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Chasing the Genetics of Ascites in Broilers using Whole Genome Resequencing

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Chasing the Genetics of Ascites in Broilers using Whole Genome Resequencing

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by

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ABSTRACT

We are using whole genome resequencing to identify chromosomal regions associated with resistance or susceptibility to ascites, a form of pulmonary hypertension syndrome, meat-type chickens. Previous Genome Wide Association Studies (GWAS) based on Single Nucleotide Polymorphisms (SNPs) have identified regions on chromosomes 2, 9 and Z. Despite several GWAS and further genotyping, there are no reliable or potential markers for ascites phenotype. We have completed screening of Copy Number Variations (CNVs) and Single Nucleotide Polymorphisms in ascites resistant and susceptible birds from the relaxed, REL, line derived from a commercial elite broiler line. DNA samples from resistant and susceptible birds were purified, quantified and pooled in two pools of 10 DNAs from each phenotype for both genders. Eight pools (2 pools x 2 phenotypes x 2 genders) were generated. Each pool was submitted for bar-coded library generation, and 2x125 paired end reads on Illumina HiSeq 2500 and with 66X genome coverage. The sequence reads were mapped onto Galgal5 using Bowtie for initial CNV mapping cn.mops (R package). Further mapping to chromosomes were done using NGen and ArrayStar (DNAStar ver 13). So far, we have identified two potential regions for CNVs and 31 regions for SNPs with potential association with ascites phenotype. CPQ gene on chromosome 2 and LRRTM4 gene on chromosome 22 have been validated for containing ascites QTLs. However, their exact role in ascites is yet to be discovered. Further, we screened the regions from REL line in DNAs from an unrelated commercial broiler line using WGR.

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It is my great pleasure and honor to acknowledge those who made this dissertation complete on time. My humble gratitude to my advisor, Prof. Dr. Douglas D. Rhoads (Director, CEMB graduate program), Biological Science Department for his incomparable and round the clock supervision during my research and the final documentation of my dissertation. This work would have never been possible without his constant guidance, and knowledgeable support. His continuous advising has helped me equip myself with research tools and work harder. Most of all, my gratitude for being a fatherly figure from the first day we met and for becoming a source of utmost care and protection.

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I cannot ever forget the opportunities and exposure that I was given at the Department of Biological Sciences during my PhD and will always remain obliged to this institution, the faculty members especially Dr. Ines Pinto, and the whole staff in SCEN 601. My sincere thanks to Dr. Nicholas Anthony and his team for maintaining the ascites research lines at the farm and providing us the samples.

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DEDICATION

To my beloved parents,

My father, a source of knowledge and hard work

My mother, a source of strength and determination

To my brothers,

Muhammad Jamil, a source of utmost care

Muhammad Tariq, a source of inspiration

Muhammad Wasim, a source of passion

To my sister,

Fozia, a source of zeal and zest

And to myephews and niece,

Sinan, a source of happiness

Majeed, a source of love

Nabeera, a source of hope

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CHAPTER 1. LITERATURE REVIEW

1.1 Ancestry of Modern Broiler

Chicken serves as a stable source of protein (meat and egg) for humans worldwide (Marangoni et al., 2015). The archaeological data proposes that chicken domestication started as early as 5400 BC (7,000-10,000 years ago) in southern China or Southeast Asia (West and Zhou, 1988; Crawford, 1990). Modern chicken's mitochondrial DNA (mtDNA) sequence-based phylogenetic tree construct revealed wild red junglefowl (Gallus gallus) to be the primary ancestor (Fumihito et al., 1994, 1996; Liu et al., 2006; Kanginakudru et al., 2008). Chicken domestication became popular in Southeast and South Asian countries over time (Granevitze et al., 2009). The early domestication of chicken was for food, decoration, cultural and religious purposes in Southeast Asia, the Middle East and Africa. From the early 1900s, chickens became important for specific traits selection (meat and egg production) and as an experimental model to study human diseases in Europe and the United States.

1.2 Poultry Industry Commercialization

The poultry industry continues to proliferate, and per capita consumption of chicken in the US has risen from 30 to 90 pounds in the last 50 years (Figure 1.1). However, beef consumption over the same time has fallen slightly with pork and turkey remaining steady (Meyer and Steiner, 2015). The combined production from broilers, eggs, turkeys, and chickens from sales in 2016 was $38.7 billion (US Poultry and Egg Association, 2016).

The evolutionary process of large-scale commercialization of the poultry industry began around 1950. Until that time, the chicken was a dual-purpose animal where meat production and reproduction were complementary traits. However, the realization of these two traits being non-

complementary led to the foundation of the modern poultry industry separating the egg and meat-type birds.

Since then, quantitative genetics has been applied to improve the growth rate, meat yield, and feed conversion by top broiler breeding companies. Significant improvement has been observed in terms of body weight with time. The bodyweight of a 42 days old bird from 1950 was 539g as compared to a modern broiler of the same age weighing ~3000g (Havenstein et al., 2003). Genetic improvements resulted in modern broilers twice the live weight on 50% less feed when compared to their performance in 1953 (Havenstein et al., 1994, 2003). Better control of diseases and improved husbandry also decreased the mortality rate over the years (Flock et al., 2005). All these improvements have resulted in negative attributes in the poultry industry over the last 30 years. Ascites syndrome (Julian, 2000), bone deformation (Julian, 1998; Cook, 2000), fat deposition (Anthony, 1998; Barbut, 1997, 1998), abnormalities in immune system (Leshchinsky and Klasing, 2001; Koenen et al., 2002; Parmentier et al., 2012) and reproductive inefficiency (Qureshi and Havenstein, 1994; Emmerson, 1997; Anthony, 1998; Julian, 1998; Cheema et al., 2003) are just a few examples of the consequences of divergent growth rates that contribute to economic losses in poultry industry.

1.2.1 Schematics of Broiler Breeding

Trap-nest and artificial incubator are two key inventions that have led to successful poultry breeding. The breeding industry uses a pyramidal breeding structure for genetic progress in addition to quantitative genetics (Figure 1.2). The pyramid scheme of breeding has resulted in the desirable production of lines with a 300% increase in the rate of growth since the 1950s (Paxton et al., 2010). Each level of the pyramid represents one generation (Figure 1.2), and at the fifth generation, approximately 50 m broilers are generated from a base unit of 1 male and ten females

(Flock et al., 2007; Laughlin, 2007). The top represents the elite, pedigree line (great-great-grandparents) where genetic progress has been achieved. A pedigree stock is maintained by breeding the selected birds, and unselected pedigree is moved down to the great grandparents (GGP) category. GPPs are mated to produce grandparent (GP) stock. Parent birds are mated to produce a commercial broiler, which is then sold out to industries that are producing consumer products such as eggs, and meat.

The pedigree level consists of two lines from each gender (Figure 1.3). All four lines from the unselected pedigree are used as GGP to produce GP. GP level (first hybrid) are then mated to generate genetic heterosis of parent stock (second hybrid). Heterosis production means that specific traits are improved, and recessive genes are prevented from affecting the offspring. The crossing of parent birds results in a double hybrid commercial stock (Figure 1.3). Since pedigree lines are crossed, companies buying parent birds cannot recreate the pure line, and pedigree is preserved.

1.3 Chicken Genome Project

The chicken genome is made up of 38 pairs of autosomes and one pair of sex chromosomes, where the female is heterogametic (ZZ male, ZW female). The autosomes include eight pairs of macrochromosomes and 30 pairs of microchromosomes (Smith et al., 2000; International Chicken Genome Consortium, 2015). The total length of chicken DNA is 1230.26 Mb, about one-third of the size of the human genome (Hillier et al., 2004; Schumtz and Grimwood, 2004) while the size of mitochondrial DNA (mtDNA) is 16,775bp (Desjardins and Morais, 1990). The chicken genetic linkage map includes 4,000 cm of total length (Groenen et al., 2000; Wang et al., 2005). Comparative analysis of chicken and human genomes has revealed around 70Mb of highly conserved segments (Burt et al., 1998; Suchyta et al., 2001). Bacterial artificial chromosome

(BAC) based library generation has contributed to the assembly of sequences along the chicken genome with excellent genome coverage (Wallis et al., 2004). Development of quantitative trait loci (QTLs) is essential for a better understanding of variations in phenotypic traits (Hocking, 2005). QTLs refer to genetic loci on a chromosome(s) in which allelic variation has a statistical association with observed phenotypes (Grisel, 2000). Integration of genetic and physical mapping has delineated macrochromosomal properties along with genomic size and distribution of chromosomes (Ladjali-Muhammad et al., 1999; Smith et al., 2000). In recent years, genome-wide association studies (GWAS) and whole-genome sequencing (WGS) have not only decrypted phylogeny, complex traits for selection, and conserved regions but also allowed the accurate QTL mapping of commercially essential features (Hillier et al., 2004; Wong et al., 2004; Rubin et al., 2010).

1.4 Genetic Markers Based Selection in Poultry

Identification of QTLs and genes that are associated with a particular phenotype in a genome is like finding a needle in a haystack. A significant number of research studies have been conducted to associate genotype with phenotypes for economically important traits in the poultry industry (Bumstead et al., 1994; Lamont et al., 1996; Smith et al., 1997). Leveque et al. (2003) found that Toll-like receptor 4 (TLR4) has an association with *Salmonella enterica* serovar Typhimurium infection. Decuypree et al. (2000) reported that genetically modern broilers, especially male broilers, are more prone to develop ascites.

1.4.1 Genome-Wide Association Studies in Chicken

Genome-wide association studies (GWAS) scan markers for a set of genomes or DNAs (case-control design) to find genetic variations related to a disease of interest. Purified DNAs from blood or tissues placed on chips are measured on lab automated machines, were strategically selected

markers of genetic changes or single nucleotide polymorphisms (SNPs) are identified. SNPs with high frequencies in disease samples have a potential association with the disease. However, these might not be causative; therefore, sequencing of the affected region can determine the exact change associated with the phenotype.

In many GWAS, SNPs have been successfully linked with disease-specific phenotypes and used as biomarkers for respective diseases (Wang et al., 2006; Sheng et al., 2013; Subedi et al., 2013). SNP is a single nucleotide change at a position in the genomic DNA in which at least one alternative exists. Klein et al. (2005) had first successful GWAS where two SNPs were found to be associated with age-related macular degeneration in humans. Since then, GWAS was conducted in many other organisms, including chicken. Meat yield and quality, growth, fertility, fat reduction, and egg production, etc., are some of the traits, where GWAS had gained popularity for QTL mapping.

Gu et al. (2011) used GWAS in an F2 cross between White Plymouth Rock and Silky Fowl to identify SNPs in a region on chromosome 4 which included LIM domain-binding factor 2 (LDB2) gene, were associated with late growth. Also, SNPs in a few genes on chromosome 1 and 18 showed significant effects on body weight at different stages (Gu et al., 2011). Another GWAS proposed four candidate genes on a region on chromosome 4 associated with carcass weight (Liu et al., 2013). These genes included lamin B receptor 2 (LBR2), leucine aminopeptidase 3 (LAP3), ligand-dependent nuclear receptor corepressor-like (LCORL) and transmembrane anterior-posterior transformation 1 (TAPT1). Sun et al. (2013) were able to identify 14 candidate genes for meat quality traits in Chinese chicken breed (Beijing-You) and a broiler line (Cobb-Vantress). The meat quality traits included the colors of meat and skin, dry matter in breast and thigh muscles, intramuscular fat content in breast muscle (IMFBr), abdominal fat percentage and weight (Sun et

al., 2013). GWA studies also found SNP in growth factor receptor-bound protein 14 (GRB14) gene that might influence the number of eggs (Liu et al., 2011).

Next-generation sequencing studies have revealed another type of structural variation called copy number variation (CNV) that can have a significant impact on phenotypes. CNVs are genomic polymorphisms where there is duplication or deletion of a segment of DNA extending from 1000 bases to several million in length. There is evidence of variation in copy numbers associated with the number of infectious and genetic diseases in humans (Clop et al., 2012; Hollox and Hoh, 2014).

1.5 Marker-Based Selection in Poultry

Marker-based selection for commercially important traits is a primary focus in animal and plant breeding industries. SNPs and CNVs that tend to show high association with the trait/disease of interest can be part of marker-assisted selection (MAS) in the poultry breeding. MAS will play a huge role in selecting traits that are difficult and expensive to measure or with low heritability and late expression in the development of a broiler. However, some diseases can be difficult to work within animals because of the continuous rise in the cost of growth and maintenance while the cost of genotyping is steadily decreasing. Even though a variety of potential markers for important traits have been identified in poultry science, the use of candidate markers still seems to be limited regarding marker-based selection for many reasons.

1.6 Introduction to pulmonary hypertension or ascites syndrome

Ascites syndrome develops because of the inability of the heart and lungs to deliver oxygen to tissues because of high oxygen (O_2) requirement during rapid growth. Ascites syndrome, pulmonary arterial hypertension (PAH), and pulmonary hypertension syndrome (PHS) are largely interchangeable (Julian, 1998; Owen et al., 1994; Wideman et al., 2007). Ascites syndrome has

been extensively studied and is considered a progressive disease that initiates with pulmonary hypertension and ends with the failure of the right ventricle (Julian, 1993, 2000; Scheele, 1996; Decuypere et al., 2000; Wideman, 2000).

Ascites has a significant impact on the world's economy since the incidence has been reported in all major poultry-producing countries. Ascites costs in 1993 were estimated at $100 million per year in the US poultry industry alone (Odom, 1993). In 2003, it was estimated that about 8% of the total broiler mortality per year could be attributed to ascites (Anthony and Balog, 2003).

Birds with ascites can be identified by the presence of fluid in their abdominal cavity (water belly), enlarged atonic heart, and frequent liver changes. During the rapid growth, O_2 requirement stimulates cardiac output, which elevates vascular pressure in the lungs, creating high pressure on the wall on the right ventricle (RV). The muscular ventricle thickens its wall (hypertrophy) to cope with this pressure, which further elevates pressure on pulmonary arteries and lung capillaries. The muscular right atrioventricular (AV) valve also experiences hypertrophic thickening. Failure of right AV causes cardiac output and decline in hypertension, but rising pressure in right arteria, vena cava, sinus venosus, and portal vein (Chapman and Wideman, 2001). Liver capillaries with increased pressure cause leakage of plasma into the hematoperitoneum space (Wideman, 2000). Production of erythrocytes in response to tissue hypoxia leads to an increase in blood viscosity and volume, thus worsening the pressure overload (Lubritz and McPherson, 1994). Hypoxia in heart muscles finally ends up causing RV failure.

Selection based on fast growth rate and meat yield has made broilers an ideal model organism for tracking the genetics of spontaneous idiopathic Pulmonary Arteriole Hypertension (IPAH) in humans. IPAH is a sub-group of pulmonary arteriole hypertension (PAH) in humans characterized by an increase in pulmonary arterial pressure right ventricular (RV) hypertrophy and ultimate

failure of the left ventricle. Around 3% of the birds can have spontaneous incidences of IPAH, which can exceed 20% upon changing the environmental conditions. Environmental and genetic factors both can contribute to ascites (Lubritz et al., 1995; Julian, 2000; Anthony et al., 2001) and intense genetic selection for meat yield, rapid growth, feed conversion, and body weight has contributed to this physiological problem in modern broiler populations.

1.6.1 Ascites Induction Methods

In order to study ascites in broilers, model lines were created and maintained because susceptibility and resistance status of birds cannot be determined unless they are challenged with the disease. Physical manipulation includes clamping of one pulmonary artery (Wideman et al., 1995; 1997), injection of micro-particles (Wideman and Erf, 2002). Environmental manipulation consists of cold stress (Wideman et al., 1995, Deaton et al., 1996; Shlosberg et al., 1996), dietary supplementation (Decuypere et al., 1994; Hassanzadeh, 1997), lighting, air quality, and high altitude (Smith et al., 1954, 1956). Ascites syndrome was first noticed in flocks grown at high altitude by Smith et al. (1954). Since then, researchers have used different ways to mimic high altitude environment for induction of ascites in birds (Burton and Smith, 1969; Jones, 1995). With advancement in the technologies, hypobaric chambers were developed which operated under partial vacuum to decrease partial pressure of oxygen, mimicking the natural high altitude (Mirsalimi et al., 1993; Owen et al., 1995, Balog et al., 2000; Anthony et al., 2001; Pavlidis et al., 2007). Hypobaric chamber (Figure 1.4) method was used to create the ascites experimental research lines used in my dissertation work.

1.6.2 Ascites Experimental Research Line Development

Divergent selection is a very successful method for ascites selection. Dr. Anthony at the University of Arkansas and his associates started an ascites selection program in 1994. Birds from a

commercial pedigree elite line (REL) were exposed to hypobaric hypoxia at 2,900 m (9,500 ft) above sea level by operating under a partial vacuum. The pedigree line went under one generation of relaxed selection before becoming the base population for ascites selection (Pavlidis et al., 2007). Based on generated mortality data, siblings were selected for breeding and to reproduce experimental lines after each generation. A rapid response was observed in broilers upon selection for ascites-resistance (RES) and ascites-susceptible (SUS) lines from the founder line, which had a 75.3% incidence of ascites (Figure 1.5). For the SUS line, 95.1% incidence of ascites was recorded in generation 8, while the ascites incidence in the RES line was about 7.1% in generation 9 (Figure 1.5).

1.6.3 Markers Associated with Ascites

Previous studies have predicted the action of 1 or 2 major genes with the incidence of ascites in broilers (Lubritz and McPherson, 1994; Wideman and French, 2000; Anthony and Balog, 2003; Navarro et al., 2006; Druyan et al., 2007). A genome-wide SNP panel also identified at least seven regions on chromosome 1, 9, 27, and Z (Krishnamoorthy et al., 2014). This project included 183 F_2 birds (SUS and RES line double reciprocal crosses) from the University of Arkansas. Statistical analyses showed a significant association between the phenotype and Gga 9 regions (11.9-13.6 and 15.5-16.3 Mbp) primarily affecting females. The potential candidate genes in these regions were Angiotensin II Type 1 Receptor (AGTR1), Urotensin 2 Domain Containing protein (UTS2D) and Serotonin Receptor/Transporter type 2B (5HT2B) with implications in some aspects of human and/or mice pulmonary hypertension (Watanabe et al., 2006; Djordjevic and Gorlach, 2007; MacLean, 2007; Chung et al., 2009). Another study has also identified regions on chromosomes 2, 5, 10, 27, and 28 in association with ascites (Rabie et al., 2005). Krishnamoorthy et al. (2014) also found that the region around 12-13 Mbp on chromosome 9 showed a strong association with

both ascites and hypertrophy in females. Two microsatellite markers or variable nucleotide tandem repeats (VNTRs) designated as PHS009 and PHS010 genotyping in three experimental research lines also revealed a significant correlation with ascites in the Gga9-13 region where the AGTR1 gene is also located. Dey et al. (2016) further conducted multi-general GWAS on chromosomes 2 and Z regions indicated in previous studies with no significant results. They instead, found a region around 70 Mbp on chromosome 2 associated with resistance in male broilers containing two candidate genes, melanocortin-4 receptor (MC4R) and cadherin 6 (CDH6). Another Genome-wide 60K panel identified regions around 70 Mbp on chromosome 2 and around 60Mbp on chromosome Z that had an association with ascites incidence (Tarrant et al., 2017).

With great advancement in high throughput sequencing, next-generation sequencing (NGS) is an attractive avenue in mapping the complex genetics of ascites in fast-growing birds. Using whole-genome resequencing (WGR), Dey et al. (2018) identified SNPs in intron 6 and exon 8 of carboxypeptidase Q (CPQ) gene on chromosome 2 (around 127 Mbp) associated with resistance in male birds. In humans, GWAS studies have shown CPQ or plasma glutamate carboxypeptidase (PGCP), is associated with blood pressure, and heart rate traits.

None of previous GWAS studies have shown CPQ as a candidate for ascites resistance in male birds, and our data from WGR does not support any of the previously identified regions on chromosome 2 using 60k SNP panels. Male birds are predisposed to ascites because of their higher body weights with more oxygen demand for fast growth. SNP frequency for CPQ was also higher for males in the WGS data. An additional 30 potential QTL regions have been identified from our WGR data (see Chapter 2). This suggests that the WGR method is more efficient and unbiased for studying complex diseases such as ascites.

1.7 Next-Generation Sequencing and Structural Variations

Significant advancement has been made in technologies for genome sequencing since the completion of the human genome project in 2003. Next-generation sequencing (NGS) provides low-cost high-throughput methods for nucleotide sequencing. The ease of whole-genome sequencing using NGS platforms has made it an attractive avenue for genomic research. Different techniques in NGS may vary in reading length (short or long), reading (single-end or paired-end), the time required and reads per run (Illumina. Inc., 2015). The first draft genome for *Gallus gallus* was released (v2.1) in 2004, and the most recent one (v6.0) in March 2018. Poultry genetics has changed drastically with the release of the first chicken draft genome (Hillier et al., 2004). One female red jungle fowl was used to generate the reference genome since it is the closest ancestor to modern chicken.

Identification of SNPs and CNVs that have an association with phenotypes or traits of interest can be achieved using WGS. It will allow the incorporation of these tools by a geneticist in the selection of plants and animals. Association of SNP or group of SNPs with disease susceptibility means that diseased individuals are expected to have a higher frequency of the SNP than resistant ones. These biomarkers can be used in improving efficient breeding, or in prediction, diagnosis, and treatment of a disease. The success of poultry genetics depends on the integration of traditional and molecular approaches for selection techniques (Emmerson, 1997).

SYNOPSIS

Both genetic and environmental factors influence the incidence of pulmonary hypertension syndrome (PHS) in broilers. Investigations in broilers have also shown high heritability of ascites in fast-growing lines. Although improved environment and better selection practices have reduced the yearly economic loss in the USA for the past decade, about 3% of the fast-growing birds manifest idiopathic arteriole hypertension (IPAH). Previous genome-wide association studies conducted in our labs that were based on SNPs have identified potential regions on chromosome 2, 9 and Z associated with the phenotype. However, the goal to identify major genes for ascites phenotype is yet to be achieved. SNP loci in previous GWAS that did not conform to Hardy-Weinberg equation (HWE) for the whole sample set were often discarded, which includes 5-8,000 SNP locations. We hypothesized that the discarded loci could be part of a copy number variation (CNV). It means that we can screen differentially represented CNVs in both genders for ascites susceptible and resistant birds from relaxed, REL, line.

The current dissertation emphasizes on understanding the genetics of ascites using whole-genome resequencing in a research line, REL, and a commercial broiler, designated as T. The primary focus of this project is the identification of novel CNVs, and chromosomal regions related to ascites phenotype. Secondly, WGR would further examine chromosomes/chromosomal regions for SNPs that were found to be associated with ascites in previous GWAS in our lab. Overall, the development of new markers for ascites-resistance will allow improvement in broiler industry via non-invasive means in broiler lines.

