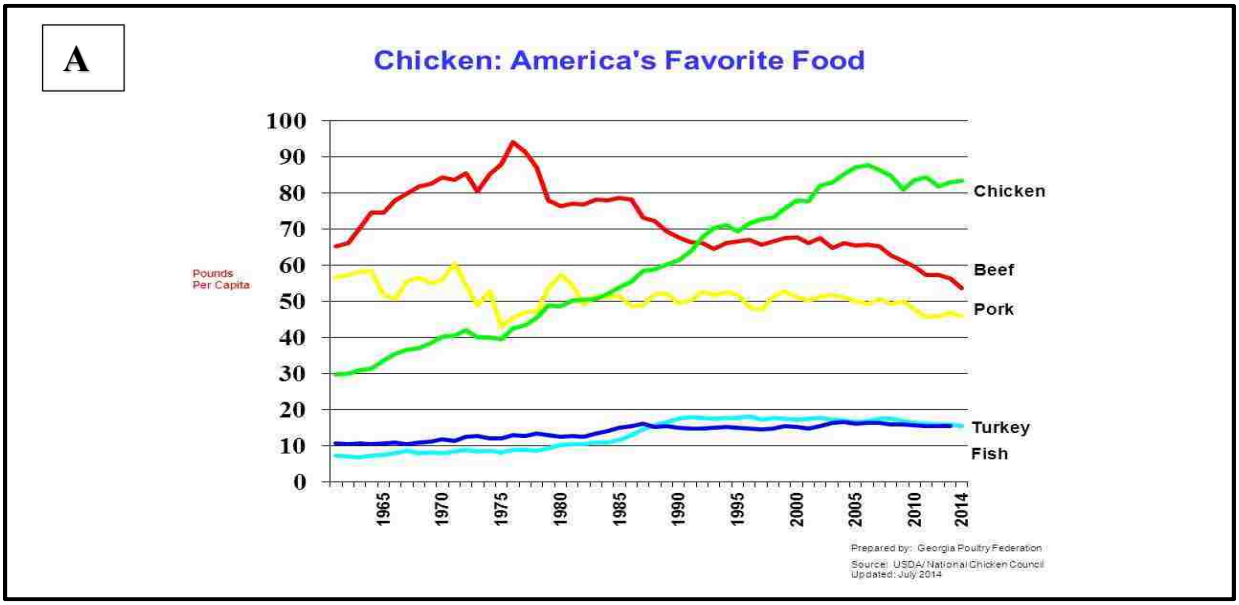
Zeng, J., D. Habier, & Fernando, R.L. (2014). Genomic prediction within family combining linkage disequilibrium and cosegregation information. *In: Proc 10th World Congress of Genetics Applied to Livestock Production* (WCGALP).

Zhao, R., Muehlbauer, E., Decuypere, E., & Grossmann, R. (2004). Effect of genotype–nutrition interaction on growth and somatotropic gene expression in the chicken. General and Comparative Endocrinology, 136:2–11.

Zheng, Q., Y. Zhang, Y. Chen, N. Yang, X. J. Wang, & D. Zhu. (2009). Systematic identification of genes involved in divergent skeletal muscle growth rates of broiler and layer chickens. *BMC Genomics*, 10:87

Zuidhof, M. J., Schneider, B.L., Carney, V.L., Korver, D.R., & Robinson, F.E. (2014). Growth, efficiency, and yield of commercial broilers from 1957, 1978, and 2005. *Poultry Science*, 93:2970–2982.

FIGURES



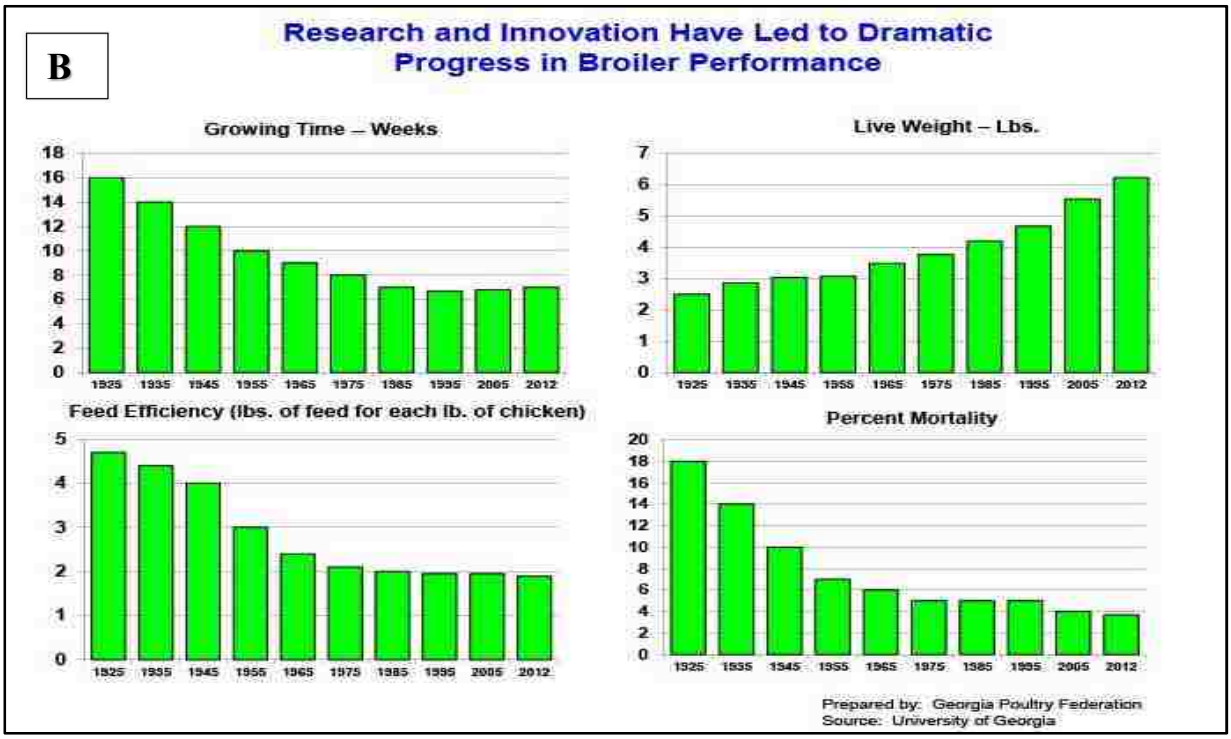


Figure 1. 1 Progress in broiler performance. A) U.S per capita consumption of various types of meat between 1965 and 2014. **B)** Trends in Broiler Performance from 1925 till 2012. Adapted by Georgia Poultry Federation, University of Georgia.

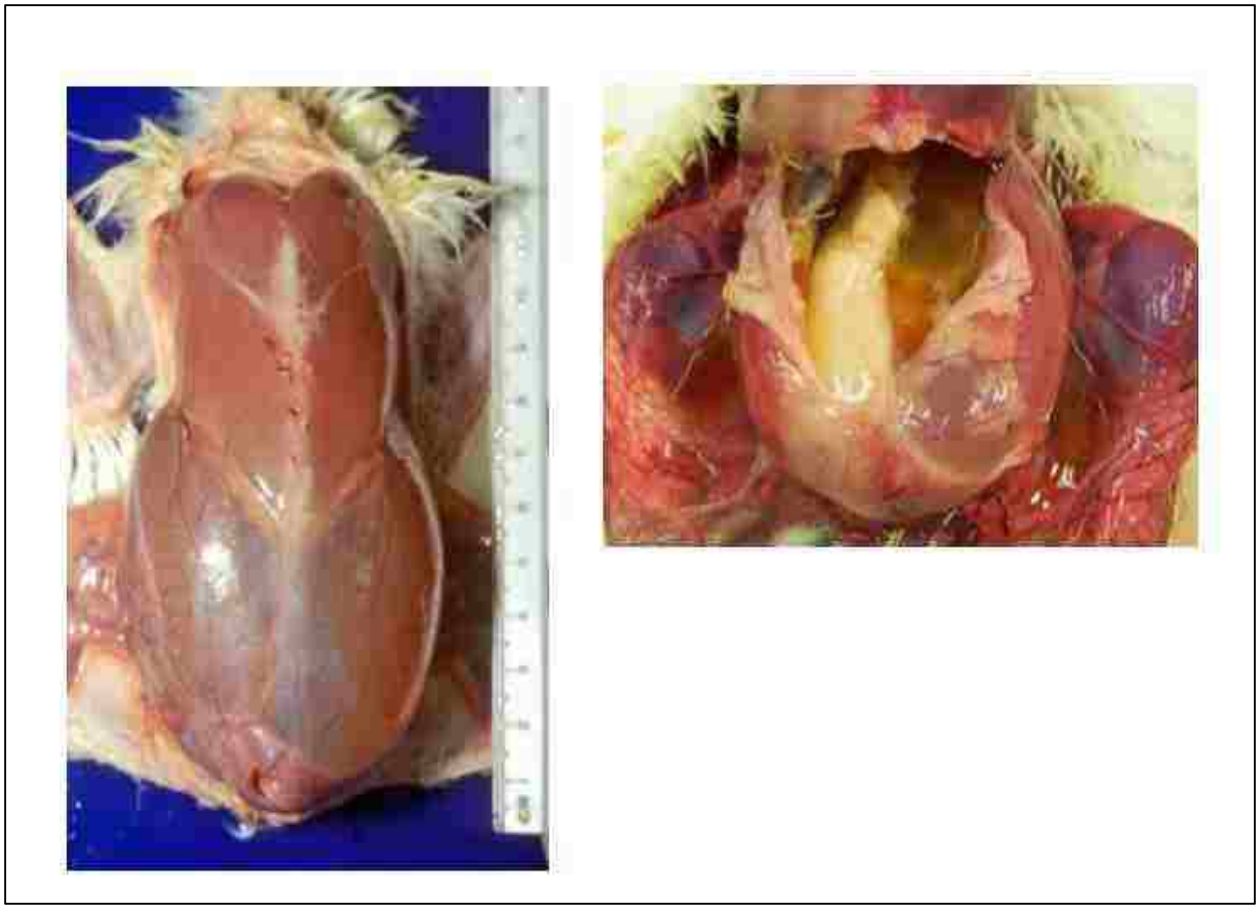


Figure 1. 2 Broiler with ascites symptoms. Accumulation of fluid in the abdominal cavity of an ascites affected bird. (Source: studyblue.com; http://neospark.com/ascites-kcp.html)

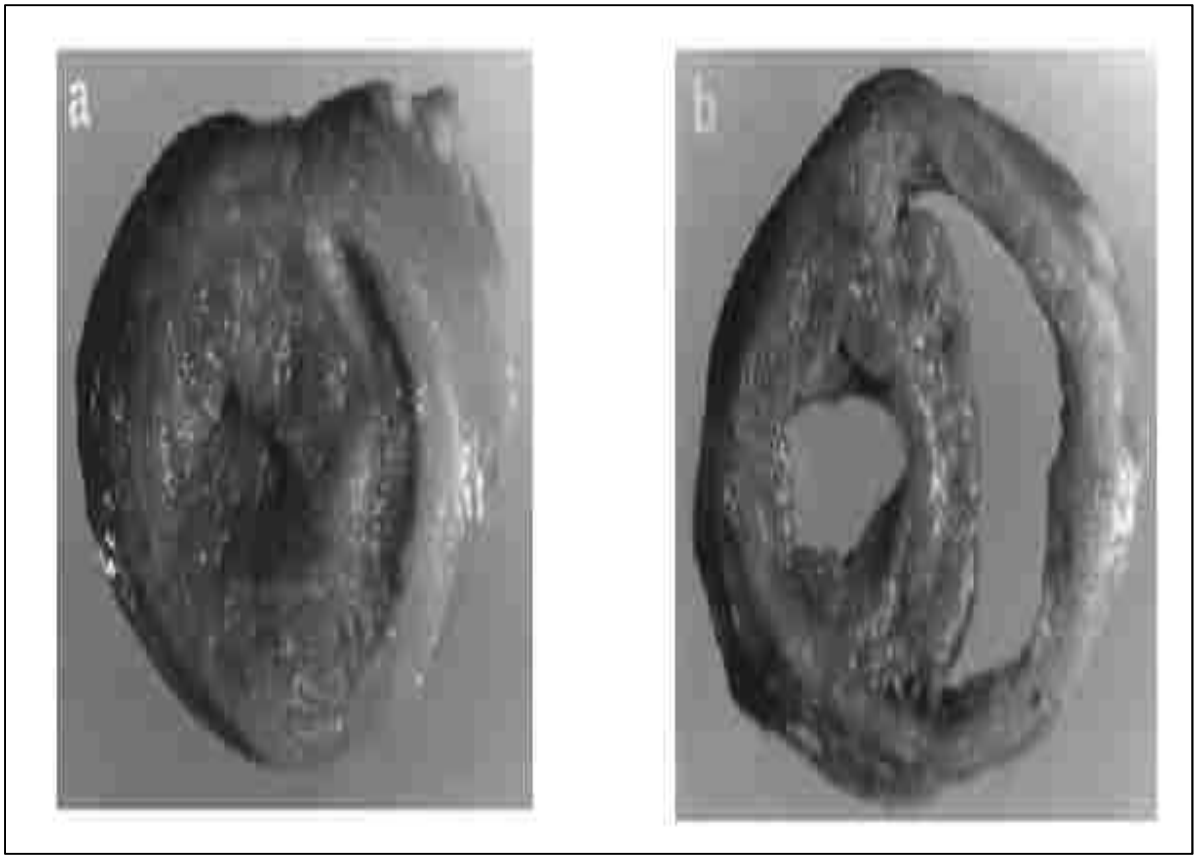


Figure 1. 3 Cross-sectional slice through ventricles of two broiler hearts. (a) a section of healthy heart; (b) Ascitic heart marked ventricular dilation (Olkowski *et al.,*2001).

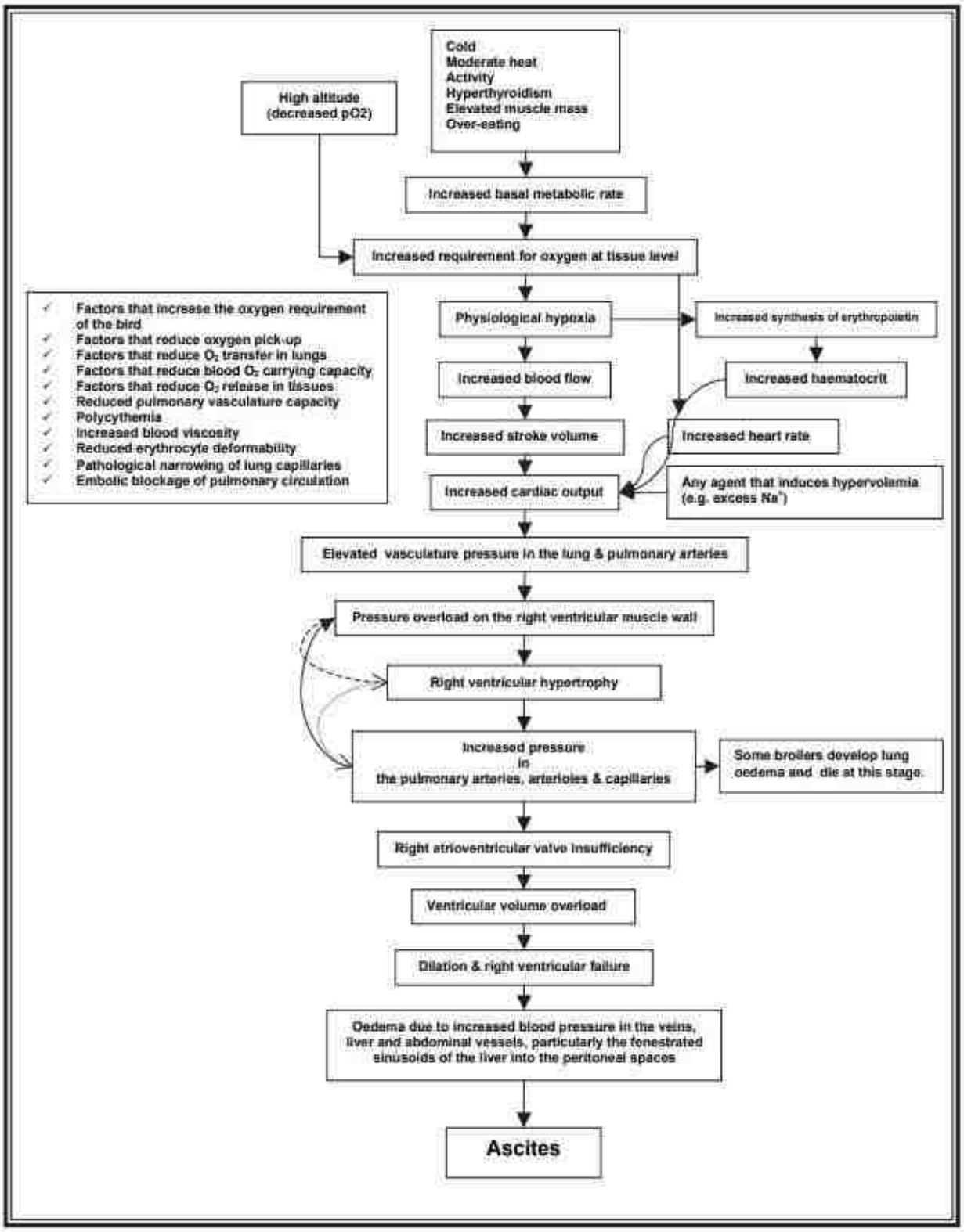


Figure 1. 4 Flow chart showing the physiological and pathophysiological factors that contribute to the development of ascites syndrome in broilers. *(*Decuypere *et al.,* 2008*).*

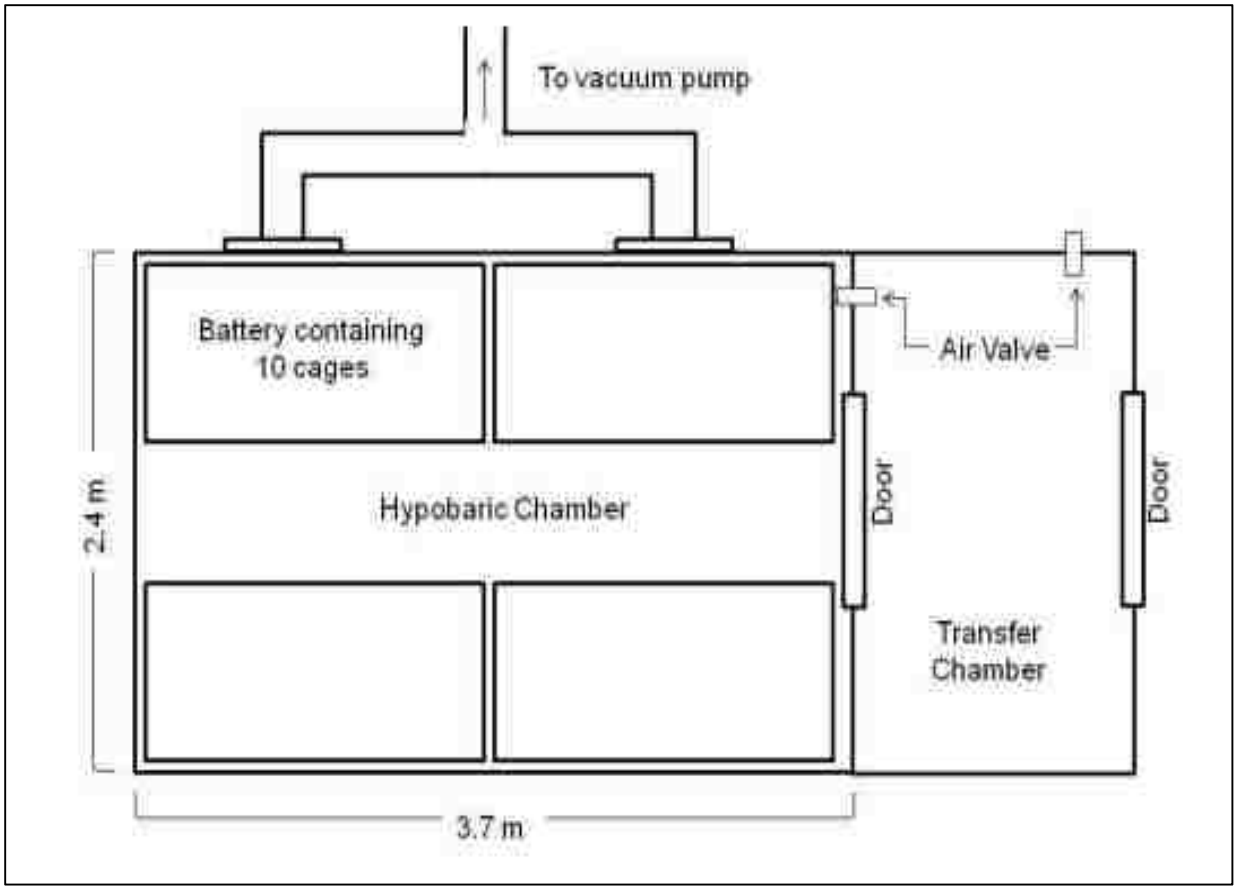


Figure 1. 5 Hypobaric chamber model for PHS induction in broilers.

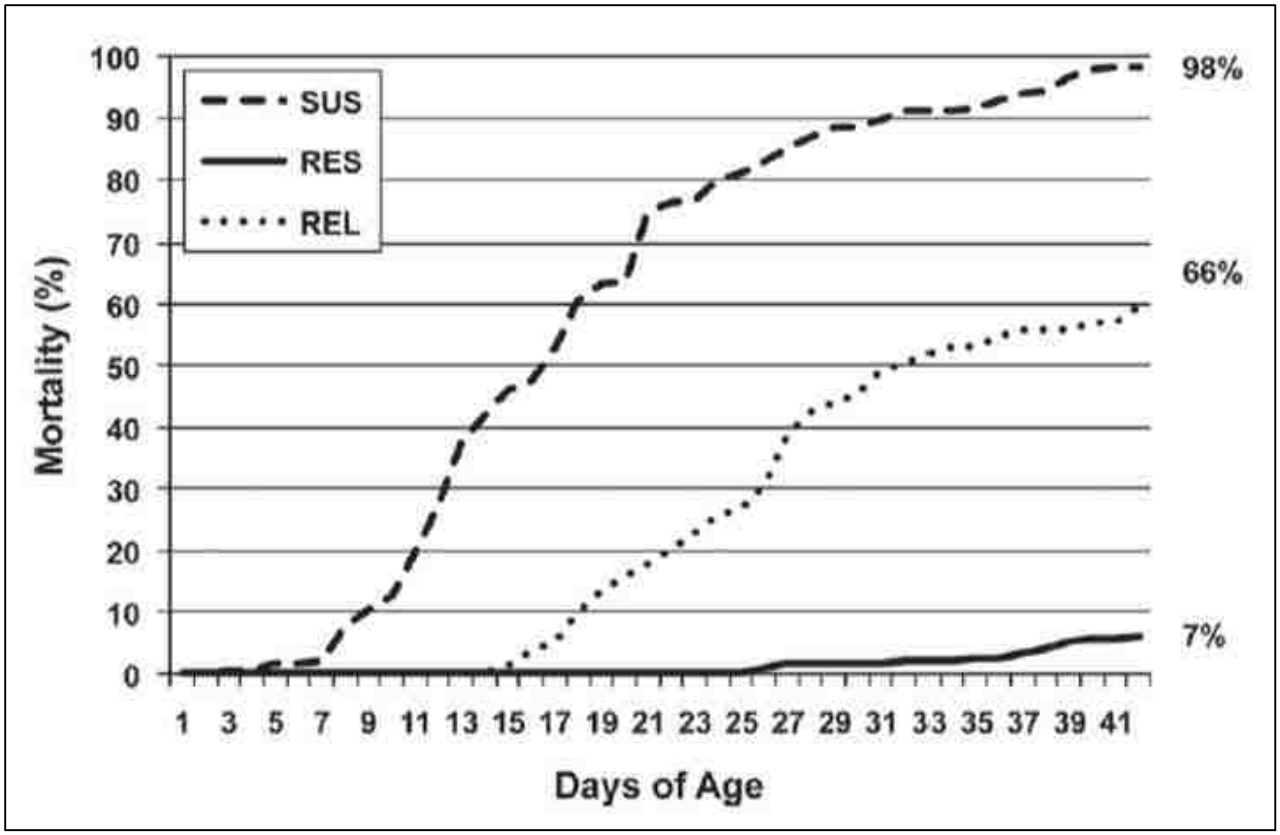


Figure 1. 6 Ascites Cumulative % mortality data for SUS, RES and REL lines at generation 14 *(*Wideman *et al.,* 2013*).*

CHAPTER 2

Polymorphism Detection and Allele- Specific Expression for the HTR2B Gene associated with Pulmonary Hypertension Syndrome in Broilers

ABSTRACT

The aim of this study was to investigate the association of HTR2B polymorphisms with pulmonary hypertension syndrome (PHS) in broilers, and to determine total gene and allele -specific differences in expression levels. PHS, or ascites is a terminal result of pulmonary hypertension, and is a significant metabolic disease of fast growing meat-type chickens. PHS results from a constant increase in pulmonary artery pressure as an immediate response to hypobaric hypoxia. A Previous genome wide association study (GWAS) had shown the association of a region on chromosome 9 (Gga9:15 Mbp) with PHS phenotype in broilers. We identified a candidate gene, HTR2B within this chromosomal region that might mediate the quantitative effect. A heterozygous (T>C rs315854205) single nucleotide polymorphism SNP located in the 3rd exon of the HTR2B gene was found be associated with PHS resistance (P=0.008) in one of three commercial broiler lines but not in our experimental lines. The results suggested that HTR2B polymorphisms are partially associated with PHS, but not a universal marker for genetic predisposition to ascites syndrome. The exonic SNP was used as a marker to measure allele specific expression (ASE) in a variety of tissues at different developmental ages. Two bird groups were used: 2 weeks and 6 weeks of age challenged in hypobaric hypoxic conditions (n=3 each). Total and allele specific gene expression was analyzed in heterozygous (T>C rs315854205) birds. We observed a statistically significant higher expression level of the C-allele vs the T-allele in hypoxic birds at 6 weeks of age in all tissues when compared with hypoxic birds at 2 weeks of age. However, the overall expression of HTR2B was reduced in hypoxic birds at 6 weeks of age as compared to hypoxic birds at 2 weeks of age.

Thus, we conclude that the hypoxia-induced decrease in HTR2B gene expression at 6 weeks of age vs 2 weeks of age results from preferential down-regulation of the T-allele. Our findings are

important for understanding the mechanisms that underlie the patterns of HTR2B expression and its potential impact on the phenotypic variation of PHS syndrome in broilers.

INTRODUCTION

Ascites, or pulmonary hypertension syndrome (PHS), is a metabolic disorder frequently observed in meat- type chickens (broilers), which is initiated when the body is insufficiently oxygenated (Julian,1993, 2000). Due to intense selection for many years for productive traits, modern broilers seem to be more prone to develop ascites. Environmental, nutritional, physiological and genetic factors affect the incidence of PHS (Decuypere *et al*., 2000; Balog, 2003; Julian, 2005). Such triggering factors of PHS lead to a series of pathophysiological changes which begins with pulmonary hypertension and ultimately leads to right ventricular failure and death. Affected birds can be identified through the presence of one or more of these symptoms: fluid in the abdominal cavity, an enlarged flaccid heart, and occasional liver changes (Decuypere *et al*., 2000; Julian, 2007). This metabolic disorder causes an estimated economic loss to the poultry industry of $100 million/year as recently as 2015 (M. Cooper and S. Gustin, personal communication, Cobb-Vantress, Inc.). In humans, idiopathic pulmonary arterial hypertension (IPAH) is a severe and progressive disease of unknown cause usually culminating in right heart failure, significant morbidity and early mortality (Firth *et al*., 2010). Despite significant advancement in the management of IPAH, yet there is no cure and it stills considered as a worldwide health burden (Taichman & Mandel, 2013). Animal models in IPAH research have contributed significantly to the current understanding of the disease pathogenesis. Broiler chickens are often used for genetic research studies for reasons such as: 1) relatively short generation interval, 2) breeding feasibility, 3) and distinct phenotypes. Our group at the University of Arkansas was able to identify a unique and a common disease characteristic in PHS broiler chickens. Dr. Wideman and others, identified the spontaneous development of complex plexiform lesions in the lungs of broiler chickens that had been genetically selected for susceptibility to PHS (Wideman *et al*., 2011). Thus, broiler

chicken model of susceptibility to IPAH provides an important experimental model to investigate the molecular and physiological mechanisms of disease pathogenesis (Wideman & Hamal, 2011). Several studies have indicated the involvement of serotonin (5-hydroxytryptamine, 5-HT) and its receptors in the etiology of PAH (Esteve *et al*., 2007; MacLean & Dempsie, 2009). Serotoninergic appetite suppressant drugs have been associated with an increased risk of developing PAH. A sustained elevation in 5-HT levels was found in the plasma of pulmonary hypertension PAH patients (Kéreveur *et al*., 2000). Isolated smooth muscle and endothelial cells from pulmonary arteries of PAH patient's express mRNAs for several serotonin receptors including HTR2B (Ullmer *et al*., 1995). Furthermore, using the chronic-hypoxic-mouse model of PH, HTR2B was found to play a role in PAH in response to hypoxia (Launay *et al.,* 2002). Subsequent treatment with HTR2B antagonists completely prevented the development of the hypoxia induced PH. Additionally, mice with knockout for HTR2B serotonin receptor were protected against hypoxic PH (Launay *et al*., 2002). In one study, HTR2B antagonism prevent the onset of heritable PAH in BMPR2 mutant mice by inhibiting the translocation and downstream activity of phosphorylated tyrosine kinase SRC, thus prevent the development of PAH (West *et al*., 2016). In broilers, 5-HT is a potent pulmonary vasoconstrictor that triggered pulmonary hypertension (PH) by activating receptors expressed on pulmonary artery smooth muscle cells PASMC (Chapman & Wideman, 2002). High levels of 5-HT have been induced in broilers using various methods such as intravenous micro-particles injection, diets containing high levels of tryptophan (a serotonin precursor) or serotonin intra venous infusion (Chapman & Wideman, 2002; Hamal *et al*., 2010; Wideman *et al*., 2013). In response to hypoxia, 5-HT is released from PASMC causing cell proliferation and irreversible vascular remodeling (Chapman *et al*., 2008). Dr. Wideman and coworkers recorded a high expression of HTR2B receptors in the lungs of broilers from a PAH-

susceptible line compared to PAH- resistant line when microparticles were injected in doses sufficient to obstruct ≥15% of the pulmonary arterioles suggesting their key role in pulmonary vasoconstriction and PASMCs proliferation (Chapman & Wideman, 2002, 2006a, 2006b). However, pretreatment with methiothepin (serotonin receptor blocker) eliminates the increase in pulmonary vascular resistance PVR and reduce pulmonary arterial pressure PAP (Hamal *et al*., 2010; Wideman *et al*., 2013). Altogether, these observations are consistent with the important role of the serotonin and serotonin receptors overexpression to susceptibility to PAH. Our lab conducted several studies to map chromosomal regions contributing to ascites susceptibility or resistance. One of the earliest studies involved a cross between an ascites resistant line (RES) with ascites susceptible line (SUS) to generate an F2 population where the birds were phenotyped as resistant or susceptible using a hypobaric chamber challenge (Pavlidis *et al*., 2007). The genome wide association study GWAS of the F2 population used a 3,072 SNP panel (Krishnamoorthy *et al*., 2014). This study identified several chromosomal regions that were associated with ascites phenotype. Further genetic analysis identified a QTL on Chromosome 9 (Gga9) around 15 Mbp (2015 genome assembly coordinates) that showed association with ascites and cardiac hypertrophy in several different broiler lines (Krishnamoorthy *et al*., 2014). A candidate gene, HTR2B was located within this QTL as a possible mediator for PHS phenotype (Figure 2.1). The objectives of the current study were to develop SNP assays for the gene region of HTR2B to examine the possible association with ascites phenotype, to analyze the expression of the HTR2B gene, and to examine allele specific expression (ASE) using a single nucleotide polymorphism (SNP) located in exon 3 of the HTR2B gene (T>C rs315854205) in different tissues at different developmental age stages under hypoxic conditions.

MATERIALS AND METHODS

Genomic Data

All genome positions indicated in this study are according to the December 2015 assembly of the *Gallus gallus* genome GenBank accession ID: GCF_000002315.4. Genomic sequences for specific chromosomal regions or genes were downloaded using UCSC genome browser (https://genome.ucsc.edu/).

Chicken Lines

All animal procedures were preapproved by the University of Arkansas Institutional Animal Care and Use Committee (under protocol 12039 and 15040). Birds used for this study were taken from an ongoing, multigenerational ascites selection study at the University of Arkansas (Pavlidis, *et al*., 2007). Three experimental lines were used in this study; the ascites- resistance (RES), ascites-susceptible (SUS), and relaxed (REL) lines were produced through 18 generation of divergent selection from a broiler elite line using sibling selection based on ascites phenotype as determined in a hypobaric challenge (Pavlidis, *et al*., 2007). Three commercial lines were used in this study. Lines W and Y represent male elite lines selected mainly for growth, yield and feed conversion, whereas line Z is a female elite line selected primarily for growth traits and reproduction.