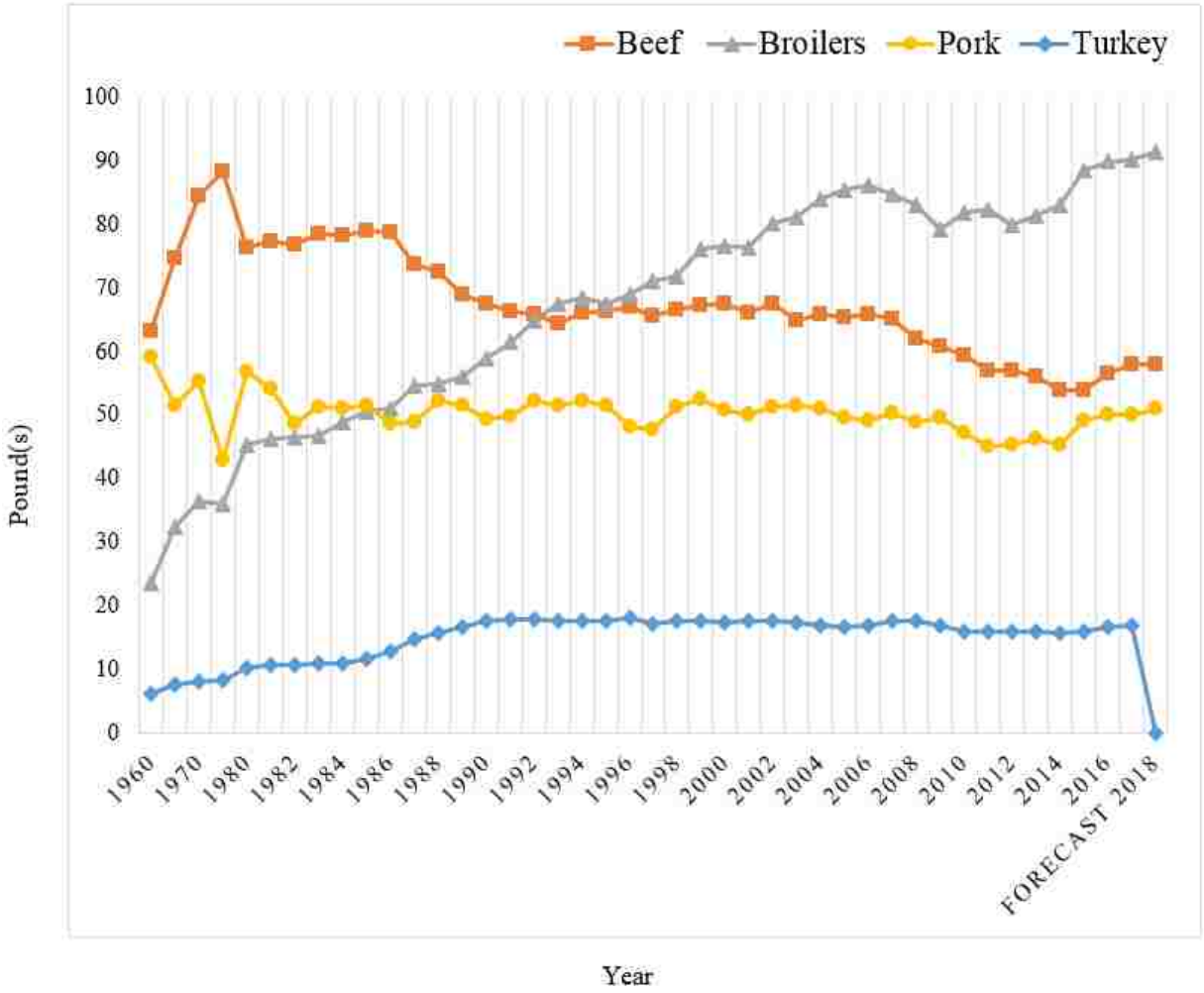

Figure 1.1. Per capita consumption of poultry and meat in the U.S. (USDA, 2017). Trends of beef, pork, broilers, and turkey consumptions (lb.) are shown in Y-axis from 1960 to 2017 (X-axis).

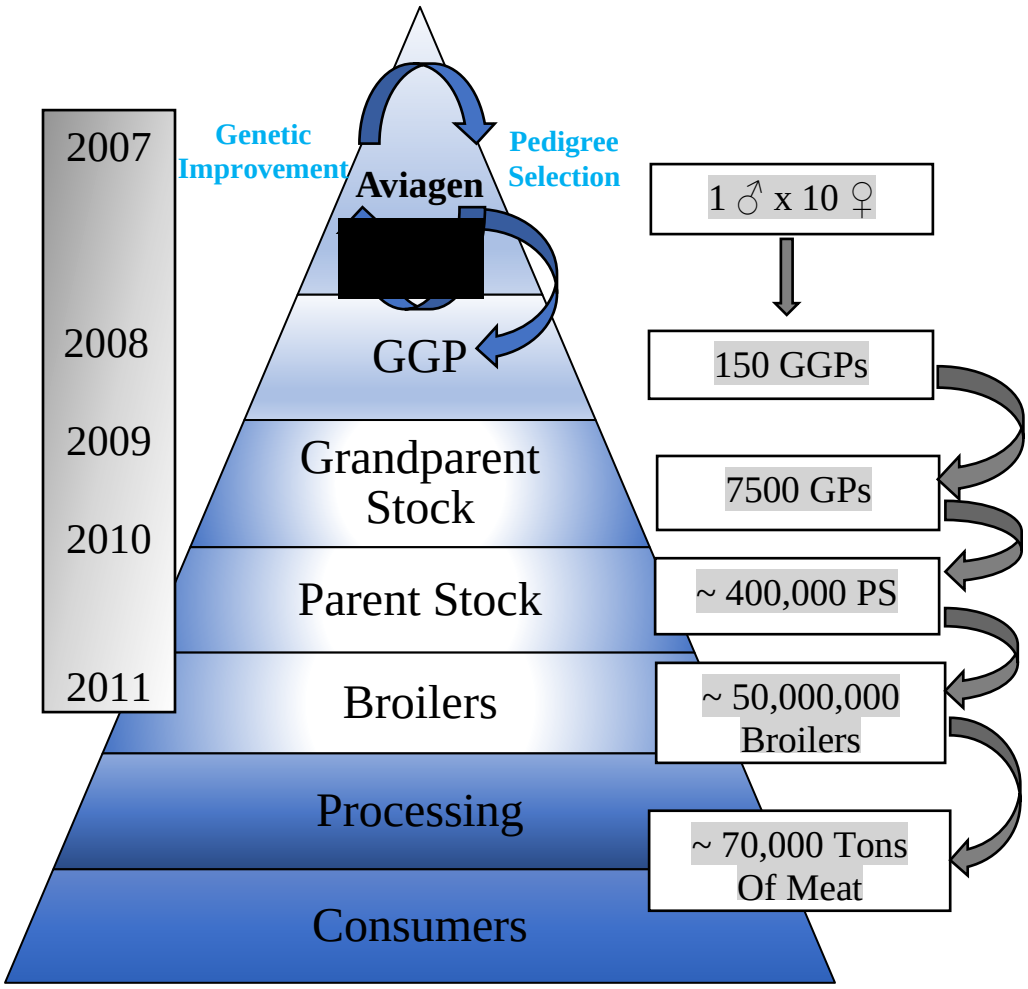

Figure 1.2. Breeding Structure in Broiler Industry (Laughlin, 2007; Stanley, 2009). The traditional breeding pyramid structure starting with the elite line at the apex, passing through great-grandparents (GGP), grandparents, parents, and commercial broiler. Pedigree selection to broilers has a time course of 4-5 years with final broiler meat around 70,000 metric tons from a base unit of 1 male and 10 females.

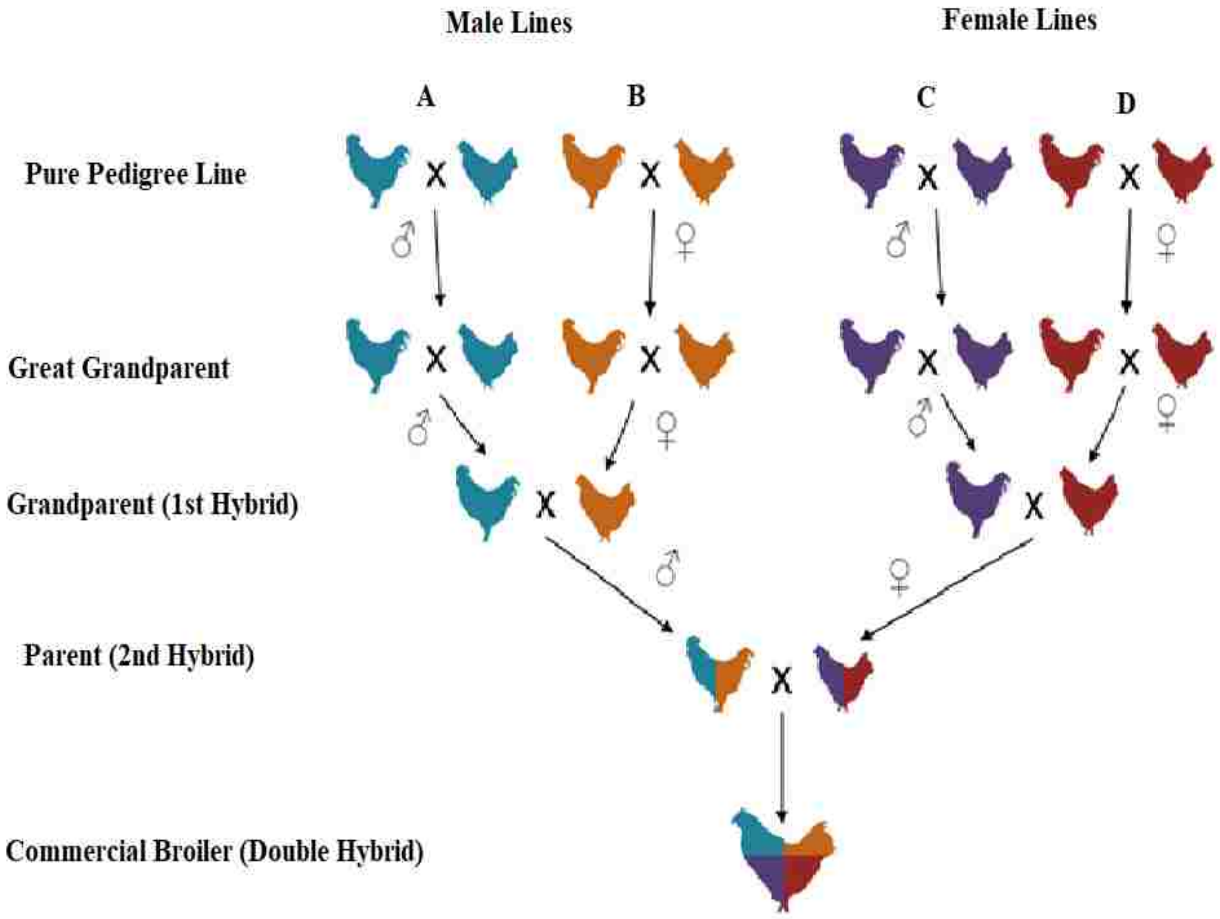

Figure 1.3. Line Crossing Schematics in Poultry Breeding Pyramid (Tarrant, 2016). Four-way crossing with the pure elite line at the top and double hybrid commercial broiler at the bottom.

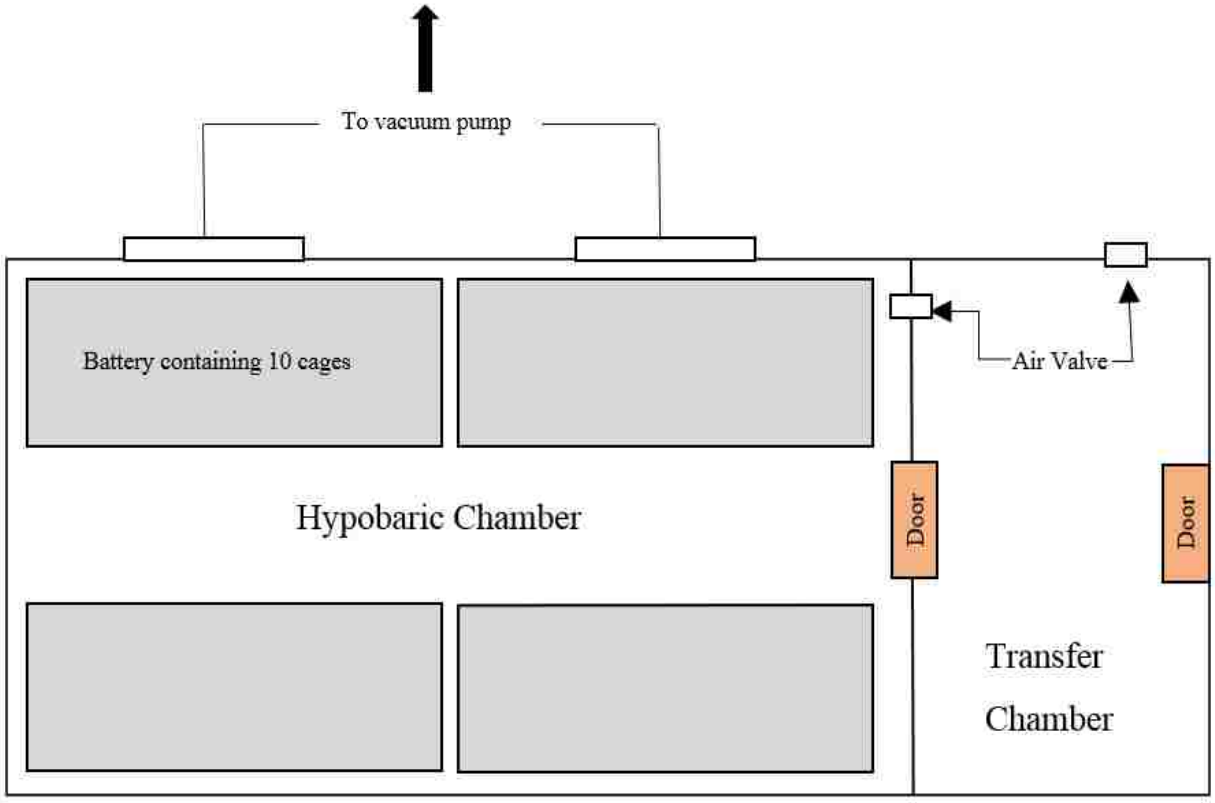

Figure 1.4. Hypobaric Chamber Model. The chamber measures 2.4m x 3.7m x 2.4m including, transfer chamber, air valves, doors, and battery. It has the capacity of housing 240 birds for six weeks.

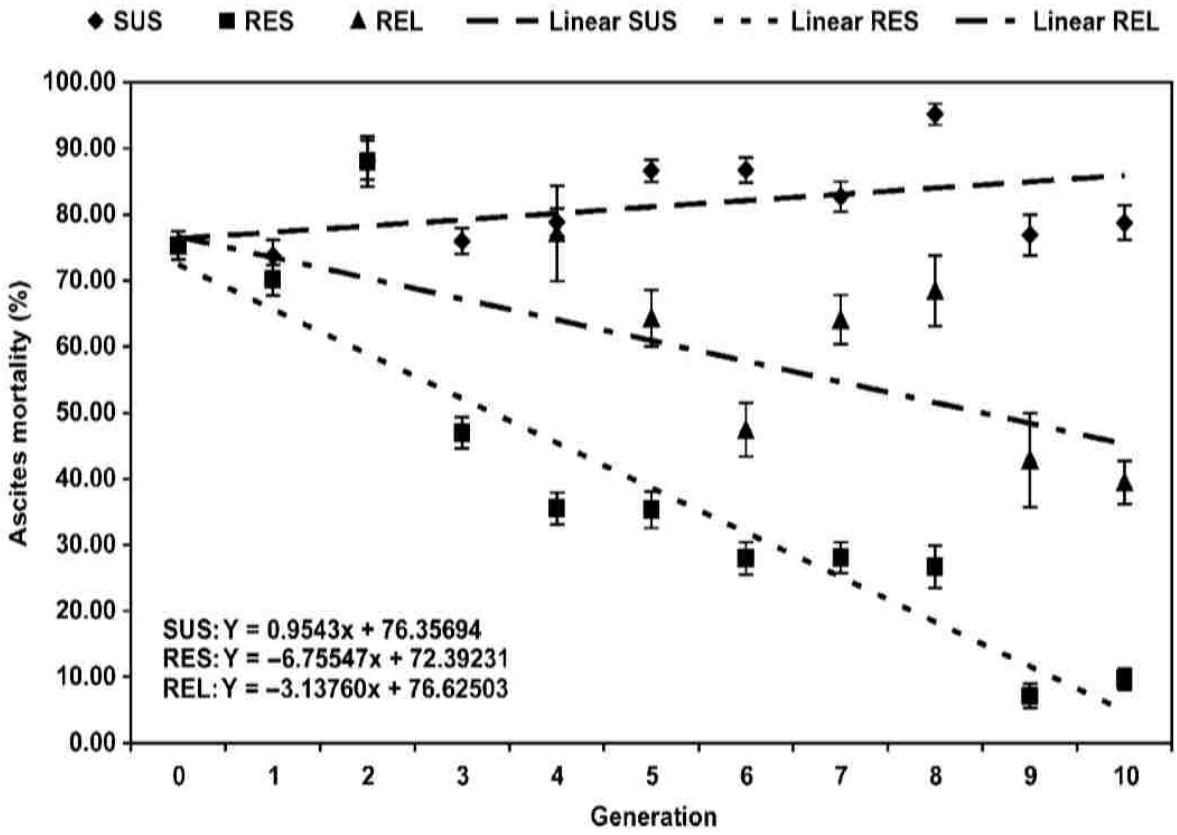

Figure 1.5. Ascites Mortality per Generation for three research lines (Pavlidis et al., 2007). The incidence of mortality in each generation was recorded for lines susceptible (SUS) and resistant (RES) to ascites and unselected, founder line (REL) as the control. All birds were reared in the hypobaric chamber set at 2,900 m above sea level.

1.8 References

Anthony, N. (1998). A Review Of Genetic Practices In Poultry: Efforts To Improve Meat Quality. *Journal of Muscle Foods, 9*(1), 25-33.

Anthony, N. B., and Balog, J. M. (2003). Divergent selection for ascites: development of susceptible and resistant lines. *Proceedings of 52nd Annual National Breeders Roundtable., St. Louis, MO,* 39-58.

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

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

Barbut, S. (1993). Colour measurements for evaluating the pale soft exudative (PSE) occurrence in turkey meat. *Food Research International,26*(1), 39-43.

Barbut, S. (1997). Occurrence of pale soft exudative meat in mature turkey hens. *British Poultry Science, 38*(1), 74-77.

Bumstead, N., Young, J. R., Tregaskes, C., Palyga, J., Dunn, P. P., Bumstead, N., . . . Dunn, P. P. (2009). Linkage mapping and partial sequencing of 10 cDNA loci in the chicken. *Animal Genetics, 25*(5), 337-341.

Burt, D. W., and Cheng, H. H. (1998). The Chicken Gene Map. *Institute for Laboratory Animal Research, 39* (2), 229-236.

Burton, R. R., and Smith, A. H. (1969). Induction of cardiac hypertrophy and polycythemia in the developing chick at high altitude. *Federation Proceedings, 28*, 1170-1177.

Chapman, M. E., and Wideman, R. F. (2001). Pulmonary Wedge Pressures Confirm Pulmonary Hypertension in Broilers Is Initiated by an Excessive Pulmonary Arterial (Precapillary) Resistance. *Poultry Science, 80*(4), 468-473.

Cheema, M., Qureshi, M., and Havenstein, G. (2003). A comparison of the immune response of a 2001 commercial broiler with a 1957 randombred broiler strain when fed representative 1957 and 2001 broiler diets. *Poultry Science, 82*(10), 1519-1529.

Chung, W. K., Deng, L., Carroll, J. S., Mallory, N., Diamond, B., Rosenzweig, E. B., . . . Morse, J. H. (2009). Polymorphism in the Angiotensin II Type 1 Receptor (AGTR1) is Associated With Age at Diagnosis in Pulmonary Arterial Hypertension. *The Journal of Heart and Lung Transplantation, 28*(4), 373-379.

Clop, A., Vidal, O., and Amills, M. (2012). Copy number variation in the genomes of domestic animals. *Animal Genetics, 43*(5), 503-517.

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 and Genetics. *Elsevier*, Amsterdam.

Deaton, J. W., Branton, S. L., Simmons, J. D., and Lott, B. D. (1996). The Effect of Brooding Temperature on Broiler Performance. *Poultry Science, 75*(10), 1217-1220.

Decuypere, E., Vega, C., Bartha, T., Buyse, J., Zoons, J., and Albers, G. A. (1994). Increased sensitivity to triiodothyronine (T3) of broiler lines with a high susceptibility for ascites. *British Poultry Science, 35*(2), 287-297.

Decuypere, E., Buyse, J., and Buys, N. (2000). Ascites in broiler chickens: Exogenous and endogenous structural and functional causal factors. *Worlds Poultry Science Journal, 56*(4), 367-377.

Desjardins, P., and 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., and Rhoads, D. D. (2018). Whole genome resequencing identifies the CPQ gene as a determinant of ascites syndrome in broilers. *PloS ONE,13*(1).

Djordjevic, T., and Görlach, A. (2007). Urotensin-II in the lung: A matter for vascular remodelling and pulmonary hypertension? *Thrombosis and Haemostasis, 98*(11), 952-962.

Druyan, S., Ben-David, A., and Cahaner, A. (2007). Development of Ascites-Resistant and Ascites-Susceptible Broiler Lines. *Poultry Science, 86*(5), 811-822.

Emmerson, D. (1997). Commercial approaches to genetic selection for growth and feed conversion in domestic poultry. *Poultry Science, 76*(8), 1121-1125.

Flock, D. K., Laughlin, K., and Bentley, J. (2005). Minimizing losses in poultry breeding and production: How breeding companies contribute to poultry welfare. *Worlds Poultry Science Journal*, *61*(2), 227-237.

Flock, D. K., and Preisinger, R. (2007). Specialization and concentration as contributing factors to the success of poultry industry in the global food market. *Archiv fur Geflugelkunde, 71*(5), 193-199.

Fumihito, A., Miyake, T., Sumi, S., Takada, M., Ohno, S., and Kondo, N. (1994). One subspecies of the red junglefowl (Gallus gallus gallus) suffices as the matriarchic ancestor of all domestic breeds. *Proceedings of the National Academy of Sciences, 91*(26), 12505-12509.

Fumihito, A., Miyake, T., Takada, M., Shingu, R., Endo, T., Gojobori, T., . . . Ohno, S. (1996). Monophyletic origin and unique dispersal patterns of domestic fowls. *Proceedings of the National Academy of Sciences,93*(13), 6792-6795.

Granevitze, Z., Hillel, J., Feldman, M., Six, A., Eding, H., and Weigend, S. (2009). Genetic structure of a wide-spectrum chicken gene pool. *Animal genetics*, *40*(5), 686–693.

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

Groenen, M. A., Cheng, H. H., Bumstead, N., Benkel, B. F., Briles, W. E., Burke, T., Burt, D. W.…. Vignal, A. (2000). A consensus linkage map of the chicken genome. *Genome Research, 10*(1), 137-147.

Gu, X., Feng, C., Ma, L., Song, C., Wang, Y., Da, Y., . . . Li, N. (2011). Genome-Wide Association Study of Body Weight in Chicken F2 Resource Population. *PLoS ONE, 6*(7).

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

Havenstein, G. B., Ferket, P. R., Scheideler, S. E., and 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.

Havenstein, G., Ferket, P., and Qureshi, M. (2003). Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. *Poultry Science, 82*(10), 1500-1508.

Hillier, L. W., Miller, W., Birney, E., Warren, W., Hardison, R. C., Ponting, C. P., Bork, P., Burt, B.W., Groenen, M. A…. Wilson, R.K. (2004). Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. *Nature, 432*(7018), 695-716.