DNA Isolation

Genomic DNAs were previously isolated from blood from phenotyped birds and stored frozen (Krishnamoorthy *et al*., 2014) using a rapid method (Bailes *et al*., 2007).

Primer Design and PCR Sequencing

The DNA region for the HTR2B gene on Gga9 at 15205069-15214131 was downloaded using the UCSC genome browser. Primer3 (http://frodo.wi.mit.edu/) was used to design PCR primers and probes, which were synthesized by Integrated DNA Technologies (IDT; Coralville, IA, USA). Primer and probe information are listed in Table 2.1. PCR was performed using either an MJ Research PTC-100 thermocycler (BioRad Laboraotories, Hercules, CA) or an Eppendorf Mastercycler Gradient (Eppendrof North America, Hauppauge, NY). PCR mixtures (40 µL) contained: 1X Taq Buffer (50 mMTris-Cl pH 8.3, 1 mM $MgCl_2$, 30 µg/ml BSA), 0.2 mM dNTPs, 0.5 µM of reverse and forward primers, 4 U of Taq polymerase and 2 µL (approximately 100 ng) of target DNA. Cycle parameters were 90°C for 30s, 40 cycles of 90°C for 15s, primer-specific soak for 30s (Table 2.1), 72°C for 60s, followed by a final extension at 72°C for 3 minutes. PCR conditions were optimized for each primer pair and used to amplify specific regions of the HTR2B gene or mRNA. PCR products were evaluated for quality on agarose gel, purified for sequencing using RapidTip pipettes tips according to the manufacturer's instructions (Diffinity Genomics, West Chester, Pennsylvania), then quantified using a TKO 100 Fluorometer (Hoefer Scientific Instruments, Livonia, Michigan). DNA samples were then mixed with single primers and submitted for capillary sequencing (Eurofins Genomics, Louisville, KY) for confirmation. The sequences ab1 files were aligned and analyzed using SeqMan Pro software (DNAStar, Madison, WI). SNPs were identified from the sequence data through comparison to the Jungle Fowl sequence.

SNP Genotyping

Primers and probes for Taqman/exonuclease assays were developed to genotype for specific SNPs of the HTR2B gene listed in Table 1. SNP genotypes were determined by quantitative-PCR (qPCR) in 96 well plates using a CFX96 real-time thermocycler (Bio-Rad Laboratories, Inc., Hercules, California). Exonuclease assays were optimized for soak temperature for genotype discrimination. Reactions (20 μL) were as for standard PCRs (see above with addition of the two probes at 0.25 μM. The cycling protocol was: 1) 90°C for 30s, 2) 90°C for 15s, 3) 64°C for 30s, 4) repeat steps 2 and 3 for 10 times, 5) 90°C for 15s, 6) 64°C for 30s + plate read, 7) Repeat steps 5 and 6 for 30 times. Amplification profiles were visually inspected to score for homozygous for either SNP pattern or heterozygous.

Statistical Methods

Genotype data (SNPlotypes) for each sample were compiled and statistically analyzed in Microsoft Excel (Microsoft Corporation, Redmond, WA). Expected allele and genotype frequencies were calculated using observed allele frequencies in the entire population. Expected genotype counts were also computed based on standard Hardy-Weinberg Equilibrium HWE ($p^2+2pq+q^2$) to assess whether the assay was performing adequately. For each allele and genotype, we calculated P-values using the ChiTest in Excel comparing the observed counts to the expected counts for the resistant and susceptible subpopulations. *P*-values for each genotype were corrected using a simple Bonferroni correction for multiple genotype assays. Significant deviation was assumed for $P<0.05$.

HTR2B gene and allele specific expression

Genotyping for Exon3 Heterozygous SNP9093

Genotyping for SNP9093 was performed on REL line birds (18th generation) by TaqMan 5'-exonuclease assays using qPCR in 96 well plates using a CFX96 real-time thermocycler (Bio-Rad Laboratories, Inc., Hercules, California). Primers and probes for these assays are shown in Table 1. Genotyping reactions (20 μL) were as for standard PCRs (see above with addition of the two probes at 0.25 μM). Cycle parameters were as indicated above. Some PCR products were purified, quantified, and submitted for sequence verification, as indicated above. Sequence ab1 files were aligned using SeqMan Pro 14 (DNASTAR Lasergene Suite 14) for editing and scoring sequence data.

Tissue Collection

For RNA expression analyses, genotyped REL line birds were raised under hypoxic challenge (simulated 9000 ft altitude in hypobaric chamber). At particular time points (2 weeks & 6 weeks) birds were euthanized by cervical dislocation. Tissues were rapidly collected and immediately placed in RNAlater^{TM}, then stored at -20 ^{o}C.

RNA Extraction and cDNA synthesis

Total RNA was purified from tissues using the standard acid guanidinum thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi, 1987). The extracted RNA was assessed for quantity and purity (A260/280) using NanoVue spectrophotometry (GE Healthcare Bio-Sciences, MA, USA). RNA integrity was evaluated by electrophoresis in 1.5% agarose gel in 0.5×TBE buffer (50 mM Tris, 1 mM Na_2EDTA, and 25 mM Borate, pH 8.3), stained by 0.5 μg/ml

ethidium bromide. Samples that did not show 3 strong and distinct bands (28S, 18S, and 5S rRNA) were discarded. Total RNA samples were further verified to be free of contaminating genomic DNA by the absence of PCR products from RNA samples using primers for genomic DNA. Chicken TATA-binding protein (*TBP*) was used as the reference gene (Radonic *et al*., 2004). First strand cDNA (Gubler & Hoffman, 1983) was PCR amplified in a 40 μL reaction for ASE in an Eppendorf Mastercycler Gradient (Eppendrof North America, Hauppauge, NY) using conditions specified above. Products were evaluated for quality on agarose gel and purified for sequencing using RapidTip pipettes tips (Chiral Technologies, West Chester, PA). Quantity was assessed by fluorimetry with Hoechst 33258 (GLOMAX Multi Jr, Promega Corp., Madison, WI) and purity (A260/280) by spectrophotometry (NanoVue, GE Healthcare Bio-Sciences, MA, USA). Expression of HTR2B and TBP was measured in parallel for each sample using RT-qPCR, CFX96 real-time thermocycler (Bio-Rad Laboratories, Inc., Hercules, California). Data from HTR2B mRNA expression were then adjusted for relative gene expression using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). For ASE analysis, cleaned cDNA amplicon samples were then submitted for capillary sequencing as above and sequence ab1 files were aligned using SeqMan Pro 14 (DNASTAR Lasergene Suite 14). Using sequence scanner v1.0, the relative mRNA expression ratios between the two alleles of the heterozygous SNP9093 were estimated by dividing the peak height of the "major" (i.e., highest) peak by the sum of both alleles.

ASE statistical analysis

Allele specific expression was performed in cDNA from heterozygous individuals. The PCR was performed in triplicate for each sample and the expression of allelic transcripts was assessed directly by dividing the ratio of the major allele by the sum of both alleles from the sequence histogram in the ab1 file. Data are expressed as means ± SD where least square means were

separated using protected least significant difference procedure (LSD) at 5% level of significance. Two-tailed student T-test was performed to compare the ASE for the 2 weeks and 6 weeks of hypoxic challenged birds. The analysis was carried out using GLIMMIX procedure in SAS (version 9.4).

RESULTS

HTR2B gene is composed of two short non-coding exons followed by exon 3 which encodes a predicted protein of 462 aa residues. For this study, we designed multiple primers to PCR amplify and sequence segments of the HTR2B gene from six RES and nine SUS samples (Table 2.1) to detect SNPs that are segregating in our research lines. We sequenced the three exonic regions of HTR2B with flanking intronic sequences. In addition, we sequenced approximately 1000 bases upstream of exon 1 to identify SNPs in possible regulatory elements and transcription factor binding sites. All sequences were aligned with the sequence for this same region of the HTR2B gene from the 2015 genome assembly for *Gallus gallus,* the Red Jungle Fowl (JF). A total of 19 SNPs were identified including of SNP875A>G (Gga9:1521455) in the 5' untranslated region (UTR), SNP6958A>G (Gga9:15208073) in intron 2, and two SNPs in exon 3 (SNP9093T>C (Gga9:15205937) and SNP9240C>A (Gga9:15205790). Based on our sequence data SNP9093 is in complete linkage with SNP9240 which is 147 bases downstream. SNP9093 is a silent, the third base substitution in a GA**Y** codon for Asp255, whereas SNP9240 is a silent, third base substitution of a TC**M** codon for Ser304. The other 15 SNPs were in the 5' flanking region of the gene, upstream of the predicted transcription start site of the HTR2B gene (Table 2.2). Five SNPs spanned an 18-base region span at 496 C>T (Gga9: 15214543), 505, 507 and 510 CCT>del3 (Gga9: 15214526, 15214524, 15214521), and 515 T>C (Gga9: 15214516). Using the GeneQuest search tool in DNAStar these SNPs affect potential binding sites for multiple transcription factors

(NFAT5, NFATc3, NF-κB, and Myb) and thus could affect expression of the HTR2B gene, and ascites susceptibility or resistance. Examination of the sequence reads determined that all "non-reference" SNPs were in complete linkage and thus define two alternative alleles. Therefore, this region was targeted for genotyping a larger collection of DNA samples using exonuclease qPCR tests (TaqMan assay). However, to avoid problems with design of exonuclease probes to this highly polymorphic region we chose to target a single SNP 71 bases downstream that was also in complete linkage, SNP586A>C (Gga9:15214444). Thus, genotype determinations were for both the promoter SNP586, and exon3 SNP9093. We then used these assays on a collection of DNAs from our ascites research lines (SUS, RES, and REL lines) and three commercial lines (Y, Z and W). All birds had been phenotyped for ascites resistance or susceptibility in the hypobaric chamber (simulated ~ 9000 ft) (Pavlidis *et al*., 2007; Krishnamoorthy *et al*., 2014).

We analyzed two generations of the REL line, generation 14 (n=192) and 18 (n=235) for both SNP586 and SNP9093. Analysis of SNP586 genotypes in both generations revealed roughly similar frequencies of all three genotypes with similar patterns of both. The CC homozygote was most frequent at 63% and 50% in generation 14, and 18 respectively. The AA homozygote was 10% and 23% in generation 14, and 18 respectively, while the AC heterozygote was 27% and 28% in generation 14, and 18 respectively (Table 2.3). All three genotypes showed similar frequency with respect to gender and phenotype. However, a significant deviation from HWE was observed in both generations because of high homozygosity for this SNP. This could signify the presence of null alleles (unidentified SNPs in the primer and/or probe regions). Since the deviation was observed consistently in two different generations, we discounted the possibility of the existence of artifacts of the PCR assay. Sequence analysis of multiple randomly selected PCR products from the genotype assays failed to identify any null alleles and confirmed the observed genotypes.

Evaluation of allele and genotype frequencies for SNP586 in both generations showed no association with resistance or susceptible phenotype in the REL line chickens for both or either gender. In the research SUS and RES (n=96 for each) lines, the genotype data for SNP586 was consistent with HWE expectations for both lines (Table 2.3). The SUS line is segregating for both alleles with nearly equal numbers of homozygotes alleles (25% CC homozygote, 29% AA homozygote, and 46% heterozygote). In RES line birds, the AA homozygote was the major allele at 49%, and the CC homozygote was the minor allele at 11%, and only 37% for AC heterozygote allele. Selection for ascites resistance was associated with an increase in the A allele in RES line birds.

The same two generations of REL line samples were genotyped for SNP9093. The genotype data for generation 14 did not confirm with HWE because of reduced heterozygosity at this locus (observed=31% vs expected 45%) but generation 18 genotype data was consistent with HWE (Table 2.3). A shift in genotype frequencies from generation 14 to generation 18 was observed in REL line where the TT homozygotes had a lower frequency in generation 18 (21%) compared with generation 14 (50%). On the other hand, the CT heterozygote allele was observed at greater frequency in generation 18 (53%) than in 14 (31%). The CC homozygotes were equally distributed in both generations (20% and 26% in generation 14 and 18 respectively). However, allele and genotype frequencies for SNP9093 showed no association with resistance or susceptible phenotype in the REL line birds for both or either gender in both generations (Table 2.3). In the research RES and SUS lines, the TT homozygote was more frequent in the SUS (30%) than the RES line (11%). Alternately, the CC allele was observed more frequent in RES line (56%) than the SUS line (23%). Therefore, selection for ascites resistance was associated with an increase in allele C in the RES line.

Analysis of three commercial lines (line W, Y, and Z) for the two SNP loci showed that these two SNPs are present at different frequencies in each line (Table 2.4, and Table 2.5). For SNP586, lines W, Y, and Z genotypes conformed with HWE. In line W, allele A was the major allele where allele C was the minor allele (84% vs 16%). The low minor allele frequency at this locus in line W may preclude detecting any association with respect to phenotype or gender. Analysis of line Y samples for the 586SNP identified the A allele at 72% while the frequency for the C allele was 28%. The CC homozygote was overrepresented in resistant females 21% and underrepresented in susceptible females 5% with statistically significant deviation (P=0.025). Thus, there might be an association to ascites in females for this line but would require analysis of additional phenotyped samples for confirmation. In line Z, there were no significant differences detected between frequencies of the resistant and susceptible birds and no association for any genotype for either gender in respect to ascites phenotype (Table 2.4).

For SNP9093, genotype data confirmed with HWE in all three commercial lines. We saw little or no association of SNP9093 with ascites phenotype in line W since this line was predominantly homozygous CC (91%). Thus, no clear association with ascites phenotype although there is one intriguing difference for the heterozygous CT; where 13% of resistant birds vs. 4% of susceptible birds were heterozygotes (P=0.008). Unfortunately, because the CT heterozygote is only 9 % of all genotypes, the counts in females and males were low (CT males: 11 resistant, 3 susceptible and CT females: 16 susceptible, 5 resistant) with no significant difference. In Line Y and line Z, genotypes data were all nearly equal in frequency. Moreover, there were no statistical associations with any allele, or genotype, for either gender with respect to ascites phenotype. Thus, in general, there was no strong statistical correlation for any genotype with phenotype for either gender in any of the three commercial lines for this locus (Table 2.5).

Next, we combined the genotype data for promoter SNP586 and exon 3 SNP9093 to generate haplotypes for further ascites association analyses. There was no association with ascites phenotype observed for either gender for any haplotype in any of the research or commercial lines (Tables 2.6, 2.7, 2.8, 2.9). In general, SNPlotype data for HTR2B gene is not consistent with this region being a QTL for ascites in different research or commercial lines.

Then, we decided to take a further step and analyze the possible effect of exon 3 SNP9093 (T>C rs315854205) on mRNA expression in two different REL line bird groups all heterozygotes for SNP9093 and across various tissues. Birds represented 2 developmental age stages: 2 weeks old hypoxic challenged (n=3), and 6 weeks old hypoxic challenged (n=3). PCR was used to quantitate relative expression levels of mRNA produced from the C-allele versus the T-allele in individuals heterozygous for SNP9093. PCR products covering the 3rd exonic SNP9093 of HTR2B were analyzed by direct sequencing to compare the expression levels between alleles. Sequences were aligned with reference T-allele or alternate C-allele and the relative mRNA expression levels between the two alleles were estimated by dividing the signal intensity of the major allele by the sum of both alleles. The results showed that this heterozygous SNP9093 can be transcribed at different ratios during different developmental ages in tissue-specific manner. First, we compared the ASE of SNP9093 between the 2 weeks and 6 weeks of age hypoxic challenged birds using two-tailed student' t-test (null hypothesis that allele fraction = 0.50) across three experimental replicates. We observed a statistically significant increase in the expression levels of the C non-reference allele vs the T reference allele at 6 weeks of age in all tissues in contrast to 2 weeks of age (Figure 2.2 & Table 2.10- 2.11). Based on the ontological shift in ASE for the heterozygotes, we used RT-qPCR to assess overall expression of the HTR2B gene for hypoxic challenged birds at 2 and 6 weeks of age. The relative expression at 6 weeks of age was determined using the TBP

gene as the reference (Radonic *et al*., 2004) and ΔΔCt computed using expression at 2 weeks of age as the calibrator. We found that HTR2B expression was almost always lower in tissues from birds of 6 weeks of age as compared with 2 weeks of age with the reduction being statistically significant in kidneys (Table 2.12). Thus, we conclude that in hypoxic birds the HTR2B gene expression is decreased from 2 to 6 weeks of age reflecting a decrease in the expression of the T reference- allele and increased expression of the C non-reference allele. In general, quantitative differences in expression patterns observed in HTR2B gene and ASE of heterozygous SNP9093 seems to play only a partial role in phenotypic variation between birds, such as susceptibility or resistance to ascites.

DISCUSSION

Genetic, epigenetic, and environmental factors determine phenotypic variation and disease risk (Cazaly *et al*., 2015). From several GWAS studies, it is becoming very clear that common traits and complex diseases are influenced by large numbers of alleles that individually have small effects (Manolio *et al*., 2009). It is also becoming apparent that most complex trait alleles can influence gene expression by modulating transcription level or transcript stability (Lo *et al*., 2003). Our previous GWAS study used a F2 cross of the resistant and susceptible lines, which were divergently selected from the predecessor of the REL line and identified a region on chromosome 9 that contains candidate gene, HTR2B for PHS incidence (Figure 2.1). (Krishnamoorthy *et al*., 2014). In the present study, we have investigated the association of two SNPs in HTR2B gene (promoter SNP586 and exon 3 SNP9093) with PHS phenotype. The allele frequencies and combinations present in our research lines differ from those in the commercial lines for both chosen SNPs. Both SNPs were conformed with HW equilibrium in all commercial lines but not

with the REL line. Failure to conform with HWE in the REL line for SNP586 in generation 14 and 18, and generation 14 for SNP9093, led us to question the reliability of our assays. However, sequence data from 10-20 PCR products representing different genotypes and randomly selected for each SNP confirmed the reliability of our assays. Therefore, the error rate of genotype mis-calls appears fairly low. In general, our genotype data for HTR2B gene appears to be only partially associated with PHS, but not a universal marker for genetic predisposition to the ascites syndrome. Bioinformatic analysis identified a cluster of SNPs within potential transcription factor (TF) binding sites in the promoter region of HTR2B gene that may affect regulatory mechanism(s) of its expression, and consequently affect the phenotype characteristics. Some of these identified transcription factors were 1) Nuclear factor of activated T-cells (NFAT5) is a transcription factor, activated under hypoxic conditions and could be a protective factor against ischemic damage (Villanueva *et al*., 2012; Dobierzewska *et al*., 2015). 2) Nuclear factor of activated T cells isoform c3 (NFATc3), which is a Ca^{2+}-dependent transcription factor plays an important role in the chronic hypoxia-induced vascular remodeling that underlie pulmonary hypertension (De Frutos *et al*., 2007; Bierer *et al*., 2011). 3) Nuclear Factor-κB (NF-κB) is a major transcription factor responding to cellular stress, and activated by hypoxia, or decreased oxygen availability in a cell (D'Ignazio & Rocha, 2016). 4) Myb avian myeloblastosis viral oncogene homolog-like 2 (MYBL2) is a transcription factor plays an essential role as a physiological regulator of cell cycle progression, survival and differentiation (Musa *et al*., 2017). Those TFs are important proteins as they have key roles in regulation of HTR2B gene expression in responses to hypoxia, including hypoxic pulmonary vascular remodeling and pulmonary hypertension.

In this study, we used 3,072 SNP panel for GWAS of F2 cross between SUS and RES lines for PHS association on Gga9, which represent only a few percent of the SNPs that are segregating

within the population (Figure 2.1). Subsequent GWAS on REL line samples have failed to show any association for ascites with HTR2B gene region on Gga9 but have identified regions on Chromosomes 2 and Z (Tarrant *et al*., 2016). Nevertheless, since the region on Gga9 showed association in an experimental cross, this region may still have limited utility for further investigations of ascites susceptibility. Furthermore, our recent high throughput whole genome resequencing study WGR was used to analyze Gga9 for ascites association (Dey *et al*., 2018). Unfortunately, we did not find any regions with clear difference in the SNP frequencies based on ascites phenotype, even for the HTR2B gene region that we had identified earlier in our GWAS (Figure 2.3). Despite this, using WGR approach, significant associations were detectable because causative variants are often in sufficient linkage disequilibrium (LD) with genotyped markers (not published). Although GWAS is a powerful tool to reconnect complex trait back to its underlying genetics, there are some important limitations that must be considered which may be drawbacks to a GWAS. Several important considerations including sample size, missing genotyping, and genetic heterogeneity to improve statistical powers and recover meaningful associations. The PHS phenotype possess a complex architecture where variants are either at low frequency or have a small effect size and even larger sample sizes may fail to discover them. Taking together, WGR approach seems to be more efficient method to identify genomic regions contributing to complex traits and diseases.

ASE (also known as allelic expression imbalance- AEI) refers to the differential abundance of the allelic copies of a transcript in heterozygous individuals (Buckland, 2004). Unequal output of allelic transcripts is a common phenomenon that may underlie disease risk influenced by disease-associated SNPs (Lo *et al*., 2003). AEI could be the outcome of the presence of at least one *cis*-regulatory element in the regulatory sequences of a gene (Campbell *et al*., 2008). This *cis*-acting

variant is likely not the transcribed SNP itself but might be in a transcription factor binding site or region that determines transcript stability. Therefore, AEI is one of the possible mechanisms underlying the effect of causative genetic variations that are not located on the translated region of a gene expression. The SNP586 is located in the HTR2B promoter region and thus cannot be used to assess differential expression of the transcribed alleles. To identify any possible SNPs in the transcribed region that can serve as a proxy for this polymorphism, we used the heterozygous T>C rs315854205 or SNP90093 located at the 3rd exon despite their imperfect linkage. We measured the allelic imbalance of this SNP in different tissues at different developmental age stages under hypoxic conditions. Although our SNPs analyses in the HTR2B gene did not establish a strong association to ascites phenotype, yet the SNP9093 can alter the mRNA structure, but not the amino acid codded for. In addition, the CT heterozygous SNP9093 was found to be partially associated with ascites resistance in one of the commercial lines, Line W (Table 2.5). Line W is the modern descendant from what was the original source from which the SUS, REL, and RES lines were originally derived (Pavlidis *et al*., 2007). Our results revealed that ASE was common and was not restricted to a specific tissue type or age. Estimations of ASE frequency vary from 5% to 80%, depending on tissue type, and developmental stage. Substantial bias in ASE ratio towards non-reference allele- C was observed in all tissues of hypoxic birds from 2 to 6 weeks of age. It was more likely as global phenomenon observed across tissues in 6 vs 2 weeks of age in hypoxic challenged birds. We subsequently looked at the overall expression of HTR2B gene in multiple tissues at 2 and 6 weeks old of hypoxic birds. Results revealed that HTR2B expression is decreased from 2 to 6 weeks of age of hypoxic birds in most tissues. Interestingly, the HTR2B overall mRNA expression is reduced corresponding to the preferential down-regulation of the T-allele from 2 to 6 weeks of hypoxic birds. It is likely that the T>C rs315854205 allele influences HTR2B

expression by reducing its transcriptional activity as the bird develops in age, yet the precise mechanism is not known. Serotonin 5-HT promotes pulmonary vasoconstriction, platelet aggregation, and pulmonary arterial smooth muscle cell proliferation, via serotonin receptors. Of the 14 different 5-HT receptors, only HTR1B, HTR2A, and HTR2B receptors show definite evidence in playing a role in the pathobiology of PAH (MacLean, 2007). Several studies have demonstrated that HTR2B receptor antagonists prevent the progression of PAH, vascular remodeling, and right ventricle hypertrophy in rat, mouse, and chicken models (Launay *et al*., 2002; Chapman & Wideman, 2002, 2006a, 2006b; Porvasnik *et al*., 2010; Dumitrascu *et al.,* 2011; Wideman & Hamal, 2011; Zopf *et al*., 2011). Our data demonstrate downregulation of HTR2B expression as the hypoxic birds develop in age which might prevent the increase in pulmonary arteries pressure and attenuate blood vessels vasoconstriction.

Recently, our group has analyzed gene expression using transcriptomics (RNA-seq) for right ventricles of normal and hypoxic birds after 2 weeks of exposure (T. Licknack, unpublished). Analysis of the RNA-seq data for HTR2B showed higher expression levels in hypoxic birds relative to the normal birds (Figure 2.4). Therefore, the RNA-seq data confirmed our qPCR assessment of changes in HTR2B gene expression pattern in broilers under hypoxic conditions. Interestingly, the transcriptome analyses also revealed significant transcription of the region upstream of the predicted exon 1 of the HTR2B gene (Figure 2.4). We confirmed transcription of the predicted upstream regions using different primers to the promoter region of HTR2B for RT-PCR and agarose gel electrophoresis. Our data do not reveal whether these "promoter transcripts" are contiguous with the HTR2B mRNA or represent separate non-coding RNAs. Considering that they are transcribed around potential transcription factor binding sites, it is possible that these RNAs play an essential role in gene regulation. Recently, it has been demonstrated in mouse and

yeast that ncRNA transcription around gene regulatory elements can enhance the binding of transcription factors (TFs) to their target sites (Sigova *et al*., 2015; Takemata *et al*., 2016). As the transcription of the upstream region relative to that for exon 1 differs between hypoxia-challenged and non-challenged right ventricles, the upstream transcription may regulate the local recruitment of TFs to their target sites in response to certain stimuli such as hypoxic conditions.

The present study has some limitations. First, the present approach does not allow us to exclude other gene polymorphisms for involvement in HTR2B expression that may be in LD with the promoter SNP586. However, when we sequenced the proximal promoter, exons, and intron flanking regions, we did not identify any other linked SNPs that affect coding sequences or putative transcription factor binding sites, which makes this scenario less likely. Second, the coding SNP9093 is not in complete LD with the promoter SNP586. Third, this study investigates the allele-specific effect on HTR2B mRNA expression levels and not protein. Finally, the limited number of replicates used in this study to measure total and ASE of HTR2B gene. However, this work is one of the first to have investigated the association of HTR2B gene polymorphisms with PHS phenotype in broilers.

In summary, ascites syndrome occurs due to the manifestation of multiple symptoms (Olkowski *et al*., 1999). Therefore, this is a complex disease whose incidence is attributed to many genetic factors. Although our study does not strongly support the association of the HTR2B polymorphisms with ascites phenotype, this gene remains as a candidate gene for future investigation of relevance for this disease and may have multiple epistatic interactions. Our findings provide important clues to understand the mechanisms that underlie the patterns of HTR2B expression and its potential impact on the phenotypic variation of PHS in broilers. Future

direction may continue searching for additional PHS common variants of small effect size but using much larger cohorts in the tens or hundreds of thousands.

REFERENCES

Bailes, S. M., Devers, J.J., Kirby, J.D., & Rhoads, D.D. (2007). An inexpensive, simple protocol for DNA isolation from blood for high-throughput genotyping by polymerase chain reaction or restriction endonuclease digestion. *Poultry Science*, 86(1):102-6.

Balog, J.M. (2003). Ascites syndrome (pulmonary hypertension syndrome) in broiler chickens: are we seeing the light at the end of the tunnel? *Avian & Poultry Biology Reviews*, 14(3), 99126.

Bierer, R., Nitta, C.H., Friedman, J., Codianni, S., de Frutos, S., Dominguez-Bautista, J.A., Howard, T.A., Resta, T.C., & Gonzalez Bosc, L.V. (2011). NFATc3 is required for chronic hypoxia-induced pulmonary hypertension in adult and neonatal mice. *American Journal of Physiology - Lung Cellular & Molecular Physiology*, 301(6), L872–L880.

Buckland, P.R. (2004). Allele-specific gene expression differences in humans. *Human Molecular Genetics*, *13 Spec No 2*, R255-60.

Campbell, C.D., Kirby, A., Nemesh, J., Daly, M.J., & Hirschhorn, J.N. (2008). A survey of allelic imbalance in F1 mice. *Genome Research*, *18*(4), 555–563.

Cazaly, E., Charlesworth, J., Dickinson, J.L., & Holloway, A. F. (2015). Genetic Determinants of Epigenetic Patterns: Providing Insight into Disease. *Molecular Medicine*, 21(1), 400–409.

Chapman, M.E., & Wideman, R.F. (2002). Hemodynamic responses of broiler pulmonary vasculature to intravenously infused serotonin. *Poultry Science*, *81*(2), 231–238.

Chapman, M.E., & Wideman, R.F.J. (2006a). Evaluation of the serotonin receptor blockers ketanserin and methiothepin on the pulmonary hypertensive responses of broilers to intravenously infused serotonin. *Poultry Science*, *85*(4), 777–786.

Chapman, M.E., & Wideman, R.F.J. (2006b). Evaluation of the serotonin receptor blocker methiothepin in broilers injected intravenously with lipopolysaccharide and microparticles. *Poultry Science*, *85*(12), 2222–2230.

Chapman, M.E., Taylor, R.L., & Wideman, R.F.J. (2008). Analysis of plasma serotonin levels and hemodynamic responses following chronic serotonin infusion in broilers challenged with bacterial lipopolysaccharide and microparticles. *Poultry Science*, *87*(1), 116–124.

Chomczynski, P., & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, *162*(1), 156–159.

Decuypere, E., Buyse, J., & Buys, N. (2000). Ascites in broiler chickens: exogenous and endogenous structural and functional causal factors. *World's Poultry Science Journal*, 56(04), 367-377.

De Frutos, S., Spangler, R., Alò, D., & González Bosc, L.V. (2007). NFATc3 mediates chronic hypoxia-induced pulmonary arterial remodeling with α-actin up-regulation. *Journal of Biological Chemistry*, 282(20), 15081–15089.

D'Ignazio, L., & Rocha, S. (2016). Hypoxia Induced NF-κB. *Cell*, *5*(1), 10.

Dey, S., Parveen, A., Tarrant, K.J., Licknack, T., Kong, B.C., Anthony, N.B., & Rhoads, D.D. (2018). Whole genome resequencing identifies the CPQ gene as a determinant of ascites syndrome in broilers. *PLoS ONE*, *13*(1), 1–17.

Dobierzewska, A., Palominos, M., Irarrazabal, C.E., Sanchez, M., Lozano, M., Perez-Sepulveda, A., Monteiro, L.J., Burmeister, Y., Figueroa-Diesel, H., Rice, G.E., & Illanes, S.E. (2015). NFAT5 Is Up-Regulated by Hypoxia: Possible Implications in Preeclampsia and Intrauterine Growth Restriction1. *Biology of Reproduction*, *93*(1), 1–11.

Dumitrascu, R., Kulcke, C., Königshoff, M., Kouri, F.M., Yang, X., Morrell, N.W., Ghofrani, H.-., Weissmann, N., Reiter, R., Seeger, W., Grimminger, F., Eickelberg, O., Schermuly, R.T., & Pullamsetti, S.S. (2011). Terguride ameliorates monocrotaline-induced pulmonary hypertension in rats. *The European Respiratory Journal, 37(5)*, 1104-18.

Esteve, J.M., Launay, J M., Kellermann, O., & Maroteaux, L. (2007). Functions of serotonin in hypoxic pulmonary vascular remodeling. *Cell Biochemistry & Biophysics, 47(1)*, 33–44.

Firth, A.L., Mandel, J., & Yuan, J. X.J. (2010). Idiopathic pulmonary arterial hypertension. *Disease Models & Mechanisms*, *3*(5-6), 268–273.

Cazaly, E., Charlesworth, J., Dickinson, J.L., & Holloway, A.F. (2015). Genetic Determinants of Epigenetic Patterns: Providing Insight into Disease. *Molecular Medicine*, 21(1), 400–409.

Gubler, U., & Hoffman, B. (1983). A simple and very efficient method for generating cDNA libraries. *Gene*, 25, 263-269.

Hamal, K.R., Wideman, R.F., Anthony, N.B., & Erf, G.F. (2010). Differential expression of vasoactive mediators in microparticle-challenged lungs of chickens that differ in susceptibility to pulmonary arterial hypertension. *American Journal of Physiology-Regulatory, Integrative & Comparative Physiology*, 298(1), R235–R242.

Julian, R. J. (1993). Ascites in poultry. *Avian Pathology*. 22:419–454.

Julian, R. J. (2000). Physiological, management and environmental triggers of the ascites syndrome: A review. *Avian Pathology*, 29(6), 519-527.

Julian, R.J. (2005). Production and growth-related disorders and other metabolic diseases of poultry*A review. *The Veterinary Journal*, 169, 350369

Julian, R.J., (2007). The Response of the Heart and Pulmonary Arteries to Hypoxia, Pressure, and Volume. A Short Review. *Poultry Science,* 86, 1006-1011.

Kéreveur, A., Callebert, J., Humbert, M., Herve, P., Simonneau, G., Launay, J., & Drouet, L. (2000). High Plasma Serotonin Levels in Primary Pulmonary Hypertension: Effect of Long-Term Epoprostenol (Prostacyclin) Therapy. *Arteriosclerosis, Thrombosis, & Vascular Biology,* 20, 2233–2239.

Krishnamoorthy, S., Smith, C.D., Al-Rubaye, A.A., Erf, G.F., Wideman, R.F., Anthony, N.B., & Rhoads, D.D. (2014). A quantitative trait locus for ascites on chromosome 9 in broiler chicken lines. *Poultry Science*, 93(2), 307-317.

Launay, J.M., Hervé, P., Peoc'h, K., Tournois, C., Callebert, J., Nebigil, C.G., Etienne, N., Drouet, L., Humbert, M., Simonneau, G., & Maroteaux, L. (2002). Function of the serotonin 5-hydroxytryptamine 2B receptor in pulmonary hypertension. *Nature Medicine*, *8*, 1129.

Livak, K.J. & Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT method. *Methods,* 25(4), 402-8.

Lo, H.S., Wang, Z., Hu, Y., Yang, H; Howard, H., Gere, S., Buetow, K.H., & Lee, M.P. (2003). Allelic variation in gene expression is common in the human genome. *Genome Research*, *13*(8), 1855.

MacLean, M.R. (2007). Pulmonary hypertension and the serotonin hypothesis: Where are we now? *International journal of clinical practice. Supplement* (Vol. 156).

MacLean, M.R., & Dempsie, Y. (2009). Serotonin and pulmonary hypertension-from bench to bedside? *Current Opinion in Pharmacology*, *9*(3), 281–286.

Manolio, T.A., Collins, F.S., Cox, N.J., Goldstein, D.B., Hindorff, L.A., Hunter, D.J., McCarthy, M.I., Ramos, E.M., Cardon, L.R., Chakravarti, A., Cho, J.H., Guttmacher, A.E., Kong, A., Kruglyak, L., Mardis, E., Rotimi, C.N., Slatkin, M., Valle, D., Whittemore, A.S., Boehnke, M., Clark, A.G., Eichler, E.E., Gibson, G., Haines, J.L., Mackay, T.F., McCarroll S.A., & Visscher, P.M. (2009). Finding the missing heritability of complex diseases. *Nature*, 461, 747.

Musa, J., Aynaud, M.M., Mirabeau, O., Delattre, O., & Grünewald, T. G. (2017). MYBL2 (B-Myb): a central regulator of cell proliferation, cell survival and differentiation involved in tumorigenesis. *Cell Death & Disease*, 8(6), e2895.

Olkowski, A.A., Korver, D., Rathgeber, B., & Classen, H.L. (1999). Cardiac index, oxygen delivery, and tissue oxygen extraction in slow and fast-growing chickens, and in chickens with heart failure and ascites: a comparative study. *Avian Pathology*, 28(2), 137-146

Pavlidis, H. O., Balog, J. M., Stamps, L. K., Hughes, J. D., Huff, W. E., & Anthony, N. B. (2007). Divergent selection for ascites incidence in chickens. *Poultry Science*, 86(12), 2517-2529.

Porvasnik, S.L., Germain, S., Embury, J., Gannon, K.S., Jacques, V., Murray, J., Byrne, B.J., Shacham, S., & Al-Mousily, F. (2010). PRX-08066, a Novel 5-Hydroxytryptamine Receptor 2B Antagonist, Reduces Monocrotaline-Induced Pulmonary Arterial Hypertension

and Right Ventricular Hypertrophy in Rats. *Journal of Pharmacology and Experimental Therapeutics*, 334(2), 364-372.

Radonic, A., Thulke S., Mackay, I.M., Landt, O., Siegert, W., & Nitschea A. (2004). Guideline to reference gene selection for quantitative real-time PCR. *Biochemical & Biophysical Research Communications*, 313:856-862.

Sigova, A.A., Abraham, B.J., Ji,X., Molinie, B., Hannett, N.M., Guo, Y.E., Jangi, M., Giallourakis, C.C., Sharp, P.A., & Young, R.A. (2015). Transcription factor trapping by RNA in gene regulatory elements. *Science (New York, N.Y.)*, *350*(6263), 978–981.

Taichman, D.B., & Mandel, J. (2013). Epidemiology of pulmonary arterial hypertension. *Clinics in Chest Medicine,* 34:619–637

Takemata, N., Oda, A., Yamada, T., Galipon, J., Miyoshi, T., Suzuki, Y., Sugano, S., Hoffman, C. S., Hirota, K., & Ohta, K. (2016). Local potentiation of stress-responsive genes by upstream noncoding transcription. *Nucleic Acids Research*, *44*(11), 5174–5189.

Tarrant, K. J., Dey, S., Kinney, R., Anthony, N.B., & Rhoads, D.D. (2016). Multi-generational genome wide association studies identify chromosomal regions associated with ascites phenotype. *Poultry Science*, 1544–1552.

Ullmer, C., Schmuck, K., Kalkman, H.O., & Lübbert, H. (1995). Expression of serotonin receptor mRNAs in blood vessels. *Federation of European Biochemical Societies (FEBS), 370*(3), 215–21.

Villanueva, S., Suazo, C., Santapau, D., Pérez, F., Quiroz, M., Carreño, J.E., Illanes, S., Lavandero, S., Michea, L., & Irarrazabal, C.E. (2012). NFAT5 is activated by hypoxia: Role in ischemia and reperfusion in the rat kidney. *PLoS ONE*, *7*(7), 1–10.

West, J. D., Carrier, E. J., Bloodworth, N.C., Schroer, A.K., Chen, P., Ryzhova, L.M., Gladson, S., Shay, S., Hutcheson, J.D., & Merryman, W. D. (2016). Serotonin 2B receptor antagonism prevents heritable pulmonary arterial hypertension. *PLoS ONE*, *11*(2), 1–18.

Wideman, R.F., Hamal, K.R., Bayona, M.T., Lorenzoni, A.G., Cross, D., Khajali, F., Rhoads, D.D., Erf, G.F., & Anthony, N.B. (2011). Plexiform Lesions in the Lungs of Domestic Fowl Selected for Susceptibility to Pulmonary Arterial Hypertension: Incidence and Histology. *Anatomical Record*, *294*(5), 739–755.

Wideman, R.F., & Hamal, K.R. (2011). Idiopathic Pulmonary Arterial Hypertension: An Avian Model for Plexogenic Arteriopathy and Serotonergic Vasoconstriction. *Journal of Pharmacological & Toxicological Methods*, *63*(3), 283–295.

Wideman, R.F., Rhoads, D.D., Erf, G.F., & Anthony, N.B. (2013). Pulmonary arterial hypertension (ascites syndrome) in broilers: a review. *Poultry Science*, 92(1), 64-83

Zopf, D.A., Neves, L.A.A., Nikula, K. J., Huang, J., Senese, P. B., & Gralinski, M.R. (2011). C-122, a novel antagonist of serotonin receptor 5-HT2B, prevents monocrotaline-induced pulmonary arterial hypertension in rats. *European Journal of Pharmacology*, *670*(1), 195–203.

TABLES AND FIGURES

Table 2. 1. Primers, Probes, and conditions for standard q-PCR. For each HTR2B SNP locus: position is the base-pair position on chromosome 9 according to the 2011 genome assembly; Primers are 5'-3' for forward (F-) and reverse (R-); Probes are 5'-3' with SNP586 allele A (tmA) labeled with FAM and allele C (tmC) labeled with HEX. SNP9093 allele C (tmC) labeled with FAM and allele T (tmT) labeled with HEX. The soak temperature (°C) used in the qPCR assay. Bold case letters indicate loci specific for SNP.

Assay	Locus	Position bp	Primers/Probes[1] (5'>3')	Soak
HTR2B promoter SNP586 genotyping	SNP586F	Chr9: 15214547-571	AGCTAGAGGGAAACAACTGGCATCC	64
	SNP586R	Chr9: 15214322-346	ATCCTGTGCCTTACTGGGTGTGATG	
	HTR2BtmA	Chr9: 15214422-446	AT**A**ATCTTCTGAGAGCTGAACCTCA	
	HTR2BtmC	Chr9: 15214421-446	AT**C**ATCTTCTGAGAGCTGAACCTCAC	
HTR2B Exon3 SNP9093 genotyping	SNP9093F	Chr9: 15205981-6005	GCCTATTTGATCAACAAGCCACCTC	68
	SNP9093R	Chr9: 15205731-755	GTTATGAAGAATGGGCACCACATCA	
	SERT9093tmC	Chr9: 15205917-936	**C**GCCACACCTGCCTGCTCACC	
	SERT9093tmT	Chr9: 15205917-936	**T**GCCACACCTGCCTGCTCACC	

[1] Nucleotides in bold italics in the probes are the SNPs being assayed

Table 2. 2. SNPs identified in the upstream promoter region of HTR2B in selected chicken DNAs. Sequences were aligned for DNAs from the RES and SUS lines (RES or SUS), White Leghorn (WL) and the published sequence for Jungle Fowl (JF). SNP positions are presented relative to the predicted transcription start site at Gga9:15214131 in the 2015 chicken assembly (GCA_000002315.3). Underscore indicates that the base is missing relative to the JF reference, and H indicates where an individual was heterozygous for the deleted base. Standard IUPAC base nomenclature is used for heterozygous SNPs

DNAs	**Promoter SNPs**						**Exon 3 SNP**
	496	**505**	**507**	**510**	**515**	**586**	**9093**
	15214534	15214526	15214524	15214521	15214516	15214444	15205937
JF	T	C	C	T	T	A	T
WL	Y	C	C	T	T	A	C
RES-1	C	C	C	T	T	M	C
RES-2	T	C	C	T	T	A	T
RES-3	T	C	C	T	T	A	C
RES-4	Y	H	H	H	Y	M	C
RES-5	Y	H	H	H	Y	M	Y
RES-6	Y	H	H	H	Y	M	Y
SUS-1	C	_	_	_	C	C	C
SUS-2	Y	H	H	H	Y	M	C
SUS-3	C	_	_	_	C	C	C
SUS-4	Y	H	H	H	Y	M	Y
SUS-5	Y	H	H	H	Y	M	Y
SUS-6	Y	H	H	H	Y	M	Y
SUS-7	Y	H	H	H	Y	M	Y
SUS-8	Y	H	H	H	Y	M	Y
SUS-9	C	_	_	_	C	C	C

.

Table 2. 3. Genotype data for HTR2B promoter SNP586 and Exon3 SNP9093 for our ascites research lines. The SUS line, RES line and generation 18 REL line genotype frequencies (freq) were determined for the entire line (All) or for the ascites resistant (R) or susceptible (S) subpopulations based on phenotype in a hypobaric challenge. The total number of genotypes (Count) is indicated below the frequencies. The REL samples were also analyzed according to gender. P-values for a simple Bonnferoni correction (see Materials and Methods) of chi square test for observed vs. expected (Adj Pval) are presented for genotypes with frequency ≥0.10.

	SUS	RES	REL				REL-Male			REL-Female		
Genotype	**All freq**	**All freq**	**All freq**	**R freq**	**S freq**	**Adj Pval**	**R freq**	**S freq**	**Adj Pval**	**R freq**	**S freq**	**Adj Pval**
SNP586												
AA	0.30	0.49	0.23	0.23	0.25	1.000	0.18	0.22	1.000	0.31	0.29	0.454
AC	0.46	0.38	0.28	0.27	0.28	1.000	0.31	0.32	1.000	0.21	0.23	0.915
CC	0.25	0.12	0.50	0.50	0.48	1.000	0.51	0.46	1.000	0.48	0.48	1.000
Count	96	95	225	135	69		83	37		52	31	
SNP9093												
CC	0.23	0.56	0.26	0.26	0.25	1.000	0.24	0.26	1.000	0.28	0.24	1.000
CT	0.47	0.32	0.53	0.50	0.56	1.000	0.55	0.54	1.000	0.43	0.58	0.915
TT	0.30	0.12	0.21	0.24	0.19	1.000	0.21	0.21	1.000	0.28	0.18	0.736
Count	77	96	234	139	37		86	39		53	33	

Table 2. 4. Genotype data for HTR2B prompter SNP586 for the commercial lines. Column and row designations areas for Tables 3.

Genotype	All freq	All R freq	All S freq	Adj Pval	Male-R freq	Male-S freq	Male Adj Pval	Female-R freq	Female-S freq	Female Adj Pval
Line W										
AA	0.70	0.70	0.71	1.000	0.64	0.69	1.000	0.77	0.71	1.000
AC	0.27	0.25	0.29	1.000	0.26	0.31	1.000	0.23	0.29	1.000
CC	0.03	0.05	0.00	0.081	0.09	0.00	0.004	0.00	0.00	0.320
Count	192	97	95		53	39		44	56	
Line Y										
AA	0.53	0.50	0.59	1.000	0.54	0.55	1.000	0.44	0.62	0.738
AC	0.37	0.37	0.36	1.000	0.39	0.39	1.000	0.24	0.32	1.000
CC	0.10	0.1	0.05	0.26	0.06	0.05	0.620	0.21	0.05	**0.025**
Count	186	111	75		59	38		52	37	
Line Z										
AA	0.33	0.35	0.29	1.000	0.35	0.39	1.000	0.33	0.20	0.556
AC	0.46	0.45	0.48	1.000	0.47	0.46	1.000	0.42	0.49	1.000
CC	0.21	0.20	0.23	1.000	0.18	0.14	0.939	0.24	0.31	0.516
Count	192	126	65		77	28		45	35	

Table 2. 5. Genotype data for HTR2B Exon 3 SNP9093 for the commercial lines. Column and row designations are as for Tables 3.

Genotype	All freq	All R freq	All S freq	Adj Pval	Male-R freq	Male-S freq	Male Adj Pval	Female-R freq	Female-S freq	Female Adj Pval
Line W										
CC	0.91	0.86	0.96	0.462	0.89	0.97	1.000	0.82	0.95	0.980
CT	0.09	0.13	0.04	**0.008**	0.11	0.03	0.440	0.16	0.05	0.143
TT	0.01	0.01	0.00	0.000	0.00	0.00	0.000	0.02	0.00	0.000
Count	192	97	95		47	38		36	53	
Line Y										
CC	0.12	0.15	0.08	1.000	0.18	0.08	0.396	0.09	0.10	1.000
CT	0.49	0.49	0.49	1.000	0.41	0.51	1.000	0.52	0.54	1.000
TT	0.39	0.36	0.43	1.000	0.40	0.40	1.000	0.38	0.35	1.000
Count	186	112	74		60	37		52	37	
Line Z										
CC	0.32	0.29	0.35	0.127	0.32	0.36	1.000	0.18	0.43	0.127
CT	0.49	0.51	0.48	1.000	0.45	0.46	1.000	0.56	0.51	1.000
TT	0.19	0.20	0.17	0.058	0.22	0.18	1.000	0.27	0.06	0.092
Count	192	126	65		77	28		45	35	

Table 2. 6. Haplotype data combining SNP586 and SNP9093 genotypes for generation 18 REL line. Haplotypes were imputed where possible, as described in materials and methods. All lines were genotyped using each assay where Genotype 1 is homozygous for allele 1, 2 is heterozygous, and 3 is homozygous for allele 2, for both SNPs. Column and row designations are as for Tables 3.

	All Freq	All-R Freq	All-S Freq	Adj Pval	Male-R Freq	Male-S Freq	Male Adj Pval	Female-R Freq	Female-S Freq	Female Adj Pval
REL Line										
11	0.01	0.01	0.01	5.421	0.00	0.03	1.327	0.02	0.00	3.018
12	0.09	0.09	0.10	7.138	0.07	0.05	2.792	0.12	0.16	1.597
13	0.12	0.13	0.13	6.760	0.11	0.14	6.151	0.16	0.13	4.028
21	0.03	0.01	0.04	2.024	0.01	0.08	0.430	0.02	0.00	2.478
22	0.18	0.18	0.20	6.202	0.23	0.24	1.746	0.10	0.16	1.332
23	0.07	0.08	0.04	2.800	0.07	0.03	3.046	0.10	0.06	3.512
31	0.21	0.23	0.20	5.726	0.23	0.16	4.117	0.24	0.26	4.827
32	0.25	0.24	0.25	6.314	0.24	0.24	7.023	0.24	0.23	6.092
33	0.03	0.03	0.01	4.580	0.04	0.03	5.421	0.02	0.00	3.017
Count	**224**	**134**	**69**		**83**	**37**		**51**	**31**	

Table 2. 7. Haplotype data combining SNP586 and SNP9093 genotypes for the commercial line W. Haplotypes were imputed where possible, as described in materials and methods. Column and row designations are as for Tables 3.

	All Freq	All-R Freq	All-S Freq	Adj Pval	Male-R Freq	Male-S Freq	Male Adj Pval	Female-R Freq	Female-S Freq	Female Adj Pval
Line W										
11	0.63	0.59	0.66	3.556	0.57	0.67	3.678	0.61	0.66	5.078
12	0.07	0.10	0.04	0.824	0.08	0.03	1.913	0.14	0.05	0.695
13	0.01	0.01	0.00	2.256	0.00	0.00	3.422	0.02	0.00	0.626
21	0.26	0.23	0.29	2.495	0.25	0.31	3.758	0.20	0.29	2.903
22	0.01	0.02	0.00	1.132	0.02	0.00	2.662	0.02	0.00	1.881
23	0.00	0.00	0.00	0.000	0.00	0.00	0.000	0.00	0.00	0.000
31	0.02	0.04	0.00	0.334	0.08	0.00	0.026	0.00	0.00	1.042
32	0.01	0.01	0.00	2.256	0.02	0.00	1.030	0.00	0.00	3.293
33	0.00	0.00	0.00	0.000	0.00	0.00	0.000	0.00	0.00	0.000
Count	**192**	**97**	**95**		**53**	**39**		**44**	**56**	

Table 2. 8. Haplotype data combining SNP586 and SNP9093 genotypes for the commercial line Z. Haplotypes were imputed where possible, as described in materials and methods. Column and row designations are as for Tables 3.

	All Freq	All-R Freq	All-S Freq	Adj Pval	Male-R Freq	Male-S Freq	Male Adj Pval	Female-R Freq	Female-S Freq	Female Adj Pval
Line Z										
11	0.08	0.08	0.09	6.917	0.08	0.11	5.768	0.07	0.06	4.572
12	0.11	0.13	0.09	4.519	0.10	0.11	6.874	0.18	0.09	1.593
13	0.06	0.08	0.02	0.720	0.06	0.04	5.221	0.11	0.00	0.347
21	0.13	0.12	0.15	4.746	0.17	0.18	2.153	0.02	0.14	0.392
22	0.23	0.23	0.25	7.439	0.19	0.18	3.118	0.29	0.29	2.935
23	0.09	0.10	0.08	5.168	0.10	0.11	6.391	0.11	0.06	3.797
31	0.10	0.08	0.15	1.176	0.08	0.07	3.347	0.09	0.23	0.192
32	0.15	0.13	0.15	6.452	0.16	0.18	5.499	0.09	0.14	2.850
33	0.04	0.05	0.02	2.420	0.05	0.04	4.287	0.04	0.00	2.200
Count	**192**	**126**	**65**		**77**	**28**		**45**	**35**	

Table 2. 9. Haplotype data combining SNP586 and SNP9093 genotypes for the commercial line Y. Haplotypes were imputed where possible, as described in materials and methods. Column and row designations are as for Tables 3.

	All Freq	All-R Freq	All-S Freq	Adj Pval	Male-R Freq	Male-S Freq	Male Adj Pval	Female-R Freq	Female-S Freq	Female Adj Pval
Line Y										
11	0.05	0.06	0.04	4.628	0.07	0.03	3.529	0.06	0.06	8.146
12	0.25	0.21	0.30	2.066	0.19	0.27	3.190	0.24	0.33	2.632
13	0.23	0.21	0.25	6.448	0.28	0.24	4.177	0.16	0.25	2.347
21	0.05	0.06	0.05	8.949	0.09	0.05	2.787	0.02	0.06	2.534
22	0.19	0.19	0.18	7.412	0.21	0.22	5.275	0.18	0.14	4.429
23	0.12	0.12	0.12	8.451	0.10	0.14	5.835	0.14	0.11	6.361
31	0.01	0.02	0.00	2.224	0.02	0.00	3.903	0.02	0.00	3.506
32	0.05	0.06	0.04	4.628	0.02	0.03	1.392	0.12	0.06	0.505
33	0.04	0.06	0.01	1.470	0.03	0.03	6.290	0.08	0.00	0.551
Count	**182**	**109**	**73**		**58**	**37**		**51**	**36**	

Table 2. 10. Percent C- allele relative Expression. Comparing ASE of SNP9093 (non -reference C- allele) across multiple tissues in hypoxic challenged birds of 2 weeks of age and 6 weeks of age (n=3 each) using two-tailed student t-Test, $P<0.05$. Data presented as Avg ± SEM (average ± standard error of the mean).

Tissue Type	2 Weeks of Age		6 Weeks of Age		P-value
	C allele	SEM	C allele	SEM	
Right Ventricle	0.46	0.012	0.58	0.003	**0.0058**
Aorta	0.45	0.005	0.64	0.01	**0.0008**
Left Lung	0.43	0.012	0.61	0.51	**0.0013**
Right Lung	0.43	0.009	0.62	0.31	**0.0214**
Left Kidney	0.54	0.002	0.74	0.06	**0.0856**
Right Kidney	0.53	0.016	0.64	0.02	**0.012**
Liver	0.49	0.008	0.59	0.003	**0.040**
Skeletal Muscle	0.55	0.013	0.63	0.03	**0.038**

Table 2. 11. Comparing ASE ratio of SNP9093 in tissues between 2 weeks and 6 weeks hypoxic challenged birds using two-tailed student t-Test. Data presented as Avg ± SEM (average ± standard error of the mean). Differences were considered significant at $P<0.05$

Tissue Type	2 Weeks Challenged Birds (n=3)				6 Weeks Challenged Birds (n=3)				P value
	Major allele	mean± SE	Minor Allele	mean± SE	Major allele	mean± SE	Minor Allele	mean± SE	
Right Ventricle	T	0.54 ± 0.011	C	0.46 ± 0.012	C	0.58 ± 0.003	T	0.42 ± 0.004	**0.0058**
Aorta	T	0.55 ± 0.006	C	0.45 ± 0.005	C	0.64 ± 0.01	T	0.36 ± 0.02	**0.0008**
Left Lung	T	0.57± 0.011	C	0.43 ± 0.012	C	0.61 ± 0.51	T	0.39 ± 0.06	**0.0013**
Right Lung	T	0.57 ± 0.001	C	0.43 ± 0.009	C	0.62 ± 0.31	T	0.38 ± 0.04	**0.0214**
Left Kidney	C	0.54± 0.002	T	0.46 ± 0.005	C	0.74 ± 0.06	T	0.26± 0.06	**0.0856**
Right Kidney	C	0.53 ± 0.016	T	0.47 ± 0.012	C	0.64 ± 0.02	T	0.36 ± 0.01	**0.0120**
Liver	T	0.51 ± 0.006	C	0.49 ± 0.008	C	0.59 ± 0.003	T	0.41 ± 0.006	**0.040**
Skeletal Muscle	C-T	0.50 ± 0.013	C-T	0.50 ± 0.014	C	0.63 ± 0.03	T	0.37 ± 0.02	**0.038**

Table 2. 12. Relative expression of HTR2B gene in different tissues of birds from REL line challenged in hypobaric chamber. ↑↓ indicates the direction of the difference for the 6 weeks old birds relative to the 2 weeks old birds. The results are shown as n-fold change (2^-ΔΔCt) of mRNA levels for the 6 weeks' birds relative to the calibrator, 2 weeks, from three birds each (n= 3) run in triplicate and presented as Avg ± SEM (average ± standard error of the mean). Statistically different results were determined using Student's *t* test for unpaired samples, P<0.05.

Tissue Type	Expression Variance ↑↓	Average ± SEM	P-value
Right Ventricle	↓	0.47 ± 1.69	0.26
Aorta	↑	1.83 ± 2.12	0.27
Skeletal Muscle	↓	0.03 ± 0.02	0.19
Liver	↓	0.58 ± 0.43	0.28
Left Kidney	↓	0.47 ± 0.81	**0.03**
Right Kidney	↓	0.16 ± 3.54	**0.03**
Left Lung	↓	0.26 ± 3.09	0.18
Right Lung	↓	0.42 ± 0.57	0.18

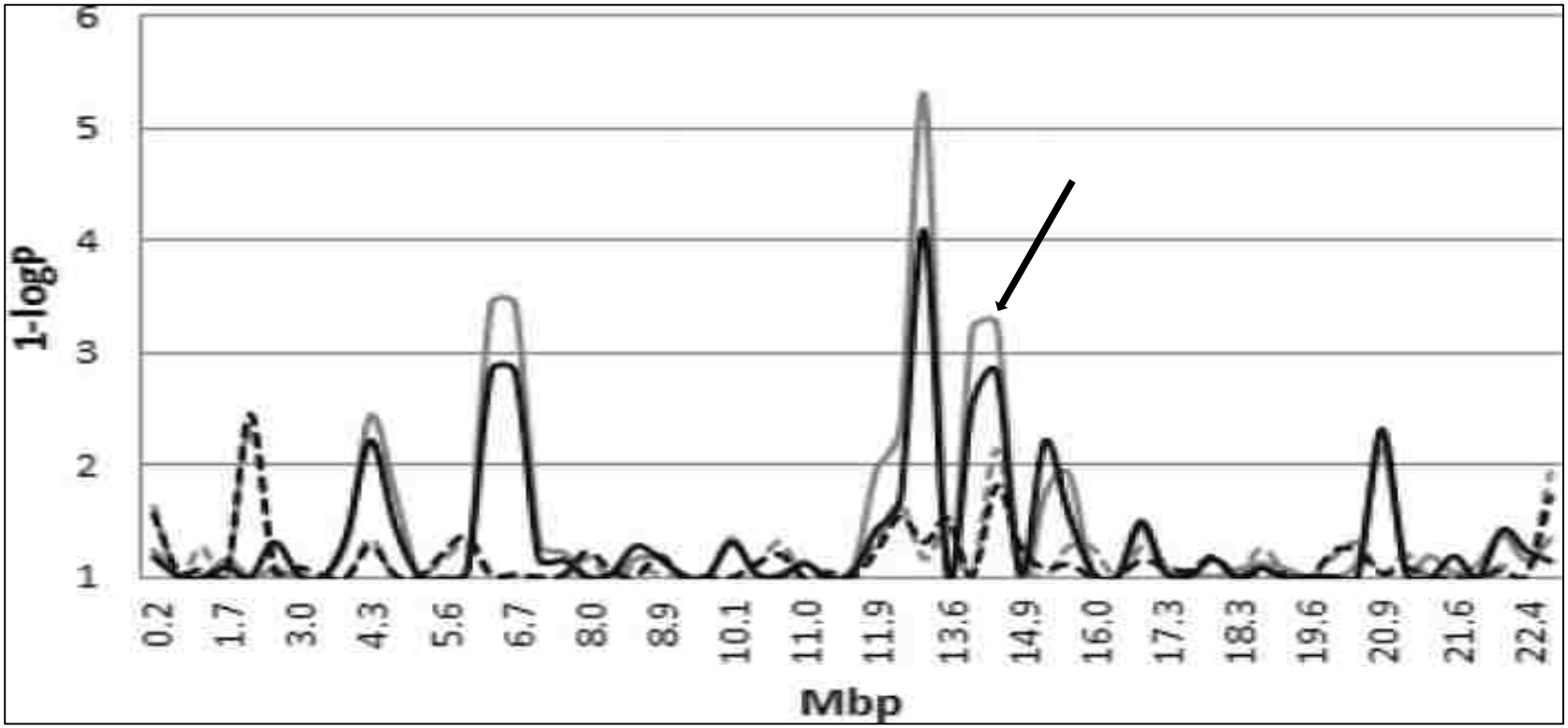


Figure 2. 1: Sex-specific SNP association analysis of chicken chromosome 9 for ascites and cardiac hypertrophy. Samples for an F_1 and F_2 cross of the ascites-resistant (RES) and ascites-susceptible (SUS) lines were genotyped using a genome-wide panel of 3,072 SNP. Chi-square *P*-values for observed vs. expected were plotted as 1 − logP for ascites phenotype (gray lines) or cardiac hypertrophy (black lines) for males (dashed lines) or females (solid lines). In this figure HTR2B is located within Gga9: 13.6-14.9 Mbp according to 2011 genome assembly as reported in our previous publication (Krishnamoorthy *et l.,* 2014). The black arrow indicates the HTR2B gene locus.

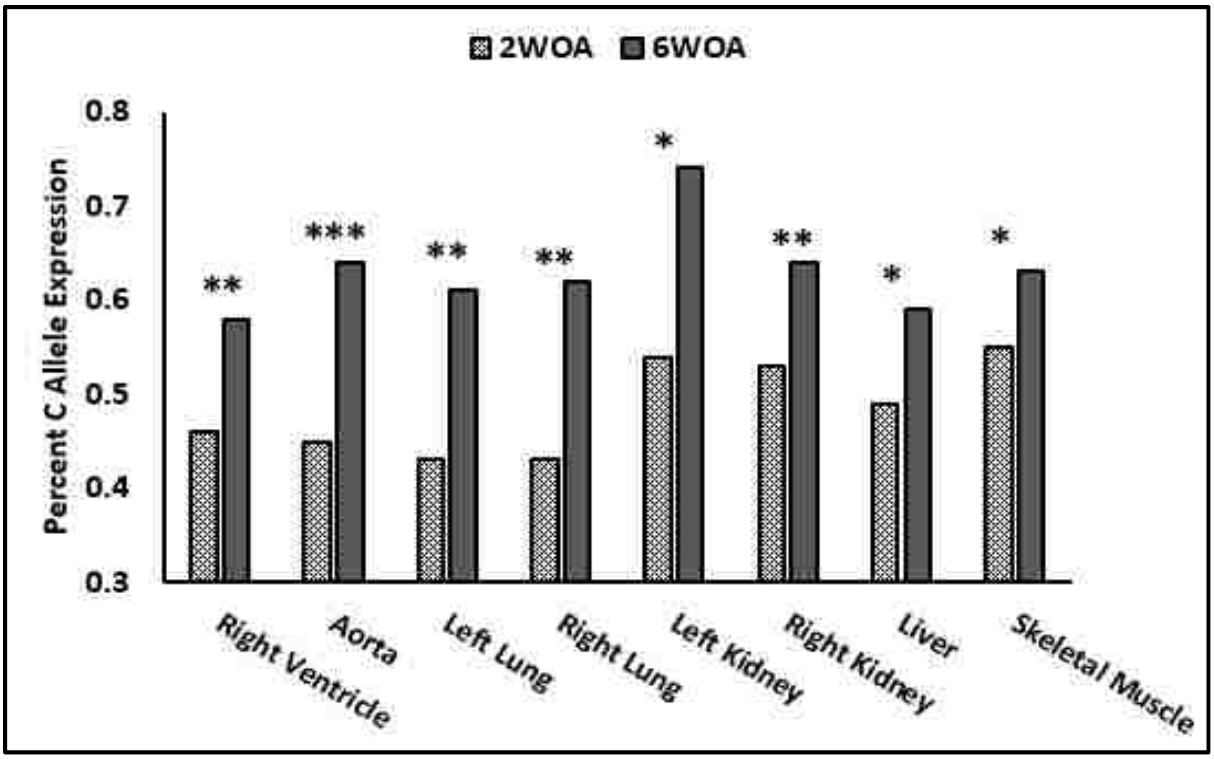


Figure 2. 2: Percent C- allele relative Expression. Comparing C non- reference allele percentage of SNP9093 across multiple tissues in hypoxic challenged birds of 2 weeks of age (2WOA, n=3) and 6 weeks of age (6WOA, n=3) using two-tailed student t-Test. Differences were considered significant at $P<0.05$.