Hocking, P. (2005). Riview on QTL mapping results in chickens. *World's Poultry Science Journal, 61*, 215-226.

Hollox, E. J., and Hoh, B. (2014). Human gene copy number variation and infectious disease. *Human Genetics, 133*(10), 1217-1233.

Illumina. Inc. (2013). An accessible, high-quality whole-genome sequencing solution for any species.http://www.illumina.com/applications/sequencing/dna_sequencing/whole_genome_sequencing.html

Julian, R. J. (1993). Ascites in poultry. *Avian Pathology, 22*(3), 419-454.

Julian, R. J. (1998). Rapid growth problems: Ascites and skeletal deformities in broilers. *Poultry Science, 77*(12), 1773-1780.

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

Kanginakudru, S., Metta, M., Jakati, R., and Nagaraju, J. (2008). Genetic evidence from Indian red jungle fowl corroborates multiple domestication of modern day chicken. *BMC Evolutionary Biology, 8*(1), 174.

Klein, R. J., Zeiss, C., Chew, E. Y., Tsai, J. Y., Sackler, R. S., Haynes, C., … Hoh, J. (2005). Complement factor H polymorphism in age-related macular degeneration. *Science, 308*(5720), 385–389.

Koenen, M. E., Boonstra-Blom, A. G., and Jeurissen, S. H. (2002). Immunological differences between layer- and broiler-type chickens. *Veterinary Immunology and Immunopathology, 89*(1-2), 47-56.

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

Ladjali-Mohammedi, K., Bitgood, J. J., Tixier-Boichard, M., Ponce de Leon, F. A. (1999). International System for Standardized Avian Karyotypes (ISSAK): standardized banded karyotypes of the domestic fowl (*Gallusdomesticus*). *Cytogenetic and Genome Research, 86*, 271–276.

Ladmakhi, M. H., Buys, N., Dewil, E., Rahimi, G., and Decuypere, E. (1997). The prophylactic effect of vitamin C supplementation on broiler ascites incidence and plasma thyroid hormone concentration. *Avian Pathology,26*(1), 33-44.

Lamont, S. J., Lakshmanan, N., Plotsky, Y., Kaiser, M. G., Kuhn, M., Arthur, J. A., Beck, N. J., and O'Sullivan, N. P. (1996). Genetic markers linked to quantitative traits in poultry. *Animal Genetics, 27*, 1-8.

Leveque, G., Forgetta, V., Morroll, S., Smith, A. L., Bumstead, N., Barrow, P., Loredo-Osti, J., Morgan, K., and Malo, D. (2003). Allelic variation in TLR4 is linked to susceptibility to Salmonella enterica Serovar Typhimurium Infection in Chickens. *Infection and Immunity, 71*(3), 1116-1124.

Laughlin, K. (2007). The Evolution of Genetics, Breeding and Production. *Aviagen, Report No. 15.*

Leshchinsky, T. V., and Klasing, K. C. (2001). Divergence of the inflammatory response in two types of chickens. *Developmental and Comparative Immunology, 25*(7), 629-638.

Liu, Y., Wu, G., Yao, Y., Miao, Y., Luikart, G., Baig, M., . . . Zhang, Y. (2006). Multiple maternal origins of chickens: Out of the Asian jungles. *Molecular Phylogenetics and Evolution,38*(1), 12-19.

Liu, W., Li, D., Liu, J., Chen, S., Qu, L., Zheng, J., . . . Yang, N. (2011). A Genome-Wide SNP Scan Reveals Novel Loci for Egg Production and Quality Traits in White Leghorn and Brown-Egg Dwarf Layers. *PLoS ONE, 6*(12).

Liu, R., Sun, Y., Zhao, G., Wang, F., Wu, D., Zheng, M., … Wen, J. (2013). Genome-wide association study identifies Loci and candidate genes for body composition and meat quality traits in Beijing-You chickens. *PloS ONE, 8*(4), e61172.

Lubritz, D. L., and McPherson, B. N. (1994). Effect of Genotype and Cold Stress on Incidence of Ascites in Cockerels. *The Journal of Applied Poultry Research, 3*(2), 171-178.

Lubritz, D. L., Smith, J. L., and 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.

MacLean, M. R. (2007). Pulmonary hypertension and the serotonin hypothesis: Where are we now? *International Journal of Clinical Practice, 61*, 27-31.

Marangoni, F., Corsello, G., Cricelli, C., Ferrara, N., Ghiselli, A., Lucchin, L., and Poli, A. (2015). Role of poultry meat in a balanced diet aimed at maintaining health and wellbeing: an Italian consensus document. *Food and nutrition research, 59*, 27606.

Meyer, S., and Steiner, L. (2011). *Daily Livestock Report. 9*, 243.

Mirsalimi, S. M., Julian, R. J., and Squires, E. J. (1993). Effect of Hypobaric Hypoxia on Slow- and Fast-Growing Chickens Fed Diets with High and Low Protein Levels. *Avian Diseases, 37*(3), 660.

Muir, W. M., Wong, G. K., Zhang, Y., Wang, J., Groenen, M. A., Crooijmans, R. P., . . . 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., Visscher, P., Chatziplis, D., Koerhuis, A., and Haley, C. (2006). Segregation analysis of blood oxygen saturation in broilers suggests a major gene influence on ascites. *British Poultry Science, 47*(6), 671-684.

Owen, R. L., Wideman, R. F., Leach, R. M., Cowen, B. S., Dunn, P. A., and Ford, B. C. (1994). Effect of Age of Exposure and Dietary Acidification or Alkalinization on Broiler Pulmonary Hypertension Syndrome. *The Journal of Applied Poultry Research, 3*(3), 244-252.

Owen, R. L., Wideman, R. F., and Cowen, B. S. (1995). Changes in Pulmonary Arterial and Femoral Arterial Blood Pressure upon Acute Exposure to Hypobaric Hypoxia in Broiler Chickens. *Poultry Science,74*(4), 708-715.

Parmentier, H. K., Verhofstad, L. P., Reilingh, G. D., and Nieuwland, M. G. (2012). Breeding for high specific immune reactivity affects sensitivity to the environment. *Poultry Science, 91*(12), 3044-3051.

Paxton, H., Anthony, N. B., Corr, S. A., and 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*.

Pavlidis, H. O., Balog, J. M., Stamps, L. K., Hughes, J. D., Huff, W. E., and Anthony, N. B. (2007). Divergent Selection for Ascites Incidence in Chickens. *Poultry Science, 86*(12), 2517-2529.

Qureshi, M. A., and Havenstein, G. B. (1994). A Comparison of the Immune Performance of a 1991 Commercial Broiler with a 1957 Randombred Strain When Fed "Typical" 1957 And 1991 Broiler Diets. *Poultry Science, 73*(12), 1805-1812.

Rabie, T. S. K. M., Crooijmans, R. P. M. A., Bovenhuis, H., Vereijken, A. L. J., Veenendaal, T., Poel, J. V. D.... and Groenen, M. A. M. (2005). Genetic mapping of quantitative trait loci affecting susceptibility in chicken to develop pulmonary hypertension syndrome. *Animal Genetics, 36*(6), 468-476.

Rubin, C. J., Zody, M. C., Eriksson, J., Meadows, J. R., Sherwood, E., Webster, M. T., Jiang, L.… and Andersson, L. (2010). Whole-genome resequencing reveals loci under selection during chicken domestication. *Nature, 464*, 587–591.

Scheele CW. (1996). Ascites in chickens: Oxygen consumption and requirement related to its occurrence. PhD Thesis. *Landbouwuniversiteit Wageningen, the Netherlands.*

Schmutz, J., and Grimwood, J. (2004). Fowl sequence. *Nature,432*(7018), 679-680.

Sheng, Q., Cao, D., Zhou, Y., Lei, Q., Han, H., Li, F., . . . Wang, C. (2013). Detection of SNPs in the Cathepsin D Gene and Their Association with Yolk Traits in Chickens. *PloS ONE, 8*(2).

Shlosberg, A., Bellaiche, M., Hanji, V., Nyska, A., Lublin, A., Shemesh, M., . . . Berman, E. (1996). The effect of acetylsalicylic acid and cold stress on the susceptibility of broilers to the ascites syndrome. *Avian Pathology, 25*(3), 581-590.

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

Smith, A. H., Wilson, W. O., and Pace, N. (1956). Growth and Reproduction in domestic birds at high altitudes. *Poultry Science, 35*, 1129-1181.

Smith, E. J., Lyons, L. A., Cheng, H. H., and Suchyta, S. P. (1997). Comparative mapping of the chicken genome using the East Lansing reference population. *Poultry Science, 76*, 743-747.

Subedi, S., Feng, Z., Deardon, R., and Schenkel, F. S. (2013). SNP selection for predicting a quantitative trait. *Journal of Applied Statistics, 40*(3), 600-613.

Suchyta, S. P., Cheng, H. H., Burnside, J., and Dodgson, J. B. (2001). Comparative mapping of chicken anchor loci orthologous to genes on human chromosomes 1, 4 and 9. *Animal Genetics, 32*, 12-18.

Sun, Y., Zhao, G., Liu, R., Zheng, M., Hu, Y., Wu, D., … Wen, J. (2013). The identification of 14 new genes for meat quality traits in chicken using a genome-wide association study. *BMC Genomics, 14*, 458.

Tarrant, Katy. (2016). Elucidating the genetic cause to ascites syndrome in broiler chickens utilizing multi-generational genome wide association studies. *Theses and Dissertations*. 1652. https://scholarworks.uark.edu/etd/1652

Tarrant, K. J., 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, 96*(6), 1544-1552.

Wallis, J.W., Aerts, J., Groenen, M. A., Crooijmans, R. P., Layman, D., Graves, T. A., Scheer, D. E., Kremitzki, C., Fedele, M. J….. Warren, W. C. (2004). A physical map of the chicken genome. *Nature, 432*, 761-764.

Wang, J., He, X., Ruan, J., Dai, M., Chen, J., Zhang, Y., … Wang, J. (2005). ChickVD: a sequence variation database for the chicken genome. *Nucleic acids research, 33*(Database issue), D438–D441.

Wang, Y., Li, H., Zhang, Y., Gu, Z., Li, Z., and Wang, Q. (2006). Analysis on Association of a SNP in the Chicken OBR Gene with Growth and Body Composition Traits. *Asian-Australasian Journal of Animal Sciences, 19*(12), 1706-1710.

Watanabe, T., Kanome, T., Miyazaki, A., and Katagiri, T. (2006). Human Urotensin II as a Link between Hypertension and Coronary Artery Disease. *Hypertension Research, 29*(6), 375-387.

West, B., and Zhou, B. (1988). Did chickens go North? New evidence for domestication. *Journal of Archaeological Science, 15*(5), 515-533.

Wideman, R. F., Ismail, M., Kirby, Y. K., Bottje, W. G., Moore, R. W., and Vardeman, R. C. (1995). Furosemide Reduces the Incidence of Pulmonary Hypertension Syndrome (Ascites) in Broilers Exposed to Cool Environmental Temperatures. *Poultry Science, 74*(2), 314-332.

Wideman, R., Kirby, Y., Owen, R., and French, H. (1997). Chronic unilateral occlusion of an extrapulmonary primary bronchus induces pulmonary hypertension syndrome (ascites) in male and female broilers. *Poultry Science, 76*(2), 400-404.

Wideman, R. F. (1998). Causes and control of ascites in broilers. *33rd National Meeting on Poultry Health and Processing, Sheraton Ocean City, Ocean City, Maryland, USA, October 14-16*, 56-85.

Wideman, R. F, Maynard, P., and Bottje, W. (1999). Venous blood pressure in broilers during acute inhalation of five percent carbon dioxide or unilateral pulmonary artery occlusion. *Poultry Science, 78*(10), 1443-1451.

Wideman, R. F., and 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. (2000). Cardio-pulmonary hemodynamics and ascites in broiler chickens. *Avian and Poultry Biology Review, 11*, 21-43.

Wideman, R. F., and Erf, G. (2002). Intravenous micro-particle injection and pulmonary hypertension in broiler chickens: Cardio-pulmonary hemodynamic responses. *Poultry Science, 81*(6), 877-886.

Wideman, R. F., Erf, G., Chapman, M., Wang, W., Anthony, N., and Xiaofang, L. (2002). Intravenous micro-particle injections and pulmonary hypertension in broiler chickens: Acute post-injection mortality and ascites susceptibility. *Poultry Science, 81*(8), 1203-1217.

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

Wong, G. K., Liu, B., Wang, J., Zhang, Y., Yang, X., Zhang, Z., … International Chicken Polymorphism Map Consortium (2004). A genetic variation map for chicken with 2.8 million single-nucleotide polymorphisms. *Nature, 432*(7018), 717–722.

CHAPTER 2. WHOLE GENOME RESEQUENCING IDENTIFIES 31 CANDIDATE QUANTITATIVE TRAIT LOCI FOR ASCITES SYNDROME IN BROILERS.

2.1 Introduction

Pulmonary hypertension syndrome (PH) or ascites syndrome is one of the consequences of selection for rapid growth and high meat yield in broilers. Ascites is associated with accumulation of fluid in the abdominal cavity leading to death (Huchzermeyer and DeRuyck, 1986; Wideman,1999; Julian, 2000; Balog et al., 2000). Ascites is a pathophysiological progression in which the rapid growing birds are unable to meet the high oxygen ($\mathbf{O_2}$) demand for elevated metabolic rates (Anthony and Balog, 2003). The hypoxic condition stimulates cardiac output leading to constriction of pulmonary arterioles causing hypertension in pulmonary circulation, right ventricular hypertrophy and terminally right ventricular failure (Julian, 1998; Chapman and Wideman, 2001; Wideman et al., 2013). The primary cause of this disease is unknown, which makes it idiopathic in nature and is an excellent model for human idiopathic arteriole hypertension IPAH. Compared to female, male birds tend to have higher body weight with more oxygen requirement for growth and therefore are predisposed to ascites. In the United States, ascites incidence is reduced through better management including: ventilation (Bottje et al., 1998), reduced lighting (Buyse et al., 1996), better temperature control (Deaton et al., 1996), feed restriction (Acar et al., 1995; Balog et al., 2000b), and nutritional control (Roch et al., 2000). However, the world-wide economic loss is still estimated to be around 100 million dollars per year (Wideman et al., 2013; communication from Cobb-Vantress Inc. 2015). Genetic components can also be a key player in PHS since ascites related traits such as cardiac hypertrophy, and abdominal fluid, which are found to possess moderate to high heritability (Lubritz et al., 1995; Maxwell and Robertson, 1997; de Greef et al., 2001). Therefore, identification of quantitative trait loci (QTL)

affecting ascites can help lower economic losses in the broiler industry, with hopefully little impact on growth rate or meat yield.

Single nucleotide polymorphisms (SNPs) linked to specific phenotypes such as fat deposition, egg quality, etc. (Wang et al., 2006; Rajasekaran et al., 2013) have demonstrated that SNPs are useful genetic markers in marker-assisted-selection (MAS). Genotype-by-sequencing (case vs. control, susceptible vs. resistant) can be used to identify potential molecular markers in genomic research (Ozaki et al., 2002; Klein et al., 2005). Many SNP-based GWAS have been conducted to understand the polygenic nature of ascites and identify potential biomarkers for resistance to this disease (Muir et al., 2008; Krishnamoorthy et al., 2014; Dey et al., 2016; Tarrant et al., 2017). The analyses from these studies identified potential regions on chromosome 1, 2, 4, 9, and Z associated with the phenotype. Unfortunately, MAS using some of these loci have only shown a marginal association between these regions and ascites (unpublished).

Dey et al. (2018) conducted whole-genome-resequencing (WGR) to investigate the regions on chromosomes 2 and 9 further. This work was as a continuation of previous work (Dey et al., 2016) to refine mapping for QTLs associated with either susceptibility or resistance in broilers. In the WGR data, we did not find any SNPs in previously identified loci from GWAS in chromosome 2 and 9 but identified a new region on chromosome 2 (127.65 – 127.75 Mbp). This new locus spans part of a gene, plasma glutamate carboxypeptidase (PGCP or CPQ), which is associated with traits such as electrocardiogram (EKG), hypertension and blood pressure in humans (https://www.ncbi.nlm.nih.gov/gap/phegeni). The homozygous, non-reference SNP pattern in intron-6 was significantly associated with resistance in male birds from our research line and a few commercial lines, consistent with the WGR preliminary data. This led to CPQ gene as the first demonstrated marker for resistance to ascites in broilers. We have now expanded our WGR

investigation for the rest of the genome to identify 30 additional regions as potential markers for the phenotype.

2.2 Methods

2.2.1 Genome Data

All genomic positions presented are relative to the December 2015 assembly of the *Gallus gallus* genome (RefSeq accession ID: GCF_000002315.4)

2.2.2 Bird Stocks and Hypobaric Chamber Trials

All animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee under protocols 15039 and 15040. This study represents birds from our research line (REL). REL line birds from the 18th generation were wing-banded and immunized for Marek's virus. The birds were then challenged for six weeks using a hypobaric chamber set at 9000 ft and 543 mm of Hg above sea level (Pavlidis et al., 2007). Mortality data were recorded daily, and a necropsy determined the gender and the cause of death. The birds were designated ascitic/susceptible when there was excessive abdominal fluid with flaccid heart, liver lesions, and right ventricle hypertrophy (right ventricle to total ventricle; RV/TV > 0.5). At six weeks, survivors were euthanized by cervical dislocation and necropsied. Birds with normal necropsy characters were scored as resistant. Only the REL line samples were used for the whole genome sequencing work.

2.2.3 DNA Extraction from Blood

Blood (10 uL) was collected via wing vein lancet puncture of chicks at 1 to 3 days of age (doa). Genomic DNAs were isolated using our rapid protocol (Bailes et al., 2007). DNAs for next-generation sequencing were further purified by phenol-chloroform extraction followed by ethanol

precipitation. DNAs were quantified by Hoechst 33258 fluorescence in a GloMax (Promega Corp., Madison, WI).

2.2.4 DNA preparation for WGR

Duplicate pools of equal weights of 10 DNAs from each phenotype were pooled for each gender to construct 8 total pools, which were submitted for Next Generation Sequencing library preparation. Libraries were sequenced, 2x125 bp paired-end on an Illumina HiSeq 2500, to generate approximately 66 Gb per library. Library construction and sequencing were performed by the Research Technology Support Facility at Michigan State University (East Lansing, MI).

2.2.5 Data Analyses and Bioinformatics

The adaptor-trimmed FASTQ sequence reads were mapped onto galGal5 using SeqMan NGen (Lasergene Suite 14; DNAStar, Madison, WI) and ArrayStar (Lasergene Suite 14) with default parameters. The pools were coded as resistant (R) or susceptible (S), male (M) or Female (F), and biological replicate pools as 1 or 2 (i.e., RM1, RM2, SM1, SM2, RF1, RF2, SF1, SF2). Separate templated alignments for each pool were used for SNP identification and tabulation in ArrayStar, and the SNP data was exported to Excel for further analyses. SNPs with low read depth (<20) for one or more replicate pools were removed. The difference in the averages of SNP frequencies of replicates for each phenotype and gender were calculated and plotted according to chromosome and chromosomal position.

2.2.6 Primers and Probes Designing

To genotype for specific SNPs, PCR primers and probes were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). Primer specificity was checked using the BLAT tool on the UCSC genome browser (University of California, Santa Cruz). Probes and primers were

synthesized by Integrated DNA Technologies (IDT; Coralville, IA). PCR conditions were optimized for each primer pair and then used to genotype individual DNAs from phenotyped birds.

2.2.7 TaqMan Assay

PCR primers and probes for quantitative real-time PCR exonuclease assays for SNP genotyping. are in Table 2.2. Reaction (20µl) included 1x Taq buffer (50 mM Tris-Cl pH 8.3, 1 mM $MgCl_2$, 30 µg/ml BSA), 0.2 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM each forward and reverse primers, 0.05 µM each probe, 2.5 units Taq polymerase, and 2 µl DNA. The PCR cycling was initial denaturation at 90°C for 3 mins, 10 cycles of 90°C for 15s, 55°C for 15s, 72°C for 1 min, followed by another 30 cycles of 90°C for 15s, 55°C for 15s, 72°C for 1 min with plate read.

For the verification of genotype calls, selected PCR products were purified using RapidTip (Diffinity Genomics, West Chester, Pennsylvania) and quantified by Hoechst fluorescence as above. DNAs were submitted for capillary sequencing by Eurofins MWG Operon (Louisville, KY). Sequence data (as .ab1 files) were aligned to the reference Jungle Fowl sequence using SeqMan Pro software (DNASTAR, Madison, WI) for further editing and SNP scoring.

2.2.8 Gene Expression Assay

Specific gene expression was assayed by reverse transcriptase quantitative PCR (RT-qPCR) for heart, liver, brain, lung, and testis RNA previously extracted from broiler tissue samples (Dey et al., 2018). Purified RNA was used to produce cDNA which was subjected to qPCR quantification. Total RNA (500 ng) was added to a mastermix consisting of 1x First Strand Buffer (Promega Corp.), 0.5 µM dNTPs, 1 µM $CT_{23}V$ primer and 100 U MML-V Reverse Transcriptase (Promega Corp.), The mixtures were incubated at 50°C for 50 min and the reaction was terminated at 65°C for 5 min. The first strand cDNA was then diluted into a PCR mixture (as described above)

containing 1x EvaGreen dye (Biotium Inc., Fremont, CA). The PCR steps included initial denaturation at 90ºC for 3 min, 10 cycles of 90ºC for 15s, 55 ºC for 15s, 72 ºC for 1 min, followed by another 30 cycles of 90 ºC for 15s, 55 ºC for 15s, 72 ºC for 1 min with plate read. All RT-qPCR were run in triplicate with TATA-box binding protein (TBP) gene as the reference, and all ΔΔCt values were relative to TBP (Radonic et al., 2004). Fold change was calculated using the ΔΔCt method (Livak and Schmittgen, 2001).

2.2.9 Statistical Methods

Genotype frequencies from Taqman assays for SNPs in the candidate region were calculated for each phenotype and gender. The expected genotype counts were computed from the observed genotype frequency for each phenotype. Microsoft Excel (Microsoft Corp., Redmond, WA) Chi-square test was performed on the count versus expected to calculate *P*-values for allele/genotype frequencies greater than 10%. *P*-values were then multiplied by the number of alleles/genotypes to generate a simple Bonferroni adjusted *P*-value. Deviation from expected was considered statistically significant where adjusted *P*-values < 0.05. Gene expression differences for alleles were based on *P*-values from t-tests (one way with unequal variance).

2.3. Results

2.3.1 Whole Genome Resequencing and Templated Assembly

Next-generation whole genome resequence data was generated for eight pools of 10 individual DNAs representing two biological replicates for each gender for both resistant and susceptible birds from our unselected, Relaxed (REL) line. The REL is descended from an elite commercial line (Pavlidis et al., 2007). Each pool was sequenced to >66x coverage using paired-end (2 x 125-bp) Illumina sequencing on a HiSeq 2500. FASTQ reads were mapped onto the galGal5 reference

genome for each pool. The average mapped read counts for all pools was 534,902,801, with ~96.2% reads successfully assembled onto the reference genome (Table 2.1).