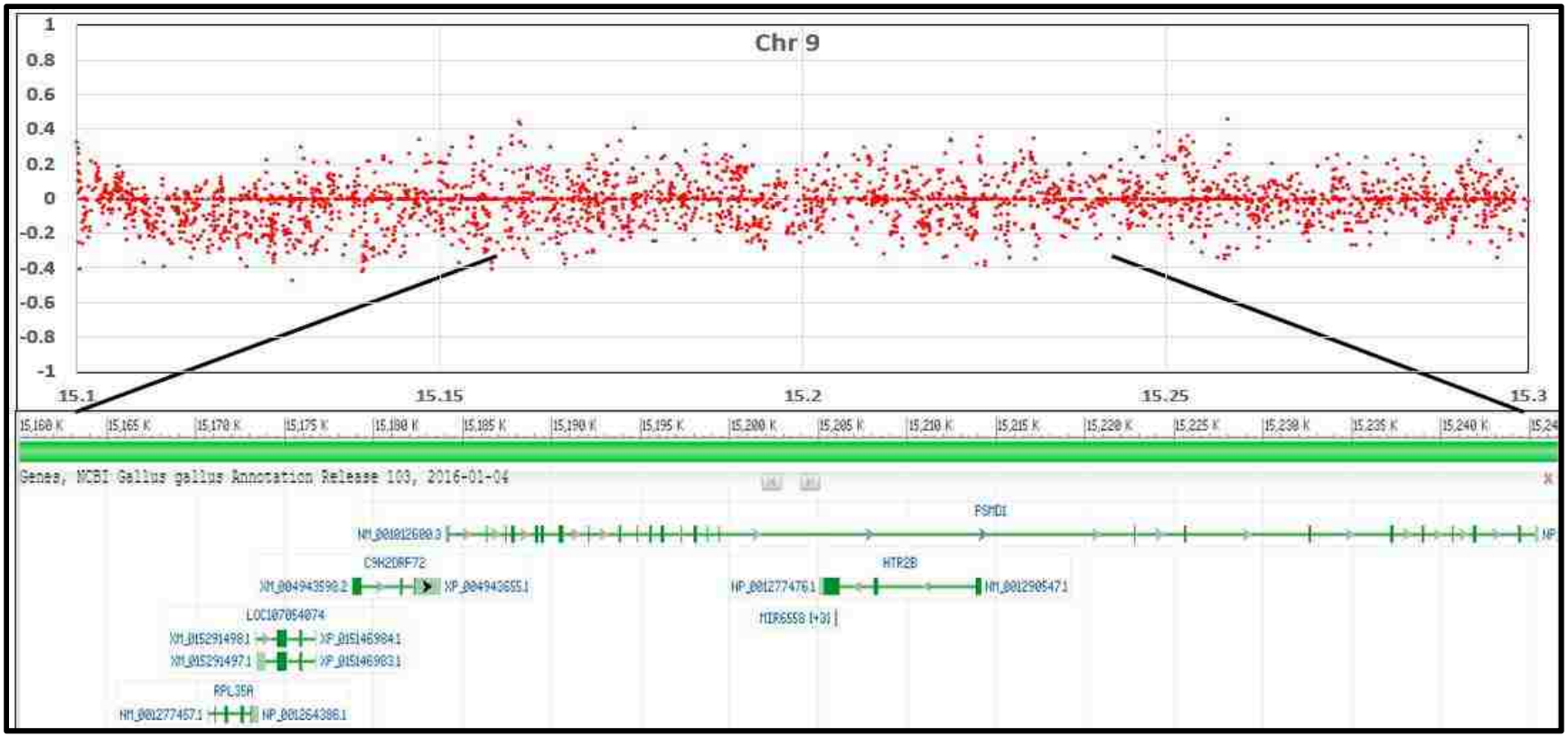


Figure 2. 3: Scatterplot representing GBS analysis of SNP frequencies on Chromosome 9 in REL line male birds. SNP frequencies has been plotted as a difference of the average non-reference SNP frequencies of the two replicates of resistant and susceptible sample reads along Y-axis. Therefore, each dot represents the variation in SNP frequencies among resistant and susceptible reads. Thus, a positive value for the SNPs at the loci along Y-axis indicates that those SNPs favor resistance, whereas a negative Y value indicates that the SNP loci favor susceptibility for ascites. X axis represents locus along chromosome 9. (A): Scatterplot representing Difference in SNP frequency (Y-axis) along chromosome 9 loci (in Mbp, X-axis) in males. the scatterplot showing SNP cluster along positive Y axis within the HTR2Bgene (black arrows) indicating the variant SNPs are equally distributed in both susceptible and resistant males. The black arrow indicates the HTR2B gene locus. (B): A zoom in version of chromosome 9 region (2015 Assembly) as graphically depicted by the NCBI presents the negative strand of HTR2B gene.

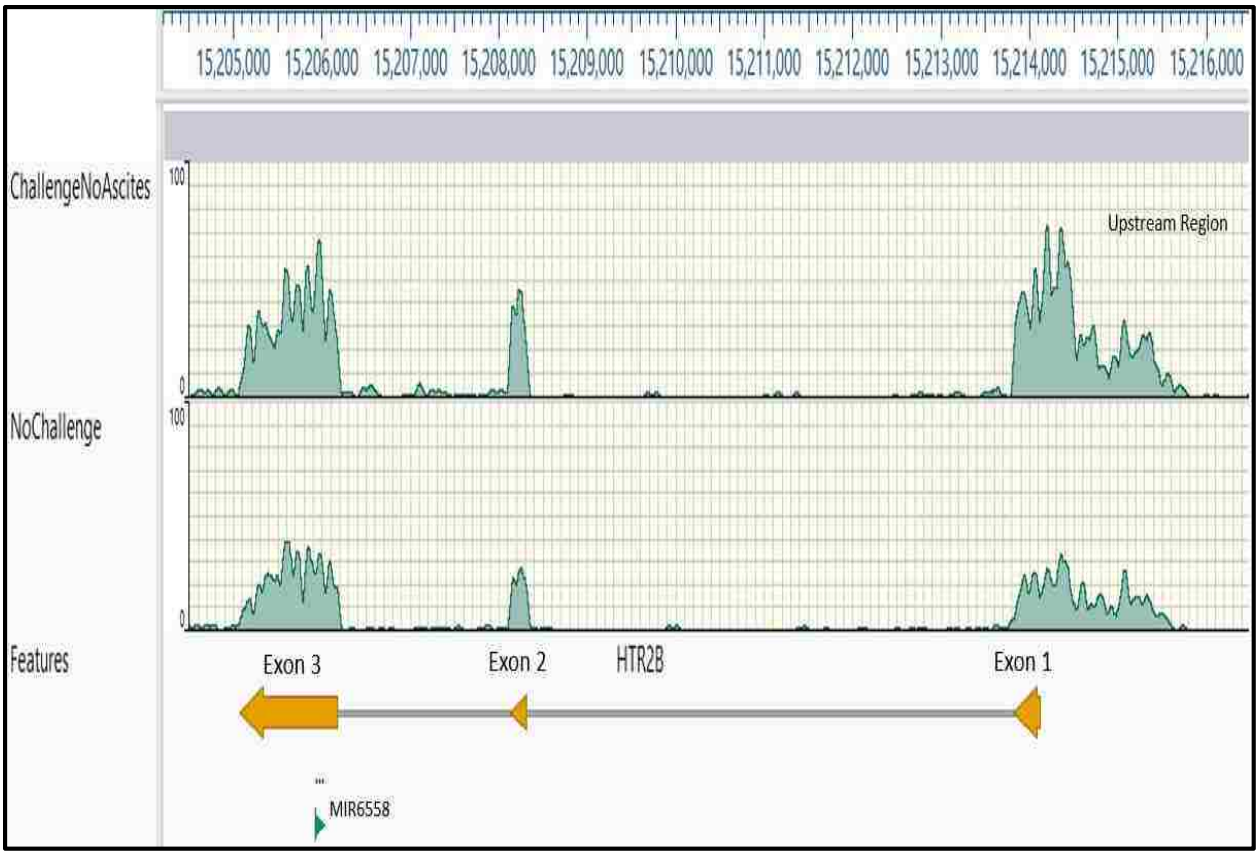


Figure 2. 4: HTR2B gene expression analysis in right ventricle. Results were obtained from RNA-seq data to compare HTR2B gene expression pattern in right ventricles from two groups of REL line birds: 1) hypobaric chamber (hypoxic) challenged with no ascites symptoms, and 2) no challenge (control). Data were generated by plotting the sums of mapped reads that overlap (cover) each nucleotide position along the genomic coordinates (x-axis), where the y-axis represents the number of reads. RNA-seq coverage and peak plots were compared between the two groups. The NCBI gene model for the HTR2B gene (3 exons and 2 introns) is shown below with exons (yellow arrows), and introns (gray lines). GeneVision Pro 14 tool (DNASTAR Lasergene Suite 14) was used for gene expression level visualization.

CHAPTER 3

Further Investigation of Mitochondrial Biogenesis and Gene Expression of Key Regulators in Ascites- Susceptible and Ascites-Resistant Broiler Research Lines

ABSTRACT

We have extended our previous survey of the association of mitochondrial prevalence in particular tissues with ascites susceptibility in broilers. We previously reported that in breast muscle of 22 week old susceptible line male birds had significantly higher mtDNA copy number relative to nuclear copy number (mtDNA/nucDNA), compared to resistant line male birds. The higher copy number correlated with higher expression of *PPARGC1A* mRNA gene. Ascites is a significant metabolic disease associated with fast-growing meat-type chickens (broilers) and is a terminal result of pulmonary hypertension syndrome. We now report the mtDNA/nucDNA ratio in lung, liver, heart, thigh, and breast of both genders at 3, and 20 weeks old. At 3 weeks the mtDNA/nucDNA ratio is significantly higher in lung, breast, and thigh for susceptible line males compared to the resistant line males. Conversely, we see the opposite for lung and breast in females. At 20 weeks of age the differences between males from the two lines is lost for lung, and thigh. Although there is a significant reduction in the mtDNA/nucDNA ratio of breast from 3 weeks to 20 weeks in the susceptible line males, the susceptible males remain higher than resistant line males for this specific tissue. We assessed relative expression of five genes known to regulate mitochondrial biogenesis for lung, thigh and breast muscle from males and females of both lines with no consistent pattern to explain the marked gender and line differences for these tissues. Our results indicate clear sex differences in mitochondrial biogenesis establishing a strong association between the mtDNA quantity in a tissue-specific manner and correlated with ascites-phenotype. We propose that mtDNA/nucDNA levels could serve as a potential predictive marker in breeding programs to reduce ascites.

INTRODUCTION

Ascites, Pulmonary hypertension syndrome PHS, or 'water belly" is a cardiovascular, metabolic disease affecting fast-growing broilers. Ascites is a complex problem resulting from many interacting factors such as genetics, environment and management, but also occurs in normal conditions as a response to high metabolic rate (Iqbal *et al*., 2001; Owen *et al*., 1990; Lubritz *et al*., 1995; Wideman & French, 2000; Balog *et al*., 2003; Hassanzadeh *et al.,* 2014). The high metabolic oxygen requirement of rapid growth, combined with insufficient capacity of the pulmonary capillaries appears to be the most important cause of ascites incidence in modern broilers (Peacock *et al*., 1990; Wideman, 2001). Inadequate oxygen levels trigger a series of events, including peripheral vasodilation, increased cardiac output, increased pulmonary arterial pressure, right ventricular hypertrophy (RVH; elevated right ventricular to total ventricular ratios-RV: TV), and ultimitly accumulation of fluid in the abdominal cavity and pericardium (Bottje *et al*,. 1995; Wideman, 2001; Balog, 2003; Pakdel *et al*., 2005; Wideman *et al*., 2013). Advances in management practices, rearing programs, and improved selection techniques have decreased ascites incidence in modern broilers. However, ascites syndrome remains an economic concern throughout the world, causing estimated losses of $100 million annually in the US (Odom, 1993; Rossi personal communication 2004, Cooper personal communication 2018). The etiology of ascites in poultry has been classified into three categories: 1) mainly pulmonary hypertension, 2) various cardiac pathologies, and 3) cellular damage caused by reactive oxygen species ROS (Currie, 1999). Mitochondria are the powerhouses of the eukaryotic cell and are the major contributor to oxidative stress through the generation of reactive oxygen species (ROS). Mitochondria are the primary oxygen consumer for energy production to sustain rapid growth in broilers (Cawthon *et al*., 2001; Chance *et al*., 1979). Mitochondria are known to be involved in the

regulation of several fundamental cellular processes, including metabolism, apoptosis, intracellular signaling, and energy production in the form of ATP via the oxidative phosphorylation. Mitochondrial biogenesis can be defined as the process of growth and division of pre-existing mitochondria to increase ATP production in response to growing demand for energy or stress conditions (Jornayvaz & Shulman, 2010; Dominy & Puigserver, 2013). During times of environmental stress (e.g., hypoxia, cold temperature, etc.), ROS levels can increase dramatically which may result in significant damage to cell structures notably the mitochondria (Dawson *et al*., 1993). Ascites can be induced at early ages by several methods such as altering the environment's temperature (Wideman *et al*., 1998; Sato *et al*., 2002), air quality (Chineme *et al.,* 1995), and altitude (Balog *et al*., 2000). Researchers at the University of Arkansas established divergently selected ascites experimental lines derived from a former full pedigreed elite line beginning in the 1990s through sibling-selection based on a hypobaric challenge (Pavlidis *et al*., 2007; Wideman *et al*., 2013). The lines are the ascites resistant (RES) line, ascites susceptible (SUS) line, and a relaxed (REL) unselected line.

Previously we reported that for a small sample set of breast muscle at 22 weeks of age for RES and SUS males, the samples from SUS males had approximately twice the ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nucDNA), and that this difference correlated with a difference in the level of expression of *PPARGC1A* (Emami *et al*., 2017). We have further investigated this apparent difference and extended our analysis to genders, multiple tissues, and additional developmental stages. We also assessed the relative expression of five genes known to regulate mitochondrial biogenesis only for those tissues that demonstrated significant sex-differences in mtDNA copy number. The results indicate a likely correlation between mtDNA/nucDNA ratios and ascites phenotype for particular tissues.

MATERIALS AND METHODS

Birds Stocks

All animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (under protocol 12039 and 15040). Birds used in this study represent the ascites-resistant (RES), the ascites-susceptible (SUS), and the relaxed unselected (REL) lines at generation 21 (Pavlidis *et al*., 2007).

Tissue Collection

Heart, lung, muscle iliotibialis (thigh), pectoralis major (breast), and liver, were collected from SUS and RES experimental lines. At three weeks of age, five male and female birds from each experimental line were randomly selected, euthanized by cervical dislocation, and samples were collected and immediately stored in RNAlater (Sigma Aldrich, St. Louis, MO). At 20 weeks of age we collected lung, thigh, and breast from five males of SUS and RES lines. For the REL line we collected breast tissue from 12 males at 3 and 20 weeks of age.

DNA Isolation

Tissue samples were homogenized in 1 ml lysis buffer (10 mM TrisCl, 1 mM Na_2EDTA pH 7.5) using a Bullet Blender homogenizer (Next Advance, Inc., Averill Park, NY) and overnight digested with 100 µg/ml pronase at 37°C. SDS was added to, then successively extracted by phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), followed by ethanol precipitation of DNA. DNAs were dissolved in 10 mM TrisCl 0.1 mM EDTA pH 7.5. DNA quantity was assessed by fluorimetry with Hoechst 33258 (GLOMAX Multi Jr, Promega

Corp., Madison, WI) and purity (A260/280) by spectrophotometry (NanoVue, GE Healthcare Bio-Sciences, MA, USA).

RT-qPCR for Mitochondrial Biogenesis

Mitochondrial DNA content was measured by quantitative, real time PCR (qPCR) in 96 well format using a CFX96 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, California, USA). The mitochondrial target was the gene for mt-tRNA^{ARG}, with the nuclear target a region of 5-Hydroxytryptamine receptor 2B (HTR2B). Specific primers (Table 1) were designed using Primer3 software (version 0.4.0; http://bioinfo.ut.ee/primer3-0.4.0/primer3/) and synthesized by Integrated DNA Technologies (Coralville, IA USA). Reactions (20 µl) were run in triplicate and consisted of 1X Taq Buffer (50 mM Tris-Cl, pH 8.3, 1 mM $MgCl_2$, 30 µg/mL BSA), 1X EvaGreen dye (Biotium Inc., Hayward, California, USA), 0.25 mM $MgCl_2$, 0.2 mM dNTP, 0.5µM each of the specific forward and reverse primers, 4 U of Taq polymerase, 2 µL of DNA (50-100ng). The cycling protocol was an intial soak at 90°C for 30 s, followed by 40-cycles of 30 s at 95 °C, 15 s at 60°C and 30 s at 72 °C followed by a plate read. Ct values from the exponential phase of the PCR were exported directly into Microsoft EXCEL worksheets for analysis. The ΔCt of mtDNA relative to the nucDNA reference SUS samples were converted to ΔΔCt values calibrated based on the ΔCt of RES samples (Livak & Schmittgen, 2001). The fold changes relative to the calibrator (RES line) was estimated as $2^{(-\Delta\Delta Ct)}$.

RNA Isolation and gene expression analyses

Total RNA was isolated from lung, thigh, and breast tissues using TRIZOL reagent (Ambion, Thermo Fisher Scientific) according to the manufacturer's instruction. The extracted RNA was assessed for quantity and purity (A260/280) using NanoVue spectrophotometry (GE Healthcare

Bio-Sciences, MA, USA). RNA integrity was evaluated by electrophoresis in 1.5% agarose gel in 0.5×TBE buffer (50 mM Tris, 1 mM Na_2EDTA, and 25 mM Borate, pH 8.3), stained by 0.5 µg/ml ethidium bromide. Samples that did not show 3 strong and distinct bands (28S, 18S, and 5S rRNA) were discarded. Gene expression for *PPARGC1A, AMPK, OPA1, SIRT1*, and *DNM1L* was performed using a two-step RT-qPCR method. RNA (up to 5µg) was combined with 2 µM $CT_{23}V$, and 0.5 mM dNTP and denatured at 70°C for 5 mins, then added to a mastermix consisting of 1X First Strand buffer (Invitrogen), 5 mM $MgCl_2$, 1 mM DTT, 20 U RNasin (Promega Corp, Madison, WI USA), and 200 U MMLV reverse transcriptase (Promega Corp) in a final volume of 20 µl. The reaction was incubated at 42°C for 60 minutes and then inactivated at 85°C for 5 minutes. Chicken TATA-binding protein (*TBP*) was used as the reference gene (Radonic *et al*., 2004). Primers (Table 1) for each gene were designed to span an intron using Primer3 software and synthesized by Integrated DNA Technologies. Second step qPCR were performed in a 20µl volume were as above for qPCR except as target 2 µL of cDNA (50-100ng). The PCR cycling was initial denaturation at 90°C for 3 mins, 10 cycles of 90°C for 15s, 60 °C for 15s, 72 °C for 1 min, followed by another 30 cycles of 90°C for 15s, 60 °C for 15s, melt curve 70°C to 90°C, finally 72 °C for 1 min with plate read. Ct values were analyzed as described above. Relative gene expression was calculated using the $2^{(-\Delta\Delta Ct)}$ method (Livak & Schmittgen, 2001) with both biological and technical replicates, and normalized to TBP as the reference gene.

Statistical Analysis

Data are presented as means ± SEM. All statistical computations were performed using EXCEL, and significant difference between lines and gender means were assessed by the Student's t-test. Probability level of $P \leq 0.05$ was considered statistically significant.

RESULTS

Previously we evaluated the mitochondrial biogenesis and *PPARGC1A* mRNA gene expression in male broiler chickens at 22 weeks of age (Emami *et al*., 2017). The analyses compared two experimental lines produced through divergent selection for ascites phenotype; the ascites-susceptible (SUS) and ascites resistant (RES) broiler lines. The comparison was based on five males from each line and the evaluation was for right ventricle and breast. Results showed that birds from SUS had significantly higher mtDNA copy number (P = 0.038) and *PPARGC1A* RNA gene (P = 0.033) in breast muscle; with no difference in right ventricle. Thus, we suggested that mitochondrial biogenesis and *PPARGC1A* mRNA gene expression differ between male boilers from RES and SUS lines in a tissue-specific manner. The present report extends our previous analyses to additional muscles and other critical tissues at additional ages and for both genders.

From each line, five birds of both sexes were sampled for right ventricle, breast, thigh, lung, and liver at 3 weeks of age. The mtDNA/nucDNA ratio was estimated by qPCR of mt-tRNA^{ARG} (mtDNA) and a single copy region of HTR2B (nucDNA). A higher mtDNA/nucDNA ratio was observed in lung (Figure 1.C), thigh (Figure 1.D), and breast (Figure 1.E) tissues of SUS line relative to the RES line in males. The breast tissue of SUS line males contained 4 times higher levels (P=0.048) of mtDNA copy number. The lung of SUS line males was 64 times higher (P=0.01) and the thigh was 16 times higher (P= 0.03). No differences were detected in mtDNA/nucDNA ratio between the males from the two lines for right ventricle (Figure 1.A), and liver (Figure 1.B). Although the right ventricle of SUS line males was higher than RES line males, the difference was not statistically significant. Inspection of the mtDNA/nucDNA ratios across tissues for males from each line revealed that the RES line males were comparable (around 1000) for right ventricle, thigh, liver and breast, but only around 100 for lung. The SUS males were

much more variable ranging from 100,000 for thigh and lung, to 5,000 to 10,000 for liver, right ventricle, and breast.

In contrast to the males, the mtDNA/nucDNA ratio at 3 weeks of age for SUS line females were lower than RES line females for lung (Figure 1.C) and breast (Figure 1. E). The breast ratio for SUS line females was half that of the RES females (P=0.03), while for lung the SUS line was 0.008x the value for the RES females (P= 0.004). No differences in mtDNA/nucDNA ratio were observed between the females for the two lines for liver (Figure 1.B), right ventricle (Figure 1.A), and thigh (Figure 1.D). Although the liver, and right ventricle of SUS line females was lower than RES line females, the difference was not statistically significant, and the RES line female values for liver were more variable. Examination of the mtDNA/nucDNA ratios across tissues for females for both lines revealed that the SUS line females were comparable (around 1000) for right ventricle, thigh, lung, and breast, and around 10,000 for liver. Unlike for males the RES female samples showed the greatest tissue variation. RES females' ratios ranged from 100,000 for liver and lung, to 10,000 for right ventricle, and 1000 for thigh and breast.

Comparison of mtDNA copy number between genders within each line at 3 weeks of age shows significant differences for some tissues. Females from the RES line had higher mtDNA copy number than males from the RES line for lung (P=0.001) and breast (P=0.006). In contrast, males from the SUS line had a relatively higher mtDNA copy number than SUS line females for lung (P= 0.05) and thigh (P=0.03). In this study, lung tissue demonstrated the most significant mtDNA/nucDNA ratio differences in respect to both gender and line.

Since only lung, thigh and breast showed differences at 3 weeks of age, we examined mtDNA/nucDNA ratios for those same tissues at 20 weeks of age. We restricted our investigation

to males since ascites mortality is consistently higher for males than for females in our research lines. This is also consistent with reports from other researchers on commercial broilers (Decuypere *et al*., 2000; Moghadam *et al*., 2001; Baghbanzadeh *et al*., 2008; Movassagh, *et al.,* 2008). Five males of both lines from the same generation were assessed for ontological changes in mtDNA copy number. As shown in Figure 3.1, we observed a decrease in mtDNA/nucDNA ratio in 20-week SUS line males compared to 3 week SUS line males for lung (P= 0.019) and thigh (P= 0.045). The reduction at 20 weeks of age results in the SUS and RES line males have comparable levels of mtDNA for lung and thigh. However, as for 3 weeks of age we continue to see a higher ratio of mtDNA/nucDNA in the breast muscle for the SUS line males compared to the RES line males. The difference between the lines for the breast decreases from 6-fold at 3 weeks of age to approximately 2-fold at 20 weeks of age but remains statistically different between the lines (P=0.02). Furthermore, consistent with our finding at a younger age, the mtDNA/nucDNA ratio in breast muscle of 20 weeks old of SUS line males was 2 times higher (P= 0.02) compared to RES line. No differences between young and old birds in mtDNA/nucDNA ratio were detected for breast tissues of SUS line males (P=0.3) indicating the consistent elevation of mtDNA/nucDNA ratio. However, we observed an increase in mtDNA/nucDNA ratio in breast tissues (P=0.03) between young and old birds of the of RES line. Thus at 20 weeks of age we see the SUS line male mtDNA copy number reduced to comparable levels as the RES line males for lung and thigh but the breast mtDNA copy number remains elevated in the SUS line compared to the RES line.

Since the REL line represents the founder population for our SUS and RES experimental lines, we decided to examine the mtDNA/nucDNA ratio in breast muscle of male birds from the REL line at 3 and 20 weeks of age. As shown in Figure 3.2, we observed an increase in mtDNA/nucDNA

ratio in 20-week REL line males compared to 3-week REL line males (P=0.03). In this study, both REL and RES line male birds had the same mtDNA/nucDNA ratio at both 3 and 20 weeks of age. Unlike the SUS male birds that have always higher mtDNA/nucDNA ratio at both ages than the RES and REL males (Figure 3.2).

A number of genes have been associated with regulation of mitochondrial biogenesis. We selected five of these genes to examine their expression levels using RT-qPCR for the tissues showing the greatest differences for gender or line. The five genes were: AMP-activated protein kinase α1 (*AMPKα1),* peroxisome proliferator-activated receptor gamma co-activator 1 alpha (*PPARGC1A),* Sirtuin 1 (*SIRT1),* optic atrophy 1 (*OPA1), and* Dynamin-1 like (*DNM1L).* The expression of these genes were assessed in lung, thigh, and breast of both lines and genders at 3 weeks of age, and breast and lung for males at 20 weeks of age. In all cases the relative expression was determined and calibrated against the expression in the RES line.

In males at 3 weeks of age, expression of all five genes were reduced in all three tissues (Table 3.2), with the reduction being statistically significant for *AMPKα1, OPA1, and DNM1L* in lung and breast ranging to half the expression in SUS relative to RES. There were no differences in the level of expression *PPARGC1A and SIRT1* genes in lung and breast between the two lines at this age. In thigh, there were no differences in expression levels for any of these five genes. Interestingly, an increase in the level of expression of *PPARGC1A, SIRT1,* and *OPA1* genes in breast of SUS males at 20 weeks of age relative to RES males was observed. No differences in the expression of *DNM1L* gene in breast while the expression of *AMPKα1* gene remained low. In the lungs of SUS males at 20 weeks of age, a reduction in the expression of all genes was observed

relative to RES males (Table 3.3), with the reduction being significant for *AMPKα1* (P=0.016), and *OPA1* (P= 0.0009). No significant differences in *PPARGC1A* expression was observed.

In females at 3 weeks of age, the expression of *AMPKα1* gene in lung was reduced (0.5x; P=0.05) in SUS line compared to RES line, while *SIRT1* mRNA expression increased by approximately 29% (Table 3.4). For thigh, only *OPA1* and *DNM1L* were reduced to 80% and 60%, respectively, in SUS vs RES females. In breast, none of the five genes were found to differ between the lines. No gene expression analyses were performed for females at 20 weeks of age.

None of these key mitobiogenesis regulators appeared to correlate with the differences in mtDNA/nucDNA ratios we observed for both genders between the two lines. However, in males, we observed a reduction in the mtDNA copy number for SUS males from 3 weeks of age to 20 weeks of age in lung, thigh, and breast, although the levels remained relatively higher in breast of the SUS males than the RES males. Consistent with that difference at 3 weeks of age we saw a decreased expression for *OPA1, and DNM1L* genes in lung and breast SUS males relative to RES males whereas at 20 weeks of age we observed a higher expression for *PPARGC1A, SIRT1,* and *OPA1* in only breast of SUS males relative to RES males. Additionally, *AMPKα1* gene was always expressed at lower levels in the breast and lung tissues of SUS males compared to RES males at both ages. In general, we found no consistent gene expression pattern to explain the marked gender and line differences in mtDNA copy number for these tissues.

DISCUSSION

Mitochondrial dysfunction is well documented in a wide array of diseases and conditions, such as Alzheimer's disease, cancer, and aging (Brown *et al*.,2001, Parr *et al*.,2006, Barazzoni *et al*, 2000). Mitochondria are central to ATP synthesis, heat production, radical oxygen species (ROS)

generation, fatty acid and steroid metabolism, cell proliferation, and apoptosis (Jornayvaz & Shulman, 2010; Ploumi *et al*., 2017). Alterations in mtDNA sequence or copy number may contribute to mitochondrial dysfunction (Malik & Czajka, 2012). Thus, it is likely that imbalances within the cell concerning mitochondria-centered metabolic pathways may contribute to ascites syndrome. Our observations indicate that variations in mtDNA copy number could be an important component in the pathoetiology of ascites syndrome in broilers. Using different tissues, we have demonstrated that mtDNA copy number can be an important biomarker during early developmental age for ascites syndrome susceptibility. Our results showed sizable tissue-specific, and gender differences in the mtDNA/nucDNA ratio at early ages of broilers. The possible existence of gender-specific differences in energy metabolism for particular tissues might be a consequence of interplay between maternally inherited mitochondria and sex chromosomes or differences in endocrine responses. In males, mtDNA/nucDNA ratio was significantly higher in lung, thigh, and breast tissues from SUS line males at 3 weeks of age in comparison with RES line males. Conversely, mtDNA levels were significantly lower in lung and breast tissues of SUS line females as compared to RES line females. The gender differences may impact ascites phenotype considering that males are documented to have higher ascites mortality than females (Decuypere *et al*., 2000; Moghadam *et al*., 2001; Baghbanzadeh *et al*., 2008; Movassagh, *et al.,* 2008). The observed elevation in the amount of mtDNA in lung, thigh and breast muscle of SUS line males might be attributable to a compensatory response to the decline in the respiratory function of mitochondria or a response to other metabolic regulatory processes. An alternate explanation is needed for the reduced mtDNA copy number in lung and breast muscle of SUS line females. One possible explanation is that differences in mtDNA content of different sexes can be attributed to imbalances in oxidative stress due to higher female estrogen levels. Previous work found that

oxidative damage to mtDNA is 4-fold higher in males than in females (Borras *et al*., 2003; 2010). The lower oxidative damage in females may be attributable to the protective effect of estrogens by upregulating the expression of antioxidant enzymes in mitochondria via intracellular signaling pathways, thus decreasing oxidative damage and increasing antioxidants defenses (Borras *et al*., 2003; 2010). Moreover, fundamental sex differences in metabolism under stressful conditions have long been observed in several organisms and may also be influenced by intrinsic differences in genomic maintenance (Demarest & McCarthy, 2015). Absent from our analysis is any determination of whether the differences in mtDNA content is associated with functional or non-functional (defective) mitochondria. Future work could involve fluorescent detection systems for visualizing mitochondria in SUS vs RES tissues to assess relative mitochondrial abundance and functional state.

Bottje and Wideman hypothesized that mitochondrial dysfunction contributes to systemic hypoxia that leads to ascites in broilers (Bottje & Wideman, 1995). They reported that mitochondrial function is defective in a variety of tissues (lung, liver, heart, and skeletal muscles) in male broilers with ascites where oxygen utilization is less efficient than in male broilers without ascites (Cawthon *et al*., 1999, 2001; Iqbal, *et al*., 2001a; Tang *et al*., 2002). They assessed the mitochondria function for both the respiratory control ratio (RCR); for electron transport chain coupling, and for the adenosine diphosphate to oxygen ratio (ADP:O); for oxidative phosphorylation. A decline in RCR and ADP:O ratio was detected in ascites mitochondria relative to the non-ascites control. This may indicate functional impairment of mitochondrial oxidative phosphorylation and less efficient utilization of oxygen than in control. On the other hand, more efficient oxidative phosphorylation and lower oxidative stress were observed in mitochondria obtained from broilers selected for genetic resistance to ascites. Accumulation of hydrogen

peroxide was observed in heart and skeletal mitochondria in broilers with ascites and of oxygen radical production in ascites liver and lung mitochondria. Therefore, there is no doubt that mitochondrial function is defective in broilers with ascites which leads to increased production of ROS. It is possible that the observed significantly lower mitochondrial biogenesis in male RES and REL lines is indicative of lower oxygen demand or ROS production. However, it is yet not clear if increased levels of ROS are a secondary effect of development of ascites or are associated with genetic susceptibility. Cisar et al. (2004) used immunoblots to quantify cardiac mitochondrial electron transport chain (ETC) protein levels in the RES and SUS lines under hypoxic challenge. ETC protein levels were similar in RES and SUS at ambient oxygen pressure but were significantly elevated only in RES under hypoxic conditions. Our data is for ambient oxygen levels only and based on mitochondrial DNA and not mitochondrial proteins sugessting the possible involvment of mitochondrial proteins with ascites phenotype.

Imbalance in mitochondrial biogenesis may only affect broilers at a young age when ascites is most likely to develop. Contrary to 3 weeks of age, at 20 weeks of age males from the RES and SUS lines showed no differences in mtDNA copy number for lung and thigh. One additional potentially confounding aspect is that the 20 week samples were from birds that had been feed restricted since 5 week post hatch. Despite this, the difference in breast mtDNA copy number was still higher for SUS males compared to RES males. Future investigations should examine females for a similar ontological shift in mitochondrial biogenesis, as well as assess the impact of feed restriction. Apparently, the consistent increase in mtDNA/nucDNA ratio between young and old birds of the two lines is restricted to breast muscle which may reflect increased energy demands or a compensatory amplification to overcome the loss of mitochondrial function or oxidative stress.

Examination of mtDNA/nucDNA ratio in breast muscle from the REL line of male birds at 3 and 20 weeks of age indicate a similar pattern as the RES line. This was surprising since our research lines, SUS and RES, were originally developed from the REL line. We expected to see a wider range of mtDNA abundance in the REL line reflecting a composite of the SUS and RES patterns. This may be the result of imbalance between the rate of biogenesis and clearance of dysfunctional or old mitochondria in SUS vs REL and RES males. Alternatively, this may be due to imbalance in mitochondrial-nuclear crosstalk is SUS vs REL and RES males. Our study strongly supports a potential decrease in the mitochondrial function with oxidative stress, yet overall mtDNA quantity increases by a feedback mechanism to compensate for general mitochondrial dysfunction and damage in ascites-SUS male birds. However, the detailed mechanism remains unclear.

We analyzed gene expression of some of the key regulators of the mitochondrial biogenesis in ascites- susceptible and ascites- resistance lines of both genders. *PPARGC1A* is the master regulator of mitochondrial biogenesis. This transcriptional coactivator coordinates the actions of several transcription factors that involved in the basic functions of the mitochondrion as well as its rate of biogenesis (Puigserver & Spiegelman 2003; Dominy & Puigserver, 2013). No changes in the *PPARGC1A* expression were detected in lung, thigh, and breast tissues of both genders and lines at 3 weeks of age. However, consistent with the observed increased mitochondrial biogenesis in in breast tissue of the SUS males at 20 weeks old, the levels of *PPARGC1A* mRNA gene expression were almost 2-fold change higher relative to the RES birds. Probably, the increased activity of *PPARGC1A* in breast muscle during sexual maturity could play a role in enhancing mitochondrial respiratory capacity which attenuates the development of ascites in SUS males. However, it has yet to be determined whether the enhanced activity of *PPARGC1A* is attributable to its promotion of mitochondrial function or its effects on nonmitochondrial gene expression.

AMPKα1 gene regulates intracellular energy metabolism in response to acute energy crises and is activated by an increase in AMP/ ATP ratio (energy depletion) and inhibited by the presence of glycogen. Thus, to maintain energy homoeostasis, *AMPKα1* switches on catabolic pathways that generate ATP, while switching off anabolic pathways that consume ATP (Dominy & Puigserver, 2013; Hardie, 2011). Interestingly, The *AMPKα1* gene activity was notably down regulated in lung, and breast tissues at 3 and 20 weeks of age of SUS line males in comparison with RES line. In females, *AMPKα1* gene was only downregulated in lung muscle of SUS line birds as compared to RES line at early age. Several studies indicate another important role of *AMPKα1* in the disposal of dysfunctional and damaged mitochondria, process known as autophagy (Hardie, 2011). Any impairment of the mitochondrial autophagy process is often accompanied by accumulation of dysfunctional or damaged mitochondria that leads to increases in mtDNA content. Therefore, it is possible that the observed *AMPKα1* downregulation in SUS line as compared with the RES line caused insufficient removal of the damaged mitochondria which may explain the increase mtDNA content in male birds of the SUS line as compared with the RES line.

OPA1 gene plays an essential role in the inner mitochondrial fusion and maintenance of the mitochondrial network architecture, which is essential for mitochondrial activity and biogenesis. *DNM1L* is the master regulator of mitochondrial division in most eukaryotic organisms (Scott & Youle, 2010). Remarkably, at 3 weeks of age, both *OPA1* and *DNM1L* mRNA expression were significantly decreased in lung, and breast tissue of SUS line males, and in thigh of SUS line females as compared with RES line. Downregulation of *DNM1L* and *OPA1* genes in these tissues at this early age may reduce the efficiency of mitochondrial autophagy and causes accumulation of dysfunctional mitochondria. Consequently, the mitochondria are not able to re-fuse with the mitochondrial network after fission leading to increase in fragmented mitochondria and mtDNA

accumulation. Conversely, at 20 weeks old, the *OPA1* was found to be significantly upregulated in breast tissue of SUS line males as compared with RES line which may reflects the enhanced activity in the mitochondrial biogenesis and the quick clearance of damaged mitochondria in breast muscle as birds advance in age.

SIRT1, a metabolic sensor that belongs to the sirtuin (NAD+ –dependent deacetylases) family and its activity can increase when NAD^+ levels are abundant, such as times of nutrient deprivation. *SIRT1* stimulates mitochondrial biogenesis via deacetylation of a variety of proteins in response to metabolic stress (Dominy & Puigserver, 2013; Tang, 2016). In our study, *SIRT1* was overexpressed in breast tissue of SUS line males at 20 weeks old and in lungs of SUS line females at 3 weeks of old compared to RES birds.

In summary, our findings indicate clear sex differences in mitochondrial biogenesis establishing a strong association between the mtDNA content and ascites-susceptibility and ascites-resistance in a tissue-specific manner. The mtDNA/nucDNA levels could serve as potential predictive markers to screen for ascites phenotype in birds at early developmental ages. Moreover, this study confirms that the consistent increase in the mtDNA/nucDNA ratio between young and old birds is only restricted to breast muscles. However, it is worth noting that mitochondrial biogenesis is tissue specific. This is because every type of cell and tissue has a specific transcriptional profile, and consequently unique features of metabolic pathways. Our study suggests the possible contribution of the lower expression of *OPA1*, and *DNM1L* genes in mitochondrial biogenesis defects in male SUS birds which leads to increase in mtDNA content in some tissues at early ages. Furthermore, our data is consistent with a possible role of *PPARGC1A* in breast tissue of SUS line males in controlling ascites syndrome progression and improved regulation of mitochondrial biogenesis at older ages. Nevertheless, we have no clear evidence for what genes or regulators are driving the

observed sizable sex-differences in mtDNA copy number at an early age. Despite our findings, the precise mechanism that explains the association between mtDNA copy number and ascites syndrome remains unknown. To address this further in the future, we need to test larger sample numbers, more tissues, and different populations/crosses. Our observations are based on a single experimental series and, although our results agreed with our previous data, we cannot wholly determine if this phenomenon is a cause or effect or limited to the tissues used in this study. Regardless of the limited number of replicates used, our study had sufficient statistical power to detect significant differences in mtDNA/nucDNA ratio and gene expression analysis. Future research should focus on finding mitochondrial biogenesis causal genetic regulators and exploring whether they are connected or unrelated to changes in the mtDNA.

REFERENCES

Baghbanzadeh, A., & Decuypere, E. (2008). Ascites syndrome in broilers: Physiological and nutritional perspectives. *Avian Pathology*, *37*(2), 117–126.

Balog, J.M., Anthony, N.B., Cooper, M.A., Kidd, B.D., Huff, G.R., Huff, W.E., & Rath, N.C. (2000). Ascites syndrome and related pathologies in feed restricted broilers raised in a hypobaric chamber. *Poultry Science,* 79(3), 318-323.

Balog, J.M., Kidd, B.D., Huff, W.E., Huff, G.R., Rath, N.C., & Anthony, N.B. (2003). Effect of cold stress on broilers selected for resistance or susceptibility to ascites syndrome. *Poultry Science*, 1;82(9):1383-7.

Barazzoni, R., Short, K.R., & Nair, K.S. (2000). Effects of aging on mitochondrial DNA copy number and cytochrome c oxidase gene expression in rat skeletal muscle, liver, and heart. *Journal of Biological Chemistry*, *275*(5), 3343–3347.

Borras, C., Sastre, J., Garcia-Sala, D., Lloret, A., Pallardo, F.V, & Vina, J. (2003). Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. *Free Radical Biology & Medicine*, *34*(5), 546–552.

Borrás, C., Gambini, J., López-Grueso, R., Pallardó, F.V., & Viña, J. (2010). Direct antioxidant and protective effect of estradiol on isolated mitochondria. *BBA Molecular Basis of Disease*, 1802(1), 205–211.

Bottje, W.G., & Wideman, R.F.J. (1995). Potential role of free radicals in the etiology of pulmonary hypertension syndrome. *Poultry & Avian Biology Reviews*, 6:211–231.

Brown, A. M., Sheu, R. K.F., Mohs, R., Haroutunian, V., & Blass, J. P. (2001). Correlation of the clinical severity of Alzheimer's disease with an aberration in mitochondrial DNA (AntDNA). *Journal of Molecular Neuroscience*, *16*(1), 41–48.

Cawthon, D.C., McNew, R., Beers, K.W., & Bottje, W.G. (1999). Evidence of mitochondrial dysfunction in broilers with pulmonary hypertension syndrome (ascites): Effect of t-butyl hydroperoxide on hepatic mitochondrial function, glutathione, and related thiols. *Poultry Science*, 78:114–124.

Cawthon, D., Beers, K., Bottje, W.G. (2001). Electron transport chain defect and inefficient respiration may underlie pulmonary hypertension syndrome (ascites)-associated mitochondrial dysfunction in broilers. *Poultry Science,* 80, 474–484.

Chance, B., Sies, H., & Boveris, A. (1979). Hydroperoxide metabolism in mammalian organs. *Physiological Reviews*, 59:527–605.

Chineme, C. N., Buyse, J., Buys, N., Hassanzadeh Ladmakhi, M., Albers, G. A. A., & Decuypere, E. (1995). Interaction of genotype, egg shell conductance and dietary T3 supplementation in the

development of heart failure syndrome and ascites in broiler chickens. *Archiv für Geflügelkunde*, 59(2), 129-134.

Cisar, C. R., Balog, J.M., Anthony, N.B., Iqbal, M., Bottje, W.G., & Donoghue, A.M. (2004). Differential expression of mitochondrial electron transport chain proteins in cardiac tissues of broilers from pulmonary hypertension syndrome-resistant and -susceptible lines 1. *Poultry Science*, 83(8), 1420–1426.

Currie, R.J.W. (1999). Ascites in poultry: recent investigations. *Avian Pathology*, 28:313–326.

Dawson, T. L., Gores, G. J., Nieminen, A. L., Herman, B., & LeMasters J. J. (1993). Mitochondria as a source of reactive oxygen species during reductive stress in rat hepatocytes. *American Journal of Physiology,* 264:C961–C967.

Decuypere, E., Buyse, J., & Buys, N. (2000). Ascites in broiler chickens: exogenous and endogenous structural and functional causal factors. *Worlds Poultry Science Journal*, 56, 367376.

Demarest, T.G., & McCarthy, M.M. (2015). Sex differences in mitochondrial (dys)function: Implications for neuroprotection. *Journal of Bioenergetics & Biomembranes*, *47*(1-2), 173–188.

Dominy, J.E., & Puigserver, P. (2013). Mitochondrial Biogenesis through Activation of Nuclear Signaling Proteins. *Cold Spring Harbor Perspectives in Biology*, *5*(7), a015008.

Emami N.K., Golian, A., Mesgaran, M.D., Anthony, N.B., & Rhoads, D.D. (2017). Mitochondrial biogenesis and PGC1α gene expression in male broilers from ascites susceptible and resistant lines. *Journal of Animal Physiology & Animal Nutrition*, 102(1): 482-485.

Hassanzadeh, M., Buyse, J., Toloei, T., & Decuypere, E. (2014). Ascites syndrome in broiler chickens: A review on the aspect of endogenous and exogenous factors interactions. *Egyptian Journal of Neurology, Psychiatry & Neurosurgery*, 51(3), 229–241.

Hardie, D.G. (2011). AMP-activated protein kinase-an energy sensor that regulates all aspects of cell function. *Genes & Development*, 25(18), 1895–1908.

Iqbal, M., Cawthon, D., Wideman, R.F., Beers, K.W., & Bottje, W.G. (2001a). Lung mitochondrial dysfunction in pulmonary hypertension syndrome. II. Oxidative stress and inability to improve function with repeated additions of ADP. *Poultry Science*, 80, 656–665.

Jornayvaz, F.R., & Shulman, G.I.G. (2010). Regulation of mitochondrial biogenesis. *Essays in Biochemistry*, 47, 69–84.

Livak, K.J. & Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods,* 25(4): 402-8.

Lubritz, D.L., Smith, J.L., & McPherson, B.N. (1995). Heritability of ascites and the ratio of right to total ventricle weight in broiler breeder male lines. *Poultry Science*, 74(7), 1237-1241.

Malik, A.N., & Czajka, A. (2012). Is mitochondrial DNA content a potential biomarker of mitochondrial dysfunction? *Mitochondrion*, 13, 481–492.

Moghadam, H.K., McMillan, I., Chambers, J.R., & Julian, R.J. (2001). Estimation of genetic parameters for ascites syndrome in broiler chickens. *Poultry Science*, 80(7), 844–848.

Movassagh, M.H., Karami, A.R. Ahmadzadeh, A.R., & Dolgharisharaf, J. (2008). Relation Between the Incidence of Ascites Syndrome and the Ventilation Factor in Broiler Chickens. *Asian Journal of Animal and Veterinary Advances,* 3: 334-336.

Odom, T.W. (1993). Ascites syndrome: Overview and update. *Poultry Science*, Dig. 50:14-22.

Owen, R.L., Wideman Jr, R.F., Hattel, A.L., & Cowen, B.S. (1990). Use of a hypobaric chamber as a model system for investigating ascites in broilers. *Avian Diseases,* 754-758.

Pakdel, A., Bijma, P., Ducro, B.J., & Bovenhuis, H. (2005b). Selection strategies for body weight and reduced ascites susceptibility in broilers. *Poultry science*, 1;84(4):528-35.

Parr, R.L., Dakubo, G.D., Thayer, R.E., McKenney, K., & Birch-Machin, M.A. (2006). Mitochondrial DNA as a potential tool for early cancer detection. *Human Genomics*, 2(4), 252–257.

Pavlidis, H.O., Balog, J.M., Stamps, L.K., Hughes, J.D., Huff, W.E., & Anthony, N.B., (2007). Divergent selection for ascites incidence in chickens. *Poultry Science,* 86, 2517–2529.

Peacock, A.J., Pickett, C., Morris, K. & Reeves, J.T. (1990) Spontaneous hypoxaemia and right ventricular hypertrophy in fast growing broiler chickens reared at sea level. *Comparative Biochemistry & Physiology,* 97A: 537-541.

Ploumi, C., Daskalaki, I. & Tavernarakis, N. (2017), Mitochondrial biogenesis and clearance: a balancing act. *FEBS Journal*, 284: 183–195.

Puigserver, P., & Spiegelman, B.M. (2003). Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. *Endocrine Reviews*, *24*(1), 78–90.

Radonic, A., Thulke, S., Mackay, I.M., Landt, O., Siegert, W., & Nitschea, A. (2004). Guideline to reference gene selection for quantitative real-time PCR. *Biochemical & Biophysical Research Communications*, 313:856-862.

Sato, T., Tezuka, K., Shibuya, H., Watanabe, T., Kamata, H., & Shirai, W. (2002). Cold-induced ascites in broiler chickens and its improvement by temperature-controlled rearing. *Avian Diseases,* 46(4), 989-996.

Scott, I., & Youle, R.J. (2010). Mitochondrial fission and fusion. *Essays in Biochemistry*, *47*(11), 85–98.

Tang, Z., Iqbal, M., Cawthon, D., & Bottje, W.G. (2002). Heart and breast muscle mitochondrial dysfunction in pulmonary hypertension syndrome in broilers (Gallus domesticus). *Comparative Biochemistry and Physiology*, 32:527–540.

Tang, B.L. (2016). Sirt1 and the Mitochondria. *Molecules & Cells*, 39(2), 87–95.

Wideman, R.F., Wing, T., Kirby, Y.K., Forman, M.F., Marson, N., Tackett, C.D., Ruiz-Feria, C.A. (1998). Evaluation of minimally invasive indices for predicting ascites susceptibility in three successive hatches of broilers exposed to cool temperatures. *Poultry Science,* 77(10), 1565-1573.

Wideman, R.F., & French, H. (2000). Ascites resistance of progeny from broiler breeders selected for two generations using chronic unilateral pulmonary artery occlusion. *Poultry Science,* 79(3), 396-401.

Wideman, R.F. (2001). Pathophysiology of heart/lung disorders: Pulmonary hypertension syndrome in broiler chickens. *World's Poultry Science Journal*, *57*(3), 304–307.

Wideman, R.F., Rhoads, D.D., Erf, G.F., & Anthony, N.B. (2013). Pulmonary arterial hypertension (ascites syndrome) in broilers: a review. *Poultry Science*, 92(1), 64-83.

TABLES AND FIGURES

Table 3. 1: Sequences of primer pairs used for RT-qPCR analysis of chicken target and reference genes. For each gene the primer sequence for forward (F) and reverse (R) are listed (5'-3'), genomic location, the annealing temperature in °C used (Ta), the amplicon product length (bp). All primer sequences were synthesized by Integrated DNA Technologies (IDT, Coralville, IA).

Gene	Location	Primer Sequences	Ta	Product length
mt-tRNA^{ARG} (mtDNA)	**chrM**	**Forward**: GCTTCTTCCCCTTCCATGAGCCATCC **Reverse**: AGAGATGAGGTGTGTTCGGTGGAATGC	60	288
HTR2B (nucDNA)	**Chr9**	**Forward**: GCCTATTTGATCAACAAGCCACCTC **Reverse**: GTTATGAAGAATGGGCACCACATCA	60	226
TBP	**Chr3**	**Forward**: GAACCACGTACTACTGCGCT **Reverse**: CTGCTGAACTGCTGGTGTGT	60	230
PPARGCA1	**Chr4**	**Forward**: ACGCAAGCAGTTTTGCAAGT **Reverse**: TCCGCTGTGCCTCTTTAAGT	60	271
OPA1	**Chr9**	**Forward**: CCTAACTGGCAAAAGGGTCCA **Reverse**: GCTCCCCCAAAAGGTAAGACA	60	206
SIRT1	**Chr6**	**Forward:** CGATGAAGGAAAATGGAACCAAC **Reverse:** CGCTCTCATCCTCCACATCT	60	270
MAPKα1	**ChrZ**	**Forward:** CGACGGAAGAATCCAGTGAC **Reverse**: TTTCCTTGTGCATCACCATCTG	60	206
DNM1L	**Chr1**	**Forward:** ATCCTTGCTGTTGGATGACCTT **Reverse**: AGCGTGGCTGGTACAGTCTT	60	218

Table 3. 2: Relative gene expression in lung, thigh, breast muscles of 3 weeks old male birds from SUS and RES lines divergently selected for ascites phenotype. ↑↓ indicates the direction of the difference for the SUS line relative to the RES line. Avg ± SEM is the average ± standard error of the mean for the n-fold change (2^-ΔΔCt) for the SUS line relative to the calibrator, RES line, from five birds (n= 5) run in triplicate. Statistically different results were determined using Student's *t* test for unpaired samples.

Gene	Lung 3 Weeks			Thigh 3 Weeks			Breast 3 Weeks		
	↑↓	Avg ± SEM	P value	↑↓	Avg ± SEM	P value	↑↓	Avg ± SEM	P value
AMPKα1	↓	0.75 ± 0.23	0.04	↑	1.21 ± 0.27	0.15	↓	0.66 ± 0.07	0.02
PPARGC1A	↓	0.78 ± 0.10	0.12	↓	0.82± 0.21	0.23	↓	0.92 ± 0.11	0.32
SIRT1	↓	0.84 ± 0.08	0.12	↓	0.58 ± 0.05	0.27	↓	0.91 ± 0.07	0.34
OPA1	↓	0.65 ± 0.07	0.01	↓	0.93 ± 0.22	0.38	↓	0.70 ± 0.05	0.04
DNM1L	↓	0.49 ± 0.03	0.00004	↓	0.92 ± 0.14	0.29	↓	0.63 ± 0.05	0.018

Table 3. 3: Relative gene expression in lung, and breast muscles of 20 weeks old male birds from SUS and RES lines divergently selected for ascites phenotype. Column headers and data representations are as described for Table 2. NC is no significant change.

Gene	Lung 20 Weeks			Breast 20 Weeks		
	↑↓	Avg ± SEM	P value	↑↓	Avg ± SEM	P value
AMPKα1	↓	0.15 ± 0.13	0.01	↓	0.83 ± 0.29	0.02
PPARGC1A	**NC**	1.01 ± 2.05	0.16	↑	1.97 ± 0.09	0.000003
SIRT1	↓	0.51 ± 0.08	0.10	↑	1.43 ± 0.11	0.004
OPA1	↓	0.62 ± 0.05	0.0009	↑	1.18 ± 0.07	0.03
DNM1L	↓	0.81 ± 0.08	0.11	↓	0.92 ± 0.23	0.19

Table 3. 4: Relative gene expression in lung, thigh, breast muscles of 3 weeks old female birds from SUS and RES lines divergently selected for ascites phenotype. Column headers and data representations are as described for Table 2. NC is no significant change.

Gene	Lung 3 Weeks			Thigh 3 Weeks			Breast 3 Weeks		
	↑↓	Avg ± SEM	P value	↑↓	Avg ± SEM	P value	↑↓	Avg ± SEM	P value
AMPKα1	↓	0.43 ± 0.50	0.05	↓	0.87 ± 0.28	0.19	**NC**	1.03 ± 0.24	0.47
PPARGC1A	↓	0.48 ± 0.18	0.12	**NC**	1.01 ± 0.13	0.49	↓	0.48 ± 0.16	0.08
SIRT1	↑	1.29 ± 0.12	0.02	↓	0.98 ± 0.07	0.41	↓	0.92 ± 0.09	0.28
OPA1	↑	1.16 ± 0.17	0.22	↓	0.80 ± 0.07	0.03	↓	0.86 ± 0.05	0.15
DNM1L	↓	0.60 ± 0.19	0.07	↓	0.60 ± 0.08	0.01	↓	0.79 ± 0.18	0.40

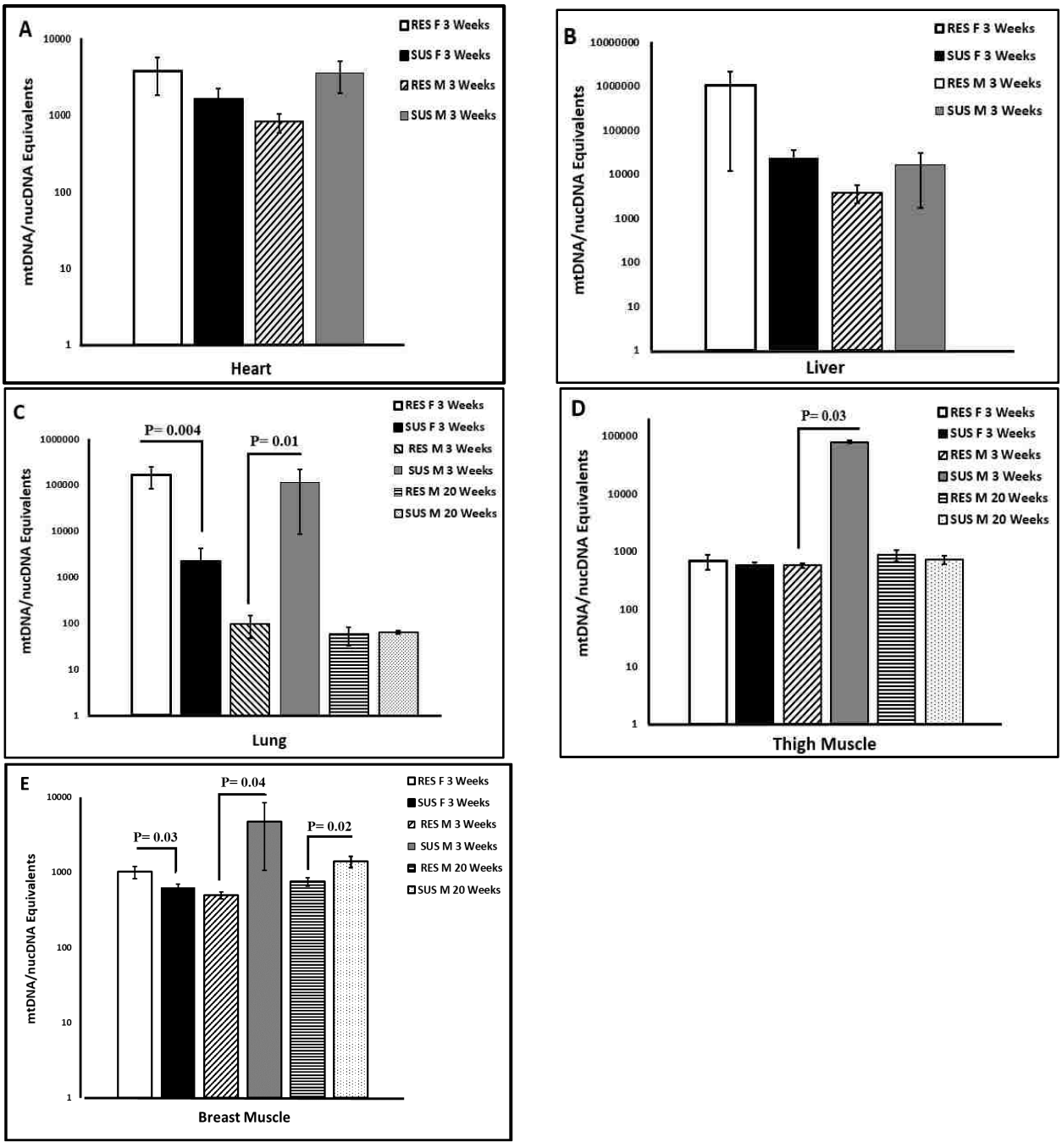


Figure 3. 1: Mean mtDNA relative to nucDNA in A, B, Heart, and Liver of the SUS and RES lines of both sexes at 3 weeks old, C, D, E, Lung, thigh, and breast muscle of the SUS and RES lines of both sexes at 3 and 20 weeks old (n=5 for each group). Error bars are SEM and P values determined by one-tailed *t*-test, *P >0.05, **P >0.01.

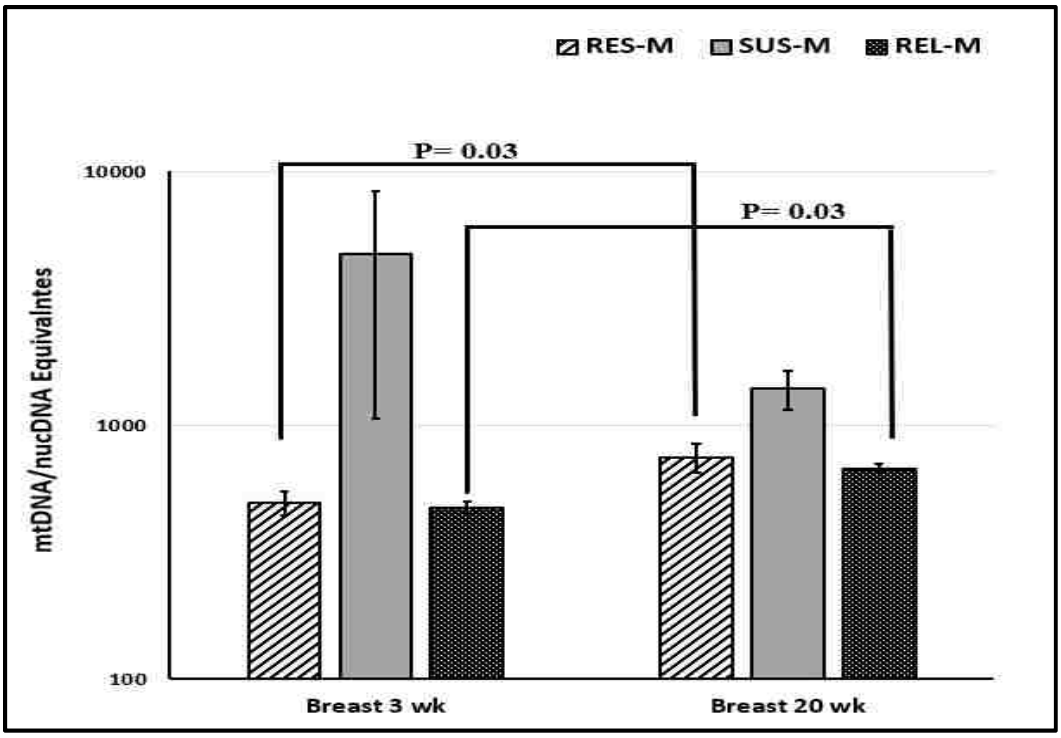


Figure 3. 2: Mean mtDNA relative to nucDNA in breast tissues of males from RES, SUS, and REL experimental lines at 3 weeks old, and 20 weeks old (n=5 for SUS and RES birds and n=12 for REL line birds). Error bars are SEM and P values determined by one-tailed *t*-test, *P >0.05.

CHAPTER 4

Differences in Promoter Accessibility Responses to Hypoxia in Pulmonary Arteries of Ascites-Susceptible and Resistance Broiler Research Lines Detected using ATAC-seq Technology

ABSTRACT

The aim of the current study was to map genome-wide changes in chromatin accessibility of ascites- susceptible and ascites- resistant bird lines during normal and hypoxic conditions. Ascites is a terminal result of pulmonary hypertension and is a significant metabolic disease of fast growing meat-type chickens. Pulmonary artery remodeling appears to be the main condition that leads to an increase of pulmonary vascular resistance, sustained arterial hypertension, right ventricular hypertrophy and ultimately death. Therefore, in this study, we investigated chromatin accessible regions in the pulmonary artery of two broiler experimental lines divergently selected for ascites phenotype of both genders via ATAC-seq technology under normal and hypoxic conditions. For the first time, transposition was completed on frozen pulmonary artery tissues. Libraries were sequenced to generate 50 million 2x150 PE reads. A total of 23,444 open chromatin regions (or peaks) were identified across all pulmonary artery samples. Our analysis showed that this method captures the tissue-specific chromatin activity of not only regulatory regions such as promoters, enhancers, and insulators, but also gene regions including exonic, intronic, and intergenic regions. Initial results demonstrate that there was a substantial increase in the chromatin accessibility throughout the genome of ascites- susceptible birds when challenged under hypoxic conditions in comparison with controls. Contrastingly, we observed reduced changes in chromatin accessibility regions in ascites-resistant birds when challenged. We focused on changes within 2 kb of transcription start sites. We identified 1324 regions that become differentially accessible. In conclusion, we showed that chromatin accessibility is a key epigenetic factor influencing transcriptional regulation and a straightforward approach to identify functional genomic regulatory regions controlling complex diseases such as ascites in birds.

INTRODUCTION

Pulmonary hypertension syndrome (PHS), or ascites is a significant metabolic disease of fast growing meat-type, broiler chickens (Wideman *et al*., 2013). In the poultry industry, the economic impact on the worldwide broiler industry is measured in tens of millions of dollars lost per year (Odom, 1993). PHS is a multifactorial disease mediated by several nutritional, management, environmental, and genetic factors, all related to the need to ensure a high level of oxygen in the tissues (Iqbal *et al*., 2001; Owen *et al*., 1990; Lubritz *et al*., 1995; Wideman & French, 2000; Balog *et al*., 2003). However, many studies have confirmed that long- term intense selection for rapid growth and meat production is the primary cause for PHS in broilers (Julian, 1993; Balog, 2003). The extremely rapid early growth performance of broilers imposes proportional challenges to their immature cardiopulmonary system. As a result, the heart and lungs are required to work very close to their physiological limit to meet the high oxygen demands required for metabolic processes. Unfortunately, cardiopulmonary system capacity cannot always meet the levels of oxygen needed for the rapid growth, which can result in internal hypoxemia and PHS development (Julian, 2000). PHS-susceptible (SUS) broilers were found to have higher pulmonary artery pressure (PAP), and pulmonary vasculature resistance (PVR) to blood flow compared with PHS-resistant (RES) broilers (Wideman *et al*., 2010). Pulmonary arterial hypertension (PAH) occurs when the right ventricle is forced to elevate the PAP to overcome increased PVR to blood flow through restrictive pulmonary arterioles (Wideman *et al*., 2007). Sustained PAH leads to a series of pathophysiological events that include pulmonary arterial remodeling, right ventricular hypertrophy (RVH), valvular insufficiency, increased hematocrit value and blood viscosity, variable changes in the liver leading to the transudation of plasma from the liver into the abdominal cavity (ascites), eventually right ventricular failure (RVF) leading to

premature death (Wideman *et al*., 2013). Pulmonary artery structural remodeling appears to be the initial early key step characterized by vascular narrowing and thickening, leading to a progressive increase in PAP and PVR in response to hypoxia. Similar pathological changes manifest in humans, and experimental animals such as: rats, dogs, and cows, with chronic pulmonary hypertension (Van Suylen *et al*., 1998; Jeffery & Wanstall, 2001; Colvin & Yeager, 2014). The pulmonary artery remodeling involves all three layers of the artery wall comprising an intima, a media, and an adventitia (Stenmark *et al*., 2006). Each layer exhibits specific functional, histological, and biochemical characteristics (Hislop & Reid, 1976; Reid, 1979; Stenmark & Mecham, 1997; Shimodao *et al*., 2013). In response to vascular stress or stimuli, each layer contributes in unique ways to regulate pulmonary arterial wall function and structure. In hypoxia-induced PAH, the pulmonary arterial remodeling is characterized by thickening of all three layers of the blood vessel wall. Such thickening is attributed to cell growth (hypertrophy) and/or proliferation (hyperplasia) of the predominant cell type within each of the layers, i.e., fibroblasts, smooth muscle cells, and endothelial cells, as well as increased production of extracellular matrix components including collagen, fibronectin, and elastin within the vessel wall (Gibbons & Dzau, 1994; Stenmark *et al*., 2009; Sakao *et al*.,2010). (Figure 4.1). As a result, the pulmonary artery lumen diameter and capacity for vasodilation are decreased causing obstruction to the blood flow, increased PVR, and sustained PAH. In spite of many years of genetic research, understanding the genetic underpinnings of PHS remains a major challenge, and there is still much work to be done. With recent advances in the techniques that allow us to sequence genomic DNA, genetics research has become more and more important over the past decades. Hundreds of genome-wide association studies (GWAS) have provided a wealth of information in which differences in the genome of individuals can be linked to various traits. However, it is becoming very clear that

complex diseases and common disease traits, such as PHS, are influenced by large numbers of variants that individually have small effects (Manolio *et al*., 2009). Moreover, most of these loci lie outside of coding genes especially in regulatory regions such as: promoters, enhancers, and insulators and act by modifying gene expression (Li *et al*., 2016). Therefore, it is difficult to identify the molecular effects of noncoding variants on gene regulation. Recently, mapping of regulatory landscapes has received considerable attention as an essential component of gene expression regulation and genome stability. Open chromatin regions are correlated with active regulatory elements and changes in chromatin accessibility patterns are thought to play a critical role in several human diseases such as cancer, diabetes, heart diseases, kidney diseases and aging-related diseases (Gomez *et al*., 2016; Francis *et al*., 2006; Wilkins *et al.,* 2015; Lewis *et al.,* 1981; Reddy *et al.,* 2015; Moskowitz *et al*., 2017; Wang *et al*., 2018). Therefore, chromatin accessibility can serve as a good indicator of gene transcription activity status. Recent technological advances, such as the Assay for Transposase-Accessible Chromatin by high- throughput sequencing (ATAC-seq), have enabled genome-wide profiling of chromatin accessibility patterns, nucleosome positioning, and transcription factor footprints at base pair resolution (Buenrostro *et al*., 2013 & 2015). Although ATAC-seq has only been reported to work with cultured cells, we aimed to adapt ATAC-seq for frozen tissues archived from prior experiments. ATAC-seq relies on the ability of hyperactive Tn5 transposase to fragment DNA and integrate into active regulatory genomic regions (Figure 4.2). Since pulmonary artery remodeling plays a key early step in PHS progression in broilers, the objective of this study was to use ATAC-seq to investigate chromatin accessible patterns in the pulmonary artery of two broiler experimental lines divergently selected for PHS phenotype, the resistant line (RES) and susceptible line (SUS) under hypoxic and non- hypoxic conditions.