We filtered the data for each SNP position for read depth >20. For each SNP position, we then calculated the difference in the frequency in resistant versus susceptible phenotype. We then plotted the data according to chromosome and chromosomal location for each gender to visually identify clusters of SNPs with frequencies skewed toward a particular phenotype. Specifically, we inspected for clusters of SNPs showing frequency differences of >20%. In total, we detected nearly 11 million SNPs (Figure 2.1) in male birds and 12 million SNPs in female birds that were suitable for identification of candidate QTL regions associated with ascites phenotype. We identified 31 regions on 15 chromosomes showing clusters of SNPs differentially represented in the different phenotypes. We identified 24 clusters in males, with 7 SNP clusters in females (Table 2.3). Most clusters showed a higher frequency of non-reference SNPs in the ascites resistant birds. For the genes in each region, we then consulted the NCBI Phenotype-Genotype Integrator, (PheGenI; https://www.ncbi.nlm.nih.gov/gap/phegeni) for whether those genes had been identified in human GWAS as associated with traits possibly contributing to ascites.

2.3.2 Details of the Potential Regions

For the 31 regions, we identified 23 regions of SNPs where non-reference SNP frequencies were associated with resistance in males for six regions on chromosome 1, four regions on chromosome 3, two regions on chromosome 6, two on chromosome 7, two on chromosome Z, and one each on chromosomes 11, 13, 15, 22, 27, and 28. There were seven regions where non-reference SNP frequencies were higher in susceptible males, including two on chromosome 4, and one each on chromosomes 3, 8, 14, 28, and Z. We found three regions, where non-reference SNP frequencies were higher in resistant females, including two on chromosome 4, and one on chromosome 6.

There were only two regions where non-reference SNP frequencies were higher in susceptible females; single regions on chromosome 3, and 14.

Examining regions identified in both genders revealed that the two regions on chromosome 4 for males and females overlap, but the non-reference SNP frequency association is opposite for phenotype in the two genders, higher non-reference SNP frequency in susceptible males and resistant females. Males and females share a region on chromosome 6, where non-reference SNP frequencies are higher in resistant birds for both genders. Both genders share a region on chromosome 14 where the frequency of non-reference SNP was higher in susceptible birds.

2.3.3 LRRTM4 Genotyping in Association with Ascites Syndrome

The male-biased region on chromosome 2 has already been extensively analyzed for association with ascites phenotype and confirmed to show an association of the non-reference SNPs with ascites resistance in males (Dey et al., 2018). We selected the male-biased region on chromosome 22 for further investigation. This region contains 166 high-quality SNPs spanning 3.80-3.90 Mbp Gga22 with a frequency difference (resistant-susceptible) between (25-61%), where 123 of these SNPs are within the gene for LRRTM4. The remaining 43 differentially represented SNPs are in the intergenic region downstream of the gene. LRRTM4 is the gene for leucine-rich repeat transmembrane neuronal protein 4 which has been suggested to play a role in the regulation of dendritic spine development in the nervous system (Lauren et al., 2003; Siddiqui et al., 2013). NCBI PheGenI associates the human LRRTM4 gene region with traits such as antihypertension, carotid artery disease, coronary heart disease, and pulmonary embolism, so an association with ascites in broilers is possible. We, therefore, developed exonuclease assays for genotyping an extensive collection of archived DNAs from ascites phenotyped birds. Two SNPs from the intergenic region were selected because the pattern for non-reference frequency differences, was

uniform within the genic portion of the gene. TaqMan qPCR assay-based genotypes in >600 REL DNAs showed a close correlation of observed genotype frequencies with the calculated genotype frequencies based on allele frequencies and the Hardy Weinberg Equilibrium (HWE) formula ($1=p^2+2pq+q^2$). Therefore, the qPCR genotyping was assumed to be accurate, and the sample of DNAs non-biased. Based on our findings with the chromosome 2 region for CPQ and similar SNP map data from the WGR for LRRTM4 (Figure 2.2) we expected to find a strong association of LRRTM4 gene with ascites resistance in male birds. These same DNAs had been used to associate the CPQ gene on chromosome 2 with ascites (Dey et al., 2018). Although we expected to detect an association of the non-reference SNP allele with resistance in male, instead the homozygous non-reference genotype for the two targeted SNPs was associated with susceptibility in female birds (P=0.047; Table 2.4). We also observed a significant adjusted *P*-value (P=.0083) for "all" sample set, but since there was no significance in male broilers, it was appropriate to focus on the females. Susceptibility was increased by 15% in females compared to both "all" and male sample sets.

To test for potential epistatic interaction between CPQ and LRRTM4, we combined the genotype data for LRRTM4 with the CPQ gene (Dey et al., 2018). Genotype associations (Table 2.5A) were performed on combined genotypes using a Bonferroni correction of nine. Male broilers with homozygous non-reference genotype for intron 6 SNPs in CPQ gene have shown approximately 20% higher overall resistance (Dey et al., 2018). The combined genotype of heterozygous for LRRTM4 and homozygous non-reference for CPQ (G8-RRCC) genotype was significantly associated with ascites resistance in males (*P*=0.033), contrary to the LRRTM4 genotype alone. Around 85% of the male birds were resistant for the combined genotype (Table 2.5A) which is a higher frequency of resistant males than for the homozygous non-reference genotype for CPQ

alone. These data suggest that one copy of the non-reference LRRTM4 allele is sufficient to amplify the effect on ascites of the homozygous non-reference CPQ genotype. Also, for homozygous reference LRRTM4, the addition of one copy of non-reference CPQ increased the resistance by 40% in both "all" sample set and male birds. Summarizing, the CPQ non-reference homozygous genotype confers approximately 2:1 odds on ascites resistance in males but with the addition of one non-reference allele for LRRTM4 improves the odds to approximately 6:1.

2.3.5 Tissue-specific Expression Assay

LRRTM4 gene expression for each of the homozygous genotypes was measured using a TaqMan RT-qPCR assay in heart, lung, liver, brain, and testis samples, with TATA-binding protein (TBP) as an internal control. No expression was detected in lung, liver, brain, or testis RNA samples, but the expression was observed in the heart. We saw no difference in the level of expression of LRRTM4 between reference and non-reference homozygous genotypes (P=0.188) (Figure 2.3).

2.4 Discussion

The poultry industry has fueled the affordable production of chicken by selecting for economically important traits such as growth rate and meat yield. This rapid growth and muscle deposition make chicken an excellent model for human idiopathic arteriole hypertension or IPAH (Wideman and Hamal, 2011). About 3% of the fast-growing broilers develop IPAH and exposure to one of the stressors such as cold temperature (Balog et al., 2003) and high altitude (Owen et al., 1995) can increase the incidence up to 20%.

Multiple investigations have concluded high heritability of ascites susceptibility which led our laboratory to look at the genetic basis of ascites. In this current study, we conducted whole-genome resequencing in ascites research line, REL, and have identified 31 candidate regions on 15 different

chromosomes of the genome (Table 2.3). Previous GWAS studies did identify candidate regions for ascites phenotype (Krishnamoorthy et al., 2014; Dey et al., 2016; Tarrant et al., 2017), however, marker-assisted selection revealed marginal association with the phenotype. Therefore, we concluded these are non-reliable QTLs for ascites (unpublished). One region with CPQ gene on chromosome 2 from our WGR has been identified as a determinant for ascites syndrome in broilers (Dey et al., 2018). Human CPQ gene is a carboxypeptidase with a function in protein hydrolysis and thyroxine synthesis (Ahmetov et al., 2016). Dey et al. (2018) showed that the non-reference homozygous genotype for CPQ has a significant association with ascites resistance in REL broilers. This is consistent with the human studies, where CPQ was associated with traits such as electrocardiography, hypertension, blood pressure, and heart rate. Recent revisions to PheGenI have reduced the linked traits, probably revisions related to publicly available data on the NCBI site.

The CPQ work led to the expansion of our investigation for the rest of the genome to identify 30 additional regions for ascites phenotype (Figure 2.4). Around 62 genes with high-quality SNPs are either residing within or flanking areas of these 30 potential regions. The PheGenI revealed that almost all these genes have somewhat association with hypertension, body weight, fat deposition, heart failure, and hypotrophy in humans (Figure 2.4).

We investigated one region on chromosome 22 containing LRRTM4 gene for a possible association with ascites phenotype. LRRTM4 is a member of leucine-rich-repeat (LRR) transmembrane protein family that are involved in the synapse development and maintenance of the nervous system (Lauren et al., 2003). The LRRTM4 non-reference homozygote contributes to the ascites susceptibility in the female broilers as compared to males. This suggests that LRRTM4 is a candidate QTL for ascites in broiler chickens despite the effect being gender specific. We also

looked for possible interaction between CPQ and LRRTM4 genes. In males, the epistatic interaction between the LRRTM4 heterozygote and intron-6 non-reference homozygote of CPQ gene amplifies the resistance in males as compared to CPQ or LRRTM4 alone. The LRRTM4 gene, along with CPQ gene, can be employed in marker-assisted selection for increasing ascites resistance in the broiler industry. However, the exact mechanisms by which these genes contribute to resistance in male broilers are still unknown.

More studies on understanding the gene networks, downstream regulation, or the interaction between their protein products in chicken are very crucial. None of 31 regions have appeared in an of our previous GWAS studies. Identification of the CPQ gene (Dey et al., 2018) and now LRRTM4 demonstrates WGR to be a more effective and robust method in finding markers for complex traits. In GWAS, the SNPs utilized are pre-specified and limited in numbers, while in WGR, the sequence data identifies the segregating SNPs with tens of millions. With decreasing costs and increasing output, the WGR approach is highly cost-effective and much higher density.

In conclusion, CPQ and LRRTM4 are gender-specific markers for ascites resistance in broilers and MAS studies will help us answer whether these genes are significant QTLs for ascites resistance. The 29 additional regions are available for further investigation as potential QTLs associated with ascites phenotype. Since we know that 3% of the fast-growing broilers develop IPAH type, we can look for a correlation between the resistance phenotype and altered allele expression for QTLs for hypertension in humans by relating with developing therapeutics.

Table 2.1 Mapped and unmapped reads to the chicken reference genome. The assembly statistics are the average of pools for each gender from the generated SeqMan NGen reports for REL line birds.

Mapping Statistics for REL DNAs	
Average number of reads generated for all pools (million)	~ 535
Average number of reads assembled (million)	~ 518
Average number of Mapped reads (million)	
Reads mapped in pairs	~ 492
Reads mapped as one	~ 12.7
Unpaired reads assembled	~ 15
Unmapped reads (million)	~ 21
Bad reads (million)	~ 0.2

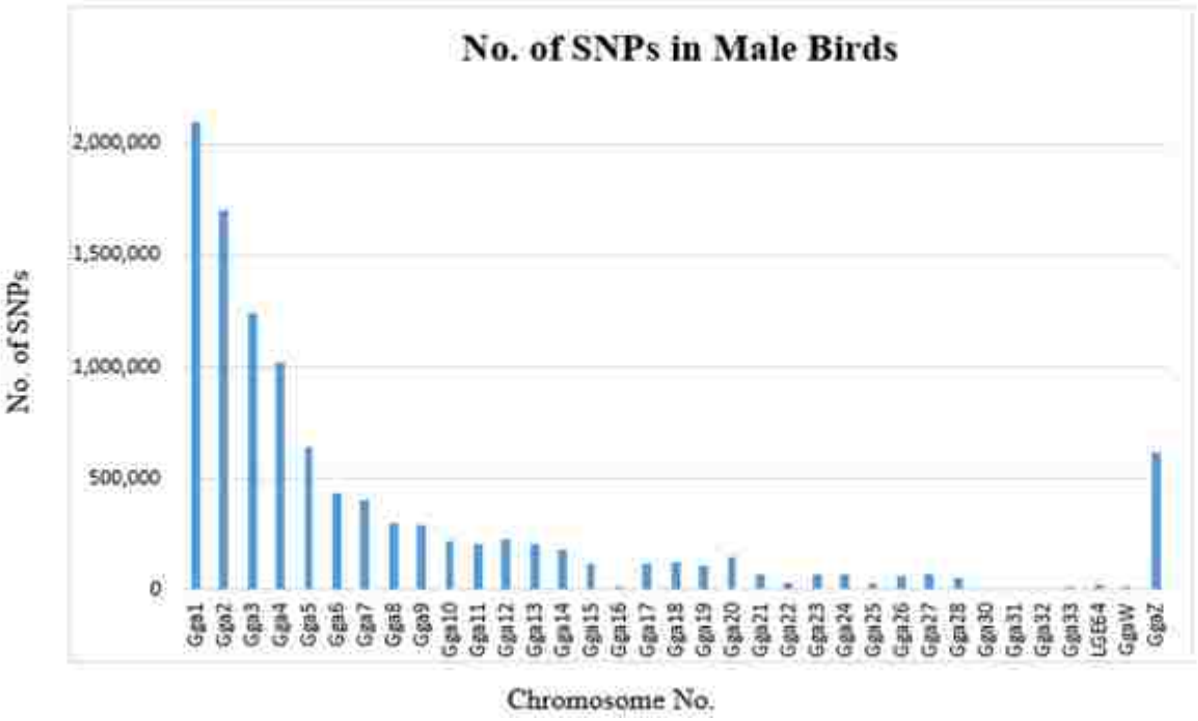

Figure 2.1. Identification of SNPs per chromosome in male REL line birds. The total number of SNPs detected in the given chromosome of male birds prior the filtration step.

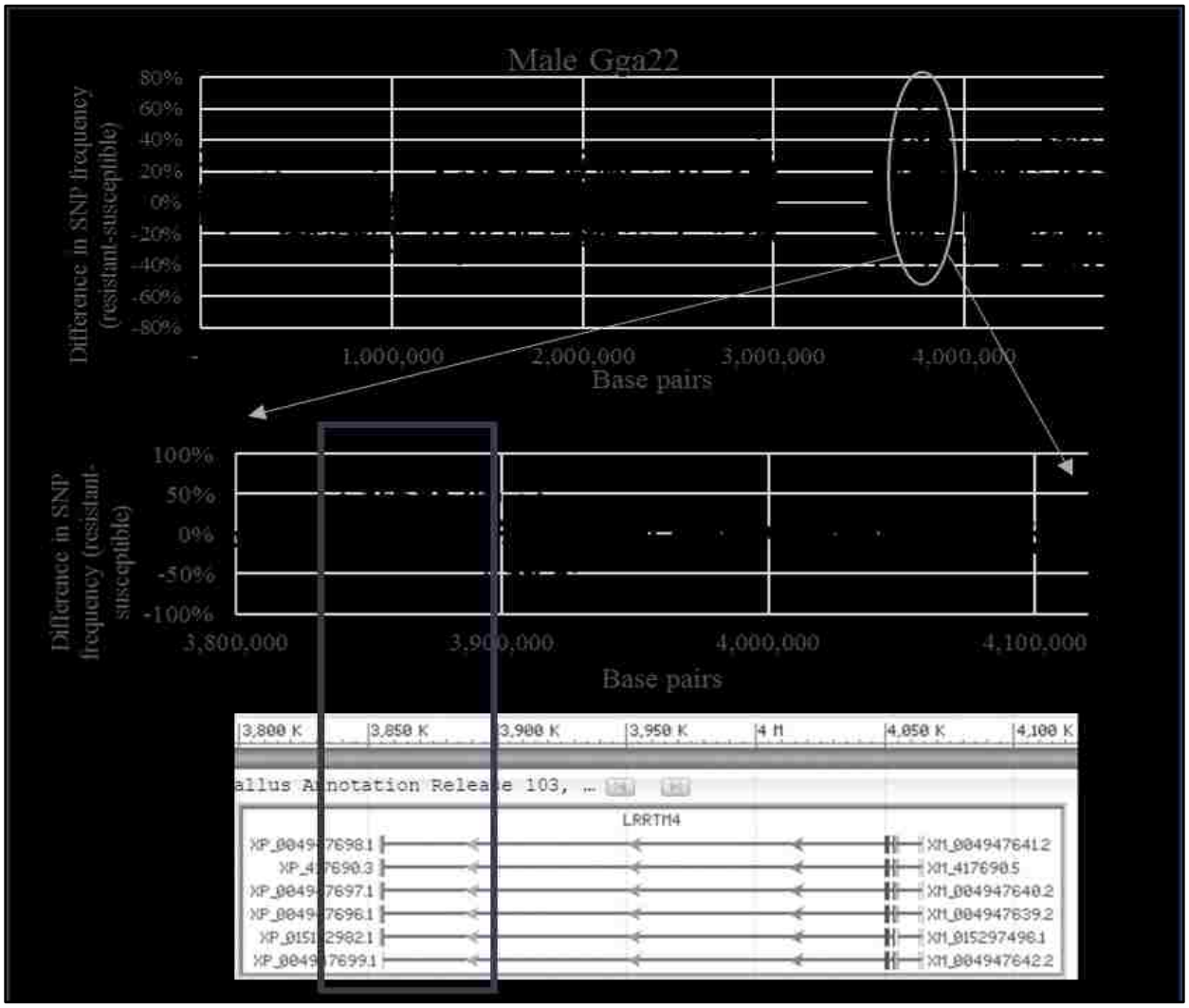

Figure 2.2. SNP map plot of the chromosome 22 of male chicken shown with enlarged region of interest. LRRTM4 gene map from NCBI aligned with SNP map to show the overlap with SNP region of interest

Table 2.2. Primers and probes for TaqMan qPCR. For SNP locus, position is the base-pair position on chromosome 22 according to the 2015 genome assembly. Primers are 5′-3′ for forward (F-) and reverse (R-); Probes are 5′- 3′ with allele 1 (Ref) labeled with HEX and allele 2 (Non) labeled with FAM.

Locus	SNP Position		Sequence
LRRTM4	3,850,947-9	Primers	F-CAGCCACTGATGCAATGAGCTGTCTGAA
F1, R1			R-CTCGGTASTAGCTGAAGCACTGCACATC
		Probes	Ref-T***G***A***A***ATCTATTACCTGCATCATCTGCCT
		(5′ →3′)	Non-T***A***A***G***ATCTATTACCTGCATCATCTGCCT
LRRTM4		Primers	F-GCCCTGCACGTATACCATCT
F2, R2			R-CGACTGAGTTCCAGGTTGGT

** Nucleotides in bold italics in the probes are the SNPs being assayed.

Table 2.3. The predicted potential regions for QTLs for ascites trait using WGR in our REL line. The Res-Sus shows the non-reference SNP frequency difference between the phenotypes of both genders for 31 regions (galGal5) on total 15 different chromosomes of chicken genome. Positive sign indicates the non-reference SNP to be associated with resistance and vice versa.

	Megabase pair (Mbp)		Res-Sus SNP%	
Chromosome No.	**Start**	**Stop**	**Male**	**Female**
Gga1	138.85	138.89	40%	0%
Gga1	173.78	173.84	40%	0%
Gga1	173.95	173.98	35%	0%
Gga1	180.67	180.73	40%	0%
Gga1	181.00	181.20	35%	0%
Gga1	188.96	189.01	35%	0%
**Gga2	127.65	127.75	60%	30%
Gga3	24.76	24.91	40%	0%
Gga3	25.32	25.41	20%	0%
Gga3	29.36	29.44	20%	-30%
Gga3	29.48	29.54	20%	0%
Gga3	72.35	72.50	-40%	0%
Gga4	33.71	33.73	-30%	20%
Gga4	33.98	34.02	-30%	20%
Gga6	33.98	34.02	20%	20%
Gga6	12.35	12.45	20%	0%
Gga7	12.60	13.30	40%	0%
Gga7	21.30	21.50	40%	0%
Gga8	26.25	26.27	-40%	0%
Gga11	18.45	18.54	50%	20%
Gga13	11.10	11.50	60%	0%
Gga14	1.50	1.56	-40%	-30%
Gga15	0.80	1.00	40%	0%
Gga22	3.80	3.90	60%	0%
Gga27	2.95	2.97	35%	0%
Gga28	0.47	0.52	-25%	0%
Gga28	0.74	0.77	20%	0%
Gga28	4.65	4.95	-20%	0%
GgaZ	50.25	50.28	30%	0%
GgaZ	63.50	63.90	-20%	0%
GgaZ	66.80	67.01	40%	0%

******Gga2 region SNPs have been already verified for a potential QTL (Dey et al., 2018).

Table 2.4. Possible contribution of LRRTM4 to ascites susceptibility in female birds. Data represented is the analyses of the SNP genotypes results from TaqMan qPCR assay. Discrepancies in totals from the lack of phenotype and/or gender data for genotyped birds.

REL line	**All**			**Male**			**Female**		
Genotype	Sus	Res	p-val.	Sus	Res	p-val.	Sus	Res	p-val.
GA (Ref)	34.5%	65.5%	1.7638	36.5%	63.5%	1.652	29.6%	70.4%	0.516
RR	31.0%	69.0%	1.1303	27.7%	72.3%	0.209	32.7%	67.3%	0.833
AG (Non-ref)	45.6%	54.4%	0.0083*	41.6%	58.4%	0.243	47.8%	52.2%	0.047*
n=	**270**	**479**		**126**	**251**		**128**	**219**	

***P<0.05**

Table 2.5. Statistical Analyses of the epistatic interaction between the LRRTM4 and CPQ genes in association with resistance in male birds. A) Combined SNP genotypes for LRRTM4 and CPQ (italicized) genes. LRRTM4 genotypes are as follows: GA is reference homozygous, RR is heterozygous, and AG is non-reference homozygous. CPQ genotypes are as follows: TA is reference homozygous, YM is heterozygous, and CC is non-reference homozygous (Dey et al., 2018). B) Statistical analyses of SNP genotype for epistatic interaction between the two genes based on HWE.

A)

G1- AG*TA*	G4- GA*TA*	G7- RR*TA*
G2- AG*CC*	G5- GA*CC*	G8- RR*CC*
G3- AG*YM*	G6- GA*YM*	G9- RR*YM*

B)

REL line	**All**			**Male**			**Female**		
Genotype	Sus	Res	p-val.	Sus	Res	p-val.	Sus	Res	p-val.
G8-RR*CC*	25.8%	74.2%	0.080	14.8%	85.2%	0.033*	36.5%	63.5%	4.818
n=	**31**	**89**		**9**	**52**		**19**	**33**	

***P<0.05**

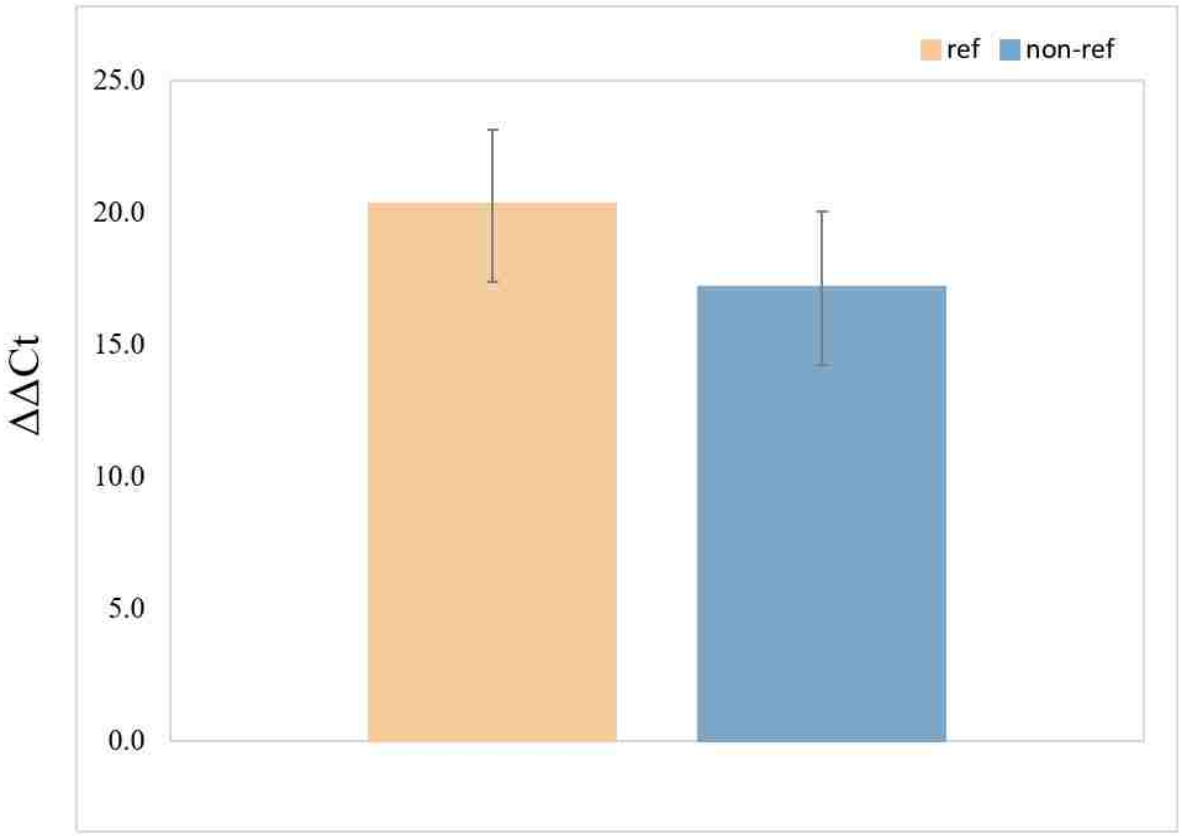

Figure 2.3. Relative expression of LRRTM4 in broiler heart samples as compared to TBP using a TaqMan RT-qPCR assay. There was no significant difference in the expression of either reference or non-reference homozygous genotypes.