MATERIALS AND METHODS

Genomic Data

All genome positions indicated in this study are according to the December 2015 assembly of the *Gallus gallus* genome GenBank accession ID: GCF_000002315.4.

Birds Stocks

All animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (under protocol 12039 and 15040). Birds used in this study represent the ascites-resistant (RES), and the ascites-susceptible (SUS) lines at generation 21 (Pavlidis *et al*., 2007). Soon after hatching, a total of 24 chicks from each line were randomly divided into a normal group (n = 12) and a hypoxic challenged group (n=12) with 6 from each gender in each group. All birds were reared at ambient pressure at room temperature (20-23°C) for 2 weeks. Starting from the third week, birds in the normal group were maintained at ambient pressure, whereas broilers in the hypoxic challenged group were transferred to the hypobaric chamber at simulated high altitude (8000 ft) by operating under partial vacuum to lower the partial pressure of oxygen. Birds remained in the hypobaric chamber under hypoxic condition for one week. All birds were allowed to have free access to the same diet *ad libitum* and had a 24h lighting throughout the trial period.

Pulmonary Arteries Collection

At the end of the third week of the trial, all birds were killed by cervical dislocation and pulmonary artery tissues were rapidly collected from both groups and immediately frozen in dry ice and stored in a -80°C freezer for later use.

ATAC-Seq Libraries Preparation

Pulmonary arteries from 2 birds of the same gender within each group were mixed together to generate a pooled sample. Thus, based on gender and phenotype, samples constituted 12 pooled samples from the control group and 12 pooled samples from the hypoxic challenged group (3 pools x 2 phenotypes x 2 genders). Nuclei were isolated from frozen pulmonary artery tissues (~ 10 mg) using the Omni-ATAC protocol as previously described (Corces *et al*., 2017). Tissue was homogenized gently with approximately 10 to 15 strokes with the loose 'A' pestle, followed by 20 to 25 strokes with the tight 'B' pestle using 1-ml Dounce homogenizer. Approximately 10,000 counted nuclei were tagged using Tn5 transposase (Nextera DNA sample prep kit; Illumina, San Diego, CA) for 30 min at 37°C as described previously (Buenrostro *et al.,* 2013; 2015), with the modification that fragmented genomic DNA was recovered using Zymo DNA Clean and Concentrator 5 columns (Zymo Research, Irvine, CA). In order to enrich small tagmented DNA fragments, DNAs were initially amplified using custom-synthesized (Integrated DNA Technologies, Coralville, IA) index primers (Ad1_noMX and Ad2.1–2.24, Table 1) barcoded primers for 5 cycles of standard PCR. Analysis of the qPCR data allowed a rough estimate of the number of additional cycles needed to generate product prior to saturation. The additional number of cycles needed was determined by plotting the Relative Fluorescence (RFU) versus Cycle and then selecting the cycle number where the reaction transitioned one-third of the maximum RFU, also known as Cycle threshold (Ct). For most samples the Ct was 10 to 12 additional PCR cycles added to the initial set of 5 cycles. After PCR amplification, libraries were purified using Zymo DNA Clean and Concentrator 5 columns. DNAs were gel separated and ~ 800 bp fragments were collected using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The quality control (QC) checkpoints consist of morphological evaluation of nuclei, agarose gel

electrophoresis of libraries, and real-time qPCR to assess the enrichment of open-chromatin sites. The selected DNA fragments were then quantified using a KAPA Library Quant Kit for Illumina Sequencing Platforms (KAPA Biosystems, Wilmington, MA). Finally, all quantified libraries were pooled and sequenced using an Illumina HiSeq X with paired- end read length of 150 bases to a minimum depth of 50 million reads per sample at MedGenome, Inc. (Foster City, CA).

ATAC-seq Data processing

ATAC-seq reads that passed quality filtering and demultiplexing were aligned to the Galgal5 assembly using NGen software (DNASTAR Lasergene Suite 15.2) with standard parameters and library insert size of 800. Alignments were analyzed using Arraystar software (DNASTAR Lasergene Suite 15.2). The relative RPM (reads per million) values for each pool were compared across libraries, genders, chicken line, and treatments. Peak calling was performed using MACs model-based implementation system in ArrayStar where the number of reads/peak was determined for each sample. Reads mapping to the mitochondrial genome were used to calculate the percentage of mitochondrial DNA in our libraries (Table 4.3). Peak-to-gene assignment were all based on RefSeq database, which was downloaded from UCSC table browser (https://genome.ucsc.edu/). Genes that were difficult to annotate via RefSeq database were annotated with non-RefSeq database. Visualizations of insertion tracks were done using GenVision Pro tool (DNASTAR Lasergene Suite 15.2). Peak classification was performed using an in-house script assigning peaks 2kb upstream to transcription start sites (TSS) as “promoter”. Transcription start site annotations were also made using RefSeq database.

RESULTS

Optimization and Construction of ATAC-Seq Libraries

ATAC-seq method is composed of three fundamental steps: isolation of nuclei, transposition, and amplification. After optimization, we found that the entire assay and library construction can be carried out in a few days. We followed the previously published ATAC-seq protocol (Buenrostro *et al*., 2013; 2015) with minor modifications. However, to isolate nuclei from frozen pulmonary arteries, we used an improved ATAC-seq method called Omni- ATAC protocol (Corces *et al.,* 2017). This is because standard ATAC-seq requires the transposition reaction to be performed on fresh cells and was found to perform poorly on snap-frozen samples. Omni-ATAC protocol improvements include 1:) the use of phosphate-buffered saline (PBS) in the transposition reaction, and 2) the use of multiple detergents (such as NP40, Tween-20, and digitonin). These improvements were reported to be important in increasing the signal-to-background ratio, improving cells permeabilization, removing mitochondria from the transposition reaction, and increasing the complexity of the library. We tested this protocol and found it suitable and broadly applicable for diverse tissue types (such as: liver, heart, lung) for samples archived by flash-freezing or stored frozen at -20^{o}C in RNAlater™. Frozen pulmonary artery samples were washed with cold PBS (pH 7.4) before use. We also note methods involving mechanical shearing such as a Bullet Blender homogenizer (Next Advance, Inc., Averill Park, NY) significantly reduced the quality of nuclei. The optimal method we found was homogenization using a 1-ml Dounce homogenizer on ice, which produced high-quality intact nuclei, were tissue was homogenized gently with approximately 10 to 15 strokes with the loose 'A' pestle, followed by 20 to 25 strokes with the tight 'B' pestle. We also modified the Omni-ATAC protocol for the density gradient loading method. Briefly, the 35% Iodixanol solution was first slowly load at the bottom of pre-

chilled 2-ml round bottom Lo-Bind Eppendorf tube. Then slowly released 29% Iodixanol solution onto the side of the tube above the 35%. Finally, the crude preparation of nuclei was carefully mixed with 50% Iodixanol solution to give a final concentration of 25% and slowly loaded onto the side of the tube above the 29%. After centrifugation, nuclei were collected at the 29% and 35% Iodixanol solutions interface. We found this method of loading technique improved the quality and definition of the interfaces between all three layers. Nuclei were counted after addition of trypan blue (10:10), to stain all nuclei. About 10,000 counted nuclei were then used for transposition reaction. The remainder of the ATAC-seq library preparation was performed as described previously (Buenrostro *et al*., 2013; 2015) with some modifications. The ATAC-seq protocol works by randomly inserting sequencing adapters into open chromatin regions via a hyperactive tagmentation Tn5 enzyme (Figure 2). Unlike the original protocol, we used only Zymo DNA Clean and Concentrator 5 columns (Zymo Research, Irvine, CA) instead of Qiagen products to purify DNA fragments after transposition reaction and libraries PCR amplification. Moreover, although Buenrostro et al., 2013, chose to avoid a size selection step to maximize the library complexity, and to identify accessible locations and nucleosome positioning simultaneously, we included size selection prior to sequencing. This is because samples that contain an excess of long fragments (>1 kb) can be hard to quantify and may result in reduced clustering efficiencies upon sequencing. Thus, we excised a relatively large fragment size of 800 bp to eliminate potently confounding long fragments, and at the same time maintain high library complexity. Then, we used a qPCR-based method, the KAPA Library Quant Kit for Illumina Sequencing Platforms to quantify our ATAC-seq libraries as Qubit can potentially give misleading and inaccurate results (Buenrostro *et al.,* 2015). Finally, we sent all 24 quantified libraries to MedGenome, where

libraries were pooled and sequenced using an Illumina HiSeq X with paired- end read length of 150 bp to a minimum depth of 50 million reads per sample.

Primary Data Analysis and Peak Calling

We generated ATAC-seq data from 24 pulmonary artery libraries as follows: RFNC (resistant female non- challenged, n=3), RFC (resistant female challenged, n=3), SFC (susceptible female challenged, n=3), SFNC (susceptible female non- challenged, n=3), RMNC (resistant male non-challenged, n=3), RMC (resistant male challenged, n=3), SMC (susceptible male challenged, n=3), and SMNC (susceptible male non- challenged, n=3). For this preliminary analysis, we restricted our investigation primarily to males as they show the greatest susceptibility to ascites (Decuypere et al., 2000; Baghbanzadeh et al., 2008). The first step in ATAC-seq data analysis was the mapping of reads to Galgal5 reference genome. Average reads varied from 29.9 million to 177.4 million (Table 4.2). Then, we assessed the proportion of reads that mapped to the chicken mitochondrial genome, given reports of mitochondrial reads being a significant source of contamination (Montefiori et al., 2017). Interestingly, there was a low contamination with reads mapping to the mitochondrial DNA typically, which made up less than 2% of all mapped reads from our libraries (Table 4.3). Thus, Omni- ATAC protocol for isolation of nuclei from frozen tissues can reduce contamination of ATAC-seq libraries from mitochondrial DNA. We also noted that ATAC-seq reads correlate well with the library concentrations (Table 4.2 & Figure 4.3). Libraries with low input tend to generate less ATAC-seq reads as they don't efficiently cluster well during sequencing and vice versa. Next, we identified accessible regions throughout the chicken genome and then assigned each ATAC-seq peak to the nearest gene based on annotated transcription start sites (TSS). The peak calling software (ArrayStar) was used to transform raw sequence alignments into regions of enrichment and background considering the false discovery rate and noise. In total,

23,444 high-confidence open chromatin regions (or peaks) were identified across all pulmonary artery samples. Peak intensities were highly reproducible and similar between biological replicates. ArrayStar peak calling algorithms generally assigned enriched regions by absolute signal values (read counts) or by significance of reads enrichment (P-values). We could assign ATAC-seq peaks into five general types based on the distribution of accessible sites: promoter-proximal, enhancer, exonic/intronic, 3′UTR, and intergenic. Promoter- proximal is defined as the region within 2 kb of the reference transcription start site (TSS), as determined by the UCSC genome browser which would also include peaks including the 5′UTR. Peaks located within 2 kb of transcription end site were included with the 3′UTR peaks. Peaks that found 10kb upstream from TSS was considered as "enhancers", and peaks that fall within the gene body as either "exon" or "intron", and any remaining peaks as "intergenic". In this study, we focused on detecting chromatin accessibility in promoter- proximal regions and used an in-house script/Excel formula assigning peaks 2kb from TSS as "promoter" where differential peaks were annotated to the nearest gene based on their distance to TSS. Overall, we identified 1324 gene regions out of the 23,444 (~ 5.6% of all peaks) that become differentially accessible within the TSS. Therefore, we conclude that most differentially accessible regions (94.4 %) were largely distal intergenic regions or located at gene intronic, and exonic regions, with relatively few promoter-proximal regions exhibiting differential accessibility. In general, we observed a nearly unidirectional substantial and intense increase in chromatin accessibility when comparing the SMC with SMNC, potentially reflecting the much greater response to hypoxic conditions (Figure 4.4). On the other hand, comparison of samples from the RMC vs. RMNC, showed decreased profiles of chromatin accessibility when challenged under hypoxic conditions (Figure 4.5). Both RMNC and SMNC showed broadly similar profiles of chromatin accessibility

(Figure 4.6). To illustrate our results, a zoom out of chr 1 of 100 million bp window showed that SMC are clearly stimulated by hypoxic conditions and contain way more active/open chromatin regions in comparison to the RMC that seem to respond to the challenge by becoming inaccessible whereas RMNC and SMNC behave the same (Figure 4.7). Moreover, ATAC-seq reveals the presence of clusters of similarly affected genes within the same chromosomal region. For example, we found a large cluster of histone genes that includes the major histone components (H2A, H2B, H3 and H4) located on chr1 ranging from ~48091382 bp to 48201409 bp (Figure 4.11). Interestingly, histone genes were stimulated by hypoxic challenge in SUS males compared to RES.

The most challenging aspect of ATAC-seq is the analysis of the sequence data, since generalized methods and tools are limited and still under development. Moreover, definitive identification of enriched regions from ATAC-seq experiments can be challenging as peaks can differ in shape and span. For example, narrow/sharp peaks (~ 100 bp), broader peaks (> 1 kb), and mixed signals. Sharp peaks have been generally found for protein–DNA binding (i.e.; transcription factors binding sites) such as *SP1* gene (Figure 4.8), whereas broad peak regions have been often associated with histone modifications that mark domains—for example, activated or repressed regions, such as the gene that codes for *RN5S* protein (Figure 4.9). Peaks that contain both narrow and broad shapes are still not very well understood such as *RPS27A* gene (Figure 4.10) (Sims et al., 2014). Additionally, the data revealed categories of peaks that are either specific to one gene or shared between a cluster of genes. For instance, a peak associated with *USP5* is shared other gene *CDCA3* (Figure 4.12), whereas a peak associated with *SP1* is specific to the gene (Figure 4.8). Furthermore, pathway- and network-level analysis were performed using KEGG Pathways tool to identify known and novel relationships among annotated genes. Top identified pathways

include: Metabolic pathways, Endocytosis, Ubiquitin mediated proteolysis, MAPK signaling pathway, Cell cycle, RNA transport, Tight junction, Cellular senescence, Peroxisome, Protein processing in endoplasmic reticulum, Lysosome, Oxidative phosphorylation, spliceosome, Ribosome, Pyrimidine metabolism, Adrenergic signaling in cardiomyocytes, and FoxO singling pathway.

Altogether, our ATAC-seq analysis provides a global landscape of high confidence open chromatin regions in chicken under hypoxic conditions. Finally, our data demonstrate that the Omni-ATAC protocol provides high quality intact nuclei from frozen tissues, and broadly applicable platform for the generation of chromatin- accessibility profiles in chicken.

DISCUSSION

Soon after the genome sequences of many organisms had been completed, it became clear that the epigenome would also be required. Based on hundreds of human GWAS studies, it was realized that 93% of the trait-associated variants were in non-coding regions, mostly within cis-regulatory elements (Maurano *et al.,* 2012). Therefore, much remains to be answered about how genetic information is interpreted, and which genes are expressed by which cell type, and when. The epigenome represents a second dimension to the genome (Rivera & Ren, 2013), and plays a significant role in directing the unique gene expression programming in each cell type or tissue at different development stages together with its genome. (Sarda & Hannenhalli, 2014). The epigenome consists of chemical changes to the DNA such as DNA methylation at CpG dinucleotides, covalent modifications of histone proteins, noncoding RNAs (ncRNAs), chromatin accessibility, nuclear localization, and higher-order chromatin architecture (nucleosome positioning and occupancy, 3D chromatin structure) (Bernstein *et al*., 2007; Berger *et al.,* 2009;

Bonasio *et al*., 2010). Epigenetic changes can be heritable through cell division. Importantly, epigenetic modification can behave similarly to genetic mutations in terms of stability, yet they are reversable, and therefore have the potential to be manipulated therapeutically (Bernstein *et al*., 2007). With the rapid development of next-generation sequencing (NGS) technology and the significant drop of cost, the field of epigenomics is enjoying a substantial increase and the number of epigenomic publications and studies has grown exponentially.

The development of the ATAC-seq method enabled the epigenomic profiling of precious or rare tissue samples to generate data from a low amount of biological sample (Buenrostro *et al*., 2015). Chromatin openness is a prerequisite for the binding of transcription factor proteins to specific active regulatory elements in the genome, and thereby control gene expression. In comparison to earlier established methods to study chromatin accessibility such as MNase-seq, FAIR-seq and DNase-seq, ATAC-seq enables rapid and efficient library construction as it is faster and easier to perform, does not require cross-linking or sensitive enzymatic digestions, has higher signal to noise ratio, and can be performed on few cell numbers. Therefore, we believe that ATAC-seq will soon become broadly applicable and the preferred method for the study of chromatin structure and nucleosome positioning. Nevertheless, to ensure a successful ATAC-seq experiment, optimizing every step and quality assurance procedures that include both wet lab and computer-based quality assessment are required. Moreover, compared with its easy-to-perform experiment, ATAC-seq data analysis may take considerable amount of time and effort. This is because tools and software necessary to process ATAC-seq data is still lacking. Currently, several software programs were developed that covers important aspects of analyzing ATAC-seq data especially for quality control assessment and downstream statistical analysis including peak calling, transcription factors

footprinting, nucleosome occupancy, and enrichment analysis such as esATAC, and ATACseqQC (Wei *et al*., 2018; Ou *et al*., 2018).

In this study, we performed ATAC-seq to provide a chromatin accessibility map of pulmonary artery tissues' regulatory elements genome wide in chicken. The unexpected finding is that there were tremendous changes in chromatin accessibility across activated promoters, body genes, and enhancers during a genome-wide change in response to hypoxic challenge. A total of 23,444 differentially accessible regions were identified across all tested samples where 1324 gene regions fall within 2kb of TSSs as "promoters". As expected, we observed no changes in chromatin accessibility in both non-hypoxic SUS and RES birds (RMNC & SMNC). However, our initial analysis reveals striking epigenetic differences between RES and SUS birds upon challenging under hypoxic conditions. We observed a significant increase in accessibility of chromatin across the genomic regions of in the SUS male birds compared with the unchallenged control. Increased accessibility during hypoxic challenge is attributed to decreased nucleosomal occupancy corresponding to genes being potentially expressed or repressed. Thus, global widespread increase in chromatin openness could be a hallmark for PHS- progression in response to hypoxia. Conversely, reduced chromatin accessibility in RES male upon challenge relative to the non-challenged control was observed. Unfortunately, we cannot determine if the decreased chromatin accessibility represents an adaptive response that may protect pulmonary arterial cells from going through vascular remodeling and therefore continue to function even in the face of hypoxia. Identifying the precise molecular mechanism that mediates the global changes in chromatin accessibility in RES vs SUS birds can provides valuable key information to understand PHS pathogenesis.

Future direction will continue to identify potential key regulatory factors responsible for differential chromatin accessibility in SMC vs SMNC and RMC vs RMNC. This can be done by searching for DNA sequence that were enriched in the peaks thorough TFs binding sites, motif enrichment, and gene ontology analysis tools. Additionally, future work may extend ATAC-seq investigations to other PHS- related tissues such as heart and lung as the epigenomic landscape of each cell can vary considerably, contributing to distinct gene expression regulations and biological functions. Moreover, our chromatin accessibility profiles can enable the interpretation of our previous GWAS and whole genome sequencing (WGS) results that have mapped PHS relevant polymorphisms to noncoding regions. In human, it was found that genetic variants associated with specific traits show epigenomic enrichments in trait-associated tissues (Roadmap Epigenomics Consortium, 2015). Therefore, simultaneous measurement of chromatin accessibility and genetic variants will need to be conducted to investigate the interplay between those two factors. Finally, our ATAC-seq dataset can be integrated with epigeneomic and transcriptomic information such as histone marks (Chip-seq), DNA methylation (MeDIP-seq), RNA expression (RNA-seq) to enhance interpretation of computational results, construct a regulatory network, and reveal potential key gene regulators of PHS in chicken. Integrative analysis of genomic and epigenomic regulation provides new insights into understanding gene regulation, cellular differentiation, and disease progression. There are several classes of mechanisms that might lead to increased or decreased accessibility of chromatin. To illustrate this, for example, reduced DNA methylation can cause increased chromatin accessibility, thereby allowing for TFs to associate with DNA in open regulatory regions and regulate gene expression. This would then predict that progressively increased chromatin accessibility should be associated with PHS progression.

Although, epigenetics is still an emerging science, the scientific community is now building new tools to study epigenetic alterations in the genome especially in human and mouse models. Unfortunately, progress in the avian epigenome is limited. However, recent international efforts on the functional annotation of animal genomes (FAANG) including chickens have been initiated (Andersson *et al*., 2015). It is expected that epigenome data generated in the next few years will greatly contribute to our understanding of gene expression regulation in chicken. In fact, the FAANG consortium is planning to use ATAC-seq as a standard for measuring chromatin accessibility. We also expect to share our ATAC-seq dataset from the pulmonary artery tissues of chicken with the FAANG consortium and database in the coming years. Collectively, this study provides the first initial insights into the *in vivo* gene regulatory network of PHS pathogenesis and should serve as a valuable resource for future studies.

REFERENCES

Andersson, L., Archibald, A., Bottema, C., Brauning, R., Burgess, S., Burt, D., Casas, E., Cheng, H., Clarke, L., Couldrey, C., Dalrymple, B., Elsik, C., Foissac, S., Giuffra, E., Groenen, M., Hayes, B., Huang, L., Khatib, H., Kijas, J., Kim, H., Lunney, J., McCarthy, F., McEwan, J., Moore, S., Nanduri, B., Notredame, C., Palti, Y., Plastow, G., Reecy, J., Rohrer, G., Sarropoulou, E., Schmidt, C., Silverstein, J., Tellam, R., Tixier-Boichard, M., Tosser-Klopp, G., Tuggle, C., Vilkki, J., White, S., Zhao, S., & Zhou, H. (2015). Coordinated international action to accelerate genome-to-phenome with FAANG, the Functional Annotation of Animal Genomes project. *GenomeBiology.com*, 16(1), 1-57.

Baghbanzadeh, A., & Decuypere, E. (2008). Ascites syndrome in broilers: Physiological and nutritional perspectives. *Avian Pathology*, 37(2), 117–126.

Balog, J.M. (2003). Ascites syndrome (pulmonary hypertension syndrome) in broiler chickens: are we seeing the light at the end of the tunnel? *Avian & Poultry Biology Reviews*, 14(3), 99126.

Balog, J.M., Kidd, B.D., Huff, W.E., Huff, G.R., Rath, N.C., & Anthony, N.B. (2003). Effect of cold stress on broilers selected for resistance or susceptibility to ascites syndrome. *Poultry Science*, 1;82(9):1383-7.

Berger, S.L., Kouzarides, T., Shiekhattar, R., & Shilatifard, A. (2009) An operational definition of epigenetics. *Genes & Development,* 23:781–783.

Bernstein, B.E., Meissner, A., & Lander, E.S. (2007) The mammalian epigenome. *Cell,* 128:669–681.

Blatti, C., & Sinha, S. (2014). Motif Enrichment Tool. *Nucleic Acids Research*, *42*(Web Server issue), W20–W25.

Bonasio, R., Tu, S., & Reinberg, D. (2010) Molecular signals of epigenetic states. *Science,* 330:612–616.

Boyle, A.P., Davis, S., Shulha, H.P., Meltzer, P., Margulies, E.H., Weng, Z., Furey, T.S., & Crawford, G.E. (2008) High-resolution mapping and characterization of open chromatin across the genome. *Cell,* 132:311.

Buenrostro, J.D., Giresi, P.G., Zaba, L.C., Chang, H.Y., & Greenleaf, W.J (2013) Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nature Methods,* 10:1213–1218

Colvin, K.L., & Yeager, M.E. (2014). Animal Models of Pulmonary Hypertension: Matching Disease Mechanisms to Etiology of the Human Disease. *Journal of Pulmonary & Respiratory Medicine*, 4(4), 198.

Corces, M.R., Trevino, A.E., Hamilton, E.G., Greenside, P.G., Sinnott-Armstrong, N.A., Vesuna, S., Satpathy, A.T., Rubin, A.J., Montine, K.S., Wu, B., Kathiria, A., Cho, S.W., Mumbach, M.R, Carter, A.C., Kasowski, M., Orloff, L.A., Risca, V.I., Kundaje, A., Khavari, P.A.,

Montine, T.J., Greenleaf, W.J., & Chang, H.Y. (2017). An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues. *Nature Methods*, 14(10), 959–962.

Decuypere, E., Buyse, J. & Buys, N. (2000). Ascites in broiler chickens: exogenous and endogenous structural and functional causal factors. *Worlds Poultry Science Journal*, 56, 367376.

Dennis, G., Sherman, B.T., Hosack, D. A., Yang, J., Gao, W., Lane, H., & Lempicki, R.A. (2003). DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biology*, 4(9), R60.

Gibbons, G.H., & Dzau, V.J. (1994). The emerging concept of vascular remodeling. *The New England Journal of Medicine*, 330(20), 1431–1438.

Giresi, P.G., Kim, J., McDaniell, R.M., Iyer, V.R., & Lieb, J.D. (2007) FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) isolates active regulatory elements from human chromatin. *Genome Research,* 17:877–885

Gomez, N.C., Hepperla, A., Dumitru, R., Simon, J.M., Fang, F., & Davis, I.J. (2016). Widespread chromatin accessibility at repetitive elements links stem cells with human cancer. *Cell Reports*, 17(6), 1607–1620.

Enkvetchakul, B., Beasley, J., & Bottje, W. (1995). Pulmonary arteriole hypertrophy in broilers with pulmonary hypertension syndrome (ascites). *Poultry Science*, *74*(10), 1677–1682.

Francis, J., Babu, D.A., Deering, T.G., Chakrabarti, S.K., Garmey, J.C., Evans-Molina, C., Taylor, D.G., & Mirmira, R.G. (2006). Role of Chromatin Accessibility in The Occupancy and Transcription of The Insulin Gene by The Pancreatic Transcription Factor PDX-1. *Molecular Endocrinology (Baltimore, Md.)*, 20(12), 3133–3145.

Hamal, K.R., Wideman, R.F., Anthony, N.B., & Erf, G.F. (2010). Differential expression of vasoactive mediators in microparticle-challenged lungs of chickens that differ in susceptibility to pulmonary arterial hypertension. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, 298(1), R235-42.

Hernández, A., & De Sandino, M. (2007). Hypoxic Pulmonary Arterial Hypertension in the Chicken Model. *Pulmonary Hypertension – From Bench Research to Clinical Challenges*

Hislop, A., & Reid, L. (1976). New findings in pulmonary arteries of rats with hypoxia-induced pulmonary hypertension. *British Journal of Experimental Pathology*, 57(5), 542–554.

Iqbal, M., Cawthon, D., Wideman, R.F., Beers, K.W., & Bottje, W.G. (2001). Lung mitochondrial dysfunction in pulmonary hypertension syndrome. II. Oxidative stress and inability to improve function with repeated additions of ADP. *Poultry Science*, 80, 656–665.

Jeffery, T.K., & Morrell, N.W. (2002). Molecular and cellular basis of pulmonary vascular remodeling in pulmonary hypertension. *Progress in Cardiovascular Diseases*, 45(3), 173–202.

Jeffery, T.K., & Wanstall, J.C. (2001). Pulmonary vascular remodeling: a target for therapeutic intervention in pulmonary hypertension. *Pharmacology & Therapeutics*, 92(1), 1–20.

Julian, R. J. (1993). Ascites in poultry. *Avian Pathology*. 22:419–454.

Koboldt, D.C., Steinberg, K.M., Larson, D.E., Wilson, R.K., & Mardis, E.R. (2013) The next-generation sequencing revolution and its impact on genomics. *Cell,* 155:27–38.

Lewis, P.N., Lukiw, W.J., De Boni, U., & McLachlan, D.R. (1981). Changes in chromatin structure associated with Alzheimer's disease. *Journal of Neurochemistry*, 37(5), 1193–1202.

Lubritz, D.L., Smith, J. L., & McPherson, B.N. (1995). Heritability of ascites and the ratio of right to total ventricle weight in broiler breeder male lines. *Poultry Science*, 74(7), 1237-124.

Montefiori, L., Hernandez, L., Zhang, Z., Gilad, Y., Ober, C., Crawford, G., Nobrega, M., Jo Sakabe, N. (2017). Reducing mitochondrial reads in ATAC-seq using CRISPR/Cas9. *Scientific Reports*, 7(1), 2451.

Moskowitz, D.M., Zhang, D.W., Hu, B., Le Saux, S., Yanes, R.E., Ye, Z., Buenrostro, J.D., Weyand, C.M., Greenleaf, W.J., & Goronzy, J.J. (2017). Epigenomics of human CD8 T cell differentiation and aging. *Science Immunology*, 2(8), eaag0192.

Maurano, M.T., Humbert, R., Rynes, E., Thurman, R.E., Haugen, E., Wang, Reynolds, H.A., Sandstrom, R., Qu, H., Brody, J., Shafer, A., Neri, F., Lee, K., Kutyavin, T., Stehling-Sun, S., Johnson, A.K., Canfield, T.K., Giste, E., Diegel, M., Bates, D.,R. Hansen, R.S., Neph, S., Sabo, P.J., Heimfeld, S., Raubitschek, A., Ziegler, S., Cotsapas, C., Sotoodehnia, N., Glass, I., Sunyaev, S.R., Kaul, R., & Stamatoyannopoulos, J.A. (2012). Systematic Localization of Common Disease-Associated Variation in Regulatory DNA. *Science (New York, N.Y.)*, 337(6099), 1190–1195.

Odom, T.W. (1993). Ascites syndrome: Overview and update. *Poultry Science*, Dig. 50:14-22.

Ou, J., Liu, H., Yu, J., Kelliher, M.A., Castilla, L.H., Lawson, N.D., & Zhu, L.J. (2018). ATACseqQC: a Bioconductor package for post-alignment quality assessment of ATAC-seq data. *BMC Genomics*, 19, 169.

Owen, R.L., Wideman Jr, R. F., Hattel, A. L., & Cowen, B.S. (1990). Use of a hypobaric chamber as a model system for investigating ascites in broilers. *Avian Diseases,* 754-758.

Reddy, M.A., & Natarajan, R. (2015). Recent Developments in Epigenetics of Acute and Chronic Kidney Diseases. *Kidney International*, 88(2), 250–261.

Reid, L.M. (1979). The pulmonary circulation: remodeling in growth and disease. The 1978 J. Burns Amberson lecture. *The American Review of Respiratory Disease*, 119(4), 531–546.

Rivera, C.M., & Ren, B. (2013) Mapping human epigenomes. *Cell,* 155:39–55.

Roadmap Epigenomics Consortium, Kundaje, A., Meuleman, W., Ernst, J., Bilenky, M., Yen, A., … Kellis, M. (2015). Integrative analysis of 111 reference human epigenomes. *Nature*, 518(7539), 317–330.

Sakao, S., Tatsumi, K., & Voelkel, N.F. (2010). Reversible or Irreversible Remodeling in Pulmonary Arterial Hypertension. *American Journal of Respiratory Cell and Molecular Biology*, 43(6), 629–634.

Sarda, S., & Hannenhalli, S. (2014) Next-generation sequencing and epigenomics research: a hammer in search of nails. *Genomics Inform,* 12: 2–11.