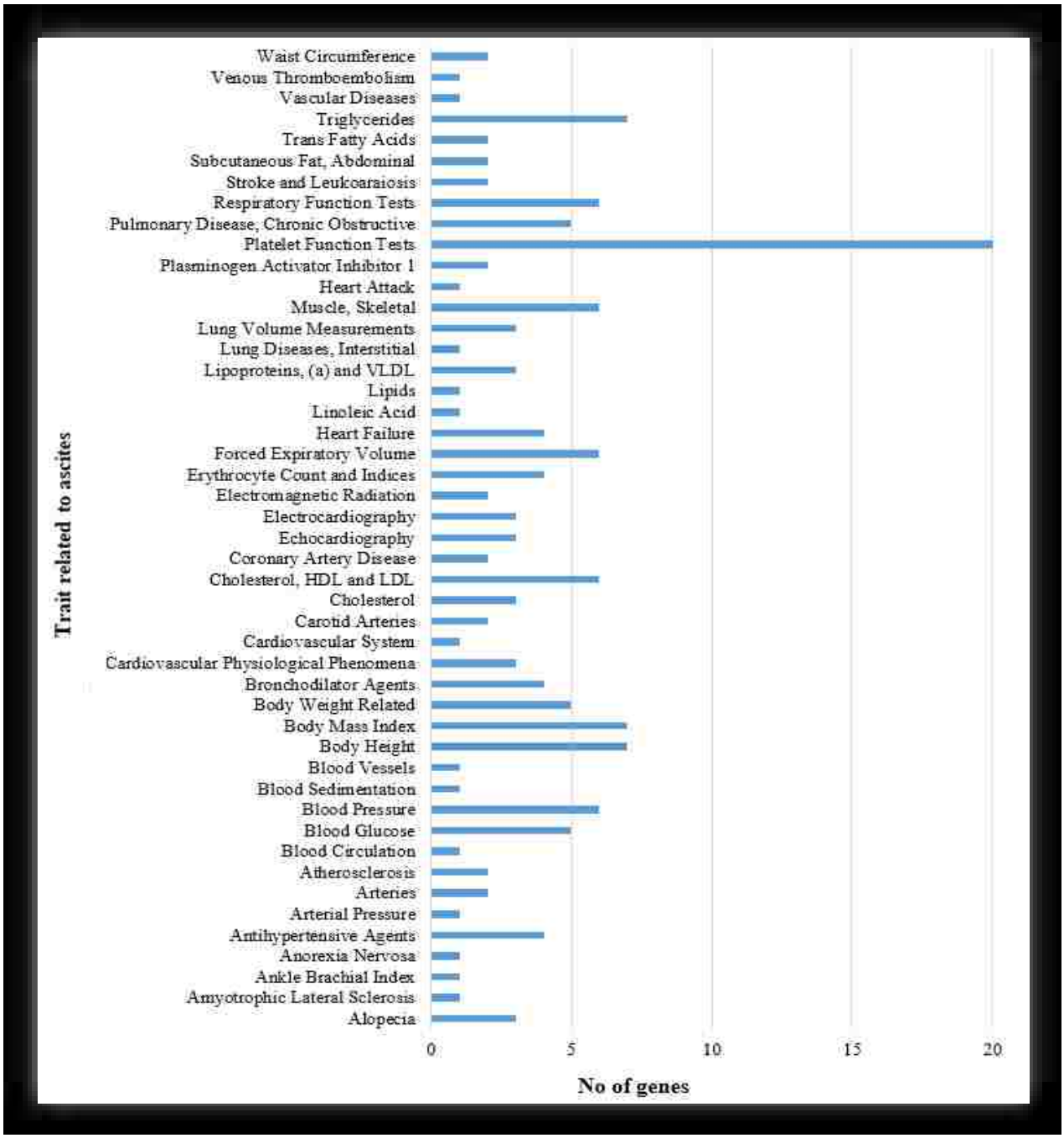

Figure 2.4. Number of genes from 31 potential regions with a role in the ascites associated traits in humans using PheGenI (NCBI). Most of the genes are reported to be associated with more than one trait in human studies.

2.5 References

Acar, N., Sizemore, F. G., Leach, G. R., Wideman, R. F., Owen, R. L., and Barbato, G. F. (1995). Growth of Broiler Chickens in Response to Feed Restriction Regimens to Reduce Ascites. *Poultry Science,74*(5), 833-843.

Ahmetov, I. I., Egorova, E. S., Gaddrakhmanova, L. J., and Fedotovskaya, O.N. (2016). Genes and Athletic Performance: An Update. *Medicine and Sports Science, 61*, 41-54.

Anthony, N. B., and Balog, J. M. (2003). Divergent selection for ascites: development of susceptible and resistant lines. *Proc. 52nd Annual National Breeders Roundtable., St. Louis, MO,* 39-58.

Bailes, S. M., Devers, J. J., Kirby, J. D., and 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), 102-106.

Balog, J. M., Anthony, N. B., Cooper, M. A., Kidd, B. D., Huff, G. R., Huff, W. E., and 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., 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*(8), 1101-1105.

Balog, J. M., Kidd, B. D., Huff, G. R., Huff, W. E., Rath, N. C., and Anthony, N. B. (2000). Effect of cold stress on broilers selected for resistance or susceptibility to ascites syndrome. *Poultry Science, 89*(9), 1383-7.

Bottje, W. G., Wang, S., Kelly, F. J., Dunster, C., Williams, A., Mudway, I. (1998). Antioxidant defense in lung lining fluid to broilers; Impact of poor ventilation conditions. *Poultry Science, 77,* 516-522.

Buyse, J., Kuhn, E. R., and Decuypere, E. (1996). The use of intermittent lighting in broiler raising. 1. Effect on broiler performance and efficiency of nitrogen retention. *Poultry Science, 75*(5), 589-594.

Chapman, M. E., and Wideman, R.F. (2001). Pulmonary wedge pressures confirm pulmonary hypertension in broilers is initiated by an excessive pulmonary arterial (precapillary) resistance. *Poultry Science, 80,* 468-473.

de Greef, K. H., Kwakernaak, C., Ducro, B. J., Pit, R., and Gerritsen, C. L. (2001). Evaluation of Between-Line Variation for Within-Line Selection Against Ascites in Broilers. *Poultry Science, 80*(1), 13-21.

Deaton, J. W., Branton, S.L., Simmons, J. D., Lott, B.D. (1996). The effect of brooding temperature on broiler performance. *Poultry Science, 75,* 1217-1220.

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,* 88–97.

Dey, S., Parveen, A., Tarrant, K. J., Licknack, T., Kong, B. C., Anthony, N. B., and 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.

Huchzermeyer, F. W., and DeRuyck, A. M. C. (1996) Pulmonary hypertension syndrome associated with ascites in broilers. *Veterinary Record, 199*, 94.

Julian R. J. (1998). Rapid growth problems: ascites and skeletal deformities in broilers. *Poultry Science, 77*,1773-1780.

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

Klein, R. J., Zeiss, C., Chew, E. Y., Tsai, J. Y., Sackler, R. S., Haynes, C., et al. (2005). Complement factor H polymorphism in age-related macular degeneration. *Science, 308*(5720), 385–389.

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

Lauren, J., Airaksinen, M.S., Saarma, M., and Timmusk, T. (2003). A novel gene family encoding leucine-rich repeat transmembrane protein differentially expressed in the nervous system. *Genomics, 81*(4), 411-421.

Livak, K. J., and Schmittgen, T. D. (2001). *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method*. *Methods, 25,* 402–408.

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.

Maxwell, M. H., and Robertson, G.W. (1997). 1993 UK broiler ascites survey. *World's Poultry Science Journal, 53*(1); 59-60.

Muir, W. M., Wong, G. K.-S., Zhang, Y., Wang, J., Groenen, M. A. M., Crooijmans, R. P. M. A., Megens, H.-J., Zhang, H., Okimoto, R., Vereijken, A., et al. (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,* 17312-17317.

Owen, R. L., Wideman, R. F., and Cowen, B. S. (1995). Changes in Pulmonary Arterial and Femoral Arterial Blood Pressure upon Acute Exposure to Hypobaric Hypoxia in Broiler Chickens. *Poultry Science,74*(4), 708-715.

Ozaki, K., Ohnishi, Y., Lida, Aritoshi., Sekine, A., Yamada, R., Tsunoda, T., Sato, H., et al. (2002). Functional SNPs in the lymphotoxin-a gene that are associated with susceptibility to myocardial infarction. *Nature Genetics, 32,* 650-654.

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

Radonic, A., Thulke, S., Mackay, I. M., Landt, O., Siegert, W., Nitsche, A. (2004) Guideline to reference gene selection for the quantitative real-time PCR. *Biochemical and Biophysical Research Communication, 313*(4), 856-862.

Rajasekaran, S., Kanna, R. M., Senthil, N., Raveendran, M., Cheung, K. M. C., Chan, D., Subramaniam,S., and Shetty, A. P. (2013). Phenotype variations affect genetic association studies of degenerative disc disease: conclusions of analysis of genetic association of 58 single nucleotide polymorphisms with highly specific phenotypes for disc degeneration in 332 subjects. *Spine Journal, 10,*1309-20.

Roch, G., Boulianne, M., De Roth, L. (2000). Effect of vitamin E and selenium on incidence of ascites, growth performance and blood parameters in cold-stressed broilers. *Poultry Science, 79*(1), 41.

Siddiqui, T.J., Tari, P.K., Connor, S.A., Zhang, P., Dobie, F.A., She, K., Kawabe, H., Wang, Y.T., Brose, N., and Craig A.M. (2013). An LRRTM4-HSPG complex mediates excitatory synapse development on dentate gyrus granule cells. *Neuron, 79*, 680-695.

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, 96*(6),1544–1552.

Wang, Y. Z., Gu, Z. L., Wang, H., and Zhang, Y. D. (2006). Analysis on Association of a SNP in the Chicken OBR Gene with Growth and Body Composition Traits. Asian-Aust. *Journal of Animal Science, 19*,1706-1710.

Wideman, R. F. (1999). Cardiac output in four-, five- and six-week-old broilers, and hemodynamic responses to intravenous injections of epinephrine. *Poultry Science, 78*, 392- 403.

Wideman, R. F., and 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., and Anthony, N. B. (2013). Pulmonary arterial hypertension (ascites syndrome) in broilers, A review. *Poultry Science, 92*, 64-83.

CHAPTER 3. IDENTIFICATION OF COPY NUMBER VARIATIONS FOR ASCITES IN REL BROILERS USING NEXT-GENERATION SEQUENCING

3.1 Introduction

Copy number variations (CNVs) refer to duplications or deletions of genomic regions ranging from 1 Kbp to over 5 Mbp in size (Feuk et al., 2006; Freeman et al., 2006). If the number of copies in a genome is different as compared to the reference assembly of that genome, then the region is said to have copy number gain or copy number loss (Wang et al., 2014). The prevalence of CNVs in healthy individuals show the importance of these genome variations (Lafrate et al.,2004; Sebat et al., 2004). CNVs have been estimated to affect approximately 12% of the human genome (Conrad et al., 2010; Malhotra and Sebat, 2012). Based on the size of the CNV, one or more genes can be part of a CNV. The variation in the numbers of these DNA fragments can disrupt a coding region or change the gene dosage (Zhou et al., 2011). It can also negatively impact the regulatory landscape of a genome by introducing positional effects or by making chimeric genes (Cook and Scherer, 2008; Chung et al., 2014).

The early method for detecting CNVs relied on experiments such as array comparative genomic hybridization (CGH). Sebat et al., (2007) found de novo CNVs that had a significant association with autism spectrum disorder (ASD). Walsh et al. (2008) reported *de-novo* CNVs associated with childhood-onset schizophrenia (COS). These findings validated the role of pathogenic CNVs as disruptors of some genes which are essential players in neurological development.

With more advancement in high-throughput sequencing, multiple studies have found CNVs that are either pathogenic (associated with disease susceptibility) or non-pathogenic (population-specific/diversity). Pfundt et al. (2017) used whole-exome sequencing to identify clinically

relevant *de novo* CNVs in patients with a specific neurodevelopmental disorder. Another study confirmed 44 CNVs in individuals with inherited retinal dystrophies (Ellingford et al., 2017).

Although CNVs can influence gene expression by being part of the deleted or duplicated regulatory elements but most obviously by changing the dosage of functional copies. It can be true for genes that either code for a protein or miRNAs. However, miRNA dosage might not always be influenced if CNVs are partially deleted (Marcinkowska et al., 2011). CNVs affecting small non-coding micro RNAs (miRNAs) genes have been shown to affect the binding and regulation of their target genes. Vaishnavi et al. (2013) showed CNV loci harboring miRNAs play a role in mediating the genetic deficit in autism suggesting an active part of miRNAs in phenotypic variability and genetic heterogeneity of complexes diseases in subsets of individuals.

3.1.1 MicroRNAs

Chen et al. (2017) have predicted that human cells can express thousands of regulatory non-coding RNAs (ncRNAs). One subclass of ncRNAs is the miRNAs. These miRNAs are evolutionarily conserved and play an essential role in regulating processes such as metabolism, apoptosis, and cell proliferation (Leak et al., 2002; Bartel, 2004, 2005; Berezikov and Plasterk, 2005; Croce and Calin, 2005; Zamore and Haley, 2005). In eukaryotes, these small RNAs negatively regulate the expression of genes at the post-translational stage. MiRNAs transcribe as part of an intron of a protein-coding gene or from an intergenic portion of the genome. They are recognized as forming a 70-nucleotide double-stranded (ds), hairpin primary miRNA (pri-miRNAs). An enzyme complex, Drosha/DGCR8, present in the nucleus then processes the pri-miRNAs (Hastings and Krainer, 2001) into smaller (22 nucleotides) precursor miRNAs (pre-RNAs). The pre-RNAs are transported into the cytoplasm in an energy-driven manner via exportins. Another enzyme, Dicer, processes these transported pre-miRNAs into mature duplex miRNAs which then interact with

RNA-induced silencing complex (RISC) to unwind and bind to the 3′ untranslated region (UTR) of one or more target mRNAs. The RISC recognition of a messenger RNA (mRNA) results in either the inhibition of translation or degradation of that target mRNA (Bilen et al., 2006).

3.1.1.1 miRNAs dysregulation in complex diseases

Over the past two decades, many studies have shown the role miRNAs play in complex human diseases such as cancer, autoimmune, pulmonary arterial hypertension (PAH) and other cardiovascular conditions (McManus, 2003; Thum et al., 2008; Urbich et al., 2008; Grant et al., 2013). Expression of miRNAs in many studies are specific to a particular tissue or cell, and expression is dysregulated during the disease (Lagos-Quintana et al., 2002; Landgraf et al., 2007). Ascites syndrome is a metabolic disorder in fast-growing chickens. It starts with hypoxia leading to a cascade of pathophysiological changes including high blood pressure in pulmonary arteries, the remodeling of pulmonary arteries, hypertrophy in the right ventricle and failure, elevated levels of free radicals and oxidative degradation of lipids (Wideman et al., 2013).

Pulmonary arterial remodeling is an irreversible pathological hallmark of PAH (Wideman et al., 2013), where epithelial and smooth muscle cells are resistant to apoptosis thus are hyperproliferative. The increased proliferation in epithelial cells results in the formation of plexiform lesions (Sakao et al., 2010), blockage of blood vessels, and blood flow loss, in the small distal arteries of pulmonary circulation (Cook et al., 1999). The remodeling of pulmonary arteries is well studied, but the exact cellular mechanisms that lead to the initiation and progression are still under investigation.

3.1.1.2 miRNA-target prediction

Most of the algorithms for searching for miRNA target genes require the sequence information of

miRNA sequence (Riffo-Campos et al., 2016). Apart from the sequence information, we also need the 3' UTR of the target mRNAs, the seed regions of the miRNA, the free energy calculation for miRNA : mRNA complex, and the 3' site of the miRNA (Mathews et al., 1999; Sethupathy and Corda, 2006; Friedman et al., 2009; Xiao et al., 2009; Schnall et al., 2011; Kozamara et al., 2014).

3.1.2 Structural Variations Detection using Next-Generation Sequencing

NGS based structural variation (SV) discovery is dependent on the sequencing reads (Volik et al., 2003; Tuzun et al., 2005). There are four types of strategies (Medvedev et al., 2009; Mills et al., 2011), which are used for detecting CNVs/SVs using NGS (Figure 3.1). These methods are: read-depth (RD), split-read (SR), read-pair (RP) and assembly (AS). The RD, RP, and SR use a reference genome for mapping reads, but AS is based on *de novo* assembly. Each strategy has its own advantages and drawbacks but combining two or more of these methods can be more accurate for CNVs detection.

3.1.2.1 Read-Depth

Read-depth refers to the number of reads that cover a specific nucleotide in each locus of a given genome. The RD strategy is based on the correlation between the depth and copy number in a region (Teo et al., 2012). Reference genome-based alignment is used to count read depths for a pre-defined window and then normalized by removing potential biases such as repeated regions or GC content (Boeva et al., 2011; Janevski et al., 2012). Finally, the calls with statistical significance are predicted, and filtration is applied. We are expected to have significantly higher RD for duplication events and reduced RD for deletions. This approach can count exact numbers of SVs and can work better on large-sized CNVs but cannot discriminate between the specific types of duplications.

3.1.2.2 Read-Pair

The RP approach assesses sequenced read-pairs to compare the average insert size using a reference genome (Korbel et al., 2007). Insert size, read length, and coverage are vital parameters for increasing sensitivity and accuracy of this strategy (Alkan et al., 2011). The insert size longer than expected is an insertion, and shorter size than expected is a deletion.

3.1.2.3 Split-Read

SR is also based on paired-end mapping where one read is completely mapped to the genome while the second fails to or partially aligns to the reference genome (Zhang et al., 2011; Jiang et al., 2012). The incompletely aligned reads are then split into fragments followed by remapping of start and end fragments of each split read independently to the genome.

3.1.2.4 Assembly

The AS constructs contigs/scaffolds based on overlapping reads without using a reference genome (Nijkamp et al., 2012; Teo et al., 2012). AS based tools require a short to long read for better coverage for their algorithms but can be less accurate for eukaryotic genomic regions with repeats and duplications (Pirooznia et al., 2015). The contig quality and computational efficiency can be improved by using a guide, reference genome.

In previous GWAS studies using SNP panels conducted at our laboratory (University of Arkansas), all the loci that did not fit to Hardy-Weinberg equilibrium (HWE) were removed through filtration and not considered for association mapping. These discarded SNP loci might have deviated from HWE because they are part of CNVs. The objective of this study is to identify CNV loci segregating in our experimental research line, REL, and whether those CNVs are associated with either resistance or susceptibility to ascites.

3.2 Methods

3.2.1 DNA Extraction

Blood (10 uL) was collected via wing vein lancet puncture of chicks at 1 to 3 days of age (doa). Genomic DNAs were isolated using our rapid protocol (Bailes et al., 2007). DNAs for next-generation sequencing were further purified by phenol-chloroform extraction followed by ethanol precipitation. DNAs were quantified by Hoechst 33258 fluorescence in a GloMax (Promega Corp., Madison, WI).

3.2.2 DNA preparation for WGS

An equal amount of 10 DNAs from each phenotype for both genders were pooled together and submitted for library preparation. Eight pools (2 pools x 2 phenotypes x 2 genders) submitted included bar-coded library generation, 2x125 paired-end reads on Illumina HiSeq 2500 with a ~66X genome coverage. Library construction and sequencing steps were performed at the Research Technology Support Facility at Michigan State University (East Lansing, MI).

3.2.3 Data Analysis and Bioinformatics

The sequence reads (FASTQ) were mapped onto galGal5 using Bowtie. Generated sam (sequence alignment map) files were then converted into bam (binary alignment map) files using SAMtools package (https://de.cyverse.org/de/) as an input for the initial CNV detection using cn.mops (Copy Number estimation by a Mixture of PoissonS), which is an R based package on Bioconductor. Counted reads were then analyzed for CNVs, and detected segments were visualized as a segmentation plot using an R based seg-plot. Each peak was then tested for whether it contained candidate genes possibly related to ascites traits in humans using NCBI Phenotype-Genotype Integrator or PheGenI (https://www.ncbi.nlm.nih.gov/gap/phegeni). Additional

mapping for FASTQ reads was on galGal5 using SeqMan NGen (Lasergene Suite 14; DNAStar, Madison, WI)) and ArrayStar (Lasergene Suite 14). CNV data (i.e., name, source sequence, position, and the difference of the average of total reads per kilobase per million-copy number or RPKM-CN for both phenotypes in each gender) from ArrayStar were then exported to Microsoft Excel (Microsoft Office 2016) for further analyses. We used miRbase (https://www.mirbase.org) for retrieving the mature miRNA sequence and annotation information. Target genes for given chicken microRNAs were predicted using used mirDB (http://www.mirdb.org) developed by Wong and Wang, (2015).

3.3 Results

3.3.1 Identification of CNV regions using cn.mops

The generated SAM files from Bowtie were converted into BAM format using SAMtools, and the counted reads were then analyzed to detect CNVs. CNVs were visualized as a segmentation plot (see Methods). Seg-plots were generated for all chromosomes for each phenotype in both genders. This approach identified potential CNV peaks on chromosome 1 and 13 (Figure 3.2). The genes in the affected region were analyzed using PheGenI to identify ascites related gene functions in the CNV region. For chromosome 1, the peak contains three genes: zinc finger and SCAN domain containing 25 (ZSCAN25), cholinergic receptor nicotinic alpha 10 subunit (CHRNA10), and ADP-ribosyltransferase (ART1). There were no ascites-associated traits found for ART1 in humans, but the intron region of ZSCAN25 has been associated with traits such as metabolism and blood in humans. For chromosome 13, the peak encompasses a gene, protocadherin beta-5 or PCDHB5. PCDHB5 is associated with traits such as lymphocytes count, and leptin in humans. We were particularly interested in chromosome Z for the fact that male birds have higher incidences

of ascites. The resultant Z seg-plot for males was strange (Figure 3.2), and we have no explanation for why it appears so.

3.3.2 Identification of CNVs using ArrayStar

We mapped the sequence reads using SeqMan NGen and quantified the reads using ArrayStar as described in methods. The RPKM-CN values were evaluated further in Microsoft Excel. The difference between the averages RPKM-CN of the two replicates (resistant vs. susceptible) for each phenotype and gender were calculated to select genes where the differences in the averages were 2-fold over-represented or 2-fold under-represented according to phenotype, for either gender (Figure 3.3). For females, this identified 27 miRNAs, four genes, and five uncharacterized loci. For males, we found a total of 32 miRNAs, one gene, and one uncharacterized locus. If we increased the threshold difference in RPKM-CN to ±3, males had only four miRNAs. Increased copies of miR-7450 and miR-1678 in ascitic-resistant and increased copies of miR-449a and miR-1708 in ascitic-susceptible male birds (Figure 3.3). In females, we saw a completely different set of miRNAs for a given phenotype. The resistant female birds had increased copies of two miRNAs, miR-6666 and miR-6621whereas susceptible females had increased copies of miR-1757 and miR-1623 with a difference in RPKM-CN ±3. The targeted genes were predicted, and their associated traits in humans were determined using PheGenI shown in Table 3.1.

There were no common CNVs in miRNAs for both genders and/or phenotypes suggesting that these CNVs are gender-specific for both phenotypes. We looked into the literature and PheGenI to find any preliminary studies or data on these miRNAs in cardiovascular diseases or related traits in both human and chicken.

3.3.2.1 miRNAs in susceptible males

In human cells, it has been found that miR-449a is an antagonist of mitochondrial biogenesis for cells exposed to T-2 toxins from the Fusarium genus (Ma et al., 2018). The suppression of miR-449 leads to the upregulation of the sirtuin 1 (SIRT1) gene, a regulator of peroxisome proliferator-activated receptor gamma and coactivator 1 alpha (PGC-1α). This would stimulate mitochondrial biogenesis but needs further investigation for the exact mechanism of miRNA suppression. A recent study (Al-Zahrani et al., 2019) revealed a correlation between mitochondrial DNA abundance in particular muscles and ascites phenotype in broilers. There were substantial differences in mitochondrial DNA copy number with respect to gender and ascites phenotype. We do not know whether the role of miR-449a for ascites phenotypes in broilers is the same as in humans and we cannot know if miR-449a even regulates the same set of genes. The predicted targets from miRDB for miR-449a in chicken (Table 3.1) are neuron navigator 3 (NAV3) and ELKS/RAB6-interacting/ CAST family member 1 (ERC1). PheGenI associates both genes with traits such as blood pressure, cardiomegaly, echocardiogram, and body weight in humans.

The miR-1708 is located in an intron of the gene for FCH and double SH3 domains 2 (FCHSD2), which is involved in promoting clathrin-dependent endocytosis. For miR-1708, one of the predicted target genes is cAMP responsive element binding protein 3 like 1, CREB3L1 (Table 3.1). PheGenI associates SNP in the upstream region of CREB3L1 with myocardial infarction or heart attack. Another target gene, protein phosphatase 2 regulatory subunit B alpha (PPP2R3A), is believed to play a role in blood flow velocity and body mass index (BMI). However, there is no literature on the role of miR-1708 in cardiovascular diseases in chicken.

3.3.2.2 miRNAs in resistant males

In vitro studies have found that in plasma-induced chicken Sertoli cells (SC), a synthetic antagomir of miR-7450 led to cell death by disrupting the membrane potential of mitochondria and decreased ATP levels. (Zhang et al., 2018). Our predicted targeted genes for this miRNA are leucine rich repeats and immunoglobulin like domain 1 (LRIG1) and AT-rich interaction domain 5B (ARID5B). PheGenI associates these genes in humans with traits such as heart function, pulmonary disease, and heart attack. For the miR-1678, one of the chicken targets is solute carrier family 39 member 10 associated with body weight and body fat distribution in humans. These traits were associated with the SNPs in the intronic region of the gene, but how exactly miR-1678 would play a role is yet to be understood.

3.3.2.3 miRNAs in ascitic-susceptible female birds

The miR-1757 in susceptible females is located in the intronic region of the solute carrier family 25A member 43 (SLC25A43). A recent study in chicken has shown SNPs in the pre-miR-1757 gene to be associated with economically important traits such as body size index (Li et al., 2015). When we looked at the predicted chicken target genes (Table 3.1), transmembrane 9 superfamily member 2, TM9SF2, is associated with blood pressure, whereas RasGEF domain family member 1C, RASGEF1C, is linked with heart failure in human.

For miR-1623, the predicted target gene, transmembrane protein 215, TMEM215, shown in Table 3.1 is linked to traits such as blood pressure and coronary artery disease in humans. The second target gene is ring finger protein 165, RNF165, found to be associated with human body weight. A study in chicken has found miR-1623 to be significantly downregulated in dwarf chickens as compared to normal birds (Lin et al., 2012).

3.3.2.4 miRNAs in ascitic-resistant female birds

The miR-6666 is located on the intronic region of protein tyrosine phosphatase receptor type S (PTPRS) gene. One of the predicted target genes, NEDD4 binding protein 3 or N4BP3, is associated with waist-hip ratio. However, we did not find literature on the association of miR-6666 with ascites related traits either in chicken or humans.

For miR-6621, we predicted multiple target genes that are associated with cardiovascular traits in humans (Table 3.1). Intergenic variations in prickle planar cell polarity protein 1, PRICKLE1, is associated with human body weight, electrocardiograph (ECG), low density lipoprotein (LDL), and high-density lipoprotein (HDL). Myocardial infarction is associated with another target gene, cullin 3 (CUL3) in humans. The protein tyrosine phosphatase receptor type E, PTPRE has been linked with human coronary disease.

3.4 Discussion

Ascites or pulmonary hypertension syndrome is one of the physiological consequences of selection for fast growth and muscle deposition in poultry industry. Progressive hypertension in the cardio-pulmonary system leads to accumulation of fluid in the abdominal cavity of the fast-growing bird (Chapter 1). The pathophysiological changes include arterial wall remodeling, hypertrophy, etc. and the genetic and molecular bases of these changes remain to be elucidated (Rothman et al., 2017). There is a recent trend in ascertaining the role of miRNAs in complex diseases such as PH. Rhodes et al. (2013) highlighted the association of circulating miRNAs in plasma with poor survival prognosis in human PAH. Another investigation used monocrotaline-induced pulmonary hypertension in rats and revealed a correlation between miR-26a expression with increased systolic pressure and hypertrophy in the RV of rats (Schlosser et al. 2013). Several other studies have reported that altering the expression of certain miRNAs shows a strong association with resistance

or susceptibility to PH (Deng et al., 2015; McLendon et al., 2015; Wallace et al., 2015). With the growing evidence of the involvement of miRNA in ascites, the detection of these changes can be achieved by looking at the miRNA in the extracellular vesicles or circulatory miRNAs in the peripheral blood.

Hardy-Weinberg equilibrium (HWE) is a quality control test of allele and genotype data in genetic based studies (Anderson et al., 2010). Genomic regions that show extensive deviation from HWE are excluded because the deviation indicates either genotyping error, biased sampling, or evolutionary influences such as selection. Deviation from HWE could also result from a recently appreciated chromosomal variation, copy number variation or CNV, where gain and loss of the genomic region effects the frequency of genotypes. CNVs that harbor miRNA can also be investigated for their possible contribution in the pathophysiology of ascites syndrome.

In our study, we identified multiple gender-specific miRNAs that reside in CNVs for both phenotypes. The predicted target genes (Table 3.1) for these miRNAs are involved in important cellular processes and associated cardiovascular traits. For example, PTPRE is a signaling molecule that regulates differentiation, mitosis, and cell growth. We know that hypertrophy and hyperplasia in arterial walls are among the initial physiological changes in ascites. CREB3L1 is another target gene involved in the vascular smooth muscle contraction pathway hence complementing the phenomenon of increased peripheral arterial resistance due to elevated vasoconstriction in PAH. ARID5B is highly expressed in heart tissues, is an epigenetic modulator, and is involved in differentiation of smooth muscle cells in humans. Therefore, ARID5B may have a role in pulmonary hypertension, where there is an increased accumulation of SMCs and fibroblast cells. Our copy number variation data in chicken ascites suggest that gender-specific miRNAs genes may be effective biomarkers in this complex disease. However, we do not know for sure if

the same traits will appear in chicken for increased copies of given miRNAs. MAS and breeding studies are required to determine the actual QTL effect of these miRNAs in the pathophysiological alterations in ascites.

In summary, miRNAs can regulate many other genes, including those associated with pulmonary hypertension syndrome. However, further validation experiments are necessary for understanding the effect of miRNAs on the expression of the predicted target genes in chicken. The potential approaches for understanding the downstream pathways of our targeted miRNA will be either using synthetic mimics or antagonists for miRNAs. The specific tissues where the miRNAs have their effect also need to be determined. Our CNV data was with blood DNA and investigations on how extensive the CNV extends to other tissues is still to be studied. Our hypothesis is that microRNAs can be an attractive avenue for selection in breeding, but more expression work is needed to be done. In future, marker-assisted selection work using the interpretation of CNVs with miRNAs will be challenging but not impossible.

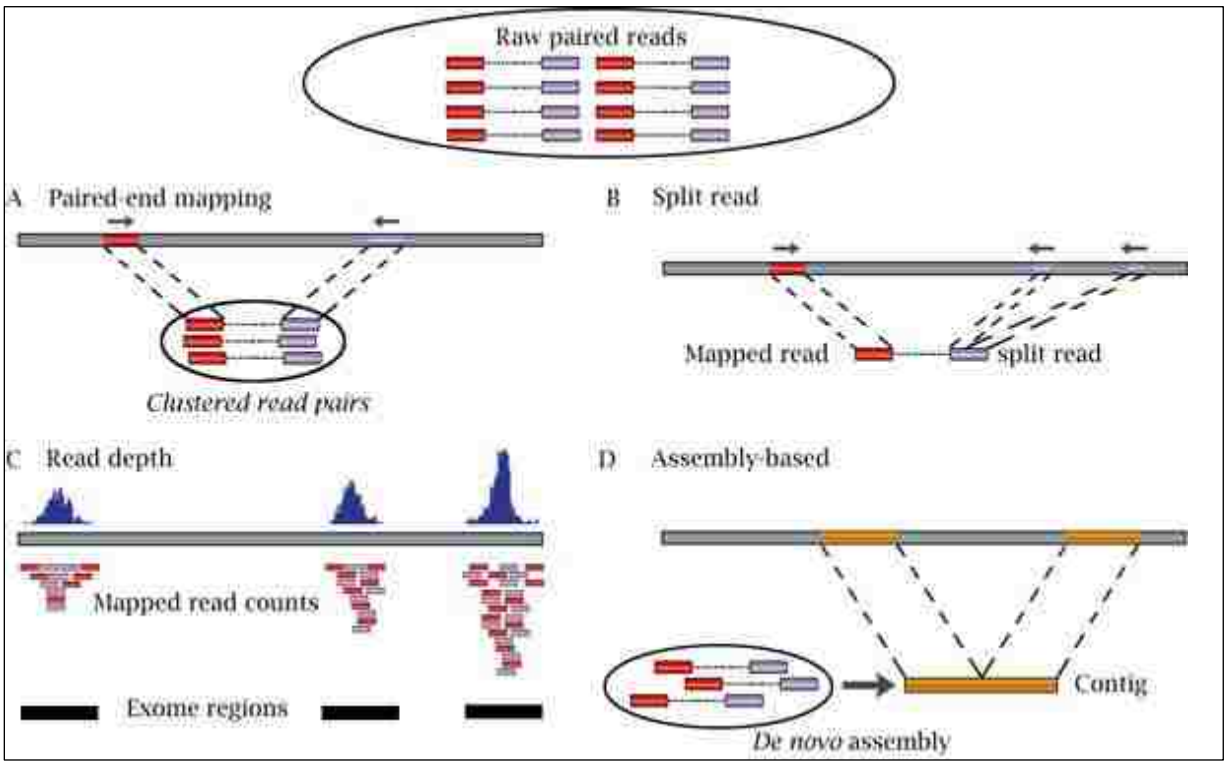

Figure 3.1. CNVs detection methods with NGS data (Zhao et al., 2013). A. Read-Pair (RP), **B**. Split-Read (SR), **C**. Read-Depth (RD), and **D**. Assembly (AS).

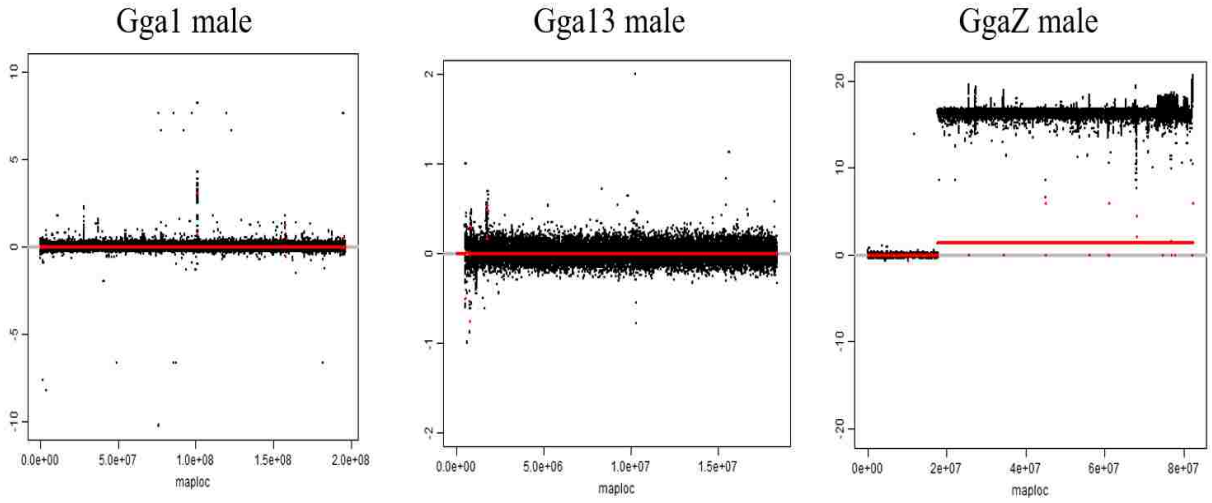

Figure 3.2. Copy number variation (CNV) segmentation plot using cn.mops. The Y-axis represents the log ratio of the read counts and the copy number call of each segment (red) on the genomic position of chromosome 1, 13, and Z in male REL birds.

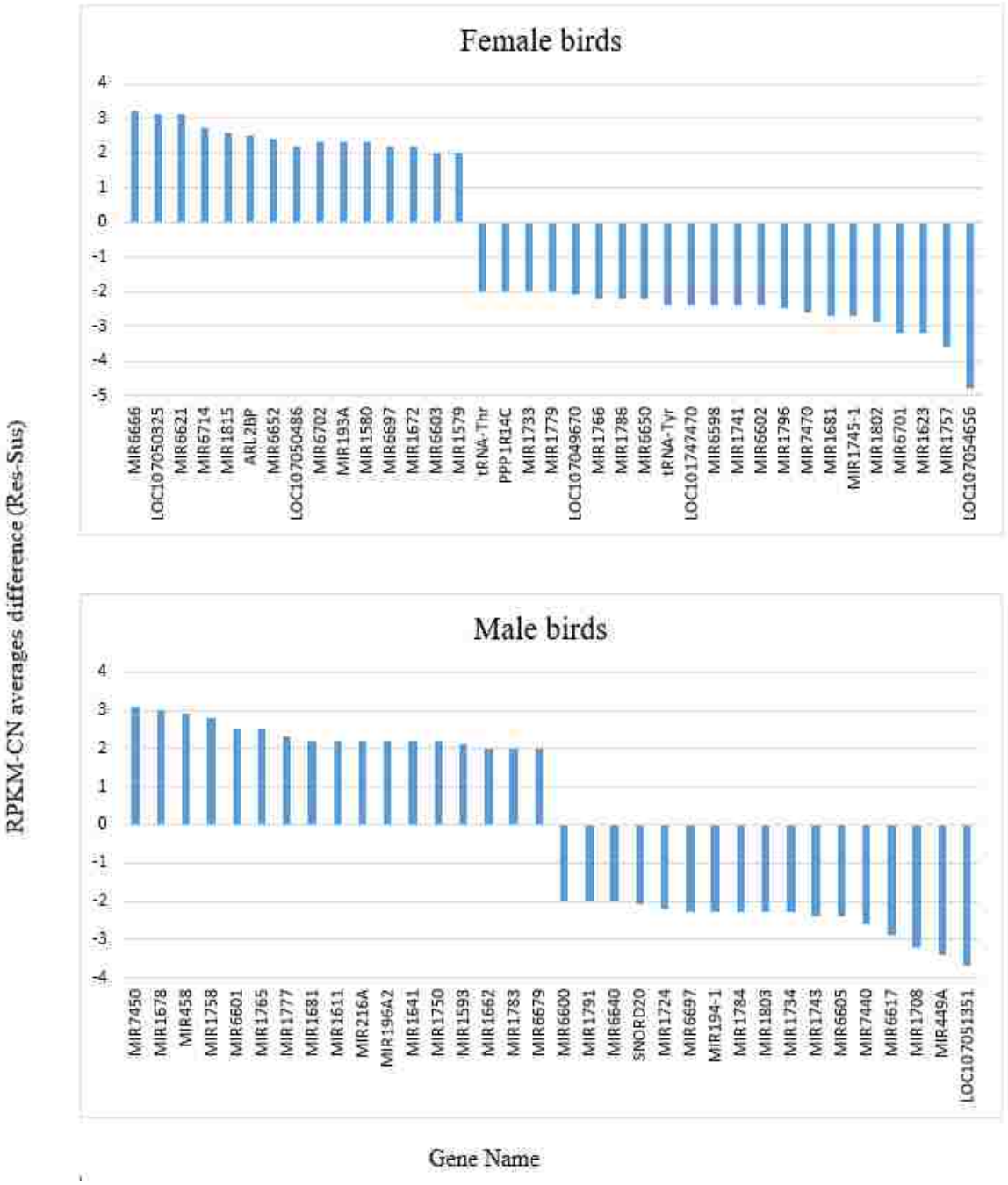

Figure 3.3. Identification of microRNAs and genes with increased copy number. The Y-axis represents the RPKM-CN averages difference (Res-Sus) for give genes and miRNAs (X-axis) in male and female birds of REL line.

Table 3.1. Predicted target genes for microRNAs. MicroRNAs were with increased copies for each phenotype in respective genders of REL line. The predicted target genes are in chicken, but the context represents gene regions with SNPs in human-based association studies.

S. No	Name	Chromosome No.	Gender	Phenotype	Predicted Target Gene (miRDB)	Associated Traits for predicted genes in humans (PheGenI)	Context
1	miR-449a	Z	Male	ascitic-susceptible	NAV3	Blood pressure, Cardiomegaly, ECG, LDL	Intronic
					ERC1	Body weight, Blood pressure	intronic
2	miR-1708	1	Male	ascitic-susceptible	CREB3L1	Heart attack, Pulmonary diseases	nearGene -5
					PPP2R3A	LDL, Blood flow velocity, BMI	intronic, intergenic
3	miR-7450	1	Male	ascitic-resistant	LRIG1	Heart function test, ECG	Intronic
					ARID5B	Arterial pressure, Blood pressure, Pulmonary diseases	Intronic
4	miR-1678	12	Male	ascitic-resistant	ADGRL4	Pulse, Blood pressure	Intergenic
					MYLIP	Cholesterol, LDL	nearGene -5
5	miR-1757	4	Female	ascitic-susceptible	TM9SF2	Blood pressure	Intronic
					RASGEF1C	Heart failure	Intronic
6	miR-1623	10	Female	ascitic-susceptible	RNF165	Body weight	Intronic
					TMEM215	Blood pressure, Coronary artery disease	Intergenic
7	miR-6666	28	Female	ascitic-resistant	N4BP3	Waist-hip ratio	Intergenic
8	miR-6621	1	Female	ascitic-resistant	PRICKLE1	HDL, ECG, Body height	Intergenic
					CUL3	Heart attack	Intergenic
					PTPRE	Coronary disease	Intergenic
					FERMT1	Inflammatory bowl disease, Arteries	Intergenic

3.5 References

Alkan, C., Coe, B. P., and Eichler, E. E. (2011). Genome structural variation discovery and genotyping. *Nature Reviews Genetics,12*(5), 363-376.

Alkan, C., Kidd, J. M., Marques-Bonet, T., Aksay, G., Antonacci, F., Hormozdiari, F., … Eichler, E. E. (2009). Personalized copy number and segmental duplication maps using next-generation sequencing. *Nature Genetics*, *41*(10), 1061–1067.

Anderson, C. A., Pettersson, F. H., Clarke, G. M., Cardon, L. R., Morris, A. P., and Zondervan, K. T. (2010). Data quality control in genetic case-control association studies. *Nature Protocols*, *5*(9), 1564–1573.

Bartel, B. (2005). MicroRNAs directing siRNA biogenesis. *Nature Structural and Molecular Biology*, *12*(7), 569–571.

Bartel, D. P. (2004). MicroRNAs: genomic, biogenesis, mechanism, and function. *Cell*, *116*(2): 281-297.

Berezikov, E., and Plasterk, R. H. (2005). Camels and zebrafish, viruses and cancer: a microRNA update. *Human Molecular* Genetics, *14* (Suppl 2), 183-190.

Bilen, J., Nan, L., and Bonini, N. M. (2006). A new role for micro RNA pathways: modulation of degeneration induced by pathogenic human disease proteins. *Cell Cycle*, *5*(24), 2835–2838.

Boeva, V., Zinovyev, A., Bleakley, K., Vert, J. P., Janoueix-Lerosey, I., Delattre, O., and Barillot, E. (2011). Control-free calling of copy number alterations in deep-sequencing data using GC-content normalization. *Bioinformatics (Oxford, England)*, *27*(2), 268–269.

Chen, X., Yan, C. C., Zhang, X., & You, Z. H. (2017). Long non-coding RNAs and complex diseases: from experimental results to computational models. *Briefings in bioinformatics*, *18*(4), 558–576.

Chung, B. H., Tao, V. Q., and Tso, W. W. (2014). Copy number variation and autism: New insights and clinical implications. *Journal of the Formosan Medical Association,113*(7), 400-408.

Conrad, D. F., Pinto, D., Redon, R., Feuk, L., Gokcumen, O., Zhang, Y., … Hurles, M. E. (2010). Origins and functional impact of copy number variation in the human genome. *Nature*, *464*(7289), 704–712.

Cool, C. D., Stewart, J. S., Werahera, P., Miller, G. J., Williams, R. L., Voelkel, N. F., and Tuder, R. M. (1999). Three-dimensional reconstruction of pulmonary arteries in plexiform pulmonary hypertension using cell-specific markers. Evidence for a dynamic and heterogeneous process of pulmonary endothelial cell growth. *The American Journal of Pathology*, *155*(2), 411–419.

Croce, C. M., and Calin, G. A. (2005). miRNAs, cancer, and stem cell division. *Cell, 122*(1), 6–7.