Shimoda, L.A., & Laurie, S.S. (2013). Vascular Remodeling in Pulmonary Hypertension. *Journal of Molecular Medicine (Berlin, Germany)*, *91*(3), 297–309.

Sims, D., Sudbery, I., Ilott, N.E., Heger, A., & Ponting, C.P. (2014). Sequencing depth and coverage: key considerations in genomic analyses. *Nature Reviews. Genetics*, 15(2), 121–132.

Stenmark, K.R., & Mecham, R.P. (1997). Cellular and molecular mechanisms of pulmonary vascular remodeling. *Annual Review of Physiology*, 59, 89–144.

Stenmark, K.R., Fagan, K.A., & Frid, M.G. (2006). Hypoxia-induced pulmonary vascular remodeling: Cellular and molecular mechanisms. *Circulation Research*, 99(7), 675–691.

Stenmark, K.R., Meyrick, B., Galie, N., Mooi, W.J., & McMurtry, I.F. (2009). Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, 297(6), L1013-32.

Thurman, R. E., Rynes, E., Humbert, R., Vierstra, J., Maurano, M. T., Haugen, E., Sheffield NC, Stergachis AB, Wang H, Vernot B, Garg K, John S, Sandstrom R, Bates D, Boatman L, Canfield TK, Diegel M, Dunn D, Ebersol AK, Frum T, Giste E, Johnson AK, Johnson EM, Kutyavin T, Lajoie B, Lee BK, Lee K, London D, Lotakis D, Neph S, Neri F, Nguyen ED, Qu H, Reynolds AP, Roach V, Safi A, Sanchez ME, Sanyal A, Shafer A, Simon JM, Song L, Vong S, Weaver M, Yan Y, Zhang Z, Zhang Z, Lenhard B, Tewari M, Dorschner MO, Hansen RS, Navas PA, Stamatoyannopoulos G, Iyer VR, Lieb JD, Sunyaev SR, Akey JM, Sabo PJ, Kaul R, Furey TS, Dekker J, Crawford GE, Stamatoyannopoulos, J. A. (2012). The accessible chromatin landscape of the human genome. *Nature*, 489(7414), 75–82.

Van Suylen, R.J., Smits, J.F., & Daemen, M.J. (1998). Pulmonary artery remodeling differs in hypoxia- and monocrotaline-induced pulmonary hypertension. *American Journal of Respiratory and Critical Care Medicine*, 157(5 Pt 1), 1423–1428.

Wang, J., Zibetti, C., Shang, P., Sripathi, S. R., Zhang, P., Cano, M., Thanh, H., Shuli, X., Hongkai, J., Shannath, L. M., Donald, J. Z., James, T.H., Debasish, S., Seth, B., & Qian, J. (2018). ATAC-Seq analysis reveals a widespread decrease of chromatin accessibility in age-related macular degeneration. *Nature Communications*, 9, 1364.

Wei, Z., Zhang, W., Fang, H., Li, Y., & Wang, X. (2018). esATAC: an easy-to-use systematic pipeline for ATAC-seq data analysis. *Bioinformatics*, bty141-bty141.

Wideman, R.F., & French, H. (2000). Ascites resistance of progeny from broiler breeders selected for two generations using chronic unilateral pulmonary artery occlusion. *Poultry Science,* 79(3), 396-401.

Wideman, R.F., Chapman, M.E., Hamal, K.R., Bowen, O.T., Lorenzoni, A.G., Erf, G.F., & Anthony, N.B. (2007). An inadequate pulmonary vascular capacity and susceptibility to pulmonary arterial hypertension in broilers. *Poultry Science*, 86(5), 984–998.

Wideman, R.F., Eanes, M.L., Hamal, K.R., & Anthony, N.B. (2010). Pulmonary Vascular Pressure Profiles in Broilers Selected for Susceptibility to Pulmonary Hypertension Syndrome: Age and Gender Comparisons. *Poultry Science*, 89(9), 1815–1824.

Wideman, R. F., Hamal, K. R., Bayona, M. T., Lorenzoni, A. G., Cross, D., Khajali, F., RHOADS, D.D., Erf, G. F., & Anthony, N. B. (2011). Plexiform Lesions in the Lungs of Domestic Fowl Selected for Susceptibility to Pulmonary Arterial Hypertension: Incidence and Histology. *Anatomical Record*, 294(5), 739–755.

Wideman, R.F., & Hamal, K.R. (2011). Idiopathic pulmonary arterial hypertension: An avian model for plexogenic arteriopathy and serotonergic vasoconstriction. *Journal of Pharmacological and Toxicological Methods*, 63(3), 283–295.

Wideman, R.F., Rhoads, D.D., Erf, G.F., & Anthony, N.B. (2013). Pulmonary arterial hypertension (ascites syndrome) in broilers: a review. *Poultry Science*, 92(1), 64–83.

Wilkins, M.R., Ghofrani, H.-A., Weissmann, N., Aldashev, A., & Zhao, L. (2015). Pathophysiology and Treatment of High-Altitude Pulmonary Vascular Disease. *Circulation*, 131(6), 582 LP-590.

TABLES AND FIGURES

Table 4. 1 ATAC-seq Primer sequences. Adapted from *Buenrostro et al., 2013*

Primer	Sequence
Ad1_noMX	AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTCAGATGTG
Ad2.1_TAAGGCGA	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGT
Ad2.2_CGTACTAG	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGT
Ad2.3_AGGCAGAA	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGGAGATGT
Ad2.4_TCCTGAGC	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGT
Ad2.5_GGACTCCT	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGGAGATGT
Ad2.6_TAGGCATG	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGGAGATGT
Ad2.7_CTCTCTAC	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGGAGATGT
Ad2.8_CAGAGAGG	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTCTCGTGGGCTCGGAGATGT
Ad2.9_GCTACGCT	CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTCTCGTGGGCTCGGAGATGT
Ad2.10_CGAGGCTG	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGGAGATGT
Ad2.11_AAGAGGCA	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGGAGATGT
Ad2.12_GTAGAGGA	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGGAGATGT
Ad2.13_GTCGTGAT	CAAGCAGAAGACGGCATACGAGATATCACGACGTCTCGTGGGCTCGGAGATGT
Ad2.14_ACCACTGT	CAAGCAGAAGACGGCATACGAGATACAGTGGTGTCTCGTGGGCTCGGAGATGT
Ad2.15_TGGATCTG	CAAGCAGAAGACGGCATACGAGATCAGATCCAGTCTCGTGGGCTCGGAGATGT
Ad2.16_CCGTTTGT	CAAGCAGAAGACGGCATACGAGATACAAACGGGTCTCGTGGGCTCGGAGATGT
Ad2.17_TGCTGGGT	CAAGCAGAAGACGGCATACGAGATACCCAGCAGTCTCGTGGGCTCGGAGATGT
Ad2.18_GAGGGGTT	CAAGCAGAAGACGGCATACGAGATAACCCCTCGTCTCGTGGGCTCGGAGATGT
Ad2.19_AGGTTGGG	CAAGCAGAAGACGGCATACGAGATCCCAACCTGTCTCGTGGGCTCGGAGATGT
Ad2.20_GTGTGGTG	CAAGCAGAAGACGGCATACGAGATCACCACACGTCTCGTGGGCTCGGAGATGT
Ad2.21_TGGGTTTC	CAAGCAGAAGACGGCATACGAGATGAAACCCAGTCTCGTGGGCTCGGAGATGT
Ad2.22_TGGTCACA	CAAGCAGAAGACGGCATACGAGATTGTGACCAGTCTCGTGGGCTCGGAGATGT
Ad2.23_TTGACCCT	CAAGCAGAAGACGGCATACGAGATAGGGTCAAGTCTCGTGGGCTCGGAGATGT
Ad2.24_CCACTCCT	CAAGCAGAAGACGGCATACGAGATAGGAGTGGGTCTCGTGGGCTCGGAGATGT

Table 4. 2 Samples and libraries submitted for ATAC-seq with read counts.

Library Identifier	Line	Gender	Treatment	Replicate	Library ng/ul	Number of reads
RFNC1	RES	Female	NoChallenge	1	0.17	2,745,153
RFNC2	RES	Female	NoChallenge	2	6.64	80,047,438
RFNC3	RES	Female	NoChallenge	3	5.59	55,009,857
RFC1	RES	Female	Challenge	1	6.18	105,386,567
RFC2	RES	Female	Challenge	2	9.14	87,294,716
RFC3	RES	Female	Challenge	3	5.38	92,319,717
SFNC1	SUS	Female	NoChallenge	1	3.67	88,057,108
SFNC2	SUS	Female	NoChallenge	2	2.76	80,488,368
SFNC3	SUS	Female	NoChallenge	3	2.14	58,712,101
SFC1	SUS	Female	Challenge	1	6.71	102,231,416
SFC2	SUS	Female	Challenge	2	4.07	115,570,137
SFC3	SUS	Female	Challenge	3	4.51	179,490,405
RMNC1	RES	Male	NoChallenge	1	2.9	72,040,415
RMNC2	RES	Male	NoChallenge	2	1.39	46,853,650
RMNC3	RES	Male	NoChallenge	3	1.7	42,000,034
RMC1	RES	Male	Challenge	1	1.07	28,879,877
RMC2	RES	Male	Challenge	2	1.15	43,287,040
RMC3	RES	Male	Challenge	3	0.56	17,572,541
SMNC1	SUS	Male	NoChallenge	1	1.36	48,023,812
SMNC2	SUS	Male	NoChallenge	2	2.51	77,509,760
SMNC3	SUS	Male	NoChallenge	3	4.02	66,427,471
SMC1	SUS	Male	Challenge	1	5.52	222,911,333
SMC2	SUS	Male	Challenge	2	6.95	160,560,907
SMC3	SUS	Male	Challenge	3	5.03	148,931,645

Table 4. 3 Proportion of ATAC-Seq reads mapped to the chicken mitochondrial genome pooling biological replicates.

Line	Gender	Treatment	Avg Reads	Total mapped reads	Total mitochondrial mapped reads	% Mitochondrial
RES	**Female**	**NoChallenge**	45,934,149	137,802,447	1,428,344	1.0
RES	**Female**	**Challenge**	95,000,333	285,001,000	3,625,428	1.3
SUS	**Female**	**NoChallenge**	75,752,526	227,257,577	743,974	0.3
SUS	**Female**	**Challenge**	132,430,653	397,291,958	3,194,105	0.8
RES	**Male**	**NoChallenge**	53,631,366	160,894,099	1,426,604	0.9
RES	**Male**	**Challenge**	29,913,153	89,739,458	394,439	0.4
SUS	**Male**	**NoChallenge**	63,987,015	191,961,044	2,048,357	1.1
SUS	**Male**	**Challenge**	177,467,961	532,403,884	10,589,523	2.0

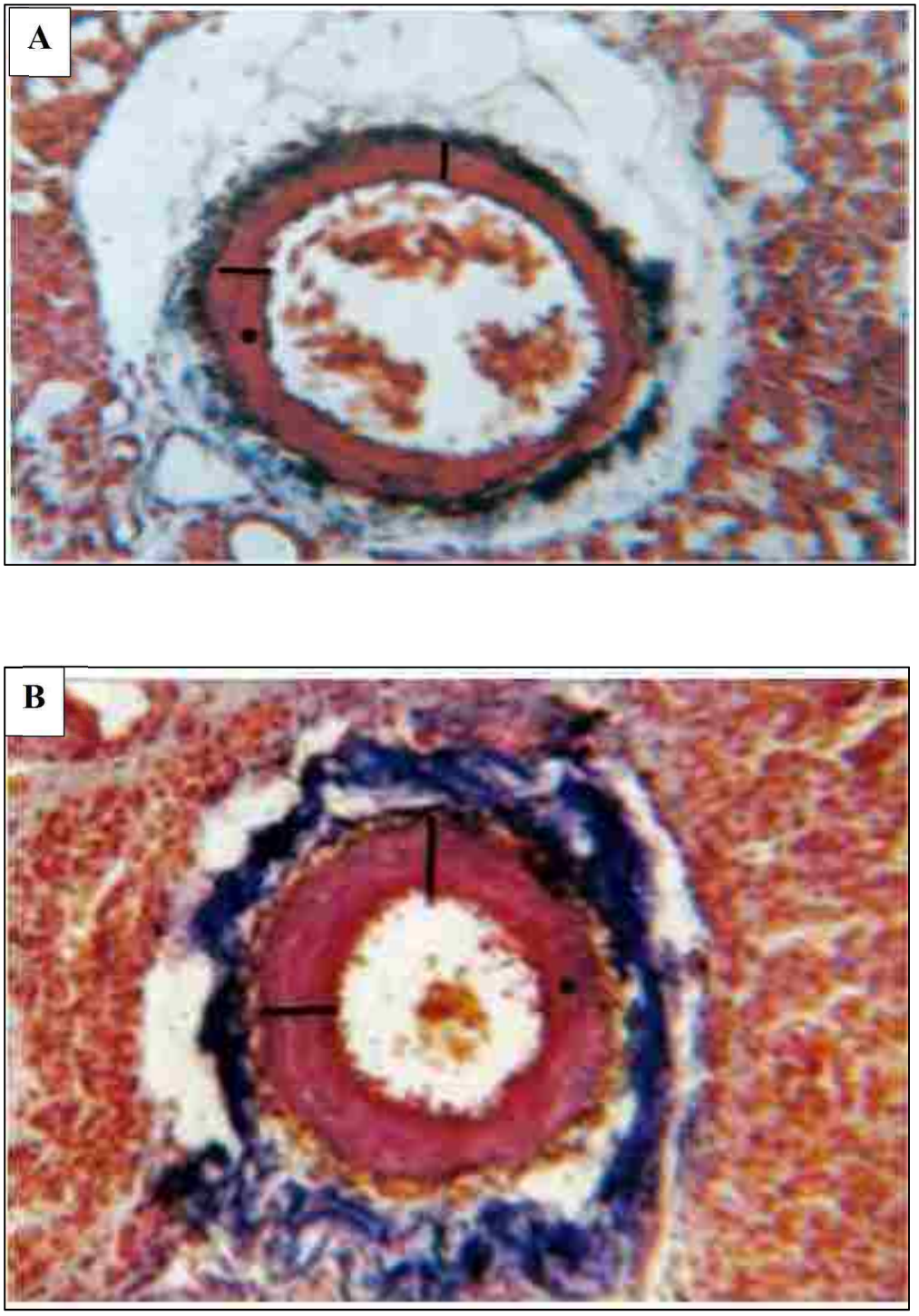


Figure 4. 1: A cross -section of normal and hypertensive chicken arteriole. A) Non-hypertensive chicken's arteriole with the thin smooth muscle (stained in red and indicated by black lines) and adventitial (dark blue) layers. B) Hypertensive chicken´s arteriole with thick smooth muscle (stained in red and indicated by black lines) and surrounding adventitial (stained in dark blue) layers. Adapted from Hernández & De Sandino (2007).

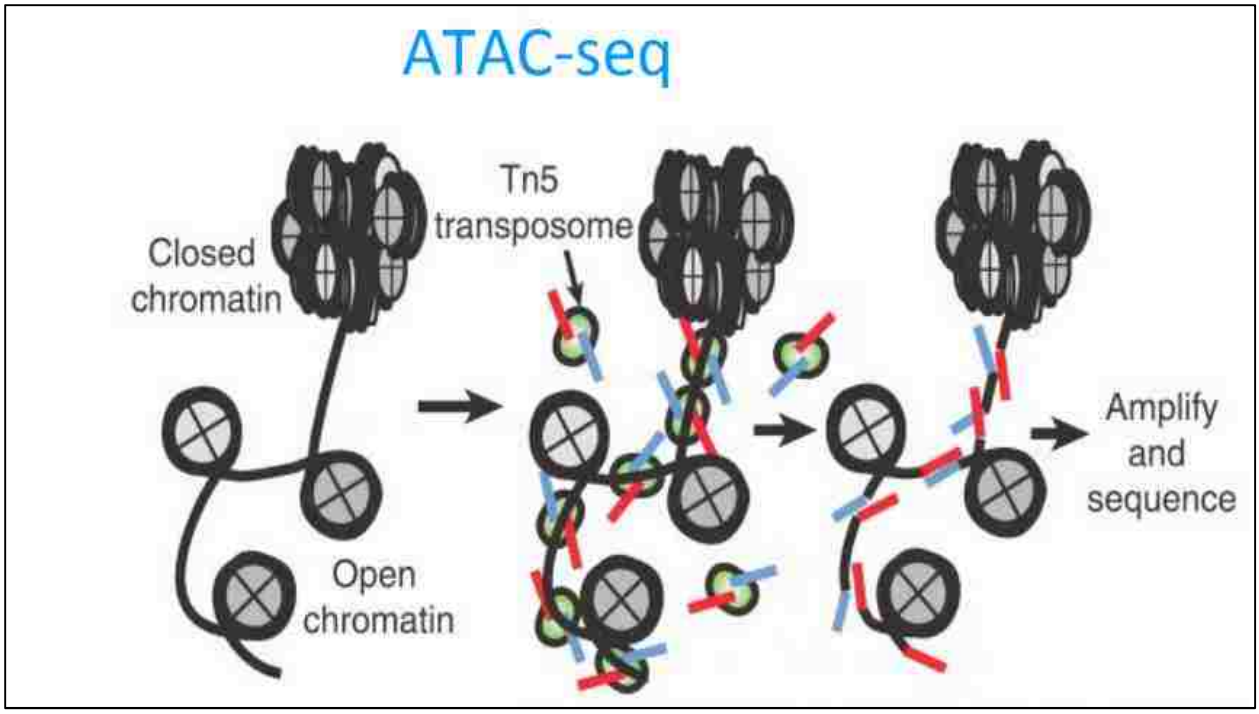


Figure 4. 2: Assaying accessible chromatin with Tn5 transposase. ATAC-seq (Assay for Transposase Accessible Chromatin with high-throughput sequencing), a method for mapping chromatin accessibility regions genome-wide. This method uses the hyperactive Tn5 transposase, which inserts sequencing adapters (red and blue) into open chromatin regions and generates sequencing library fragment followed by PCR amplification and sequencing. Adapted from *Buenrostro et al., 2015.*

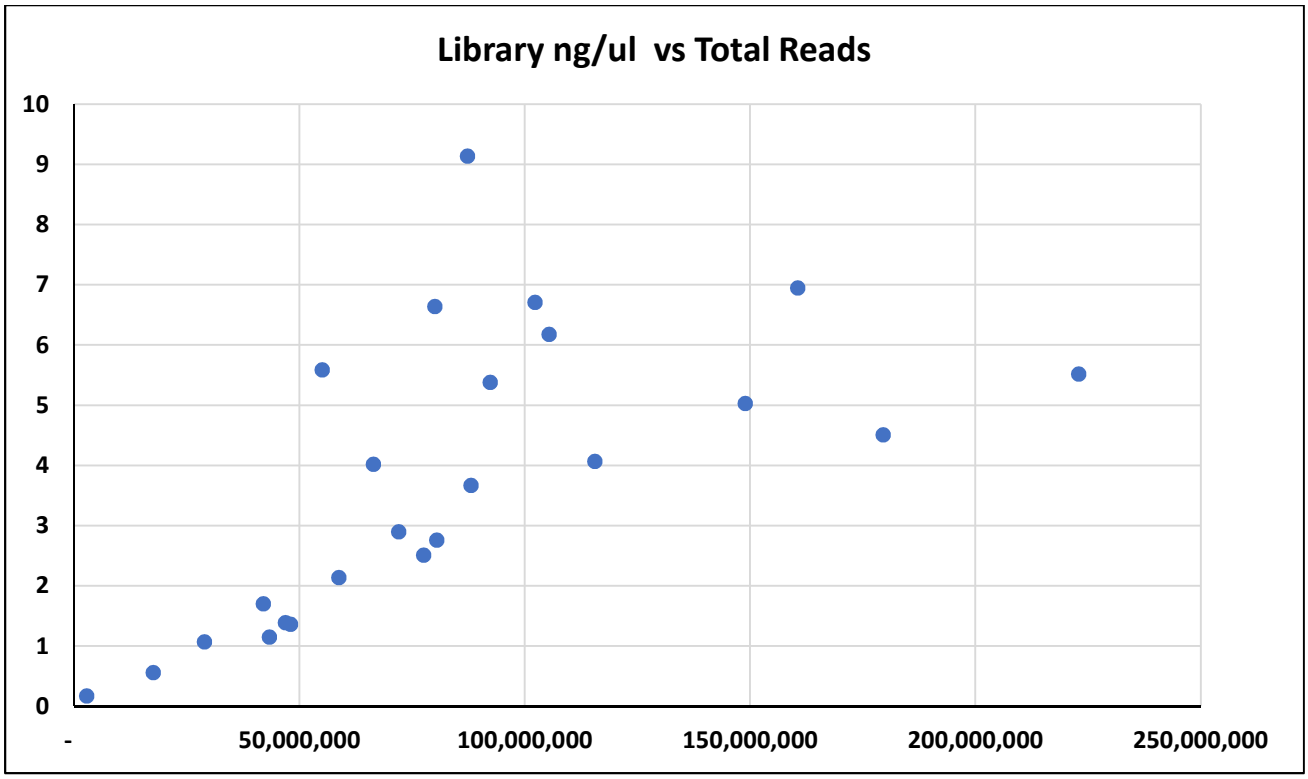


Figure 4. 3: Correlation between library concentration and total reads for each ATAC-seq library. Data from Table 1.

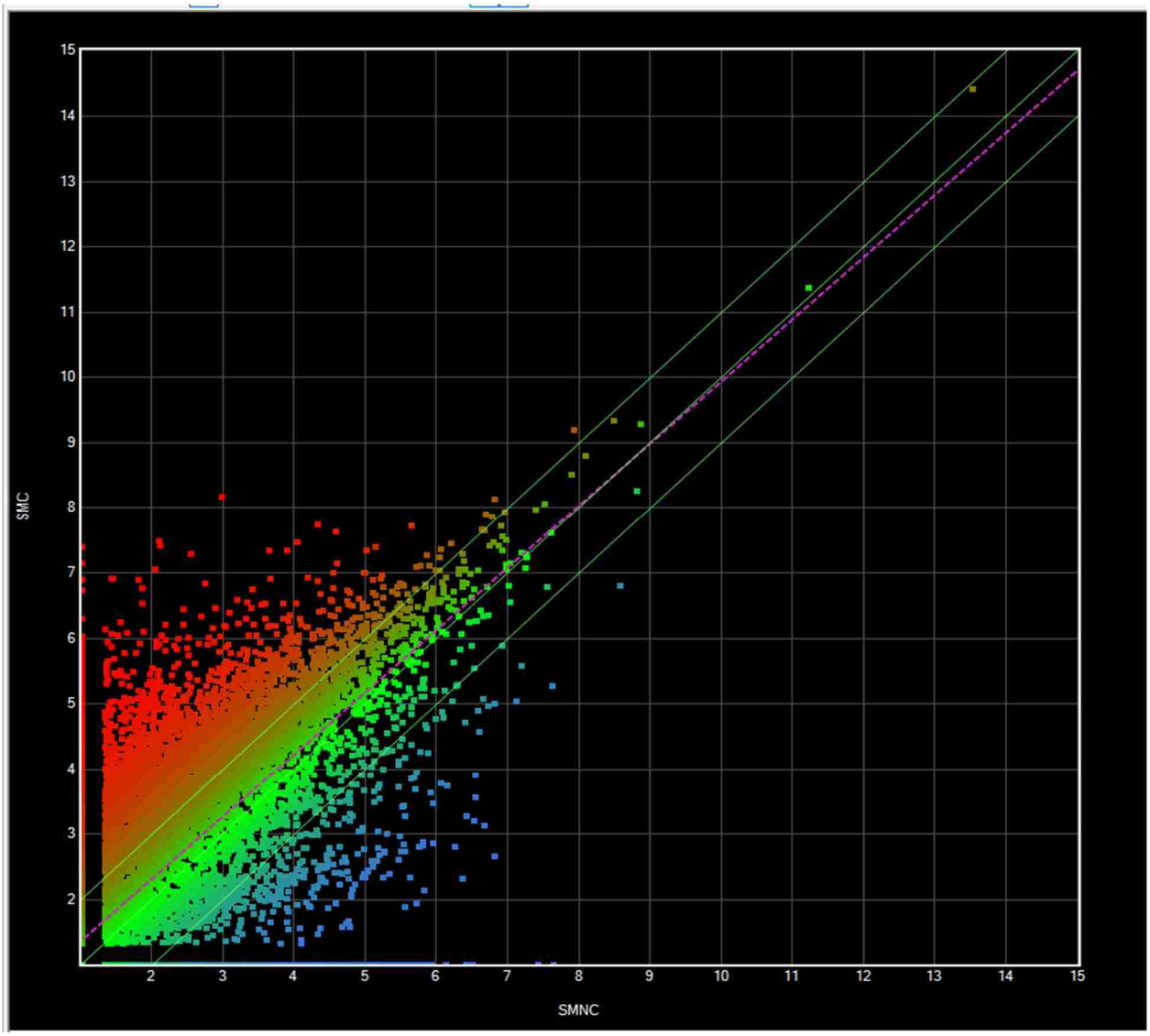


Figure 4. 4: Scatterplot of ATAC-seq reads demonstrating changes of chromatin accessibility in SMC vs SMNC where each dot represents one ATAC-Seq peak

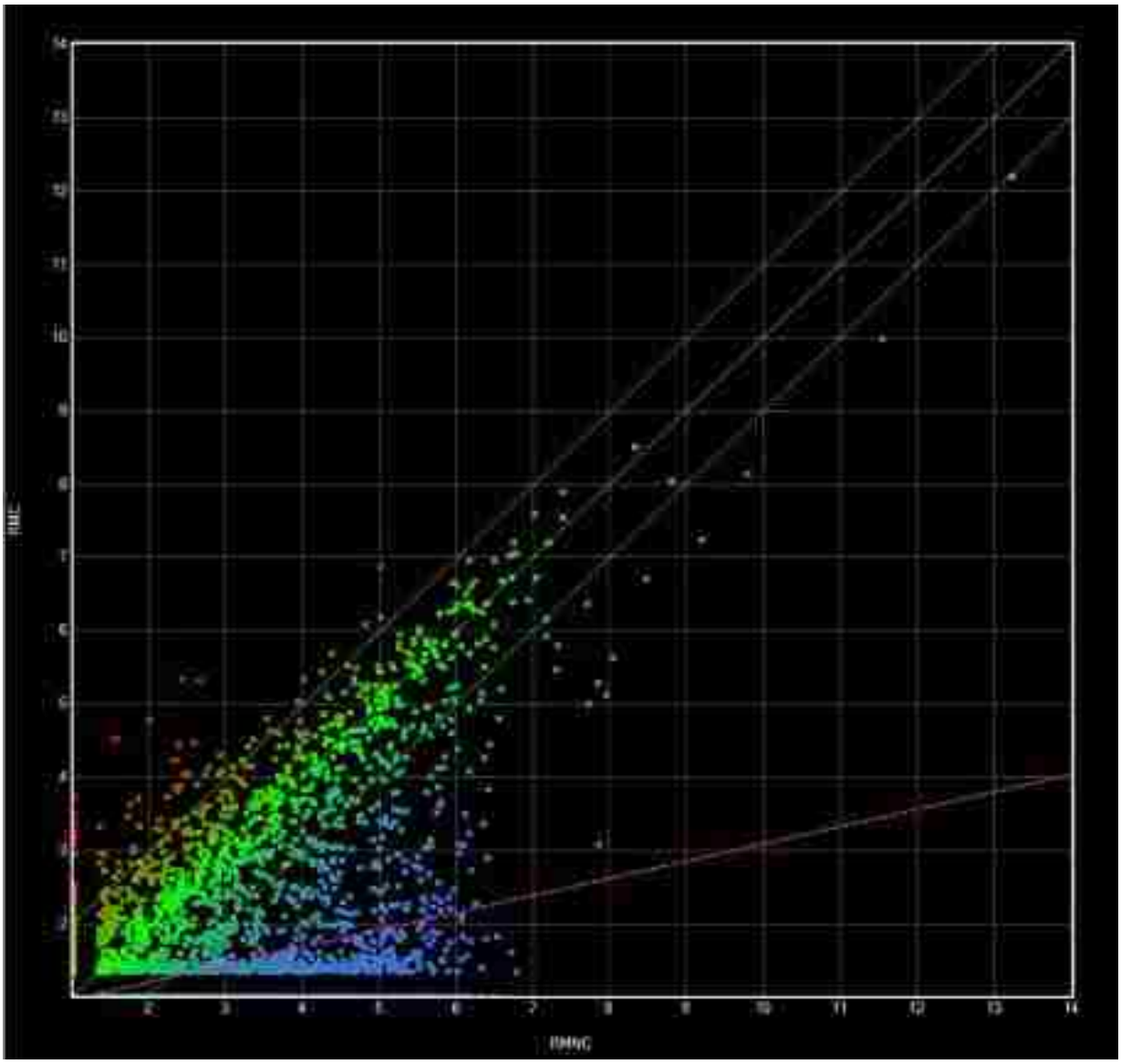


Figure 4. 5: Scatterplot of ATAC-seq reads demonstrating changes of chromatin accessibility in RMC vs RMNC where each dot represents one ATAC-Seq peak.

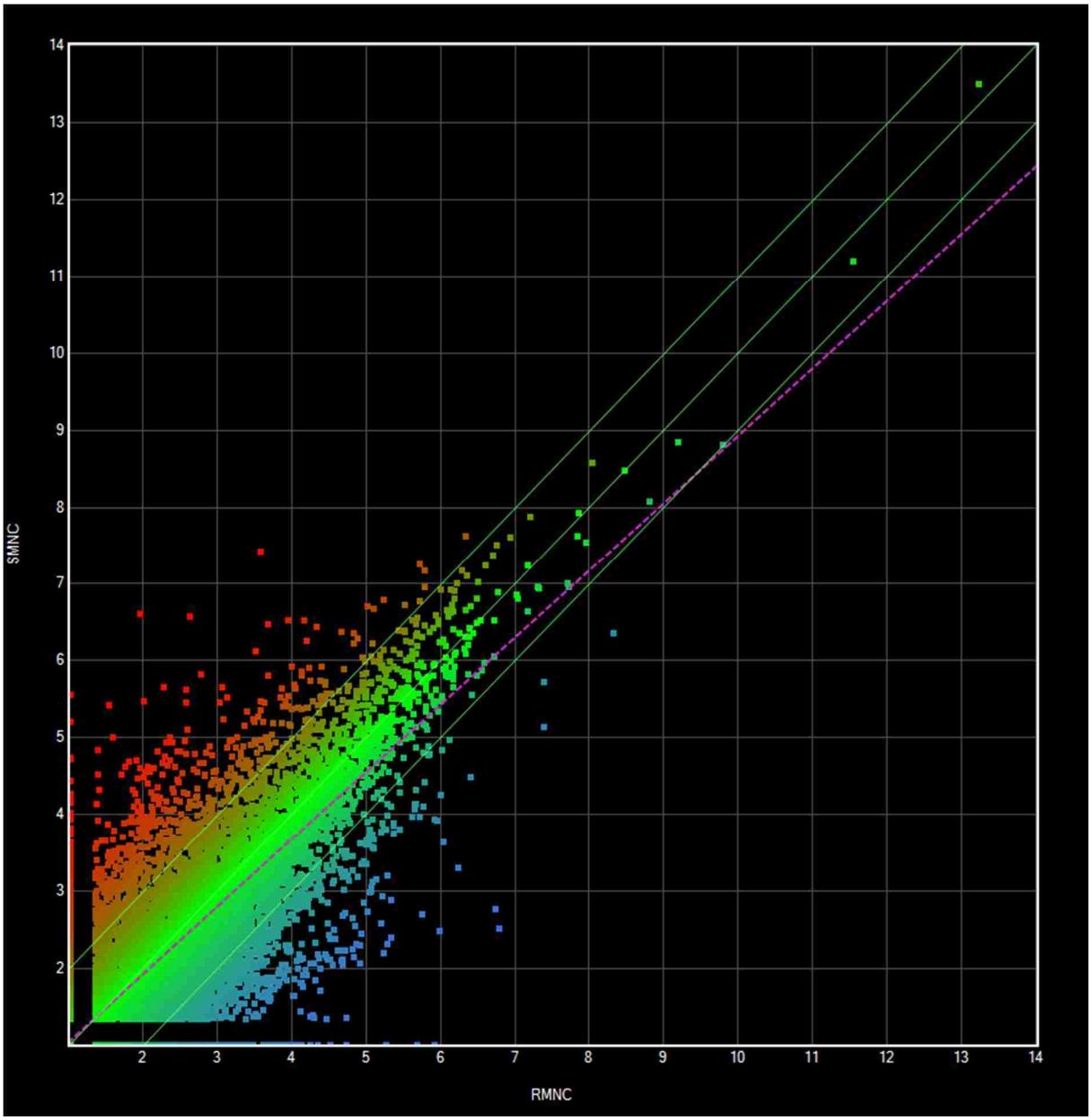


Figure 4. 6: Scatterplot of ATAC-seq reads demonstrating changes of chromatin accessibility in RMNC vs SMNC where each dot represents one ATAC-Seq peak.