Deng, L., Blanco, F. J., Stevens, H., Lu, R., Caudrillier, A., McBride, M., … Baker, A. H. (2015). MicroRNA-143 Activation Regulates Smooth Muscle and Endothelial Cell Crosstalk in Pulmonary Arterial Hypertension. *Circulation Research*, *117*(10), 870–883.

Feuk, L., Carson, A. R., and Scherer, S. W. (2006). Structural variation in the human genome. *Nature Reviews Genetics, 7,* 85-97.

Friedman, R. C., Farh, K. K., Burge, C. B., and Bartel, D. P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Research*, *19*(1), 92–105.

Grant, J. S., White, K., MacLean, M, R., and Baker, A. H. (2013). MicroRNAs in pulmonary arterial remodeling. Cellular and Molecular Life Sciences, 70(23): 4479-4494.

Griffiths-Jones, S., Grocock, R. J., van Dongen, S., Bateman, A., and Enright, A. J. (2006). miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Research*, *34*(Database issue), D140–D144.

Hastings, M. L., and Krainer, A. R. (2001). Pre-mRNA splicing in the new millennium. *Current Opinion in Cell Biology*, *13*(3), 302–309.

Janevski, A., Varadan, V., Kamalakaran, S., Banerjee, N., and Dimitrova, N. (2012). Effective normalization for copy number variation detection from whole genome sequencing. *BMC Genomics*, *13 Suppl 6*(Suppl 6), S16.

Jiang, Y., Wang, Y., and Brudno, M. (2012). PRISM: pair-read informed split-read mapping for base-pair level detection of insertion, deletion and structural variants. *Bioinformatics, 28* (20), 2576–2583

Korbel, J. O., Abyzov, A., Mu, X. J., Carriero, N., Cayting, P., Zhang, Z., … Gerstein, M. B. (2009). PEMer: a computational framework with simulation-based error models for inferring genomic structural variants from massive paired-end sequencing data. *Genome Biology*, *10*(2), R23.

Korbel, J. O., Urban, A. E., Affourtit, J. P., Godwin, B., Grubert, F., Simons, J. F., … Snyder, M. (2007). Paired-end mapping reveals extensive structural variation in the human genome. *Science (New York, N.Y.), 318* (5849), 420–426.

Kozomara, A., and Griffiths-Jones, S. (2014). miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Research*, *42*(Database issue), D68–D73.

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W., and Tuschi, T. (2002). Identification of tissue-specific microRNAs from mouse. *Current Biology*, *12*(9), 735-739.

Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., … Tuschl, T. (2007). A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell*, *129*(7), 1401–1414.

Leak, L. V., Petricoin, E. F., Jones, M., Paweletz, C. P., Ardekani, A. M., Fusaro, V. A., and Liotta L. A. (2002). Proteomic technologies to study disease of the lymphatic vascular system. *Annals of the New York Academy of Sciences, 979,* 211–228.

Lin, S., Li, H., Mu, H., Luo, W., Li, Y., Jia, X., … Zhang, X. (2012). Let-7b regulates the expression of the growth hormone receptor gene in deletion-type dwarf chickens. *BMC Genomics, 13,* 306.

Ma, S., Zhao, Y., Sun, J., Mu, P., & Deng, Y. (2018). miR449a/SIRT1/PGC-1α Is Necessary for Mitochondrial Biogenesis Induced by T-2 Toxin. *Frontiers in Pharmacology, 8,* 954.

Malhotra, D., & Sebat, J. (2012). CNVs: harbingers of a rare variant revolution in psychiatric genetics. *Cell, 148*(6), 1223–1241.

Marcinkowska, M., Szymanski, M., Krzyzosiak, W. J., and Kozlowski, P. (2011). Copy number variation of microRNA genes in the human genome. *BMC Genomics, 12,* 183.

Mathews, D. H., Sabina, J., Zuker, M., Turner, D. H. (1999). Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *Journal of Molecular Biology, 288,* 911–940.

McLendon, J. M., Joshi, S. R., Sparks, J., Matar, M., Fewell, J. G., Abe, K., … Gerthoffer, W. T. (2015). Lipid nanoparticle delivery of a microRNA-145 inhibitor improves experimental pulmonary hypertension. *Journal of Controlled Release: official journal of the Controlled Release Society, 210,* 67–75.

McManus, M. T. (2003). MicroRNAs and cancer. *Seminars in Cancer Biology, 13*(4), 253–258.

Medvedev, P., Stanciu, M., and Brundo, M. (2009). Computational methods for discovering structural variation with next-generation sequencing. *Nature Methods, 6,* S13-20.

Mills, R. E., Walter, K., Stewart, C., Handsaker, R. E., et al. (2011). Mapping copy number variation by population-scale genome sequencing. *Nature, 470,* 59-65.

Nijkamp, J. F., van den Broek, M. A., Geertman, J. M., Reinders, M. J., Daran, J. M., and de Ridder, D. (2012). De novo detection of copy number variation by co-assembly. *Bioinformatics, 28* (24), 3195–3202.

Riffo-Campos, Á. L., Riquelme, I., and Brebi-Mieville, P. (2016). Tools for Sequence-Based miRNA Target Prediction: What to Choose?. *International Journal of Molecular Sciences, 17*(12), 1987.

Rhodes, C. J., Whaton, J., Boon, R. A., Roexe, T., Tsang, H., Wojciak-Stothard, B., Chakrabarti, A…. Wilkins, M. R. (2013). Reduced microRNA-150 is associated with poor survival in pulmonary arterial hypertension. *American Journal of Respiratory Critical Care Medicine, 187*(3), 294-302.

Rothman, A., Restrepo, H., Sarukhanov, V., Evans, W. N., Jr, R. G., Williams, R., . . . Mann, D. (2017). Assessment of microRNA and gene dysregulation in pulmonary hypertension by endoarterial biopsy. *Pulmonary Circulation,7*(2), 455-464.

Sakao, S., Tatsumi, K., and Voelkel, N. F. (2010). Reversible or irreversible remodeling in pulmonary arterial hypertension. *American Journal of Respiratory Cell and Molecular Biology*, *43*(6), 629–634.

Schnall-Levin, M., Rissland, O. S., Johnston, W. K., Perrimon, N., Bartel, D. P., and Berger, B. (2011). Unusually effective microRNA targeting within repeat-rich coding regions of mammalian mRNAs. *Genome Research*, *21*(9), 1395–1403.

Sebat, J., Lakshmi, B., Troge, J., Alexander, J., Young, J., Lundin, P., Maner, S., Massa, H., Walker, M …. Wigler, M. (2004). Large-scale copy number polymorphism in the human genome. *Science, 305*(5683), 525–528.

Sethupathy, P., Corda, B., and Hatzigeorgiou, A. G. (2006). TarBase: A comprehensive database of experimentally supported animal microRNA targets. *RNA (New York, N.Y.)*, *12*(2), 192–197.

Teo, S. M., Pawitan, Y., Ku, C. S., Chia, K. S., and Salim, A. (2012). Statistical challenges associated with detecting copy number variations with next-generation sequencing. *Bioinformatics, 28,* 2711–2718.

Thum, T., Catalucci, D., and Bauersachs, J. (2008). MicroRNAs: novel regulators in cardiac development and disease. Cardiovascular Research, 79(4), 562-570.

Urbich, C., Kuehbacher, A., and Dimmeler, S. (2008). Role of microRNAs in vascular diseases, inflammation, and angiogenesis. *Cardiovascular Research,79*(4), 581–588.

Wallace, E., Morrell, N. W., Yang, X. D., Long, L., Stevens, H., Nilsen, M., … MacLean, M. R. (2015). A Sex-Specific MicroRNA-96/5-Hydroxytryptamine 1B Axis Influences Development of Pulmonary Hypertension. *American Journal of Respiratory and Critical Care Medicine*, *191*(12), 1432–1442.

Wang, J., Mullighan, C. G., Easton, J., Roberts, S., Heatley, S. L., Ma, J., … Zhang, J. (2011). CREST maps somatic structural variation in cancer genomes with base-pair resolution. *Nature Methods*, *8*(8), 652–654.

Wang, L. Y., Abyzov, A., Korbel, J. O., Snyder, M., and Gerstein, M. (2009). MSB: a mean-shift-based approach for the analysis of structural variation in the genome. *Genome Research*, *19*(1), 106–117.

Wong, N., and Wang. (2015). miRDB:an online resource for microRNA target prediction and functional annotations. *Nucleic Acid Research, 43*(D1), 146-152.

Xiao, F., Zuo, Z., Cai, G., Kang, S., Gao, X., & Li, T. (2009). miRecords: an integrated resource for microRNA-target interactions. *Nucleic Acids Research*, *37*(Database issue), D105–D110.

Zamore, P. D., and Haley, B. Ribo-genome: the big world of small RNAs. *Science*,*309*(5740), 1519–1524.

Zhang, J., Wang, J., and Wu, Y. (2012). An improved approach for accurate and efficient calling of structural variations with low-coverage sequence data. *BMC Bioinformatics*, *13 Suppl 6*(Suppl 6), S6.

Zhang, Z. D., Du, J., Lam, H., Abyzov, A., Urban, A. E., Snyder, M., and Gerstein, M. (2011). Identification of genomic indels and structural variations using split reads. *BMC Genomics*, *12*, 375.

Zhao, M., Wang, Q., Wang, Q., Jia, P., and Zhao, Z. (2013). Computational tools for copy number variation (CNV) detection using next-generation sequencing data: features and perspectives. *BMC Bioinformatics, 14 Suppl 11*(Suppl 11), S1.

Zhou, J., Lemos, B., Dopman, E. B., and Hartl, D. L. (2011). Copy-number variation: the balance between gene dosage and expression in Drosophila melanogaster. *Genome Biology and Evolution*, *3*, 1014–1024.

Zhu, M., Need, A. C., Han, Y., Ge, D., Maia, J. M., Zhu, Q., … Goldstein, D. B. (2012). Using ERDS to infer copy-number variants in high-coverage genomes. *American Journal of Human Genetics*, *91*(3), 408–421.

CHAPTER 4: WHOLE GENOME RESEQUENCING FOR MAPPING ASCITES RELATED LOCI IN A COMMERCIAL BROILER LINE.

4.1 Introduction

Ascites or pulmonary hypertension syndrome of fast-growing broilers is a pathophysiological, metabolic condition, where progressive hypertension results in accumulation of fluid in the abdominal cavity leading to death (Wideman et al., 2013). We used whole-genome resequencing (WGR) in our ascites research line, REL, to identify 31 SNP islands (Chapters 2) and 10 CNVs (Chapter 3) as potential loci influencing ascites phenotype. Dey et al., (2018) confirmed one of these regions as a QTL for ascites phenotype not only in our research line but also in elite commercial lines. Chapter 2 confirms an additional region as a QTL for ascites. The REL is the unselected descendant of an elite commercial line from 1998. The goal of this present study is to apply WGR to an unrelated commercial line to determine whether the regions identified in REL are shared in this unrelated commercial broiler line.

4.2 Material and Methods

4.2.1 Genome Data

All genomic positions presented are relative to the March 2018 assembly of the *Gallus gallus* genome (RefSeq accession ID: GCF_000002315.6)

4.2.2 Bird Stocks and Hypobaric Chamber Trials

All animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee under number protocols 15039 and 15040. This study represents birds from a commercial broiler, designated T. The birds were challenged for four weeks using a hypobaric chamber set at 9000 ft and 543 mm of Hg above sea level (Pavlidis et al., 2007). Mortality was recorded daily, and necropsy was used to determine gender and the cause of death. The birds were designated ascitic/susceptible when there was excessive abdominal fluid with flaccid heart, liver

lesions, and right ventricle hypertrophy (right ventricle to total ventricle; RV/TV > 0.5). At four weeks, survivors were euthanized by cervical dislocation and necropsied. Birds with normal necropsy characters were scored as resistant.

4.2.3 DNA Extraction from blood

Blood (10 uL) was collected via wing vein lancet puncture from birds that were 2-3 days old. Genomic DNAs were isolated using our rapid protocol (Bailes et al., 2007). DNAs for next generation sequencing were further purified by phenol-chloroform extraction followed by ethanol precipitation. DNAs were quantified by Hoechst 33258 fluorescence in a GloMax (Promega Corp., Madison, WI).

4.2.4 DNA preparation for WGR

A total of 24 DNAs from each phenotype for male birds were used to construct libraries using a RIPTIDE™ library kit according to the manufacturer's protocol (iGenomX, Carlsbad, CA). Pooled barcoded libraries were sequenced, 2 x 150 paired-end, on an Illumina HiSeqX through Genohub Inc. (Austin, TX) in collaboration with Admera Health (South Plainfield, NJ), to generate an average of 5x coverage.

4.2.5 Bioinformatics and Data Analyses

Demultiplexing of the raw FASTQ reads was done using the DemuxFastqs tool in fgbio (https://fulcrumgenomics.github.io/fgbio/) as a Docker container on a Windows 10 CPU. Some reads had barcodes where the first base was "N" so the read structure was input as forward: 1S7B8M134T and reverse: 8M142T, where "S" stands for skip, "B" for sample barcode, "M" for molecular barcode, and "T" for template (Figure 1). Because we reduced the barcode reads from 8 to 7 bases, max-mismatches was set to 0 and min-mismatch-delta to 1.

Demultiplexed FASTQ reads were mapped onto the galGal6 reference genome using NGen (Lasergene Suite 15.2; DNAStar, Madison, WI) and SNP frequencies computed using ArrayStar in the same package. For read mapping, the 48 samples were grouped into four pools, coded as ascites/susceptible (Y) and normal/resistant (N) with each group having 12 samples and biological replicate 1 and 2. Pool 2 (Y2 and N2) contained the 12 samples from each phenotype with the highest read depth, while pool 1 contained the 12 samples from each phenotype with the lowest read depth. Separate templated alignments for each pool were used for SNP identification and tabulation in ArrayStar and the SNP data for only chromosomes 2 (CPQ), 22 (LRRTM4) and Z, were exported to Excel for further analyses. To look for regions associated with ascites phenotype we generated X-Y plots, for chromosomal position vs. the difference in the SNP% value for the high read depth pools (N2-Y2 in graphs) for only those SNPs with read depths in both N2 and Y2 greater than 60. For SNP positions where Y1 and N1 both had read depths greater than 60 we also plotted the average of the differences (N-Y in graphs) for the paired pools (i.e., average of N2-Y2 and N1-Y1).

ArrayStar CNV graph was used to detect possible CNVs in the phenotypes (Figure 4.2). The genes with increased copy number were tested for possibly related to ascites traits in humans using NCBI Phenotype-Genotype Integrator or PheGenI (https://www.ncbi.nlm.nih.gov/gap/phegeni). We used miRbase (https://www.mirbase.org) for retrieving the mature miRNA sequence and annotation information (Griffiths-Jones et al., 2006). Target genes for given chicken microRNAs were predicted using used mirDB (http://www.mirdb.org) developed by Wong and Wang, (2015).

4.3 Results

4.3.1 Templated Assembly and Whole Genome Resequencing

Individually barcoded libraries were constructed for a total of 48 male birds, 24 samples from each phenotype of commercial broiler line T. Pooled libraries were sequenced to generate an average

of 5x coverage, with a minimum of 0.1x and a maximum of ~13x. Demultiplexed FASTQ reads were grouped (See Material and Methods) before mapping onto the galGal6 reference genome. A total of 663.7 million reads were counted, with 608.8 million assembled reads and 54.9 million unassembled reads for the grouped assemblies (Table 4.1). Since we were interested in the validated SNPs from WGR in REL, we focused on chromosome 2, and 22. We included chromosome Z because the work was solely in males. We identified a total of 14,095,066 SNPs for the grouped assemblies, with 1,996,001 SNPs on chromosome 2, 56,610 SNPs on chromosome 22, and 779,150 on chromosome Z (Figure 4.1).

4.3.2 CPQ gene on Chromosome 2

Dey et al. (2018) identified the region of the CPQ gene on chromosome 2 of REL line birds as significantly associated with ascites phenotype in multiple broiler lines. We were particularly interested in determining whether the same CPQ region would be relevant to ascites phenotype in the unrelated commercial line T. The extensive genotyping for this gene in REL and other commercial lines, was primarily based on two SNPs in intron-6 and one in exon-8. We inspected the SNP data for those specific regions and found that the SNPs in intron 6 were missing or poorly represented in the ArrayStar output for chromosome 2 from line T. The SNPs had been identified by read mapping on to galgal5. Therefore, we aligned the region for intron-6 from galgal5 with the same region from galgal6. The multiple alignment revealed gaps for the CPQ intron-6 SNP positions for galGal6a assembly (Figure 4.3). As Dey et al. (2018) had extensively sequenced this region from PCR products from REL, this suggests that Jungle Fowl which is the basis of the chicken genome assemblies may differ from the broilers in our studies. This makes it difficult to construct haplotypes combining intron-6 SNPs with exon-8. The CPQ exon-8 SNP position was much more clearly conserved in both assemblies a consensus but the difference between the SNP frequency for resistant and susceptible pools in line T does not support this region being associated

with ascites phenotype in line T (Figure 4.4). This argues that CPQ is a QTL for ascites in some but not all broiler lines.

4.3.3 LRRTM4 gene on Chromosome 22

We have validated LRRTM4 on chromosome 22 in several broiler lines (Chapter 2). We, therefore, examined the same region in line T from the WGR data (Figure 4.5). We looked for LRRTM4 gene region that was associated with ascites resistance in male birds of REL line when combined with CPQ (Chapter 2). However, no such peak or any other peaks were found on chromosome 22 (Figure 5) and we conclude that LRRTM4 cannot be a QTL for ascites in this commercial broiler line.

4.3.4 Candidate SNPs on Chromosome Z

According to studies, ascites incidence is higher in male broilers as compared to females. Previous GWAS work identified few regions on chromosome Z that were in association with ascites phenotype (Tarrant et al., 2017). We also found three candidate regions on Z of male REL birds from WGR work, where two of the regions showed association with resistance and the third one with susceptibility (Chapter 2). We examined the same regions in line T (Figure 4.6) and none of the regions appeared to have association with ascites phenotype in line T. This again argues that chromosome Z potential QTLs for ascites are specific for some not all broiler lines.

4.3.5 Candidate miRNAs as CNVs

We found only two microRNAs, miR-99A and miR-1756B (Figure 4.2), which have increased number of copies in ascitic-resistant males. We did not find any ascites associated traits for these genes in humans using PheGenI. However, looking at the predicted target genes and literature for these chicken miRNAs tells us a different story.

Hu et al. (2012) have shown that leptin treated chickens upregulated miR-99A along with 4 other miRNAs suggesting their potential role in hepatic lipid metabolism. MicroRNA 99A was one of the predicted miRNAs targeting 3-hydroxy-3methylglutaryl-CoA reductase, HMGCR, and sterol regulatory element binding protein 1c, SREBP-1c. Looking at these genes in PheGenI, we found only HMGCR linked with blood pressure, heart failure, stroke, atrial fibrillation, and myocardial ischemia in humans.

On the other hand, our predicted target gene, kelch repeat BTB domain containing 8 (KBTBD8), is found to be associated with traits such as heart failure, and electrocardiography in humans (Table 4.2). Another target gene, platelet activating factor acetylhydrolase 1b regulatory subunit 1 (PAFAH1B1) was associated with vascular diseases in human.

For miR-1756B, both of the target genes, glypican 6 (GPC6) and GRB2 associated regulator of MAPK1 subtype 1 (GAREM1) are associated with blood pressure in humans. However, we did not find any literature on the potential role of miR-1756B in chicken pulmonary diseases.

4.4 Discussion

Whole genome resequencing is an unbiased approach to find QTLs associated with phenotypes across different species including chicken. Our previous work on REL line (Chapter 2) has successfully identified potential QTLs for ascites. Extension of this approach to an entirely unrelated broiler line, line T, was aimed at determining whether the regions identified in REL are shared in this unrelated commercial broiler line. CPQ and LRRTM4 (Chapter 2) have been extensively analyzed in REL birds for ascites phenotypes but we did not see any deviations in SNP frequency differences for these gene regions with ascites in line T. One possible answer is that because the genetic background is different, the QTLs for ascites are in other regions and therefore, we need to systematically work through the rest of the genome to find those alternative regions.

Another possibility is that galGal6 assembly is not a good template for our WGR and we need to return to galGal5. Alternatively, since we used the iGenomX kit on individual barcoding rather than pooling DNAs for single barcoding that we have introduced sufficient "noise" in the system that the WGR data is not good enough for identification of regions associated with ascites.

For the CNV work, we were successful in identifying two miRNAs that appear to be associated with resistance in these male birds. The literature on the miRNAs and predicted target genes in human imply the possible correlation between genes and phenotype. However, the effect of the miRNAs on the target genes and further verification would be through additional genotyping and examining miRNA expression levels. This will help us understand whether they play a direct role in ascites and how they contribute to the disease in broilers? This is important because the target genes are predicted, and associated traits studies were conducted in humans.

In conclusion, CPQ and LRRTM4 and other QTLs on chromosome Z cannot be used as makers for ascites in all broiler lines. In future, the other 26 potential QTL regions from REL line still need to be validated in this unrelated commercial line, T. We will also resequence the DNAs to get a uniform coverage for our samples and align against a better version of galGal6 assembly.

Table 4.1. Mapped and unmapped reads to the chicken reference genome (galGal6). The assembly statistics are the grouped assemblies with two replicates from each phenotype in total 48 bird samples from the SeqMan NGen reports in commercial broiler line T.

Mapping Statistics for the grouped DNA assemblies	
Total number of reads generated for pooled (million)	663.7
Total number of reads assembled (million)	608.8
Total number of reads unassembled (million)	54.9
Total % of unassembled reads	8

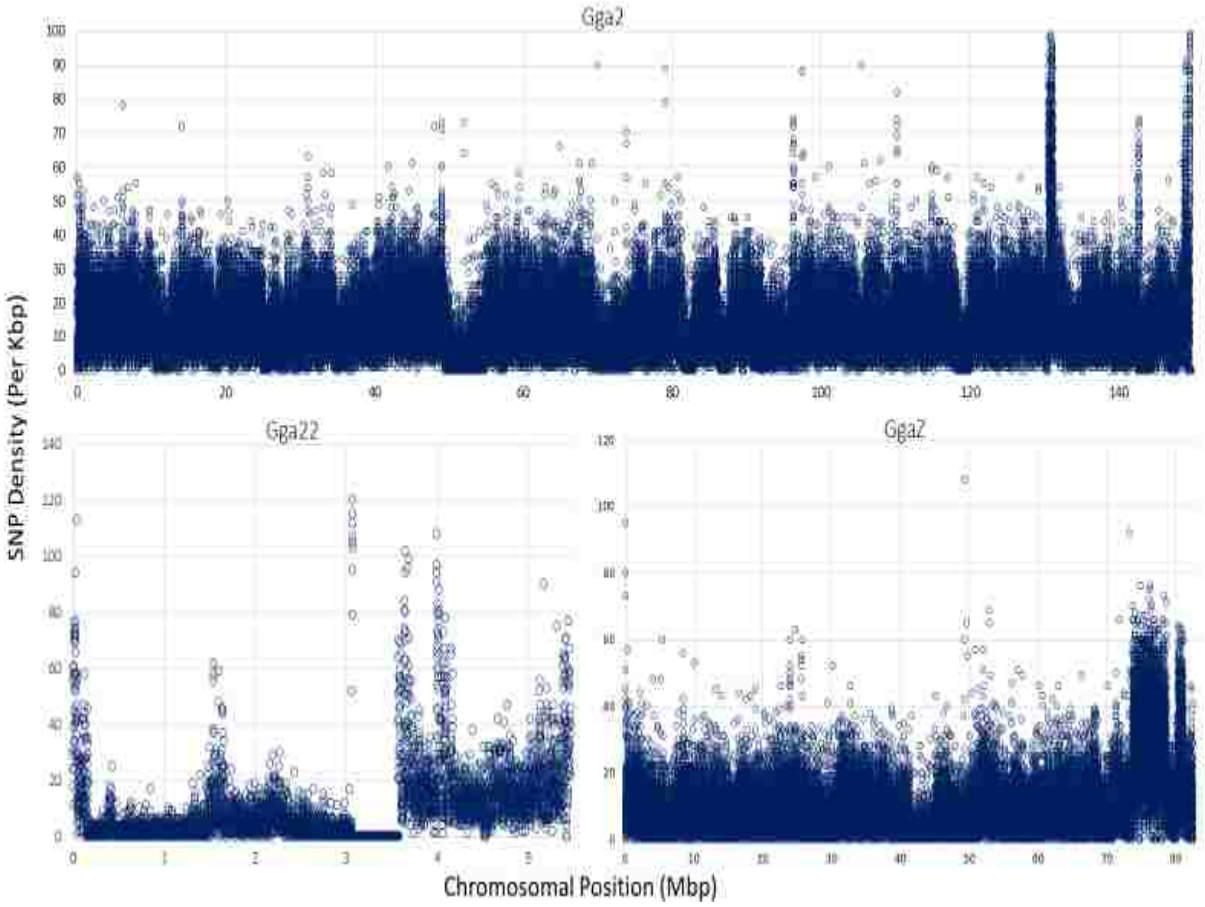

Figure 4.1. SNP density across chromosomes 2, 22, and Z for T line. The plots were generated using SNP density per 1000 bases (Y-axis) for Mbp positions on each chromosome (X-axis).

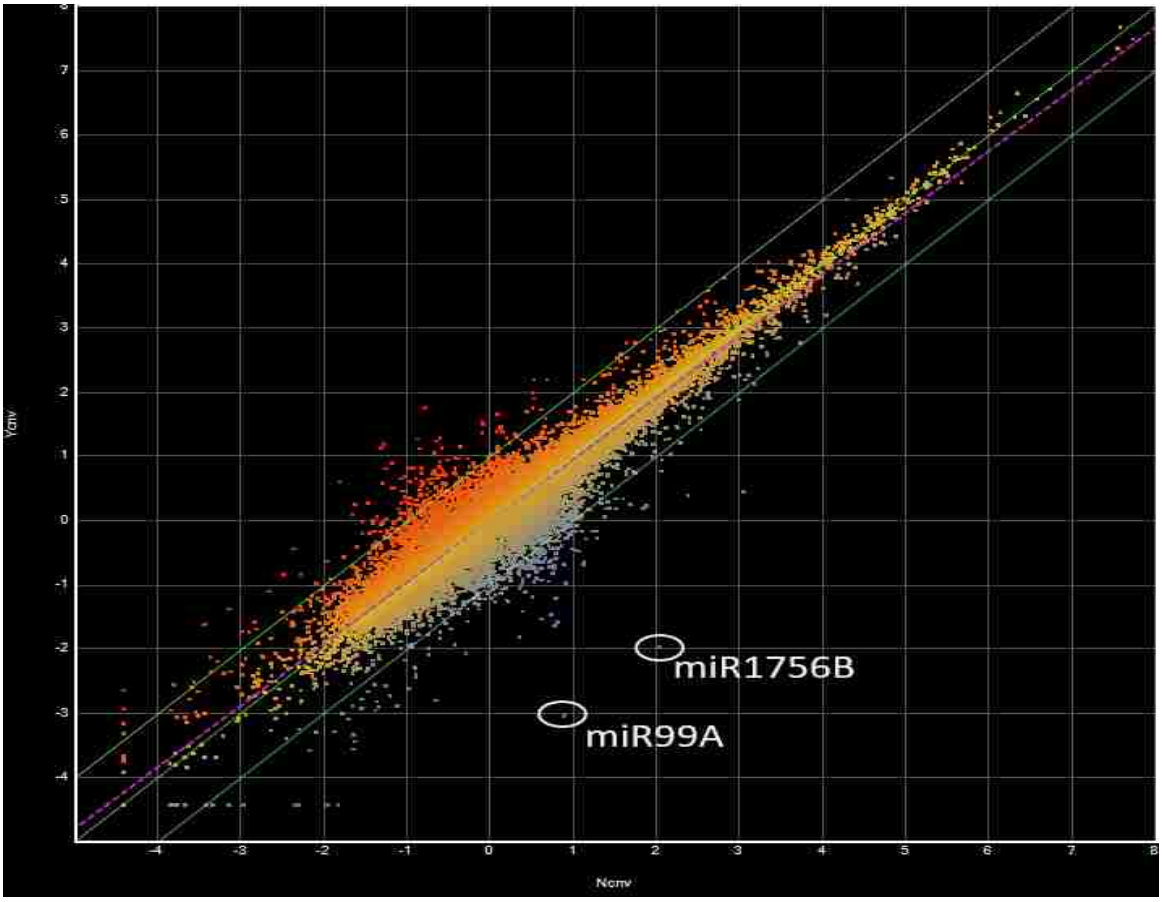

Figure 4.2. Identification of copy number variations in line T. The scatterplot was generated by plotting the CNV values in ascites-susceptible birds (Y-axis) against CNV values in ascites-resistant birds (X-axis).

Figure 4.3. Multiple sequence alignment of the probes for CPQ gene from Dey et al. (2018) with galGal5 and galGal6. 2A) MSA results for intron-6 of CPQ gene on chromosome 2 reveals the gap in the galGal6a. **2B)** MSA results for exon-8 of CPQ gene on chromosome 2 are an exact match.

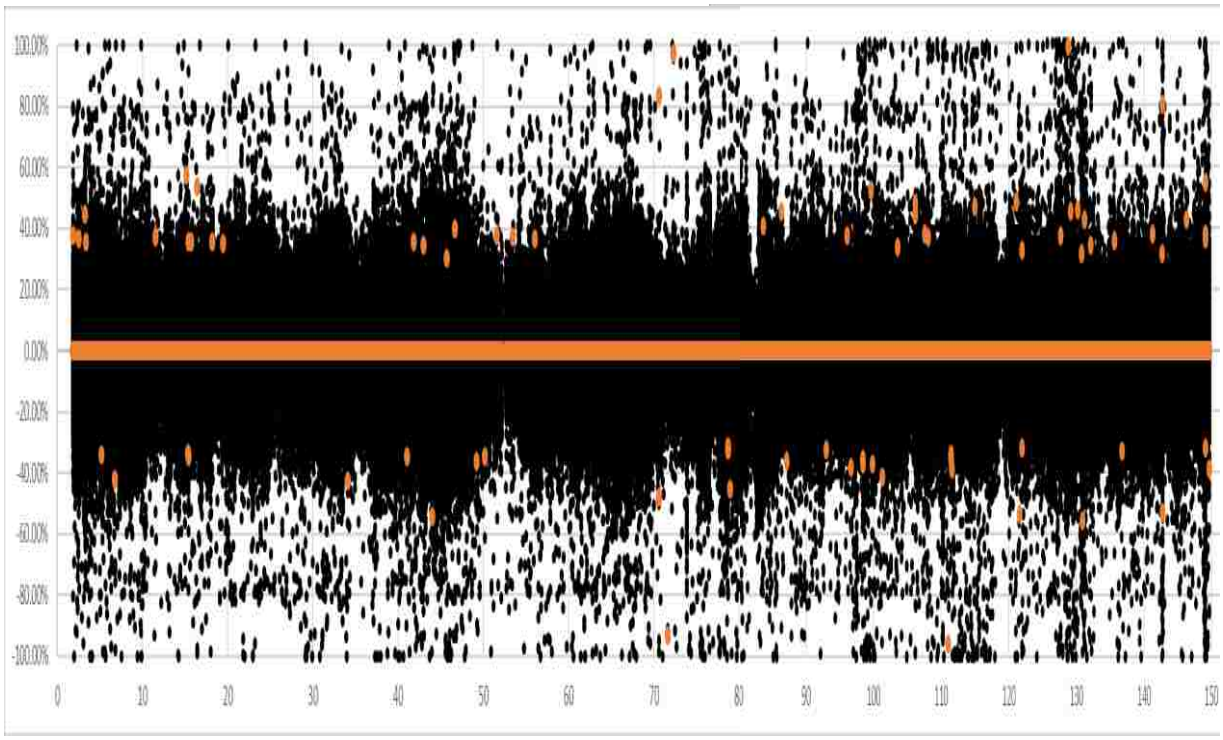

Figure 4.4. SNP frequency plots from WGR for chromosome 2 and including the CPQ gene. Data plotted was the difference in the SNP% (normal-ascites) vs. chromosomal position (Mbp). Black dots represent only those SNPs in pool 1 with read depths >60. The orange dots represent SNPs in paired pools with read depths> 60 where the difference was <-0.3 or >0.3.

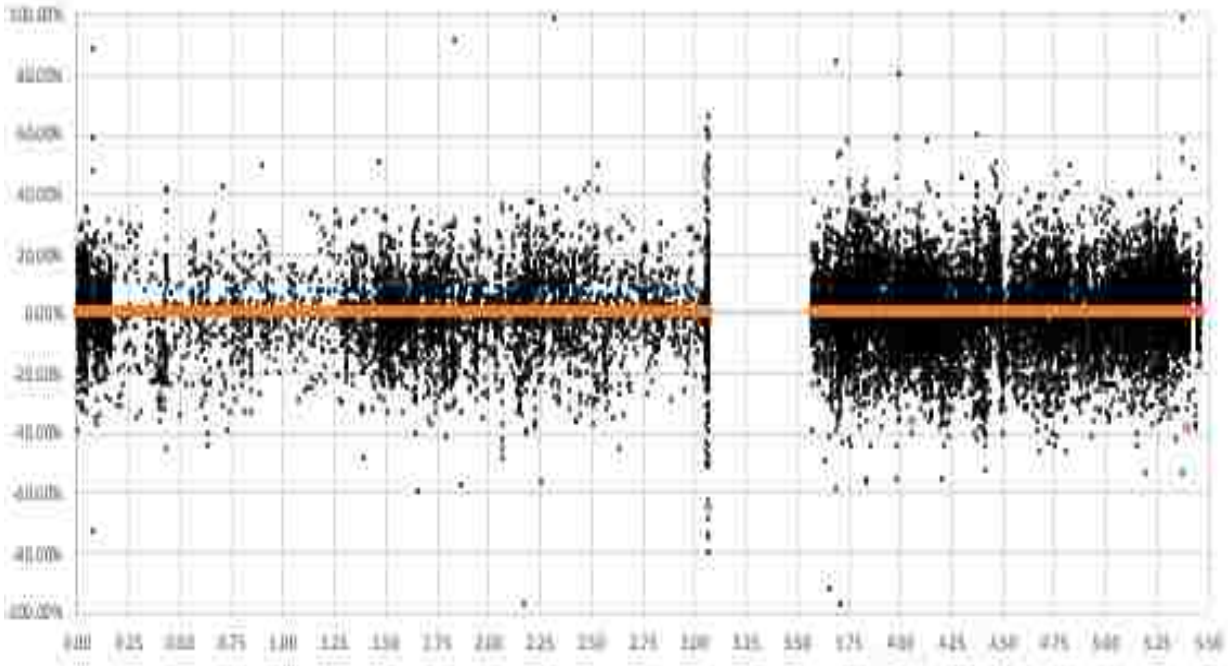

Figure 4.5. SNP frequency plots from WGR for chromosome 22 and including LRRTM4 gene. Data plotted was the difference in the SNP% (normal-ascites) vs. chromosomal position (Mbp). Black dots represent only those SNPs in pool 1 with read depths >60. The orange dots represent SNPs in paired pools with read depths> 60 where the difference was <-0.3 or >0.3.

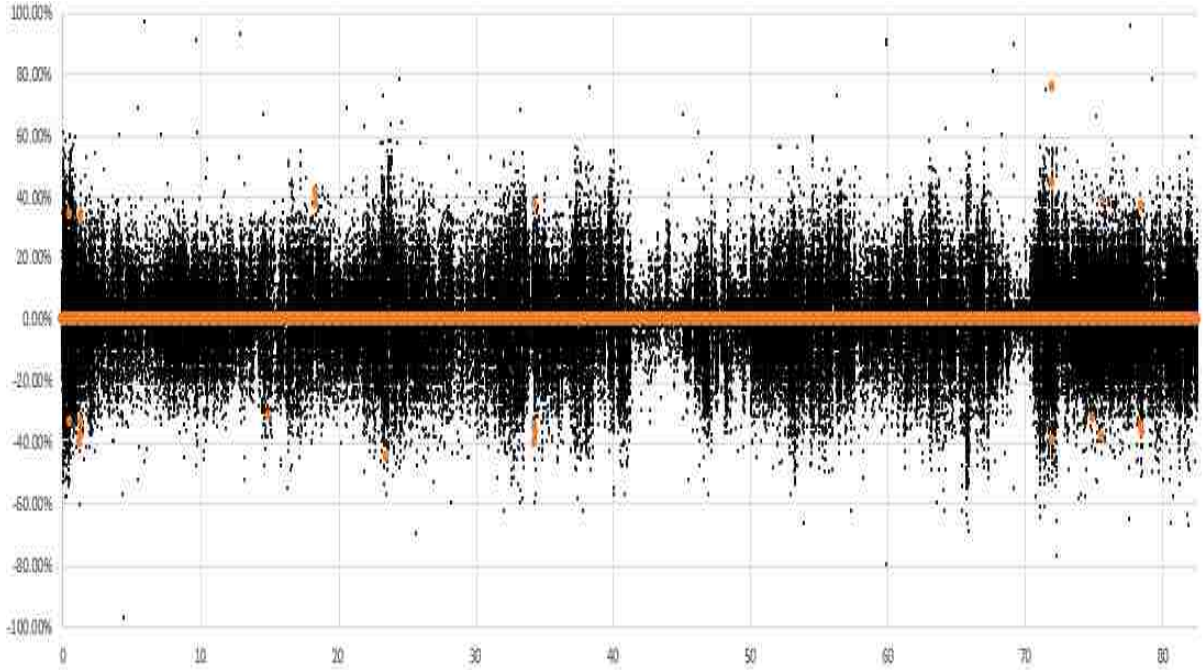

Figure 4.6. SNP frequency plots from WGR for chromosome Z. Data plotted was the difference in the SNP% (normal-ascites) vs. chromosomal position (Mbp). Black dots represent only those SNPs in pool 1 with read depths >60. The orange dots represent SNPs in paired pools with read depths> 60 where the difference was <-0.3 or >0.3.

Table 4.2. Predicted target genes for microRNAs. MicroRNAs were with increased copies in resistant male birds of a commercial line, T. The predicted target genes are in chicken, but the context represents gene regions with SNPs in human-based association studies.

Gene Name	Chr. No.	Phenotype	Predicted Target Genes (miRDB)	Associate traits for predicted genes in humans (PheGenI)	Context
miR-99A	1	ascitic-resistant	KBTBD8	heart failure, ECG	intergenic
			PAFAH1B1	vascular diseases	intronic
miR-1756B	3	ascitic-resistant	GPC6	blood pressure, body mass	intergenic
			GAREM1	blood pressure, heart rate, ECG	intronic

4.5 References

Dey, S., Parveen, A., Tarrant, K. J., Licknack, T., Kong, B. C., Anthony, N. B., and 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.

Griffiths-Jones, S., Grocock, R. J., van Dongen, S., Bateman, A., & Enright, A. J. (2006). miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Research, 34*(Database issue), D140–D144.

Hu, Y., Zhang, R., Zhang, Y., Li, J., Grossmann, R., & Zhao, R. (2012). In ovo leptin administration affects hepatic lipid metabolism and microRNA expression in newly hatched broiler chickens. *Journal of Animal Science and Biotechnology, 3*(1), 16.

Tarrant, K. J., 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, 96*(6), 1544-1552.

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

Wong, N., & Wang, X. (2015). miRDB: an online resource for microRNA target prediction and functional annotations. *Nucleic Acids Research, 43*(Database issue), D146–D152.

CHAPTER 5. CONCLUSION

Poultry meat is a valuable source of protein, minerals (iron, calcium, phosphorus), and essential vitamins such as A, B16, B12, C, D, and folic acid (Grashorn, 2007). It has less fat content as compared to red meat and comprises mainly monosaturated fatty acids (Marangoni et al., 2015; Mottet and Tempio, 2017). Beneficial Omega-6 (n-6) essential lipids (such as linoleic acid and arachidonic acid) are also precursors of biological effectors (such as eicosanoids, prostaglandins) in the regulation of inflammation, hypertension, and cardiovascular system. Broiler breeding industries have been successful in the selection of economically important traits, such as growth rate and meat yield. The resulted affordability has increased the consumption of poultry products as the common animal food source around the globe, making them one of the keys to food security and nutrition.

Ascites or pulmonary hypertension syndrome is one of the consequences of intense genetic selection for fast growth and is a major cause of mortality in broilers with 5-8 % prevalence worldwide (Balog, 2003: Pavlidis et al., 2007). Better management has reduced the incidence of ascites in heavier broiler flocks but still costs millions of dollars to the US poultry industry. Therefore, a better understanding of the genetics of ascites will help identify the biomarkers to reduce the risk in breeding industries.

Several studies have been conducted in our lab to investigate the genetic bases of ascites in broilers. Krishnamoorthy et al. (2014) used GWAS to identify AGTR1 and UTS2D genes in a region around 12-13 Mbp (galGal4 assembly) on chromosome 9 as potential candidates for the disease. However, further analyses found the SNPs to be not significantly associated with ascites (Dey et al., 2016). Another region around 70 Mbp on chromosome 2 and one around 60 Mbp on chromosome Z (galGal4 assembly) were found to be potential QTL regions with no association on further

investigation (Dey et al., 2016; Tarrant et al., 2017). With great advancement in high throughput sequencing, whole genome resequencing (WGR) study by Dey et al. (2018) revealed another region on chromosome 2, spanning 127.62 to 127.75 Mbp (galGal5 assembly). This region encompasses part of carboxypeptidase Q (CPQ) associated with cardiovascular trait (https://www.ncbi.nlm.nih.gov/gap/phegeni). Further analyses revealed an association of CPQ with resistance in male birds suggesting alleles to be used as genetic markers for ascites (Dey et al., 2018).

Chapter 2 is summarizing the WGR data, where we sequenced the whole genome for our eight pools to an aligned sequence depth of ~66X using paired-end (2 x 125-bp) Illumina sequencing (HiSeq 2500), and reads were mapped to the galGal5 reference genome. A total of 31 potential regions were identified to be associated with either biased for resistance or susceptibility and one region, CPQ gene, has already been validated (Dey et al., 2018). Another region 3.80-3.90 Mbp on chromosome 22 is encompassing mostly intergenic and the end portion of a gene, leucine-rich repeat transmembrane neuronal protein 4 (LRRTM4), ascites resistant. LRRTM4 can be a potential candidate for the heritable trait for ascites syndrome as it has also shown association with several ascites-related phenotypes including antihypertensive agents, carotid arteries, and venous thromboembolism in humans. Further investigation of SNPs on chromosome 22, make it a strong QTL for ascites in broiler chickens despite the effect being gender specific. In males, the LRRTM4 heterozygote interacts with the CPQ gene on chromosome 2 in an epistatic way to increase resistance in males. The LRRTM4 gene, along with CPQ gene seems to be the most prominent candidates and be employed in marker-assisted selection for increasing ascites resistance in broilers without compromising on the commercially important traits in broiler breeding industry.

Chapter 3 discusses the potential copy number variations that are showing biasedness for ascites phenotypes in each gender. We identified potential CNV peaks on chromosome 1 and 13 using *cn.mops*. In chromosome 1, the peak resides in the region of zinc finger and SCAN domain containing 25 (ZSCAN25), cholinergic receptor nicotinic alpha 10 subunit (CHRNA10), and ADP-ribosyltransferase (ART1) genes. For chromosome 13, the peak resides within a gene, protocadherin beta-5 (PCDHB5), which is found to be associated with traits such as lymphocytes count, platelet function tests, and leptin in humans. We also used ArrayStar to identify more CNV region. In males, we found two miRNAs (miR-7450 and miR-1678) with more copies in ascitic-resistant and another two (miR-449a and miR-1708) in ascitic-susceptible. In resistant female birds, two miRNAs, miR-6666 and miR-6621 were with more copies while in susceptible females miR-1757 and miR-1623 with increased numbers. The targeted genes for these miRNAs were identified using miRDB, and human associated traits for these predicted genes were determined using PheGenI. Most of the target genes are somewhat involved in the traits associated with ascites in human and therefore are worth looking at for future work.

Chapter 4 is summarizing the WGR data, where we sequenced the whole genome for 48 male birds (24 resistant vs. 24 susceptible) from an unrelated commercial line, designated as T. An average of ~5x sequence depth was achieved using paired-end (2 x 150 bp) Illumina sequencing (HiSeq 2500) and reads were mapped to the galGal6 reference genome. We looked into CPQ gene on chromosome 2, and LRRTM4 on chromosome 22 that have already been validated as QTL for ascites in REL line. None of these genes showed association with ascites phenotypes in line T. The candidate regions on chromosome Z was also not found in line T. CPQ and LRRTM4 cannot be used as a QTL for line T despite being associated with other commercial lines such as REL. In future, the rest of the 26 potential QTLs from REL line (Chapter 2) are still be looked into in WGS

data of line T. Two miRNAs, miR-99A and miR-1756B, showed increased copy number in resistant male birds and are, therefore, worth looking at in future work.

5.1 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 and Poultry Biology Reviews,14*(3), 99-126.

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., and Rhoads, D. D. (2018). Whole genome resequencing identifies the CPQ gene as a determinant of ascites syndrome in broilers. *PloS ONE,13*(1).

Grashorn, M. A. (2007). Functionality of Poultry Meat. *The Journal of Applied Poultry Research,16*(1), 99-106.

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

Marangoni, F., Corsello, G., Cricelli, C., Ferrara, N., Ghiselli, A., Lucchin, L., & Poli, A. (2015). Role of poultry meat in a balanced diet aimed at maintaining health and wellbeing: an Italian consensus document. *Food & Nutrition Research, 59,* 27606.

Mottet, A., & Tempio, G. (2017). Global poultry production: Current state and future outlook and challenges. *Worlds Poultry Science Journal,73*(2), 245-256.

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.

Tarrant, K. J., 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, 96*(6), 1544-1552.

CHAPTER 6. APPENDIX

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

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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

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UNIVERSITY OF ARKANSAS

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From: **Douglas Rhoads**
Program Director
Interdisciplinary graduate Program in Cell and Molecular Biology
University Professor of Biological Sciences

September 11, 2019

To: **Whom so ever it concerns**

Re: **Dissertation work of Alia Parveen**

This dissertation submitted by Alia Parveen is based on analysis of DNAs and RNAs extracted from tissue or blood samples collected by other persons under the guidance and compliance certification of Dr. Nicholas Anthony. Alia Parveen was not a party to those protocols which were submitted by Dr. Nicholas Anthony and approved by the Institutional Animal Care and Use Committee of the University of Arkansas. Copies of those approval letters have been included in the appendices.