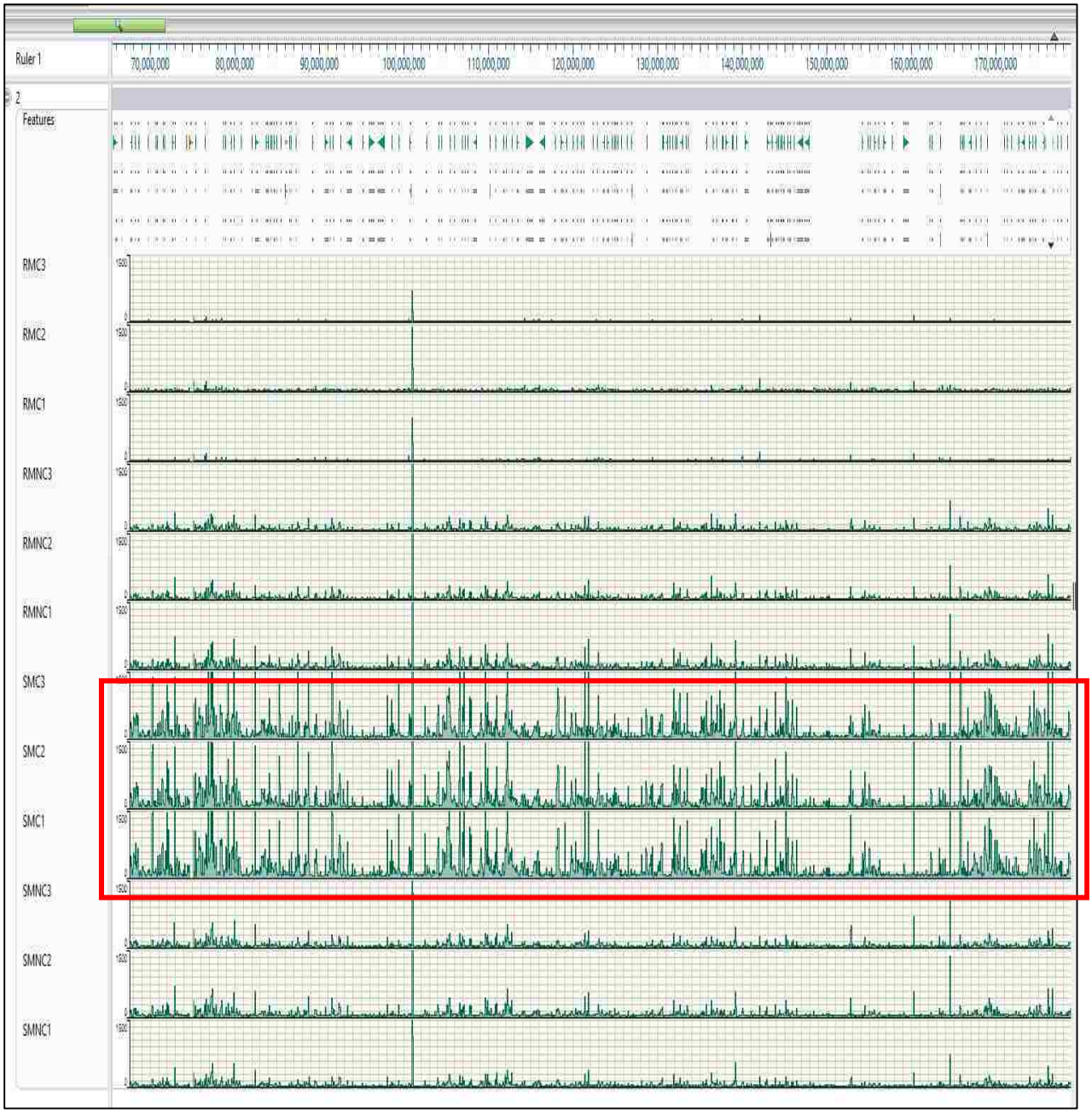


Figure 4. 7: Representative ATAC-seq differential open chromatin regions (peaks) among tested groups for a 100 MB window of Chr 1. ATAC-Seq tracks were visualized with the GenVision Pro tool (DNASTAR).

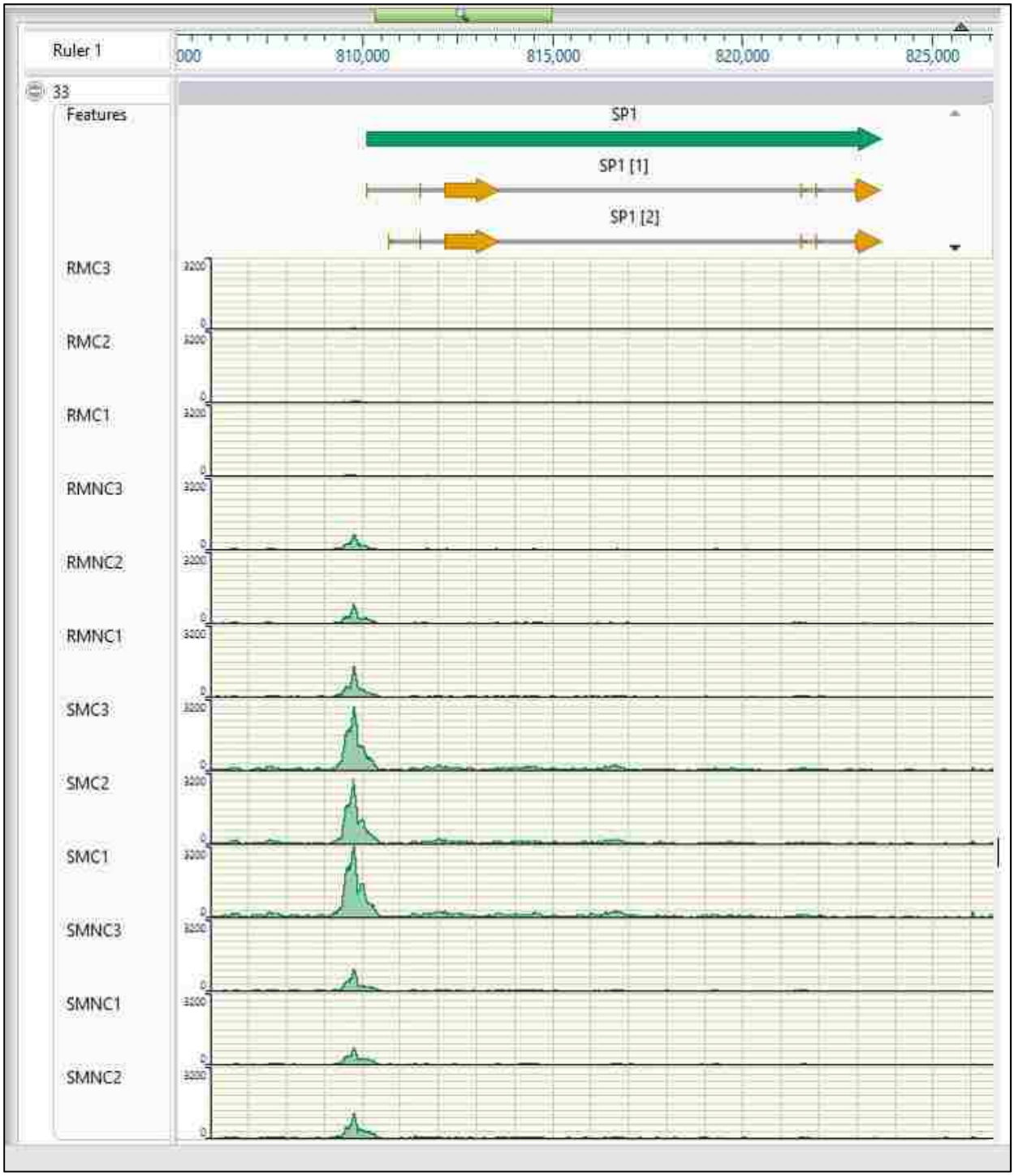


Figure 4. 8: Genomic region upstream TSS of the SP1 gene shows a sharp peak such as sequence-specific transcription factor binding site. The position of SP1 gene, direction of transcription, and exon locations are indicated.

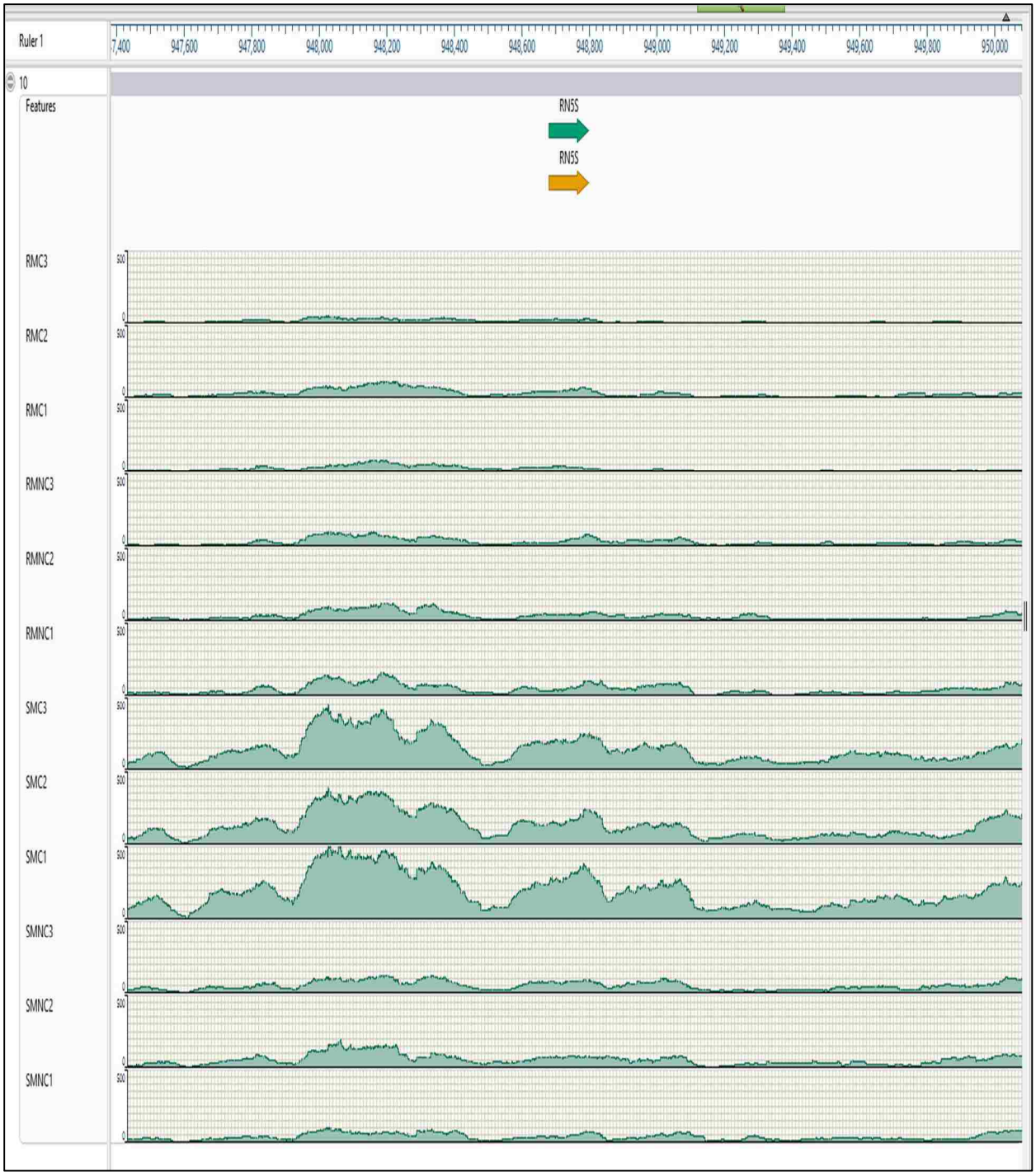


Figure 4. 9: Genomic region of RN5S gene shows a broad peak indicative of a large open chromatin region, possibly associated with histone modifications. The position of RN5S gene, direction of transcription, and exon locations are indicated.

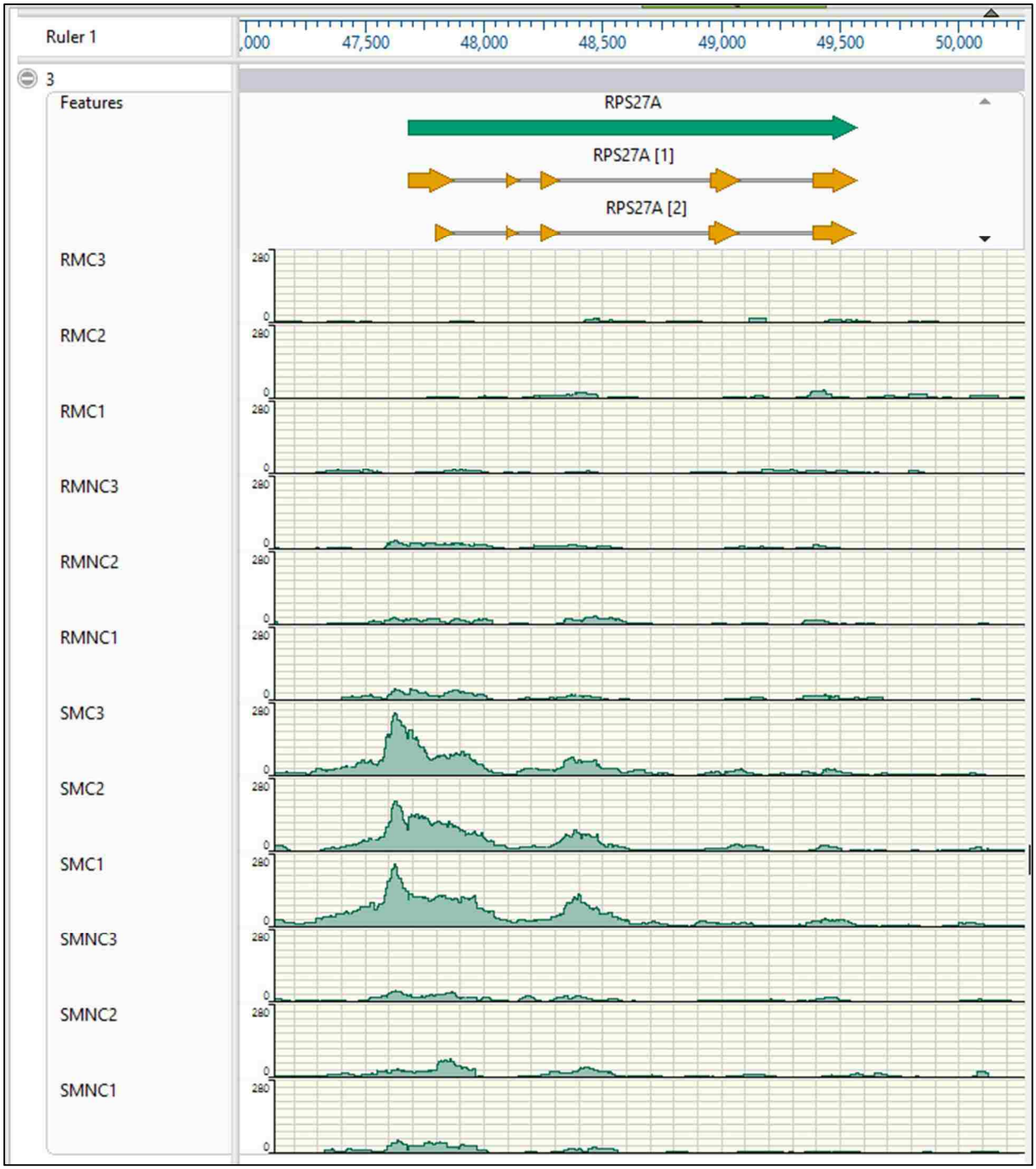


Figure 4. 10: Genomic region for RPS27A gene show overlapping/mixed peaks. The position of RPS27A gene, direction of transcription, and exon locations are indicated.

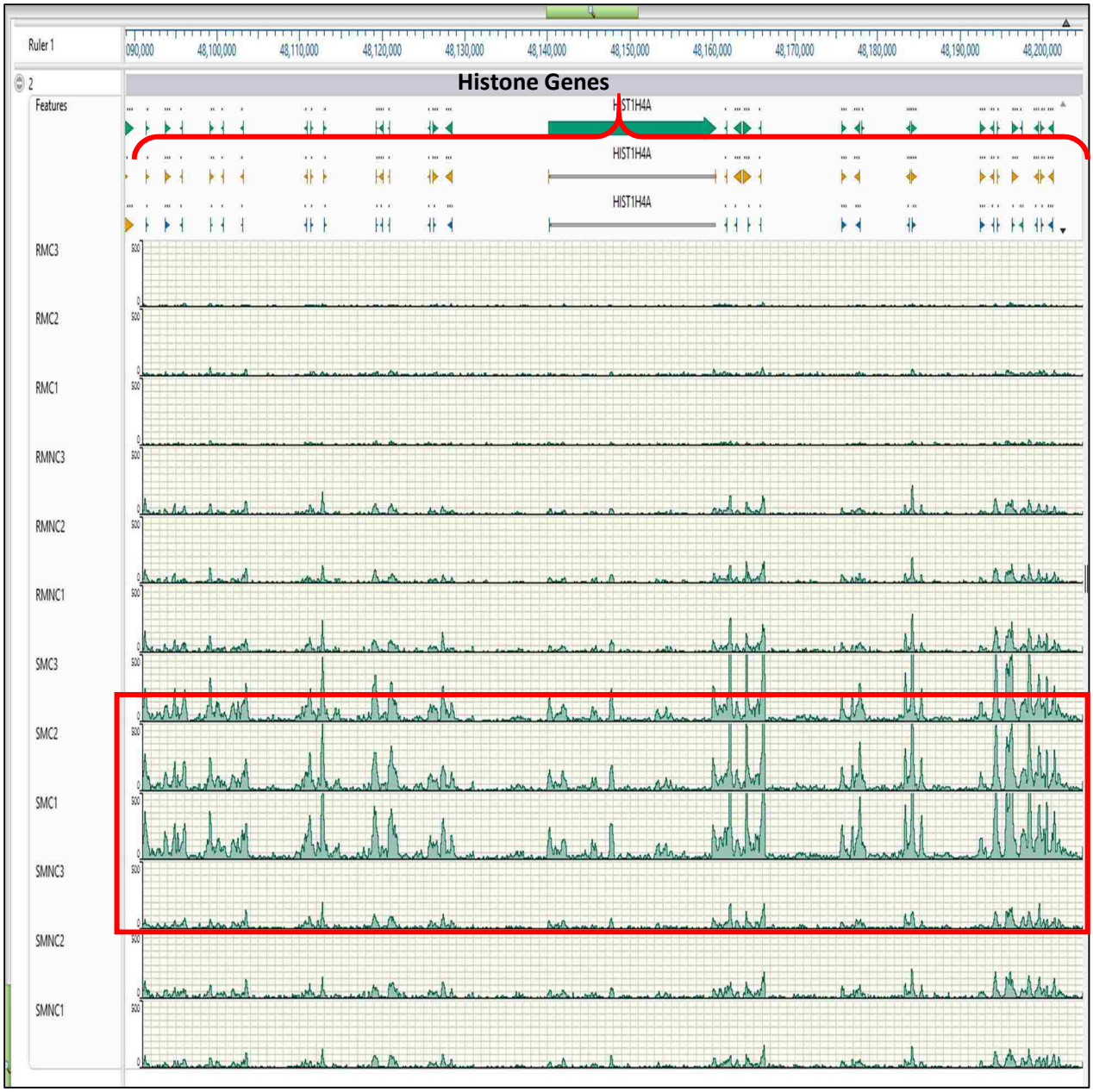


Figure 4. 5: Genome browser view of a large cluster of histone genes located on Chr1. ATAC-Seq tracks were visualized with the GenVision Pro tool (DNASTAR).

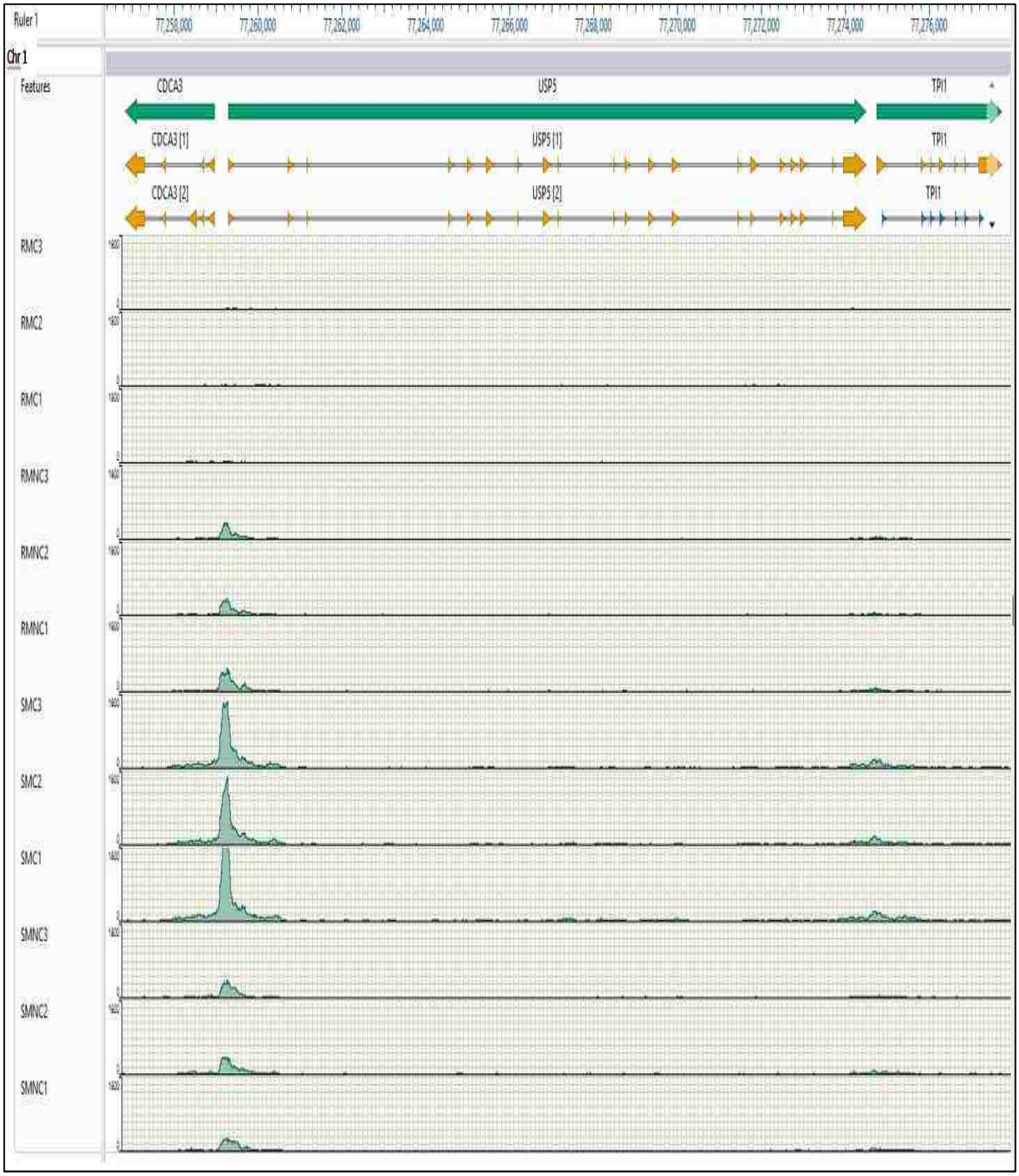


Figure 4. 6: Genomic region of USP5 and CDCA3 genes show an example of shared open chromatin site between the two genes. The positions of the two genes, direction of transcription, and exon locations are indicated.

CHAPTER 5

Summary & Conclusions

The increasing importance of chicken products has led to intensive selection on traits relevant to the consumer market such as rapid growth rate, higher feed conversion rate, and higher muscle mass. (Havenstein et al. 1994; Julian 2000; Decuypere et al., 2000; Zhao *et al*, 2004). This continues practice has brought different broiler than the broiler being marketed in the 1950s in the size and performance. However, selection for production traits has led to an increase in various metabolic disorders and diseases including pulmonary hypertension syndrome PHS, or ascites (Julian, 1998). PHS is not a disease but is a cascade of events caused by environmental, nutritional, genetic, physiological, and management factors all related to the need to ensure a high level of oxygen in the tissues (Decuypere *et al*., 2000; Balog, 2003; Julian, 2005). Although proper housing and management techniques can considerably reduce the incidence of PHS, the key to disease prevention lies in the availability of a genetic test for some of the major genetic components that confer resistance to PHS. Selection against the incidence of the PHS disease has not been implemented in commercial breeding since PHS was difficult and expensive to measure in practical breeding programs. Based on these considerations we attempted to investigate and elucidate some of the genetic and epigenetic components of the disease to gain a better understanding and identify major biological markers to reduce PHS incidence.

Our lab conducted several studies to map chromosomal regions contributing to PHS using large experimental broiler populations. One of the earliest GWAS used a F2 cross of the resistant (RES) and susceptible (SUS) lines had shown the association of a region on chromosome 9 (Gga9:15 Mbp) with PHS phenotype in broilers (Krishnamoorthy et al., 2014). HTR2B gene was then identified within this chromosomal region that might mediate the quantitative effect. Therefore, chapter 2 of this dissertation considered HTR2B gene as a potential candidate for contributing to the PHS disease due to their functional relevance with pulmonary hypertension especially in

human and mouse model as a potent vasoconstrictor (Ullmer *et al*., 1995; Launay *et al.,* 2002) However, our SNP and haplotype- based analyses results suggested that HTR2B polymorphisms are partially associated with PHS, but not a universal marker for genetic predisposition to PHS. Overall HTR2B gene expression was assessed and a heterozygous (T>C rs315854205) SNP located in the 3rd exon was also used as a marker to measure allele specific expression (ASE) in a variety of tissues at different developmental ages. We observed a statistically significant higher expression level of the non- reference C-allele vs the reference T-allele in hypoxic birds as they develop in age in all tissues when compared with younger birds. However, the overall expression of HTR2B was reduced. Therefore, we concluded that the downregulation of HTR2B expression as the bird develop in age might prevent the increase in pulmonary arteries pressure and attenuate blood vessels vasoconstriction. Our findings are important for understanding the mechanisms that underlie the patterns of HTR2B expression and its potential impact on the phenotypic variation of PHS syndrome in broilers.

Chapter 3 was an extended investigation to our previous survey of the association of mitochondrial prevalence in multiple tissues with ascites susceptibility and resistance in broilers. Previously we reported that for a small sample set of breast muscle at 22 weeks of age for RES and SUS males, the samples from SUS males had approximately twice the ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nucDNA), and that this difference correlated with a difference in the level of expression of *PPARGC1A*. Here, we further investigated this apparent difference and extended the analyses to both genders, multiple tissues, and different developmental stages. The mtDNA/nucDNA ratio in lung, liver, heart, thigh, and breast of both genders at 3, and 20 weeks old. At 3 weeks the mtDNA/nucDNA ratio is significantly higher in lung, breast, and thigh for susceptible line males compared to the resistant line males. Conversely, we see the

opposite for lung and breast in females. At 20 weeks of age the differences between males from the two lines is lost for lung, and thigh. Although there is a significant reduction in the mtDNA/nucDNA ratio of breast from 3 weeks to 20 weeks in the susceptible line males, the susceptible males remain higher than resistant line males for this specific tissue. Furthermore, the relative expression of five genes known to regulate mitochondrial biogenesis were assessed for lung, thigh and breast muscle from males and females of both lines with no consistent pattern to explain the marked gender and line differences for these tissues. Our results indicate clear sex differences in mitochondrial biogenesis establishing a strong association between the mtDNA quantity in a tissue-specific manner and correlated with ascites-phenotype. Therefore, we propose that mtDNA/nucDNA levels could serve as a potential predictive marker in breeding programs to reduce ascites incidence in poultry.

The aim of chapter 4 was designed to map genome-wide changes in chromatin accessibility associated with hypoxic relative to ambient conditions in broilers. The analysis used ATAC-seq technology (Assay for Transposase accessible Chromatin with high-throughput sequencing) to identify changes in promoter accessibility for pulmonary artery tissue in PHS- susceptible and PHS- resistant lines. Our initial analysis identified a total of 23,444 open chromatin regions (or peaks) across all pulmonary artery samples. We further identified 1324 regions that become differentially accessible within 2 kb of transcription start sites. Tissue-specific chromatin activity was captured not only in the regulatory regions such as promoters, and enhancers, but also gene regions including exonic, intronic, and intergenic regions. There was a substantial increase in the chromatin accessibility throughout the genome of ascites- susceptible birds when challenged under hypoxic conditions in comparison with controls. Conversely, we observed reduced changes in chromatin accessibility regions in ascites-resistant birds when challenged. In conclusion, we

showed that chromatin accessibility is a key epigenetic factor influencing transcriptional regulation and a straightforward approach to identify functional genomic regulatory regions controlling complex diseases such as ascites in birds.

The findings described in this dissertation could provide important clues to discern the genetics and epigenetics of PHS in broilers. Global poultry meat production is rapidly increasing with the continues growth in the world population. In fact, global poultry production is expected to grow at 2.4% per year over the next 20 years (Services, 2013). Therefore, efficient poultry production will be possible by better balance in selection programmed between welfare and broiler production traits to minimize the losses afflicted by disease-related traits. We know now that understanding the genetic aspect of the trait controlling the incidence of the disease is not enough. There will be a need to better understand how the interactions of genes, proteins, mechanisms, and the external environment to produce the phenotype of an animal. The results from our studies not only unravel essential components of ascites metabolic, genomics and epigenomics aspects in broilers but may also have some usefulness in pulmonary hypertension in humans.

REFERENCES

Balog, J.M. (2003). Ascites syndrome (pulmonary hypertension syndrome) in broiler chickens: are we seeing the light at the end of the tunnel? *Avian & Poultry Biology Reviews*, 14(3), 99126.

Decuypere, E., Buyse, J., & Buys, N. (2000). Ascites in broiler chickens: exogenous and endogenous structural and functional causal factors. *World's poultry science journal*, 56(04), 367377.

Havenstein, G. B., Ferket, P. R., Scheideler, S. E., & Larson, B. T. (1994). Growth, livability, and feed conversion of 1957 vs 1991 broilers when fed "typical" 1957 and 1991 broiler diets. *Poultry Science*, 73(12), 1785-1794.

Julian, R.J. (2000). Physiological, management and environmental triggers of the ascites syndrome: a review. *Avian Pathology: Journal of the W.V.P.A*, *29*(6), 519–527.

Julian, R.J. (2005). Production and growth-related disorders and other metabolic diseases of poultry*A review. *The Veterinary Journal*, 169, 350369

Krishnamoorthy, S., Smith, C.D., Al-Rubaye, A.A., Erf, G.F., Wideman, R.F., Anthony, N.B., & Rhoads, D.D. (2014). A quantitative trait locus for ascites on chromosome 9 in broiler chicken lines. *Poultry science*, 93(2), 307-317.

Launay, J.-M., Hervé, P., Peoc'h, K., Tournois, C., Callebert, J., Nebigil, C. G., Etienne, N., Drouet, L., Humbert, M., Simonneau, G., & Maroteaux, L. (2002). Function of the serotonin 5-hydroxytryptamine 2B receptor in pulmonary hypertension. *Nature Medicine*, 8, 1129.

Services, R. (2013). Study of the impact of genetic selection on welfare of chicken bred and kept for meat production. *European Commission.*

Ullmer, C., Schmuck, K., Kalkman, H.O., & Lübbert, H. (1995). Expression of serotonin receptor mRNAs in blood vessels. *FEBS Letters*, 370(3), 215–21.

Zhao, R., Muehlbauer, E., Decuypere, E., & Grossmann, R. (2004). Effect of genotype–nutrition interaction on growth and somatotropic gene expression in the chicken. *General and Comparative Endocrinology,* 136:2–11.

CHAPTER 6

Appendix

All animal work was through approved IACUC protocols for Dr. Nicholas Anthony. Any collections by Khaloud Al-Zahrani were tissues from deceased animals from the approved protocols (15039 and 15040).

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Nicholas Anthony

FROM: Craig N. Coon, Chairman

DATE: Apr 3, 2015

SUBJECT: IACUC Approval

Expiration Date: Apr 5, 2018

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your Protocol: 15039 "General Rearing of Selected Chicken and Quail Populations" to begin April 6, 2015

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond Apr 5, 2018 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Nicholas Anthony

FROM: Craig N. Coon, Chairman

DATE: Apr 8, 2015

SUBJECT: IACUC Approval

Expiration Date: Apr 8, 2018

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your Protocol: 15040 Utilization of Hypobaric Hypoxia to induce Ascities in Broiler Chickens. The start date is listed as April 9, 2015.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond Apr 8, 2018 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian