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Genome Wide Association and Next Generation Sequencing Approaches to Map Determinants of Ascites in Broiler Chickens

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Genome Wide Association and Next Generation Sequencing Approaches to Map Determinants of Ascites in Broiler Chickens

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ABSTRACT

These studies have investigated different candidate genomic regions for their contributions to ascites in broilers. Ascites syndrome is a manifestation of idiopathic pulmonary arteriole hypertension that concerns the poultry industry worldwide. Investigations have demonstrated the disease to be genetically regulated and to exhibit moderate to high heritabilities. Although previous studies have indicated a few chromosomes to be involved with ascites, no genes have been identified to date with direct links to the disease. This dissertation presents a collection of studies that determine the genomic and genetic interactions for regions on chromosome 2 and 9 for ascites phenotypes in broiler chickens. The dissertation further focuses on developing genetic markers for selection of broilers for ascites-resistance. To achieve these aims, the studies described here use two high throughput approaches- genome-wide association and whole genome re-sequencing. A previously identified region on chromosome 9 was further investigated with fine mapping using SNPs. A region on chromosome 2 was investigated using 2 different SNPs. Neither of these regions was found to contain more than a minor association with ascites. Re-evaluation of chromosome 2 and 9 by whole genome resequencing identified multiple regions. One of the most intriguing regions was further evaluated and found to contain the most significant association with ascites ever identified. The region contains the CPQ gene. The data support three different alleles for CPQ with two of those alleles associated with resistance to ascites. Those two alleles appear to be expressed at higher levels in three tissues critical for oxygen homeostasis. Taken together, this collection of studies provides an insight and adds to our current understanding of the molecular correlates of the disease. The markers developed in these studies will be useful in breeding schemes for ascites resistance in the broiler industry.

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DEDICATION

This dissertation is dedicated to the loving memories of my father, Asoke Kumar Dey, and grandfather, Ranajit Kumar Baksi.

I built on the knowledge that you first seeded in me. I know you are there in all that I do.

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CHAPTER 1

Introduction

Ancestry of Modern Chickens

Chicken is one of the most popular animal protein sources around the world today (Alnahhas et al., 2015). The United States, being the largest producer of chicken meat, the poultry industry is one of the most successful agricultural sectors in the country (National Chicken Council, 2016). However, archaeological and genetic evidence suggest that domestication of chicken first began 7,000- 10,000 years ago (as early as 5400 BC) in Southeast Asia, where the bird used to be an integral part of the cultural development of the human race (West et al., 1988; Tixier-Boichard et al., 2011). Molecular evidence played a vital role in generating the phylogenic tree of modern chickens, and suggests several subspecies of the wild red Jungle Fowl (*Gallus gallus*) to be the original ancestor of modern day chicken breeds (Fumihito et al., 1996; Liu et al., 2006; Muchadeyi et al., 2006). Eventually, domestication of chickens became increasingly popular in countries such as China, Japan, Korea, Thailand, and India (West et al., 1988; Liu et al., 2006; Granevitze et al., 2009). Although early domestication of chickens primarily focused on food, entertainment, religious and decorative purposes, later, starting from the 1900s, chickens are selected for specific traits such as meat production, egg production, experimental models especially in the Unites States and Europe.

Commercialization of Chickens: Artificial Selection

In the 1800s, chickens were primarily produced in small flocks in the households as a part of backyard farming in the United States. These chickens served for dual purposes of meat and egg production. In the early 1900s, commercialization of poultry began with a broiler house that accommodated as many as 10,000 birds. But it wasn't until the 1970s that great advancements took place in automation technology, management techniques, breeding programs, nutritional

discoveries, genetic selection and disease eradication programs, that allowed commercial poultry industry to generate high-quality poultry products at affordable rates (Hutt, 1949; Chambers et al., 1981; Chambers, 1990; Griffin and Goddard, 1994; Pollock, 1999; Havenstein et al., 2003). With emerging success of commercial poultry industry and increasing consumer demand for affordable poultry products, new breeding programs evolved where chickens were genetically selected for desirable production traits for a single purpose. For example, specialized chicken lines called broilers were generated specifically for meat production, while layer hens were generated for production of eggs. Artificial genetic selection programs designed to generate genetically improved chicken lines with highly heritable qualitative and quantitative production traits became increasingly popular. Pedigree selection of genetically improved chicken lines depends on the specific purpose for which the particular flocks are generated. In case of layer hens, genetic strategies are deployed to select chickens with emphasis on economically desirable characteristics such as number of eggs per day, egg weight, egg shape, egg shell thickness, yolk color and index, albumin index and Haugh unit, age of sexual maturity of the hens and clutch time (Suma et al., 2007; Bahmanimehr, 2012; Lukanov, 2014). On the other hand, broiler selection is primarily focused on growth-related traits such as growth rate, breast meat yield, feed conversion efficiency, carcass quality, fertility, hatchability, survivability at market age, skeletal development, feathering rate, feather and skin color, heat and disease resistance (Pym et al., 1979; Cooper et al., 1998; Berri et al., 2001; Okeno et al., 2011). Modern commercial broilers are highly specialized lines generated by crossing pedigree (pure) lines over multiple generations for an extensive selection of several beneficial traits associated with meat production (Griffin et al., 1994; Deeb et al., 2002; Paxton et al., 2010). Such sustained and scientific breeding programs

have largely been accomplished with the advent of modern technology and infrastructure coupled with great advances in population genetics.

Modern Broiler Breeding Schemes

Generation of a robust, superior commercial broiler line from a pedigree selection of pure lines generally takes 4-5 years depending on the reproductive lifetime of the broilers in each generation. Traditionally a typical broiler breeding scheme is represented in the form of a pyramid (Figure 1.1). Each level of the pyramid is representative of one generation. At the apex of the production pyramid are the pure lines (great-great grandparent lines) with genetically selected desired traits. At each segment of the pyramid, generally, there are male and female lines selected for specific traits. Selection of traits in male and female broiler lines are slightly different; males are mostly selected for heritable growth and feed efficiency related traits while female selection is more specific towards reproductive performance (Flock et al., 2007; Laughlin et al., 2007; Olori, 2008; Paxton et al., 2010). A constant gene flow takes place with each level, and the subsequent level is generated as a result of a cross of genetically most suitable males and females at that level. The desired commercial broiler product is the fifth generation resulting from the great-great-grandparent lines (pure lines) to great grandparents, grandparents and parent lines (Figure 1.1). The expansion at each level is such that with a base unit of 1 male and 10 females, approximately 50 million broilers (70,000 tons of meat) can be obtained at the fifth (commercial broiler) generation (Flock et al., 2007; Laughlin, 2007). Such modern breeding schemes, over more than 60 years using artificial selection, have resulted in extremely desirable, robust lines which have undergone approximately 300% increase in growth rate with desired changes in conformation (Paxton et al., 2007).

Genetic Markers in Broiler Breeding

The chicken genome is made up of 39 chromosomes that include eight pairs of macrochromosomes, a pair of sex chromosomes (Z and W) and 30 pairs of microchromosomes. In contrary to mammalian systems, females are heterogametic (ZW) in birds while males are homogametic (ZZ). The size of the chicken genome, at 1.2×10^9 bp, is one-third of that of humans (Bloom et al., 1993). The chicken genetic map spans 4000 cM and contained over 2000 genetic markers seventeen years ago (Groenen et al., 2000). Comparative genome maps for chicken and human genomes reveal highly conserved segments in the two genomes with conserved synteny groups in their gene orthologs (Suchyta et al., 2001). To understand the variation in phenotypic traits, development of QTL (Quantitative Trait Loci) maps is essential. In addition to genetic maps, construction of physical maps using cytogenic techniques have been used to delineate chicken macrochromosomal properties as well as the genomic size and chromosomal distributions (Ladjali-Mohammedi et al., 1999). Another breakthrough in physical mapping was the integration of BAC (Bacterial Artificial Chromosome) fingerprinting technique. BAC-based maps, consisting of large contigs of overlapping clones, confirm sequence assembly alignments along the chicken genome, and provide excellent coverage of the genome (Wallis et al., 2004; Romanov et al., 2006). Recent approaches using high throughput methodologies, such as whole genome sequencing and comparative mapping strategies, have not only facilitated deciphering phylogeny, complex evolutionary trait selection, and conserved gene regions of modern domestic chickens. These have also allowed developing more accurate QTL mapping spanning over different chromosomes and genes for the selection of economically relevant traits (Hillier et al., 2004; Wong et al., 2004; Rubin et al., 2009).

Molecular marker technology for detection and mapping of QTLs has seen rapid advancement from restriction fragment length polymorphisms (RFLPs), to microsatellites, and now single-nucleotide polymorphisms (SNPs). The enormous potential and efficacy of these DNA-based markers have become exceedingly evident (Lander et al., 1989; Boulliou et al., 1991; Zhang et al., 2002; Emanuelli et al., 2013; Scozzari et al., 2014; Stainton et al., 2016). QTL mapping for phenotypic selection and disease resistance in chickens has a broad range of applications in animal genetics and has revolutionized poultry breeding. Microsatellites have been widely used as molecular markers because of their highly repetitive sequence motifs (Dib et al., 1996; Powell et al., 1996; Milan et al., 1998; Hillel et al., 2003; Varshney et al., 2005; Dalvit et al., 2008; Ragognetti et al., 2015). Microsatellites are highly polymorphic, multiallelic (usually three to ten alleles) and exhibit co-dominance (unlike amplified fragment length polymorphism and random amplification of polymorphic DNA) and hence are extremely useful not only in detecting rare alleles and heterozygosity in a given population. Microsatellites are also efficient to substantiate genetic variability among divergent populations (Vanhala et al., 1998; Romanov et al., 2001; Bellinger et al., 2003; Chen et al., 2004). Microsatellites have been used in constructing consensus linkage maps for the chicken genome (Groenen et al., 2000). A high-density consensus map was generated combining genotyping data of three mapping populations, which contained 801 microsatellite markers. However, one of the limitations of microsatellite-based marker-assisted selection (MAS) studies is that multiallelic microsatellites may result in genotyping errors, such as replacement of an allele with another because of biochemical errors (Vignal et al., 2002). Other limitations of microsatellites include a) homoplasy and excessive number of alleles at some loci demanding larger sample sizes for accurate analyses; b) presence of null alleles; and c) mutations in microsatellite flanking regions resulting in identical length

variants that compromise phylogenetic and population level studies (Abdul-Muneer, 2014). Current approaches in MAS widely deploy identification of SNP-based markers for association with the studied traits. SNP markers are generally biallelic. Therefore, there are only two nucleotides at a specific position due to a single base change. That is why SNP-based markers are advantageous and easier to work with compared to microsatellite markers. In addition, the density of SNPs provides a dense and thorough coverage of the entire genome. Currently, identification of SNP markers is performed by analyzing whole genome data obtained using high throughput genome-wide association studies (GWAS) and Next-Generation Sequencing (NGS) approaches. This allows a more intuitive understanding of the complex phenotypes and traits that are regulated by a combination of the effects of linkage and pleiotropy.

Impact of Artificial Selection in Broiler Welfare

Genetic selection of broilers for increased production of the birds with efficient economical traits and high heritability has become quite successful (Havenstein et al., 1994; Pollock, 1999; Berri et al., 2001; Schmidt et al., 2009; Bahmanimehr, 2012). With the implementation of advanced technologies in the farms and increasing knowledge of the gene networks associated with production traits, breeding programs with highly accurate breeding value estimation are possible in modern days. Despite the success in culminating the beneficial effects of genetic selection, many dramatic, undesired and unexpected outcomes have become apparent. Much of these outcomes are of concern to animal welfare programs. Selection of broilers for performance traits is at risk to developing behavioral, physiological and immunological disorders.

Genetic correlation between traits has been attributed to a net effect of linkage and pleiotropy (Rauw et al., 1998). Genetic linkage is the phenomenon when two or more closely located loci in

genes regulating different traits, on the same chromosome, are inherited together due to lack of independent segregation during meiosis. Pleiotropy, on the other hand, is when a single gene contributes to the regulation of multiple phenotypic traits. Thus, the integrated molecular and biochemical mechanisms involved in the selection of the production traits, also lead to the manifestation of co-related undesirable genetic outcomes as side-effects owing to the combined influence of linkage and pleiotropy. For example, fast-growing broilers selected for increased body weight have been found to be predisposed to hyperphagia due to aberrant hypothalamic satiety mechanisms (Rauw et al., 1998). Broilers under intense selection pressure for body weight have increased body fat as a result of decreased lipolysis rates (Calabotta et al., 1985) and increased insulin and glucagon concentrations in the plasma (Sinsigalli et al., 1987). One of the major interests and concerns in broiler production is the fertility trait of the birds. Fertility is negatively correlated with growth (Dunnington, 1990), not only in naturally mated flocks (Chambers, 1990) but also in artificially inseminated broiler lines that are selected on growth for generations (Dunnington, 1990; Decuypere, 2003; Brillard, 2004). Egg fertility is a function of the genotype of the embryo, and is dependent on the genetic and non-genetic factors originating from both the parents (male and female). Increased body weight in males affects sperm quality traits such as semen concentration, sperm motility, sperm metabolism and percentage of dead or abnormal cells (Marini et al., 1969; Wilson et al., 1979). Behavior of males from leg problems can prevent successful mating (Brillard, 2003). Increased body weights in females not only affect the reproductive behavior and physiology of the bird, such as the prevalence of sperm storage tubules, but also the egg quality (Brillard, 2003). Although birds with increased body weight produce a high number of eggs as compared to those selected for low body weight, the percentage of defective eggs is higher in these birds (Anthony et al., 1989). Defective eggs (for

example, double yolk, soft shelled, no shelled, extra-calcified) result from the loss of synchrony of ovulation and packaging of the eggs (Dunnington et al., 1995). In addition to fertility problems, selection of broilers for increased body weight results in several health-related issues. For example, broilers selected for growth traits exhibit lower antibody responses (Qureshi and Havenstein, 1994) and higher mortality rates at a certain age (such as 9.7% at 42 days of age as compared to 2.2% in random-bred population; Havenstein et al., 1994). Rapid growth in broilers results in a disproportion between the increased body mass and the rate of elongation of the leg bones (such as femora and tibiae) subjecting the bones to increasing mechanical stress thereby disrupting structural integrity of the skeleton (Wideman, 2016). This phenomenon is directly related to the onset of lameness in these broilers. In many such cases, the immoderate torque creates osteochondrotic clefts in the chondrocytes of the exposed growth plates, where an opportunist bacteria colonize resulting in the development of bacterial chondronecrosis with osteomyelitis (BCO) (Wideman, 2016). Fast growing broilers are also predisposed to environmental stresses, which is attributed to changes in the metabolic characteristics of the birds' muscles. Muscle properties such as low glycolytic fiber content, good blood capillary supply, high oxidative and endurance capacity, and smaller muscle fibers, allow energy conservation aiding the organism handling environmental stresses (Henckel, 1992). Observation of high percentages of glycolytic fast twitch fibers and low percentages of oxidative slow-twitch fibers in different muscles in broilers selected for rapid growth, may be indicative of the susceptibility of the birds to environmental stress (Henckel, 1992). Another serious health concern in fast-growing broilers lies in the inability of the vital organs, such as the heart and the lung, to expand output in proportion to the body mass of the broilers, and optimally perform their functions to meet the requirements of the birds' body. The manifestation of the failure of the

cardiovascular system to support the increasing oxygen requirements in these birds is the development of idiopathic pulmonary arteriole hypertension (IPAH) or pulmonary hypertension syndrome (PHS). PHS results in accumulation of edematous fluid in the abdominal cavity of broilers, a condition known as ascites, or "water belly." The terms PHS and ascites are used interchangeably in poultry. The disease has both environmental and genetic components with moderate to high heritabilities ranging from 0.1 to 0.7 (Lubritz et al., 1995; de Greef et al., 2001; Moghadam et al., 2001; Pakdel et al., 2002; Druyan et al., 2007). The current dissertation aims to elucidate some of the genetics of PHS.

PHS or Ascites in Broilers

As discussed before, PHS or ascites syndrome is an undesirable result of selection of broilers for rapid growth in modern poultry. The selection affects many major organ systems including cardiovascular, muscular, digestive and immune systems. Rapidly growing broilers with increasing muscle mass (especially breast muscle) have a higher metabolic rate. The pulmonary vasculature in these broilers is inadequate to accommodate the cardiac output to supplement their increased metabolic demands. The focus during selection of broilers is increased growth rate of the birds, which is not accompanied by a proportional increase in the organ sizes for the heart and the lung, during the birds' development (Julian, 2000). Moreover, the broiler lungs are rigid and fixed to the thoracic cavity allowing little room for expansion of the capillary beds to accommodate the increased blood flow. The capacity of the pulmonary vasculature consists of the metabolic constraints of the primary resistance vessels (pulmonary arterioles) to maintain tone (degree of contraction) as well as anatomical restrictions for maintaining the effective volume in the blood vessels (Wideman et al., 2007). Due to the lack of functional elasticity of the pulmonary vasculature in broilers, even at the normal cardiac output, the vessels remain

engorged with blood. This implies that in broilers, there are minimal mechanisms for dilation of the arterioles, expansion of the capillaries and recruitment of under-perfused vascular channels, which are responsible for minimizing pulmonary vascular resistance (PVR), (Wideman et al., 2007). This pre-disposes broilers to the development of pulmonary arterial hypertension (PAH) and subsequently terminal pulmonary hypertension syndrome (PHS). In addition, the pulmonary and cardiac capacity of modern broilers is very similar to that of older broiler lines despite the increased mass of the modern broilers (Table 1.1; Schmidt et al., 2009). Thus, selection for an increased mass in the modern broilers has resulted in a relative decrease in the size and weight of the heart and the lung in these birds, much enhancing their risk of developing PHS (Dunnington and Siegel, 1995).

Etiology of PHS (Ascites) in Broilers

PHS or ascites syndrome has been studied for many years now, yet the primary cause of the disease is still unclear making the disease idiopathic in nature. Both physiological and/or environmental factors are thought of play a role in the increased production and/ or decreased removal of the peritoneal lymph (Balog, 2003). Ascites was first reported in broilers reared in the high-altitude Bolivian hills (Hall and Machicao, 1968). The decreased partial pressure of oxygen at high altitude creates hypoxia and hypoxemia, thereby triggering the occurrence of the disease. However, since the 1980s, ascites has become common even at sea level (Julian, 1993). In addition to altitude, there are also other external factors that induce ascites. For example, cold temperatures enhance higher metabolic and oxygen requirements of the birds due to cold stress, resulting in increased cardiac output, pulmonary arterial pressure, cardiac hypertrophy and ascites (Stolz et al, 1992; Wideman and Tackett, 2000; Balog, 2003). A continuous lighting schedule maximizes broiler growth rate, predisposing the birds to ascites. It has been reported in

several studies that limited or intermittent lighting, will slightly slow the growth rate of broilers, and effectively reduce the birds' metabolism and oxygen consumption, improving the feed efficiency and reducing ascites incidence (Julian, 1990a, b; Wideman, 1998; Julian, 2000). Additionally, is has been suggested that poor environmental conditions and housing of the birds may have negative effects on the respiratory and cardiovascular systems of the birds promoting ascites incidence (Maxwell, 1988; Wideman, 1988; Wideman, 2000; Balog, 2003). Poor conditions may include poor ventilation with low environmental oxygen, presence of toxic fumes like carbon dioxide, carbon monoxide or ammonia, presence of dust, bacteria and viruses in the air. Any, or all of these factors, may trigger one or more of the four major physiological conditions responsible for the disease. These physiological conditions are (i) *vascular damage*, (ii) *lymphatic blockage*, (iii) *plasma oncotic pressure*, and most importantly (iv) *vascular hydraulic pressure* (Balog, 2003). Vascular damage is the result of impairment of the vascular endothelium caused by viral or bacterial infections, or chemical toxins allowing the plasma fluid and proteins to escape triggering ascites (Julian, 1985; Anjum, 1990; Julian, 1990a). Ascitic fluid buildup can also be attributed to lymphatic blockage, which is usually caused by neoplasia or right ventricular failure with valvular insufficiency (Julian, 1985; Julian et al., 1989). Ascites owing to a decreased plasma oncotic pressure occurs as a result of reduction of the plasma protein and albumin levels due to right ventricular hypertrophy (Wise and Evans, 1975; Cárdenas et al., 1985). Ascites may also be attributed to an increased vascular hydraulic pressure, which may occur due to several reasons such as mycotoxins and pyrrolizidine alkaloids), right atrioventricular pathologies (genetic or due to adenoviral or Staphylococcal infections) and pulmonary hypertension causing right ventricular hypertrophy and right ventricular failure (Balog, 2003).

The histopathology of ascites in broilers is depicted in Figure 1.2. What predisposes the modern fast growing broilers to ascites syndrome is the general anatomy and physiology of the birds. The avian heart is four chambers with two atria (right and left) and two ventricles (right and left), similar to that in mammals. However, unlike mammals, the left ventricle in avian species is thick-walled, while the right ventricle is thin-walled. Oxygenated blood from the left atrium reaches the left ventricle upon arterial systole through the left atrioventricular valve, which is structurally similar to the mammalian atrioventricular valve, except that the avian valve is tricuspid. But the right atrioventricular valve, which allows passage of deoxygenated blood from the right atrium to the right ventricle, in avians is distinctly different than that in mammals. Mammalian right atrioventricular valve is tricuspid with a fibrous structural characteristic. In avians, the valve consists of a single spiral flap of myocardium attached obliquely to the free wall of the right ventricle. This anatomy of the valve is responsible for susceptibility of the birds to valvular insufficiency (Julian et al., 1987; Julian 1993). In fast growing broilers with increased deoxygenated blood flow to the right atrium, the right atrioventricular valve, because of it's anatomy, becomes thick and leaky (hypertrophic), which reduces the effective blood transportation to the right ventricle (Balog, 2003). This results in valvular failure and hypertrophy of right ventricular wall. With the continued overflow of deoxygenated blood to the relatively low capacity heart, due to increased workload in the fast growing broilers, the right ventricle has to pump harder to push more blood to the pulmonary arteries to cope with the increased pressure. This leads to thickening of the right ventricular walls, and the ventricle tends to enlarge and expand to accommodate the excess blood. This not only reduces the pumping efficiency of the right ventricle, but also results in distortion of the right atrioventricular valve causing a backflow of the blood in the right atrium with each ventricular contraction, eventually

causing right ventricular failure. Consequently, the increased pressure and over-abundance of deoxygenated blood in the pulmonary artery results in pulmonary hypertension, leading to ascites and death of the birds. The progression of the disease is therefore marked with a dilation of the heart, especially the right ventricle (Figure 1.3), which can be measured as the ratio of the right ventricle to total ventricle weights (RVTV ratio), and is used as a marker for the disease at necropsy. A higher value of the RVTV ratio, therefore, indicates increased cardiac hypertrophy and a more advanced stage of PHS or ascites. Figure 1.4 compares the heart anatomy (cross-section) of a normal broiler and a broiler with fulminant heart failure and ascites (Olkowski et al., 2001).

When the venous blood reaches the right ventricle through the right atrioventricular valve, the right ventricle pumps at low pressure, just enough to push the blood out to the blood vessels for transportation to the lungs for gaseous exchange. The lungs in chickens are rigid, and fixed volume and do not expand and contract with respiration, as do the alveolar lungs in mammals because the lungs in broilers are fixed in the thoracic cavity of the birds. In avian species, the blood and air capillary networks are such that only a little dilation of the tiny blood capillaries in the lungs is allowed so as to accommodate the increased blood flow to the lung through the pulmonary arteries. Thus, insufficient exchange of deoxygenated blood takes place in the lungs, and the pulmonary artery remains continuously engorged with the venous blood from the right ventricle. The pulmonary artery under such intense pressure constricts over time worsening the situation and leading to PHS. According to Poiseuille's Law, volume flow (Q) of a fluid depends on pressure difference (P1 -P2) along a tube with radius (r) and length (L), and is given by

$$Q = (P1 - P2) \times \frac{\pi r^4}{8\mu L}$$

where μ= viscosity of blood.

Vascular resistance (R) is given by

$$R = \frac{(P1 - P2)}{Q}$$

Therefore, it is evident that a slight constriction of the pulmonary artery can drastically affect the blood flow through the artery and increase the pulmonary ventricular resistance. This, in turn, enhances the pressure overload in the right ventricle, subsequently causing ventricular hypertrophy and ascites.

The increased blood pressure in the heart, lung and pulmonary artery eventually causes a congestion in the systemic circulatory system when all the veins in the body are saturated with venous blood. This results in leakage of ascites fluid promoting vascular permeability and increased susceptibility to ascites at an early age in the broilers (Julian, 2005). This ascitic fluid is a combination of lymph or edema fluid and blood plasma that are leaked from the liver, liver surface, and blood vessels. Depending on the cause of the disease, the ascitic fluid is accumulated at different parts of the abdominal cavity. Majority of the fluid is accumulated in the ventral hepato-peritoneal spaces, cardiac coelomic space (pericardial sac) and the intestinal peritoneal space (Julian, 1993) causing ascites syndrome or “water belly” (Figure 1.5). Yellow colored clumps or strands of fibrin can be observed in the fluid if the lymph or edema fluid is highly rich in proteins. This is most commonly observed in the fluid deposited in the hepato-peritoneal spaces, as liver edema lymph is highly proteinaceous (Julian, 1993). One to three ml of fluid in the pericardial sac of healthy broilers is usual, however, an accumulation of over 4 ml fluid in the cavity is indicative of the disease phenotype (Julian, 1993).

Broilers have limited efficiency in saturating hemoglobin with oxygen as compared to white leghorn chicken or other avian species because of the presence of a thicker respiratory membrane in the broilers. Fast growing broilers exhibit a lower oxygen saturation and higher hematocrit value than slow growing broilers suggesting that these broilers are incapable of fully oxygenating their hemoglobin even at low altitude conditions (Baghbanzadeh and Decuypere, 2008). This can be attributed to the increased rate of blood flow through the lung capillary bed that does not allow sufficient time to the hemoglobin to pick up enough oxygen to be fully saturated. This further worsens the situation of the fast-growing broilers with high metabolic rates, when there is always a dearth of oxygen in the tissues accompanied by decreased oxygen saturation and increased hematocrit values. The increase in the hematocrit values in these birds could be a result of diminished plasma volume, due to exudation of fluid out of the blood system into the abdominal cavity, or enhanced erythropoiesis (Baghbanzadeh and Decuypere, 2008).

Genetics of Ascites

(i) Genetic Parameters

Several studies have indicated the involvement of a genetic component for the development of ascites (Lubritz et al., 1995; Rhoads et al., 1995; Cahaner, 1996; Wideman and French, 1999, 2000; de Greef et al., 2001). Genetically, in the modern broilers, males are more susceptible to ascites development due to extreme selection for growth rate and feed conversion ratio which demands high metabolic rates and oxygen requirements (Decuypere et al., 2000). Interestingly, increased oxygen requirement is also governed genetically. Several studies have demonstrated that broilers that are selected for both growth rate and feed conversion are the most susceptible to ascites, and have a higher pCO_2 and a lower pO_2 as compared to slow growing broilers even at

low ambient temperatures (Decuypere et al., 2005). Selection for any one of the two traits reduces the occurrence of the disease. Some of the most common clinical traits of ascites include right ventricular hypertrophy, RVTV ratio, abdominal fluid accumulation and increased hematocrit value. All these traits were observed to have moderate to high heritability and can be used as indicators for susceptibility to ascites (Lubritz et al., 1995; Shlosberg et al., 1996; Wideman et al., 1997; Moghadam et al., 2001). However, the heritability of the traits, in some cases, depends also on environmental factors. For example, the heritability estimates of RVTV ratio under normal and cold conditions were observed to be 0.12 and 0.45, respectively, while that of hematocrit values were 0.17 and 0.45, respectively (Pakdel et al., 2005). The difference in heritability at different temperature conditions may be because the genetic differences in ascites susceptibility are predominant at colder temperatures. Ascites has moderate to high heritability. The heritability of ascites in a broiler line selected for susceptibility was 0.30 ± 0.05, and that in a line selected for resistance was 0.55±0.05 (Wideman et al., 2013). To date, the precise genetic mechanisms or the specific genes involved in the regulation of the disease are unknown. Some groups argue for the involvement of one or a few dominant genes (Druyan and Cahaner, 2007; Wideman and French, 2000), whereas others suggest ascites to be a multigenic disease because of the rapid response to selection for the disease (Rabie et al., 2005; Hamal et al., 2010; Wideman et al., 2013). Although genetic selection of broilers for ascites resistance has been accomplished successfully, as evidenced by numerous studies, the correlated responses to economically important traits have not been satisfactory (Wideman et al., 2013). For instance, broilers selected over multiple generations for ascites resistance have been observed to be approximately 163 g lighter in weight as compared to those selected for susceptibility at 42 days of age (Pavlidis et al., 2007).

(ii) Genetic Selection of Broilers for Ascites Phenotype

The propensity for development of IPAH/ascites is genetically regulated with moderate to high heritabilities. Therefore, it is feasible to successfully select broilers for resistance and susceptibility. Strategies for the development of ascites-susceptible and ascites-resistant birds include (a) exposure to hypobaric chamber with reduced partial pressure of oxygen O_2 to create hypoxic conditions (Owen et al., 1990, 1995; Anthony and Balog, 2003); (b) surgical occlusion of one pulmonary artery (unilateral occlusion) which directly reduces the pulmonary vascular capacity by 50% and increases the cardiac output to unoccluded lung by 100% (Wideman et al., 1997; Wideman and French 1999, 2000); and (c) intravenous microparticle injections triggering proportional occlusion of the pulmonary arterioles causing blood flow resistance and initiation of focal inflammatory responses in the lung parenchyma (Wideman and Erf, 2002; Wideman et al., 2002).

Dr. Anthony and his associates at the University of Arkansas successfully developed ascites-resistant and ascites-susceptible broiler lines utilizing hypobaric chambers and sib-selection (Anthony, 1998; Anthony and Balog, 2003; Wideman et al., 2013). Birds were raised in the hypobaric chambers with simulated high altitude conditions that was achieved by operating under partial vacuum, which lowered the partial pressure of oxygen. Breeding for these broilers was performed using siblings of the birds in the hypobaric chambers based on their ascites mortality data. Twenty-four sire families, 3 dams per sire, were used to reproduce each line per generation and provide sibs to generate mortality data for selection. The initial parent population came from a pedigreed commercial line. The response to selection for the generation of ascites-resistant (RES) and ascites- susceptible (SUS) lines was observed to be very rapid. While the base population exhibited an ascites incidence of 75.3%, the SUS line in the 8^{th} generation had

an ascites incidence of 95.1%, while that in the 9th generation RES line was 7.1% (Figure 1.6). An unselected relaxed (REL) line representing the original parent population was also maintained alongside the selected ascites lines. The SUS line is observed to have a rapid cumulative mortality as early as 3 days post hatch. Although the RES line exhibits some ascites related mortality, it is substantially delayed as compared to the SUS (Figure 1.7). The current generation (15th generation) of the SUS line, reared at 8,000ft simulated altitude, has a cumulative mortality of 98%, while the RES line reared at 12,000ft simulated altitude exhibit a mortality of 7% (Wideman et al., 2013; Figure 1.7). The heritabilities for ascites in the SUS and RES lines were estimated to be 0.30 ± 0.05 and 0.55 ± 0.05 respectively, which are consistent with the values measured using other conditions (Wideman and French, 1999, 2000; Moghadam et al., 2001; Deeb et al., 2002; Pakdel et al., 2002). Thus, the rapid response to selection for ascites (Figure 1.6) coupled with moderate to high heritabilities is indicative of the involvement of a few major genes with the disease (Owen et al., 1995; Anthony et al., 2003; Wideman et al., 2013). It was further observed that clinically healthy broilers from the SUS line had higher pulmonary arterial pressure and pulmonary vascular resistance as compared to healthy individuals from the RES line (Wideman, 2000; Chapman and Wideman, 2001; Anthony and Balog, 2003; Wideman et al., 2013), although the lung volumes as percentage of body weights remained the same for the birds from both the lines. These cumulative evidences suggest that the constant and rigorous selection pressures, aimed at challenging the pulmonary vascular capacity, promptly reveal the genetic basis for spontaneous IPAH in broilers (Wideman et al., 2007, 2011, 2013).

(iii) Chromosomal Associations

A few studies have been performed to map chromosomal regions contributing to ascites susceptibility. One of the studies crossed an ascites susceptible line (SUS) with ascites resistant line (RES) to generate an F2 population where the birds were phenotyped as resistant or susceptible (Pavlidis et al., 2007). A GWAS was performed to genotype the DNAs of the F2 population using 3,072 SNP panel (Krishnamoorthy et al., 2014). This study identified seven regions on four chromosomes that were associated with ascites phenotype. Three of these regions (Gga9:13.5-14.8 Mbp, Gga9: 15.5-16.3 Mbp and Gga27:2-2.3 Mbp; map positions according to *Gallus gallus* v2.1 genome assembly, May 2006) were associated with ascites in independent broiler lines, including commercial lines, in a gender-specific manner (Wideman et al., 2013). Krishnamoorthy et al. (2014) performed a microsatellite-based study and predicted the association of a few candidate genes on chromosome 9 with susceptibility to ascites based on the physiological and functional relevance of the genes in birds and mammals. These genes are AGTR1, Angiotensin II Type 1 Receptor; UTS2D, Urotensin 2 Domain Containing (Gga9:13.5-14.8 Mbp region), 5HT2B, Serotonin Receptor/Transporter Type 2B (Gga9:16Mbp); and ACE, Angiotensinogen Cleaving Enzyme (Gga27:2 Mbp). All these genes are associated with hypertension, hypoxia and blood pressure regulation in humans and mice (Benetos et al., 1996; Nebigil et al., 2001; Launay et al., 2002, 2012; Henderson et al., 2004; Kim et al., 2006; Billet et al., 2007; Forty et al., 2012; Debiec et al., 2013). Rabie et al. (2005) identified many chromosomal regions in association with ascites including chromosomes 2, 5, 8, 10, 27, and 28. Notably, chromosome 9 was not implicated in this study. In addition, the study also evidenced an association of a region on chromosome 2 with RVTV ratio. It is interesting to note that clinically healthy broilers from the SUS line exhibit higher pulmonary arterial pressure and

higher vascular resistance as compared to RES birds (Wideman et al., 2002; Chapman and Wideman 2006; Lorenzoni et al., 2008). Taken together, all these studies demonstrate the importance of genetic association and predisposition of broilers to ascites.

Synopsis

The incidence of ascites in broiler populations depend on environmental factors, such as increased elevation and temperature stress (Owen et al., 1990; Balog et al., 2003). In addition, genetic components greatly influence the predisposition to the disease (Wideman and French, 2000; Wideman et al., 2013; Krishnamoorthy et al., 2014). Physiological alterations in broilers, specifically due to rapid growth, tend to alter the genetic conformation of the ascites phenotype. Better management (such as feed, lighting, air quality, ventilation) and selection practices have been set in place to reduce the estimated $100 million per year economic loss in the United States seen since 2007 (Pavlidis et al., 2007). Yet, ascites continues to be a relevant and economically important disease internationally. To manage and control the prevalence of the disease, it will be essential to understand the gene and genetic interactions that govern susceptibility towards ascites. Although several chromosomal regions have been indicated in association with ascites incidence, no major gene has been confirmed to date for ascites susceptibility.

The current dissertation focuses on first, identifying new chromosomal regions for their association with ascites. One of the primary emphases of this study is to identify novel genes and chromosomal regions related to ascites. Second, the study further examines the association of two predicted candidate genes (AGTR1 and UTS2D) on chromosome 9 for association with ascites. Third, the study develops a new genetic marker for selection of broilers for resistance

towards ascites, which would allow a non-invasive and effective method for improving ascites-resistance in broiler lines.

Table 1.1. Comparison of the average body mass (in gms) of a heritage broiler line of the 1950s with a modern broiler line of 2009 by day (Adapted from Schmidt et al., 2009).

Day	Heritage Broiler Line	Modern Broiler Line
2	38.4	36.4
7	91.7	149.3
14	233.6	431.8
21	450.3	855.9
28	699.2	1,411.3
35	1,046.6	1,804.2

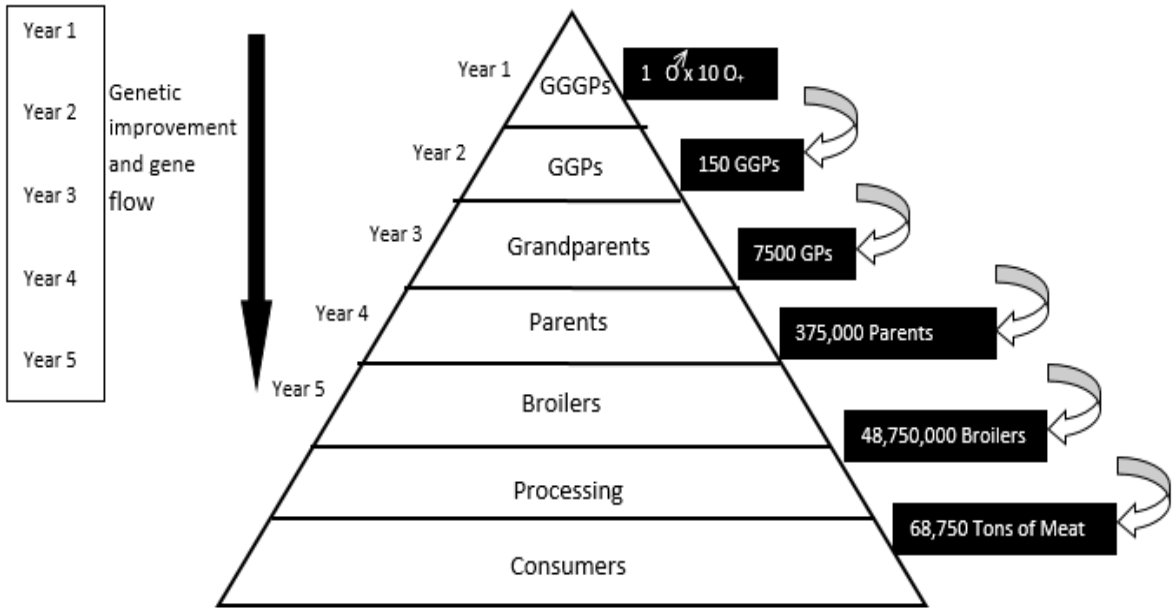

Figure 1.1. Representation of a typical modern broiler breeding scheme. GGGPs = Great-great grandparents (pedigree line/ pure line), GGPs= Great grandparents, GPs= Grandparents.

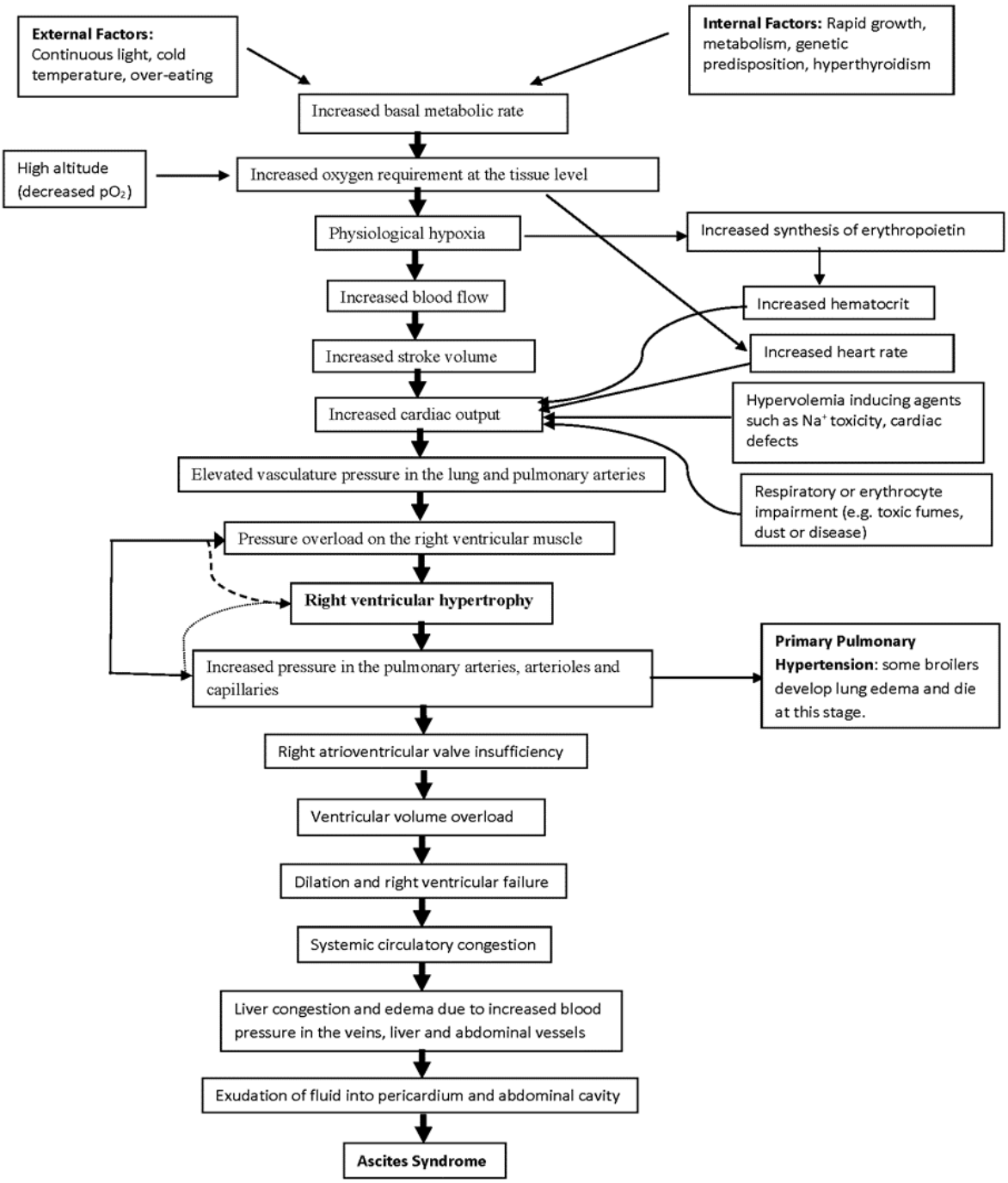

Figure 1.2. Histopathology of ascites syndrome in broilers (Balog, 2003; Baghbanzadeh and Decuypere, 2008).

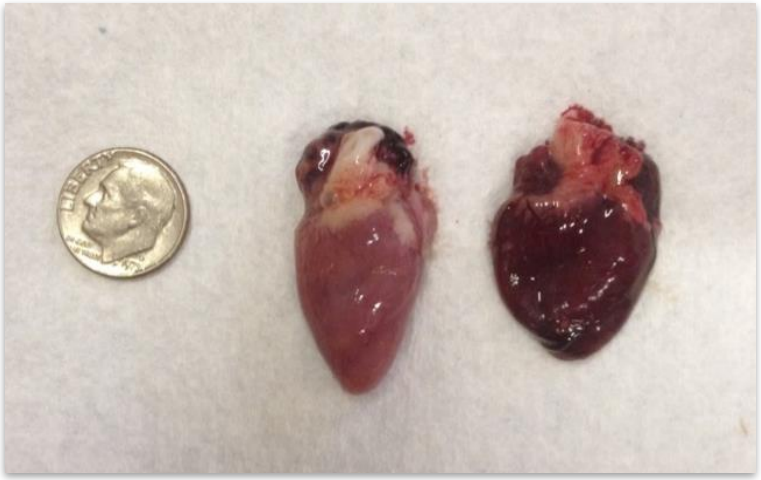

Figure 1.3. Heart of a normal broiler (left) vs. heart of a broiler suffering from ascites (right). The flaccidity of the ascitic heart is the result of dilation of the right ventricle due to right ventricular hypertrophy. The ascitic heart (right) will have a higher RVTV ratio as compared to the normal heart (left).

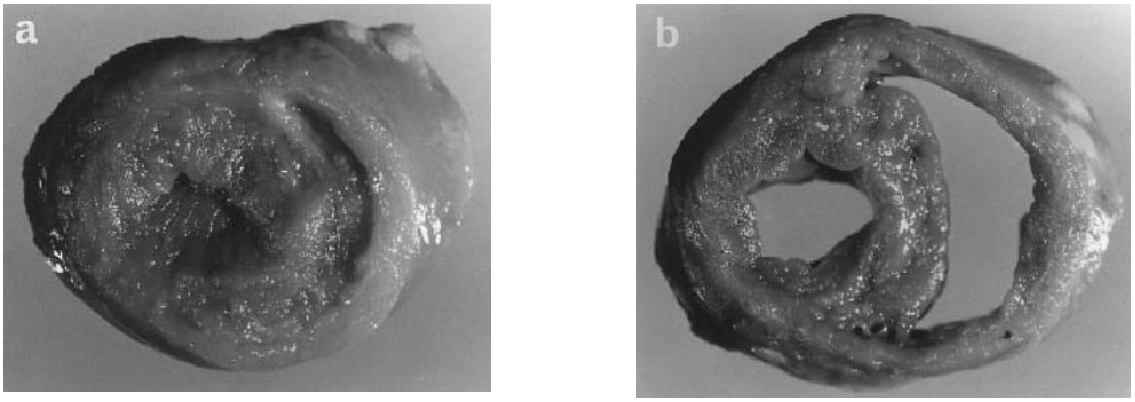

Figure 1.4. Cross-section of broiler heart. (a) Normal heat; (b) Ascitic heart, marked ventricular dilation can be observed in the ascitic heart (Olkowski et al., 2001).

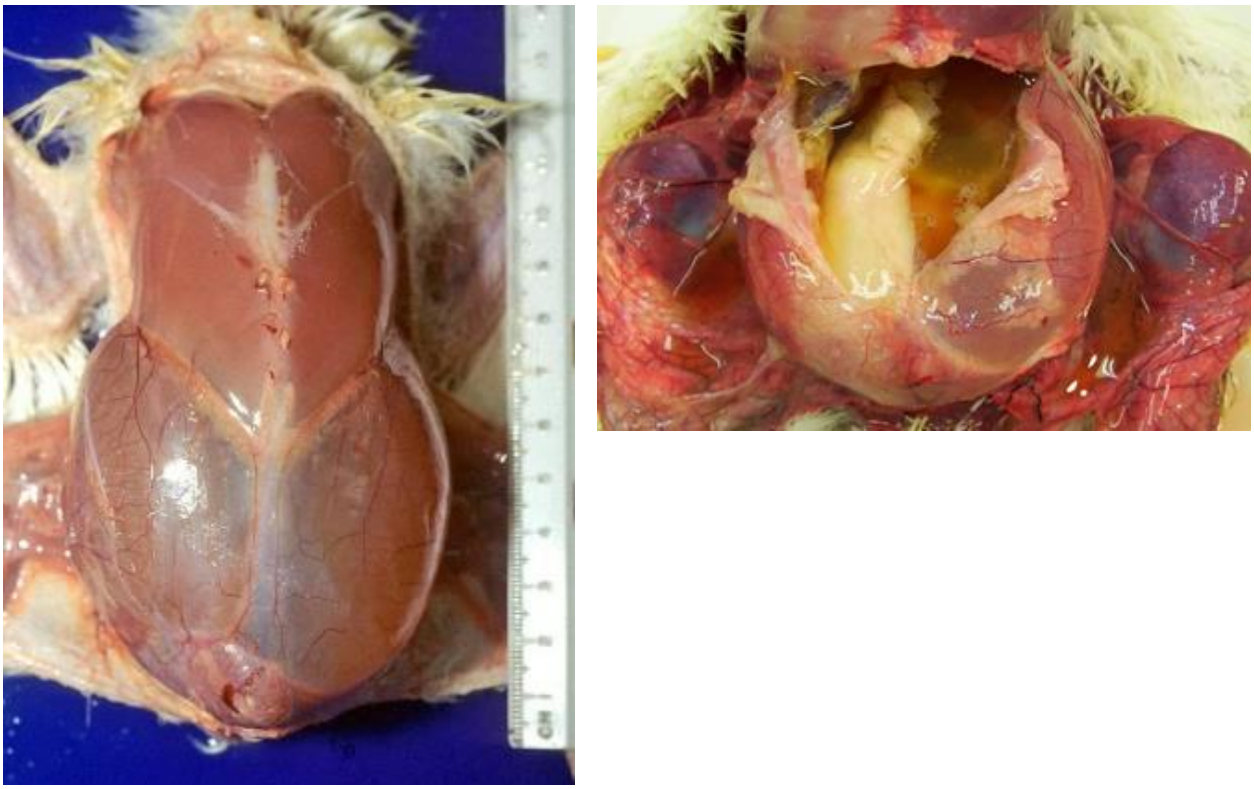

Figure 1.5. Broiler with ascites. (a) The body cavity of the broiler is distended due to the accumulation of ascitic fluid. (b) Straw colored ascitic fluid can be viewed after opening the abdominal cavity of a broiler suffering from ascites. (Sources: *www.studyblue.com*; *www.neospark.com/ascites-kcp.html*)

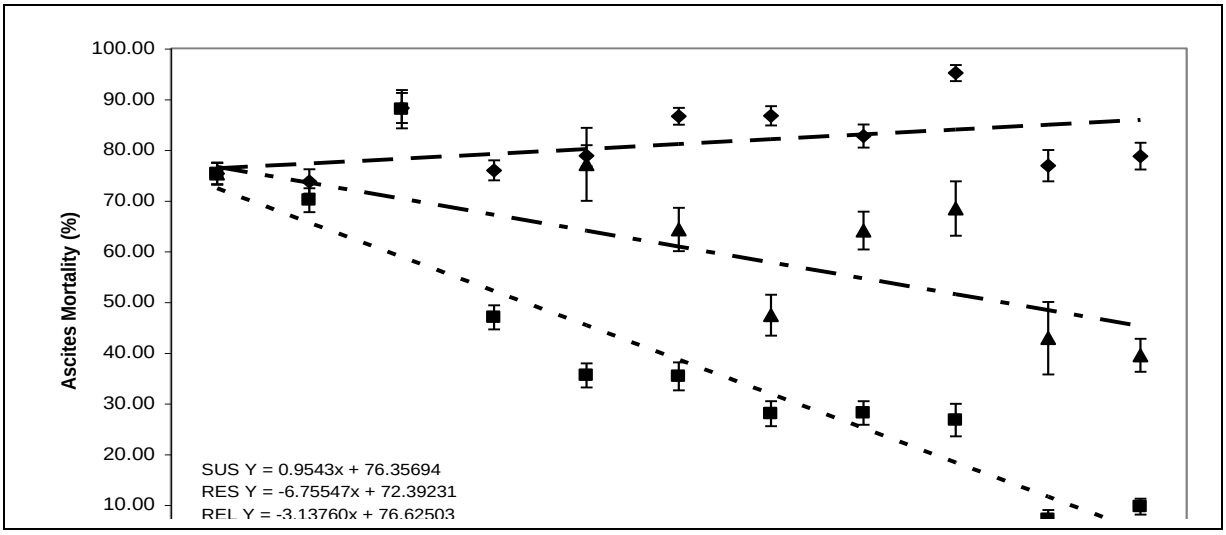

Figure 1.6. Incidence of ascites mortality per generation in the SUS, RES and REL lines.

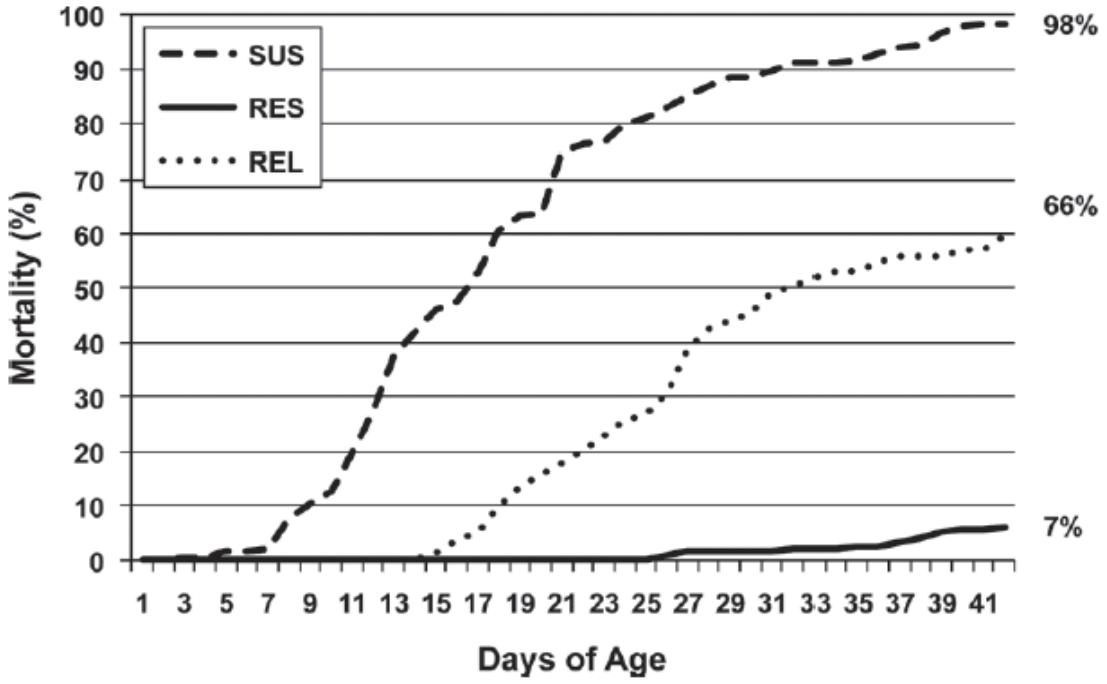

Figure 1.7. Ascites mortality curve for SUS, RES and REL lines at generation 15 (Wideman et al., 2013)**.**

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CHAPTER 2

Further investigation of a quantitative trait locus for ascites on chromosome 9 in broiler chicken lines

ABSTRACT

Previously, we reported a genome wide association study (GWAS) that had shown association of a region between 11.8 and 13.6 Mbp on chromosome 9 with ascites phenotype in broilers. We had used microsatellite loci to demonstrate an association of particular genotypes for this region with ascites in experimental ascites lines and commercial broiler breeder lines. We identified two potential candidate genes, AGTR1 and UTS2D, within that chromosomal region for mediating the quantitative effect. We have now extended our analysis using SNPs for these genes to assess association with resistance or susceptibility to ascites in these same broiler lines. Surprisingly, in contrast to our previous GWAS and microsatellite data for this region, we find no association of the SNP genotypes or haplotypes in the region suggesting that the two genes might have limited association with the disease phenotype.

INTRODUCTION

Idiopathic pulmonary arteriole hypertension (IPAH) in humans is a disease characterized by progressive pulmonary vascular remodeling that results in right-ventricular hypertrophy (RVH), right-ventricular failure and premature death (Hoeper et al., 2007). In the chicken industry, IPAH is commonly referred to as ascites. Chickens develop hypertension resulting in right ventricular hypertrophy, which results in leakage of fluids from the liver and organs accumulating in the peritoneal cavity producing a "water belly" (Julian, 1993). Most studies have indicated ascites to be a complex polygenic trait with significant genotypic and phenotypic variability between ascites resistant and susceptible chickens (de Greef et al., 2001; Moghadam et al., 2001; Pakdel et al., 2005; Rabie et al., 2005; Druyan and Cahner, 2007; Hamal et al., 2010 a, b). Ascites has also been reported to have moderate to high heritability (Lubritz et al., 1994; Lubritz et al., 1995; Wideman et al., 1999, 2000; Druyan and Cahner, 2007; Druyan et al., 2007; Pakdel et al., 2005; Pavlidis et al., 2007).

Pathogenesis of ascites syndrome in chickens has been attributed to a number of environmental, nutritional, physiological and genetic factors (Julian, 1988, 1993; Balog, 2003; Baghbanzadeh and DeCuypere, 2008). Lungs in chickens, being rigid and fixed with the thoracic cavity, do not expand with increased airflow during respiration. Hence, in healthy birds, the pulmonary circulatory system maintains a low blood flow resistance, and operates at low hydrostatic pressures in order to prevent fluid accumulation in the alveoli that would otherwise give rise to pulmonary edema (Wideman et al., 2013). However, in fast-growing broilers, due to increased demands of oxygen in the body, the pulmonary vascular channels remain engorged with de-oxygenated blood increasing the pressure in the right ventricle, and thereby causing right ventricular hypertrophy (elevated right to total ventricular ratios; RV:TV ratios). Because of the

increasing pressure, the right ventricle's enhanced cardiac output results in rapid blood flow through the lungs. The increased flow of blood through the lungs compromises complete diffusive exchange of oxygen (O_2) and carbon dioxide (CO_2) in the alveoli. As a result, the blood leaving the lungs has a lower partial pressure of the O_2 (hypoxemia) and higher partial pressure of CO_2 (hypercapnia). Iterative cycles of hypoxemic blood circulation through the lungs of healthy, fast growing broilers induce a cascade of events, primarily causing pulmonary vasoconstriction, increased vascular resistance, pulmonary hypertension, liver congestion, leakage of fluids in the lungs (edema), peritoneum and abdomen of the birds (ascites) finally triggering right ventricular failure (Wideman et al., 2007, 2013).

Hypoxia (low partial pressure of O_2 in the blood) has been shown to be one of the major factors responsible for acute pulmonary vasoconstriction and pulmonary hypertension in broilers (Peacock et al., 1989; Ruiz-Feria et al., 2001). Increase in altitude (accompanied by reduced levels of O_2) generates hypoxic conditions. Taking advantage of this, researchers at the University of Arkansas used high altitude simulation in a hypobaric chamber (creating hypoxic conditions), and sib-selection, to establish divergently selected lines derived from a former full-pedigreed elite line of the 1990s, which includes an ascites resistant (RES) line, ascites susceptible (SUS) line, and a relaxed (REL) unselected line (Pavlidis et al., 2007; Wideman et al., 2013). Previously, we used a low coverage (3700 SNP; Muir et al., 2008) genome wide SNP association panel to examine 183 offspring from an F2 reciprocal cross of the SUS and RES lines to identify an association of the 11.8 to 13.9 Mbp region on chromosome 9 (Gga9) with ascites (Krishnamoorthy et al., 2014). Two microsatellite loci from this region demonstrated female-specific association of particular genotypes with ascites susceptibility for both the experimental RES and SUS lines and for two of the three modern commercial elite broiler lines. Within the

associated region shown in the GWAS, we identified two candidate genes, AGTR1 and UTS2D as possible mediators of ascites susceptibility.

The renin-angiotensin system (RAS) pathway plays a vital role in developmental processes and organogenesis of the heart and kidney (Wolf et al., 1993; Price et al, 1997). RAS also regulates the pathophysiology of hypertension, cardiac hypertrophy and vascular diseases (Kometiani et al., 1998; Palatini et al., 2009). Angiotensin II (AngII) hormone is a key regulator of the RAS pathway, and plays an important role in systemic vascular remodeling, blood pressure control and cardiovascular homeostasis (Pousada et al., 2015). AngII binds to two G-coupled receptor subtypes- AngII type 1 receptor (AGTR1) and AngII type 2 receptor (AGTR2), which act antagonistically to regulate the RAS endocrine pathway (Greindling et al., 1996). Signaling through AGTR1 has both cardiac and systemic effects, and is known to trigger vasoconstriction, cellular hypertrophy and catecholamine release; whereas AGTR2 inhibits vasoconstriction, cellular hypertrophy and catecholamine release (Cuffe et al., 2014; Palatini et al., 2009). Increased levels of AGTR1 have also been suggested to have shown vasotonic and hypoxic responses, as well as distal muscularization in pulmonary vessels (Chassagne et al., 2000; Pousada et al., 2015). Polymorphisms of AGTR1 have been associated with diastolic heart failure in humans (Wu et al., 2009).

UTS2D belongs to the urotensin II family, along with urotensin 2 (UTS2) and urotensin 2 receptor (UTS2R) (Douglas et al., 2004). UTS2 is a potent vasoconstrictive peptide that plays a role in modulating both endothelial independent as well as dependent vasodilation (Ong et al., 2008). Elevated plasma levels of the peptide are also associated with susceptibility to diabetes, renal failure, hypertension, congestive heart failure and carotid atherosclerosis (Ong et al., 2008).

UTS2D is the endogenous and functional ligand of UTS2, which binds and activates the peptide (Jiang et al. 2008).

As discussed earlier, healthy chickens are pre-disposed to ascites when alveolar hypoxia takes place. In fast-growing broilers, hypoxia may arise due to abnormal ventilation/perfusion (V/Q) ratio (Balog, 2003). A decrease in the V/Q ratio takes place when ventilation is low and perfusion of deoxygenated blood is high, resulting in inadequate CO_2 / O_2 exchange in the lungs. In high altitude conditions, low partial pressure of O_2 results in an increase in physiological dead space within the bird's parabronchi, which further aggravates the hypoxic situation. Chronic hypoxia is a major contributor to vasoconstriction, pulmonary vasculature remodeling, increased vascular resistance and right ventricular hypertrophy (Peacock et al., 1989; Karamsetty et al., 1995; Wideman et al., 1997; Raj et al., 2002). Therefore, given the roles of AGTR1 and UTS2D in hypoxia and vasoconstriction, the two genes are strong candidates for the heritable, polygenic trait of ascites.

The objectives of our current study were to develop SNP assays for the gene regions of AGTR1 and UTS2D to examine trait association of these candidate genes to better comprehend the molecular basis of resistance/susceptibility and gender bias for ascites phenotype.

MATERIALS AND METHODS

Genomic Data

All genome positions indicated in this report are according to the November 2011 assembly of the *Gallus gallus* genome GenBank accession ID: GCA_000002315.2. Genomic sequences for

specific chromosomal regions or genes were downloaded using the UCSC genome browser (http://genome.ucsc.edu).

Genomic DNAs

DNAs isolated from chicken blood were those described in our previous report (Krishnamoorthy et al., 2014).

PCR Sequencing

PCR primers to amplify targeted gene regions were designed using Primer3 (*http://bioinfo.ut.ee/primer3-0.4.0/primer3/*), then synthesized by Integrated DNA Technologies (IDT; Coralville, IA). PCR conditions were optimized for each primer pair, and then used to amplify the region from multiple individual DNAs from the RES and SUS lines. Quality of PCR products were evaluated on agarose gels, purified using RapidTip® functional pipette tips (Diffinity Genomics, West Chester, Pennsylvania), quantified using a Model TKO 100 fluorimeter (Hoefer Scientific Instruments, Livonia, Michigan), then submitted for capillary sequencing by the University of Arkansas DNA Resource Center. Sequence data were aligned to the reference Jungle Fowl sequence using SeqMan Pro software (DNASTAR, Madison, WI) to identify SNPs.

SNP Genotyping

Primers and probes developed for exonuclease assays for particular SNPs are listed in Table 2.1. Probes incorporating Zen modifications quenched with Iowa Black were obtained from IDT. SNP genotypes were determined by quantitative-PCR (qPCR) in 96 well plates using a CFX96 real-time thermocycler (Bio-Rad Laboratories, Inc., Hercules, California). Each 20µL reaction

consisted of 1X Taq Buffer (50mM Tris-Cl, pH 8.3, 1mM $MgCl_2$, 30µg/mL of BSA), 0.25mM $MgCl_2$, 0.2mM dNTP, 0.5µM each of the specific forward and reverse primers, 0.25 µM each of the two probes, 4 units of Taq polymerase, and 2µL of DNA (50-100ng). Cycle parameters were 90°C for 30s, 10 cycles of 90°C for 15s, and primer-specific soak for 30s (Table 2.1), followed by 30 cycles of 90°C for 15s, primer-specific soak for 30s (Table 2.1), and plate read.

Statistical Methods

Analysis of genotype and allelic distributions was performed using Microsoft Excel (Microsoft Corporation, Redmond, WA). Single nucleotide polymorphism loci for each chicken line was evaluated for deviation from Hardy-Weinberg (HW) Equilibrium using the CHITEST function comparing observed counts with the computed expected counts based on allele frequencies for the line. Genotype data for dead birds with inconclusive necropsy for ascites were included, however, those genotypes were excluded from computations in resistant and susceptible subpopulations. HW equation ($1=p^2+2pq+q^2$) was used to calculate the expected genotype counts. To combine loci, we imputed haplotypes where either or both of the loci were homozygous. Haplotype frequencies for the entire population were used to calculate the expected haplotype counts for resistant and susceptible subpopulations. Observed counts for resistant and susceptible subpopulations were compared to expected counts using the CHITEST function of MS Excel to compute P-values for each genotype or haplotype. P-values were determined only for those genotypes or haplotypes that occurred at ≥ 10%. A simple Bonferroni correction was used to generate adjusted P-values, by multiplying each calculated P-value by the number of calculated P-values for that locus analysis. Statistical significance was defined as adjusted P-value < 0.05.

Linkage disequilibrium (LD) between microsatellite and SNP markers was measured using Lewontin's normalized disequilibrium co-efficient D' (Lewontin, 1967) modified for multiple alleles (Hedrick, 1987). For two allele loci, Lewontin's standardized measure is calculated as

$$D'_{ij} = D_{ij} / D_{\max} ,$$

where

$D_{ij} = x_{ij} - p_i q_j$; $D_{max} = min\ [p_i q_j, (1- p_i)(1- q_j)]$ when $D_{ij} < 0$ or $D_{max} = min\ [p_i (1- q_j), (1- p_i) q_j]$ when $D_{ij} > 0$; x_{ij} is the frequency of haplotypes with allele i at the first marker locus and allele j at the second marker locus; p_i and q_j are the allele frequencies at the first and second marker loci respectively. With multiple alleles at the two loci, the disequilibrium between the two markers is measured as

$$D' = \sum_{i=1}^{k} \sum_{j=1}^{l} p_i q_j \left| D'_{ij} \right| ,$$

where k and l are the number of alleles at the first and second marker loci.

RESULTS

Our previously reported genome wide association study (GWAS) and variable number tandem repeat (VNTR) study had demonstrated significant association of chromosome 9 (Gga9) from the 11.8 to 13.6 Mbp region (2011 genome assembly coordinates) with ascites in our ascites research lines and two of the three selected commercial broiler elite lines (Krishnamoorthy et al., 2014). The study also identified two candidate genes, AGTR1 (Gga9: 11,859,037-11,880,012 bp) and UTS2D (Gga9: 13,178,354-13,185,135 bp), within this region as possible mediators of this

quantitative trait. In order to further validate any role for these genes we used PCR sequencing of selected regions of these genes to identify SNPs that are segregating in our divergently selected lines. For AGTR1 we had previously sequenced all three exons along with some flanking intronic sequences (Burks et al., 2011). For UTS2D, we sequenced two specific non-coding sequences to identify SNPs for further genotype analyses. We chose to target the promoter (1191 bases upstream of exon 1) to possibly identify SNPs in transcription factor binding sites, and intron 3 (1945 bases), which was short enough for PCR amplification using exonic primers and would be more variable than exonic sequences.

We then chose individual SNPs, or clusters of SNPs, for development of exonuclease qPCR tests (TaqMan™ assays) for additional association studies. SNP targets included a span of 5 SNPs within 11 bases in intron 1 of AGTR1, a single SNP upstream of exon 1 for UTS2D, a pair of SNPs in intron 3 of UTS2D, and a single base SNP at 13,193,937 bp (Table 2.1). Reasons for selecting these particular SNPs are as follows.

UTS2D intron 3 sequences from six RES and nine SUS samples identified 15 SNPs defining as many as six potential haplotypes (Table 2.2). The exonuclease assay we developed (UTS2Di3, Table 2.1) targeted two SNPs (*GAG* vs. *TAA*) early in intron 3. We targeted these two SNPs because our sequence data suggested there were only two alleles that were segregating in both RES and SUS (with different apparent frequencies, Table 2.2), and a pair of SNPs would provide better probe discrimination in the exonuclease assay. For the UTS2D promoter region, sequence data from six RES and nine SUS identified 10 SNPs (Table 2.2), with three SNPs affecting four potential transcription factor binding sites. The *C*/*G* SNP at 13,177,951 bp affects a binding site for MLTF-HMG Co Ared transcription factor, a negative regulator of cholesterol biosynthesis (Osborne et al., 1987). The *C/T* SNP at 13,177,542 bp affects overlapping sites for (i) c-fos

SRE, a serum response factor associated with cell proliferation, differentiation and survival (Rouviere et al., 1990), and (ii) GR-MT IIA, a glucocorticoid receptor responsible for chondrocyte activity (Karin et al., 1984). The *T*/*G* SNP at 13,177,470bp is in a site for IRF2 a transcription factor that regulates cell growth (Tanaka et al., 1993; Fujii et al., 1999). Evaluation of the upstream regions of UTS2D in mouse and human suggested only the IRF2-binding site appears in those mammalian promoter regions in a comparable position, implying that this binding site might function in regulating this gene in all three species. We therefore developed genotyping assays for this SNP (UTS2Dp1, Table 2.1). For AGTR1 we targeted an intron 1 region containing 5 SNPs in a 13 base span (AGTR1i1; Table 2.1). Sequences for this region from 12 DNAs had identified only two haplotypes for the 5 SNPs (Burks, 2011). We also developed a SNP assay (s13.193, Table 2.1) for the *G*/*A* SNP at 13,193,937 bp. This SNP had shown the most statistical deviation in our original GWAS analyses that identified this region as associated with ascites in females in an F_2 double reciprocal cross of our RES and SUS lines (Krishnamoorthy et al., 2014). This particular SNP was therefore reasonably a strong candidate for detecting association with ascites for this region in additional lines. In the original 3k SNP panel this SNP was designated as snp-41-245-53961-S-2 (Muir, et al., 2008). The exonuclease assays we designed were then used to genotype the same collection of DNAs from our ascites experimental lines (SUS, RES and REL) and three commercial lines used in our previous VNTR genotype study (Krishnamoorthy et al., 2014). DNA samples from REL line and commercial lines W, Y and Z, were from birds phenotyped for ascites resistance or susceptibility using the hypobaric chamber challenge.

Analysis of the genotypes for AGTR1i1 in REL showed no significant deviation from Hardy-Weinberg (HW) equilibrium including expected and observed heterozygosity (Table 2.3). In the

experimental RES and SUS lines, significant deviation from HW was observed because of high homozygosity for both alleles for this locus. In SUS line, allele 2 was observed at 96% for 51 samples, so statistical deviations were mainly in the differences observed for the infrequent heterozygotes and homozygous allele 1. In the RES, we observed nearly equal numbers of homozygotes for both alleles, but only 7% heterozygotes suggesting that there is a null allele in this populations. We have no explanation for the near absence of heterozygotes in the RES. However, sequence analysis of multiple PCR products from the genotype assays failed to identify any null alleles and confirmed the genotypes observed. The genotype data for REL were consistent with HW which further supports the validity of the genotype assay. Accepting that the analysis in RES and SUS may have some as yet unexplained artifact, at face value we would conclude that selection for susceptibility has dramatically increased the frequency of allele 2 in the SUS. However, allele and genotypic frequencies for AGTR1i1 showed no association with resistance or susceptible phenotype in the REL line chickens for both or either gender (Table 2.3).

Data from UTS2Dp1 conformed with HW for the REL, SUS and RES lines. Allele 2 was the major allele more so in the RES and REL lines than in the SUS line, suggesting a shift in allele frequency with selection of the SUS (Table 2.3). However, we did not see any significant association of any particular genotype with ascites phenotype in the REL (Table 2.3). Allele 1 is a minor allele (7.5%) in the REL. The low genetic diversity at this locus in REL may preclude detecting any association.

For UTS2Di3, the genotypes for RES, SUS and REL lines conformed with HW equilibrium. In RES line birds, allele 1 was the major allele at 69%. For SUS and REL, allele 1 and 2 were nearly equal in frequency. Selection for ascites resistance was associated with an increase in

allele 1 in RES line birds (Table 2.3). Analysis of ascites phenotype showed no association in males in REL. Allele 1 was 49% in resistant females and 36% in susceptible females in REL, but was not significantly different (P=0.160). No specific genotype showed statistical deviation (Table 2.3).

The SNP genotypes at locus s13.193 conformed with HW equilibrium for SUS and REL lines but the RES line genotypes were reduced for heterozygotes (observed=21% vs expected 32%) with concomitant increases in both homozygotes (Table 2.3). Allele 2 was the major allele in REL (84%) and RES (80.5%) but was the minor allele in SUS (3%). This suggests a predominant shift to allele 1 with selection for susceptibility. However, evaluation within the REL line for phenotype for each gender did not identify any statistical association of ascites phenotype with any genotype for this locus.

Analyses were performed for the four SNP loci in the three commercial elite lines W, Y, and Z, analyzed previously (Krishnamoorthy et al., 2014). For AGTR1i1, lines W, Y and Z conformed with HW equilibrium (Table 2.4). Allele 2 was the major allele in lines W, Y and Z at 84.5%, 63.5% and 65.5% respectively. Allele and genotype counts for resistant or susceptible phenotypes were not statistically different from expected for any line or for either gender within the line (Table 2.4).

For the UTS2Dp1 locus, the genotypes conformed with HW equilibrium for commercial line Y, but not for line Z due to high allelic homozygosity. Line W was homozygous for allele 2 (Table 2.5). Allele 2 was the major allele at 100% in line W, 92.5% in line Y and 67% in line Z, similar to the ascites experimental lines. There was no statistical correlation for any genotype with phenotype for either gender in any of the three commercial lines for this locus.

For the three commercial lines, the genotypes for the UTS2Di3 locus conformed with HW equilibrium only in line Y, but not in lines W (low homozygosity) and Z (high homozygosity). Allele 1 was the major allele in line W (71%), while for lines Y and Z both alleles were nearly equal in frequency (Table 2.6). There were no statistical associations with any allele, or genotype, for either gender with respect to ascites phenotype.

The genotypes for s13.193 did not conform with HW equilibrium in any of the three commercial lines due to an over-abundance of homozygotes for both alleles. Allele 1 was the major allele in lines W (61.5%) and Z (70%), while in line Y both alleles were nearly equal in frequency (Table 2.7). Again, we saw no statistical association with respect to ascites phenotype for either gender in any of the three commercial lines for this locus (Table 2.7).

Next, we determined the linkage of the specific alleles for AGTR1, UTS2D promoter, UTS2D intron 3 and s13.193 SNP alleles for further ascites association analyses. We imputed haplotypes for each pair of loci where possible (when one or both loci are homozygous) as we had performed previously for VNTR loci (Krishnamoorthy et al., 2014). Each of the four possible haplotypes (11, 12, 21, 22) was then analyzed for association with ascites in the REL, and the three commercial lines. There were no associations with ascites phenotype observed for either gender for any haplotypes for AGTR1i1 + UTS2Dp1 (Table 2.8), AGTR1i1 + UTS2Di3 (Table 2.9), AGTR1i1 + s13.193 (Table 2.10), UTS2Dp1 + UTS2Di3 (Table 2.11), UTS2Dp1 + s13.193 (Table 2.12), or UTS2Di3 + s13.193 (Table 2.13).

In our previous analyses of this chromosome 9 region using VNTRs, the B allele, and combined PHS009-PHS010 haplotype BB showed association with ascites resistance in commercial line Y. For line Z there was association with resistance for the PHS009 FF genotype, the PHS010 BB

genotype, and the PHS009-PHS010 FB haplotype. Therefore, we aimed to determine whether the SNP genotypes for the two candidate genes further refined VNTR genotypes with respect to ascites. For this, first, we estimated LD for the two VNTRs with each of the four SNP loci in the two commercial lines Y and Z where the VNTRs showed association with ascites, and in the experimental REL line. Our analysis indicated that the SNP loci of AGTR1i1, UTS2Dp1 and UTS2Di3 were in LD with both PHS009 and PHS010 in the REL, Y, and Z lines (D' >0.5; Table 2.14). However, s13.193 did not show LD for any of the VNTRs in the three lines, except for PHS010 in REL at D' >0.5 level (Table 2.14). Second, we examined the distribution of SNP genotypes with respect to ascites phenotype for the specific VNTR genotypes that had previously shown association with resistance. These analyses examined SNP genotypes for the PHS009 and PHS010 BB genotypes birds in line Y, and the PHS009 FF genotype and PHS010 BB genotype birds for line Z. However, we did not observe a significant association with ascites for any SNP genotypes ($P<0.05$) for any of the four SNP loci within these specific VNTR genotypes (data not shown). Third, we tabulated SNP haplotypes for pairs of SNP loci with respect to the same VNTR genotypes in line Y, and line Z. Again no significant association was observed for any of these haplotypes with ascites (Tables 2.15 and 2.16).

DISCUSSION

In our previous GWAS study using an F2 cross between SUS and RES lines, a region between 11.8 and 13.6 Mbp on Gga9 was identified as possibly containing a QTL for ascites incidence (Krishnamoorthy, et al., 2014). Using VNTRs we were able to associate the region with ascites in multiple commercial lines, but not in REL line birds. We identified AGTR1 and UTS2D as

potential candidate genes in this region that are involved in cardio-pulmonary function, and therefore could mediate the disease phenotype. Moreover, these two genes represent the extremes of the chromosomal region (AGTR1 at 11.8 Mbp, and UTS2D at 13.2 Mbp). The present study, therefore, employs SNP loci spanning the region to determine which, if any, of the candidate genes are more closely linked to the ascites QTL. Remarkably, genotype data for SNP assays for AGTR1 and UTS2D did not show any significant association in the REL or any of the three commercial lines previously determined in Krishnamoorthy et al. (2014). The SNP assays spanned the region from 11.86 to 13.19 Mbp, whereas the VNTR assays had targeted 11.81 and 11.93 Mbp. None of the SNP assays showed any statistical association even in commercial line Y which had shown the strongest association using VNTRs in Krishnamoorthy et al. (2014). Line Y is the modern descendant from what was the original source from which the SUS, REL, and RES lines were originally derived (Pavlidis et al., 2007).

In our current study, all SNP loci conformed with HW equilibrium in REL. AGTR1i1 and UTS2Dp1 conformed with HW in two of the three commercial lines. Failure to conform with HW in most of the commercial lines, led us to question the reliability of the assays for the UTS2Di3 and s13.193 loci. Sequence data from 10-16 PCR products representing different genotypes for each of the loci corroborated the exonuclease-based SNP genotypes, indicating that our assays were reliable. Therefore, the error rate of genotype mis-calls appears fairly low.

We used Hedrick's multiallelic extension of Lewontin's standardized D' to examine LD between the SNP and VNTR loci (Lewontin, 1964; Hedrick, 1987). For biallelic markers, D' >0.33 has been posited to indicate loci in LD (Abecasis et al., 2001), while others have suggested that LD is indicated only when D' is greater than 0.5 (Reich et al., 2001). D' >0.3 has been used as threshold for useful measure of LD between SNP and VNTR loci (Schulze et al., 2002). In our

study, comparison of the AGTR1i1, UTS2Dp1 and UTS2Di3 SNP loci with VNTR loci in REL, Y and Z lines manifested D' values greater than 0.5, suggesting that the SNP and microsatellite loci were in LD in the given lines. However, s.13.193 was generally in much lower LD for the VNTRs in most of the lines (Table 2.14). Given that these loci occur within a region spanning approximately 1.4 Mbp on chromosome 9, we would have expected higher LD for all the SNP loci, including s13.193. For AGTR1i1 and UTS2Dp1, we could identify a SNP allele that was predominantly associated with particular VNTR alleles that had previously shown association. AGTR1i1 allele 2, and UTS2Dp1 allele 2 were overwhelmingly associated with the BB genotype for PHS009-010 in line Y, and FF and BB genotypes for PHS009 and PHS010 respectively in line Z. Even though those VNTR genotypes were associated with ascites resistance, the corresponding SNP alleles or the combined SNP haplotype did not show any association with ascites resistance.

Since the UTS2Dp1 probes are for SNPs that affect an IRF2 binding site, we have pursued an allele specific expression assay targeting the 3'-UTR. We PCR amplified and sequenced the 615bp long 3'-UTR (13184523-13185137 bps) for UTS2D from DNA samples for five heterozygotes and two homozygotes for UTS2Dp1 (based on qPCR and verified by PCR sequencing). We identified five SNPs in the 3'-UTR. Both UTS2Dp1 homozygotes were heterozygous for all five SNPs, while only one UTS2Dp1 heterozygote was heterozygous for these five SNPs. When we included the UTS2Di3 genotypes, from these seven DNAs we identified five different combined (UTS2Dp1-UTS2Di3-3'UTR) genotype patterns (211, 221, 232, 322, and 332) which would require a minimum of five distinct alleles to produce these five patterns. Therefore, although we chose apparently biallelic SNP regions for our exonuclease genotype assays, each of those alleles may have a number of subtypes based on SNPs in flanking

regions. This is evidenced by the number of haplotypes we impute for the three SNP positions we investigated here, despite the three SNP regions spanning only 7700 bases on chromosome 9. It is quite likely that s13.193 (which is 21 kb 3' to UTS2D) is also affected by the same diversity of alleles. The presence of the subtypes of SNP alleles could also explain why the UTS2D promoter and s13.193 do not conform with HW equilibrium in some of the tested lines.

Therefore, our current study suggests that apparently there is no association of the AGTR1 and UTS2D polymorphisms/haplotypes with ascites, so we are unable to ascribe any QTL function to a specific candidate gene or gene polymorphism for this region of Gga9 based on SNP genotypes. Our data are most consistent with high rates of recombination events within this region that re-assort the SNP loci generating multiple haplotypes and compromising LD across a small chromosomal region. Thus, it is not a simple task to develop a useful genetic test for marker assisted selection for this region. We selected common SNPs whose alleles represented approximately 50% frequency in REL for our investigations, and it may be that less frequent SNPs would be more diagnostic. Combining SNPs into haplotypes using 10 or more SNPs might also identify particular chromosomal segments associated with ascites. Subsequent GWAS on REL line samples have failed to show any association for ascites with this region on Gga9 but have identified regions on Chromosomes 2 and Z. Nonetheless, because the region on Gga9 showed association in an experimental cross, and VNTR data showed an association in some commercial lines as described in Krishnamoorthy et al. (2014), this region may still have limited utility for further investigations of ascites susceptibility. Although our study does not support association of the polymorphisms in the two genes with ascites phenotype, AGTR1 and UTS2D still remain candidates for future investigation of relevance in this disease. This work is one of the first to have investigated the association of these two gene polymorphisms with ascites

phenotype in broilers. Our null findings provide important clues and cautions for future studies involving the genes contributing to ascites. Future studies may need to account for environmental and epistatic effects, as well as perform genome wide studies to identify additional candidate gene regions for ascites phenotype.

Table 2.1. Primers, probes, and conditions for qPCR. For each SNP locus: position is the base-pair position on chromosome 9 according to the 2011 genome assembly; Primers are 5'-3' for forward (F-) and reverse (R-); Probes are 5'-3' with allele 1 (P1) labeled with FAM and allele 2 (P2) labeled with HEX; and the soak temperature (°C) used in the qPCR assay.

Locus	Position	Primers	Probes[1]	Soak
AGTR1i1	11860182-94	F-CGAGTTGTATGGCAATAAGTGACAGCAGG R-TCTTCTCCTGCTCCCTGCCTCCA	P1-ACT***G***TTGT***G***TTA***CA***T***T***GCATGGGGCA P2-CT***A***TTGT***T***TTA***TG***T***A***GCATGGGGCAGTC	63.0
UTS2Dp1	13177469	F-AGGMAGGCTAGCGCTGTTACTGCT R-GAAGGCCAGATGCCAGTTCTGTCT	P1-TA***T***AAAACTAGTAGCAGGTCCCTGCTC P2-TA***G***AAAACTAGTAGCAGGTCCCTGC	66.9
UTS2Di3	13182517-19	F-CTTTGCAGACTGGGAGCTGGCAAAGA R-GCAGCTGATGCAATGCACAGGCAG	P1-AGC***TTA***GCTAGGGTATTATTCATGCAACAG P2-AGC***CTC***GCTAGGGTATTATTCATGCAA	63.7
s13.193	13193937	F-GTGACCAGGTGGGAATCTTTCAGC R-GGCAAGAGAAGGGTCAGCAAAGAG	P1-CA***A***GTTCAGCCCCAGGGAAGAATT P2-CA***G***GTTCAGCCCCAGGGAAG	67.5

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

Table 2.2. SNPs identified in the UTS2D promoter and intron 3 from SUS and RES chickens, Jungle fowl (JF) and White Leghorn (WL). For the UTS2D promoter, the SNP chromosomal locations (bp) are: 13,177,222, 13,177,287, 13,177,384, 13,177,418, 13,177,419, 13,177,463, 13,177,470, 13,177,470, 13,177,542, 13,177,951, and 13,178,095, respectively. For the UTS2D intron 3, the locations are: 13,182,464, 13,182,520-13,182,522, 13,182,532, 13,182,569, 13,182,575, 13,182,646, 13,182,659, 13,182,677, 13,182,693, 13,182,808, 13,182,872, 13,182,905, 13,182,916, and 13,182,943, respectively.

UTS2D promoter	Identified SNPs (5'-3', positive strand)[1]	UTS2D intron 3	Identified SNPs (5'-3', positive strand)[1]
Chicken Lines		**Chicken Lines**	
JF	A G A G C G G T C C	JF	G TAA – C A G A T T G G C C T
WL	G G A A A G G T C C	WL	G GAG A C G A A T C A G C A C
RES-1	R R R R M G G Y S C	RES-1	A KAR R C G R A Y T G A Y C T
RES-2	A G A G C G G T C C	RES-7	A KAR R Y G A A Y Y R G Y M Y
RES-3	G A G A A G G C G C	RES-8	A TAA – T G G A C T G A C C T
RES-4	A G A G C G G T C C	RES-9	A TAA – T G G A C T G A C C T
RES-5	A G A G C G G T C C	RES-10	A KAR R Y G R A Y Y R R C M Y
RES-6	A G A G C G G T C C	RES-11	A KAR R C G R A Y T G R C C T
SUS-1	G R R A A G G Y S T	SUS-1	G KAR R Y G R A Y T G R C C T
SUS-2	G R R R M R K Y S Y	SUS-2	A KAR R Y G R A Y T G A Y C T
SUS-3	G R R A A G T Y S Y	SUS-10	A KAR R C G A R Y T G G C C T
SUS-4	G G A A A G G T C T	SUS-11	A TAA – T G G A C T G A C C T
SUS-5	R G A R M R K T C T	SUS-12	R TAA – Y G R R C T G R C C T
SUS-6	G R R A A G T Y S Y	SUS-13	G TAA – C G A G C T G G C C T
SUS-7	G G A A A G G T C T	SUS-14	G KAR R C G A R Y Y G R C C T
SUS-8	R G A R M R K T C T	SUS-15	G TAA – C G A G C T G G C C T
SUS-9	G R R A A G G Y S Y	SUS-16	R TAA – Y G R R C T G R C C T

[1] IUPUC nomenclature has been used for heterozygous SNP nucleotides: R= A/G; Y= C/T; M= A/C; S=C/G; K= G/T. For In/Dels where the base is missing is indicated as '-'.

Table 2.3. Genotype data for AGTR1i1, UTS2Dp1, UTS2Di3, and s13.193 for the experimental ascites lines. The SUS, REL and RES lines were genotyped using each assay where Genotype 1 is homozygous for allele 1, 1+2 is heterozygous, and 2 is homozygous for allele 2 (Table 2.1). Genotype frequencies (Freq) were determined for the entire line (All) or for the ascites resistant (R) or susceptible (S) subpopulations based on phenotype in a hypobaric challenge. The total number of genotypes (Count) is indicated below the frequencies. The REL samples were also analyzed according to gender. P-values for a simple Bonferroni correction (see Materials and Methods) of chi square test for observed vs. expected (Adj Pval) are presented for genotypes with frequency ≥0.10.

	SUS	RES	REL				REL Male			REL Female		
Genotype	All Freq	All Freq	All Freq	R Freq	S Freq	Adj Pval	R Freq	S Freq	Adj Pval	R Freq	S Freq	Adj Pval
AGTR1i1												
1	0.02	0.53	0.25	0.27	0.22	1.233	0.29	0.22	1.156	0.25	0.21	1.800
1+2	0.04	0.07	0.51	0.54	0.50	1.827	0.51	0.48	2.065	0.58	0.53	1.436
2	0.94	0.41	0.24	0.19	0.28	0.346	0.20	0.31	0.524	0.17	0.26	0.825
Count	51	76	279	139	113		80	65		59	47	
UTS2Dp1												
1	0.27	0.03	0.02	0.02	0.03		0.00	0.00		0.05	0.06	
1+2	0.35	0.28	0.11	0.09	0.11	1.213	0.12	0.13	1.096	0.05	0.09	0.497
2	0.38	0.69	0.87	0.89	0.86	1.684	0.88	0.87	1.866	0.90	0.85	1.647
Count	37	93	196	99	80		59	45		40	34	
UTS2Di3												
1	0.29	0.52	0.24	0.27	0.20	0.935	0.28	0.27	1.131	0.24	0.10	0.160
1+2	0.37	0.34	0.50	0.50	0.50	2.711	0.51	0.48	2.560	0.50	0.52	2.423
2	0.33	0.13	0.26	0.23	0.30	0.828	0.21	0.25	1.032	0.26	0.38	0.385
Count	81	96	278	139	113		81	64		58	48	
s13.193												
1	0.94	0.10	0.04	0.07	0.02		0.05	0.00		0.11	0.05	
1+2	0.06	0.21	0.24	0.26	0.19	0.647	0.26	0.20	0.517	0.26	0.19	0.972
2	0.00	0.70	0.72	0.67	0.78	0.705	0.69	0.80	0.930	0.63	0.76	0.990
Count	96	92	208	100	88		62	50		38	37	

Table 2.4. Genotype data for AGTR1i1 for the commercial lines. For each line the SNP genotypes were analyzed by ascites phenotype for All and for each gender. All other column headings and row descriptions are as for Table 2.3.

Genotype	All Freq	All R Freq	All S Freq	Adj Pval	Male R Freq	Male S Freq	Male Adj Pval	Female R Freq	Female S Freq	Female Adj Pval
Line W										
1	0.03	0.01	0.04		0.02	0.03		0.00	0.06	
1+2	0.27	0.28	0.25	1.429	0.22	0.18	0.461	0.35	0.31	0.484
2	0.71	0.71	0.70	1.942	0.76	0.79	0.868	0.65	0.63	0.920
Count	180	89	91		49	39		40	52	
Line Y										
1	0.17	0.16	0.18	2.049	0.17	0.18	2.418	0.14	0.16	1.820
1+2	0.35	0.37	0.33	1.886	0.41	0.26	0.664	0.32	0.40	1.524
2	0.48	0.47	0.49	2.586	0.41	0.55	0.952	0.54	0.43	1.343
Count	189	113	76		63	38		50	37	
Line Z										
1	0.21	0.24	0.17	1.008	0.23	0.20	2.009	0.24	0.15	1.236
1+2	0.31	0.29	0.37	1.113	0.33	0.40	1.265	0.21	0.36	0.640
2	0.48	0.48	0.47	2.752	0.44	0.40	1.437	0.55	0.48	1.471
Count	166	105	60		64	25		38	33	

Table 2.5. Genotype data for UTS2Dp1 for the commercial lines. Column and row designations are as for Tables 2.3 and 2.4.

Genotype	All Freq	All R Freq	All S Freq	Adj Pval	Male R Freq	Male S Freq	Male Adj Pval	Female R Freq	Female S Freq	Female Adj Pval
Line W										
1	0.00	0.00	0.00		0.00	0.00		0.00	0.00	
1+2	0.00	0.00	0.00		0.00	0.00		0.00	0.00	
2	1.00	1.00	1.00	1.000	1.00	1.00	1.000	1.00	1.00	1.000
Count	184	94	90		52	38		42	52	
Line Y										
1	0.05	0.05	0.05		0.05	0.05		0.06	0.05	
1+2	0.05	0.04	0.06		0.05	0.05		0.02	0.08	
2	0.90	0.91	0.89	0.836	0.90	0.89	0.964	0.92	0.86	0.788
Count	188	112	76		62	38		50	37	
Line Z										
1	0.15	0.13	0.20	0.712	0.17	0.22	1.050	0.06	0.20	0.426
1+2	0.34	0.36	0.32	2.128	0.34	0.37	2.395	0.38	0.30	1.837
2	0.50	0.52	0.47	2.168	0.49	0.41	1.427	0.56	0.50	1.908
Count	155	96	59		59	27		32	30	

Table 2.6. Genotype data for UTS2Di3 for the commercial lines. Column and row designations are as for Tables 2.3 and 2.4.

Genotype	All Freq	All R Freq	All S Freq	Adj Pval	Male R Freq	Male S Freq	Male Adj Pval	Female R Freq	Female S Freq	Female Adj Pval
Line W										
1	0.45	0.54	0.37	0.169	0.56	0.39	0.406	0.51	0.35	0.429
1+2	0.52	0.42	0.61	0.138	0.42	0.59	0.527	0.42	0.63	0.286
2	0.03	0.04	0.02		0.02	0.02		0.07	0.02	
Count	190	95	95		52	41		43	54	
Line Y										
1	0.08	0.08	0.08		0.05	0.08		0.12	0.08	
1+2	0.74	0.73	0.75	1.807	0.79	0.79	1.097	0.66	0.70	0.948
2	0.18	0.19	0.17	1.628	0.16	0.13	0.840	0.22	0.22	0.793
Count	189	113	76		63	38		50	37	
Line Z										
1	0.24	0.27	0.19	0.908	0.22	0.07	0.197	0.35	0.23	0.556
1+2	0.46	0.42	0.54	0.779	0.42	0.63	0.506	0.41	0.50	1.676
2	0.30	0.31	0.27	1.908	0.36	0.30	1.048	0.24	0.27	1.515
Count	172	112	59		73	27		37	30	

Table 2.7. Genotype data for s13.193 for the commercial lines. Column and row designations are as for Tables 2.3 and 2.4.

Genotype	All Freq	All R Freq	All S Freq	Adj Pval	Male R Freq	Male S Freq	Male Adj Pval	Female R Freq	Female S Freq	Female Adj Pval
Line W										
1	0.46	0.45	0.47	2.459	0.44	0.39	1.549	0.46	0.53	1.413
1+2	0.31	0.35	0.27	1.128	0.33	0.34	1.980	0.37	0.23	0.611
2	0.23	0.20	0.25	1.434	0.23	0.26	1.942	0.17	0.25	1.253
Count	180	89	91		48	38		41	53	
Line Y										
1	0.46	0.44	0.48	1.404	0.51	0.47	0.949	0.34	0.50	0.432
1+2	0.09	0.08	0.11		0.05	0.14		0.13	0.08	
2	0.45	0.48	0.41	1.030	0.43	0.39	1.125	0.53	0.42	0.753
Count	182	109	73		62	36		47	36	
Line Z										
1	0.57	0.58	0.54	2.269	0.60	0.46	1.223	0.56	0.58	2.757
1+2	0.26	0.22	0.33	0.590	0.19	0.43	0.108	0.26	0.26	2.940
2	0.17	0.20	0.13	0.927	0.21	0.11	0.861	0.18	0.16	2.550
Count	178	116	61		73	28		39	31	

Table 2.8. Haplotype data combining AGTR1i1 and UTS2Dp1 genotypes for the REL and commercial lines. Haplotypes were imputed where possible, as described in materials and methods. Column and row designations are as for Tables 2.3 and 2.4.

Haplotypes	All Freq	All R Freq	All S Freq	Adj Pval	Male R Freq	Male S Freq	Male Adj Pval	Female R Freq	Female S Freq	Female Adj Pval
REL										
11	0.02	0.03	0.01		0.02	0.01		0.04	0.02	
12	0.50	0.54	0.44	0.466	0.54	0.44	0.601	0.53	0.43	0.848
21	0.03	0.02	0.04		0.02	0.04		0.01	0.05	
22	0.45	0.42	0.50	0.571	0.42	0.51	0.685	0.42	0.50	0.991
Count	360	188	142		112	80		76	60	
Line W										
12	0.16	0.15	0.18	0.845	0.13	0.13	0.449	0.17	0.22	0.306
22	0.84	0.85	0.82	1.447	0.88	0.88	1.185	0.83	0.78	1.057
Count	344	172	172		96	72		76	100	
Line Y										
11	0.02	0.01	0.03		0.02	0.03		0.01	0.03	
12	0.32	0.32	0.32	1.928	0.35	0.29	0.921	0.29	0.33	1.118
21	0.05	0.05	0.05		0.04	0.05		0.06	0.06	
22	0.61	0.61	0.60	1.738	0.59	0.63	1.453	0.64	0.58	1.249
Count	370	220	150		120	76		100	72	
Line Z										
11	0.12	0.09	0.17	0.331	0.10	0.23	1.838	0.08	0.14	1.499
12	0.25	0.32	0.16	0.067	0.30	0.18	2.784	0.35	0.16	1.670
21	0.18	0.17	0.19	2.986	0.18	0.20	2.716	0.15	0.20	2.372
22	0.45	0.42	0.48	1.913	0.48	0.40	1.557	0.42	0.50	1.987
Count	240	114	94		88	40		52	50	

Table 2.9. Haplotype data combining AGTR1i1 and UTS2Di3 genotypes for the REL and commercial lines. Haplotypes were imputed where possible, as described in materials and methods. Column and row designations are as for Tables 2.3 and 2.4.

Haplotypes	All Freq	All R Freq	All S Freq	Adj Pval	Male R Freq	Male S Freq	Male Adj Pval	Female R Freq	Female S Freq	Female Adj Pval
REL										
11	0.30	0.34	0.26	0.675	0.35	0.29	1.116	0.31	0.19	0.336
12	0.21	0.22	0.20	3.009	0.21	0.15	0.704	0.23	0.28	0.566
21	0.19	0.20	0.19	3.263	0.21	0.23	1.386	0.18	0.14	1.025
22	0.30	0.25	0.35	0.282	0.23	0.33	0.511	0.28	0.39	0.580
Count	402	188	172		110	96		78	74	
Line W										
11	0.11	0.11	0.10	2.450	0.12	0.06	0.599	0.10	0.14	1.119
12	0.01	0.01	0.01		0.00	0.00		0.01	0.01	
21	0.63	0.66	0.59	1.270	0.67	0.63	1.715	0.65	0.56	1.209
22	0.26	0.22	0.30	0.490	0.21	0.31	0.6069	0.24	0.29	1.448
Count	314	160	154		92	68		68	86	
Line Y										
11	0.10	0.10	0.11	3.426	0.13	0.08	1.553	0.07	0.12	1.231
12	0.18	0.18	0.19	3.623	0.20	0.18	3.058	0.16	0.18	2.642
21	0.33	0.33	0.33	3.758	0.29	0.38	1.342	0.36	0.28	1.664
22	0.39	0.39	0.38	3.226	0.38	0.35	2.609	0.41	0.42	2.552
Count	272	160	112		86	60		74	50	
Line Z										
11	0.08	0.08	0.08		0.06	0.06		0.09	0.09	
12	0.25	0.26	0.24	2.129	0.29	0.31	0.910	0.21	0.20	1.057
21	0.36	0.37	0.34	2.146	0.32	0.22	0.416	0.45	0.36	0.812
22	0.31	0.29	0.35	1.410	0.32	0.41	0.967	0.26	0.34	1.274
Count	238	156	80		96	32		58	44	

Table 2.10. Haplotype data combining AGTR1i1 and s13.193 genotypes for the REL and commercial lines. Haplotypes were imputed where possible, as described in materials and methods. Column and row designations are as for Tables 2.3 and 2.4.

Haplotypes	All Freq	All R Freq	All S Freq	Adj Pval	Male R Freq	Male S Freq	Male Adj Pval	Female R Freq	Female S Freq	Female Adj Pval
REL										
11	0.07	0.08	0.06		0.07	0.06		0.10	0.06	
12	0.41	0.42	0.43	1.526	0.44	0.45	0.969	0.39	0.38	1.143
21	0.05	0.07	0.03		0.05	0.01		0.10	0.06	
22	0.47	0.43	0.48	0.985	0.44	0.48	1.363	0.42	0.50	1.002
Count	352	162	156		100	88		62	66	
Line W										
11	0.09	0.10	0.08		0.09	0.03		0.11	0.11	
12	0.05	0.03	0.06		0.02	0.05		0.03	0.07	
21	0.54	0.55	0.54	1.846	0.53	0.53	1.768	0.56	0.54	1.673
22	0.33	0.33	0.32	1.849	0.35	0.39	0.652	0.30	0.28	0.740
Count	310	152	158		86	64		66	94	
Line Y										
11	0.15	0.16	0.14	2.467	0.20	0.10	0.381	0.12	0.19	1.025
12	0.17	0.16	0.18	3.005	0.17	0.17	3.538	0.15	0.16	2.494
21	0.35	0.32	0.39	0.962	0.34	0.44	0.734	0.29	0.36	1.226
22	0.33	0.36	0.29	1.099	0.29	0.29	1.191	0.45	0.30	0.171
Count	358	216	142		122	70		94	70	
Line Z										
11	0.23	0.23	0.23	3.695	0.27	0.19	1.356	0.16	0.28	0.768
12	0.13	0.14	0.12	2.756	0.10	0.17	1.274	0.18	0.09	0.794
21	0.48	0.48	0.49	3.459	0.44	0.53	1.628	0.59	0.43	0.799
22	0.16	0.16	0.16	3.837	0.19	0.11	0.938	0.07	0.20	0.266
Count	266	170	94		108	36		56	54	

Table 2.11. Haplotype data combining UTS2Dp1 and UTS2Di3 genotypes for the REL and commercial lines. Haplotypes were imputed where possible, as described in materials and methods. Column and row designations are as for Tables 2.3 and 2.4.

Haplotypes	All Freq	All R Freq	All S Freq	Adj Pval	Male R Freq	Male S Freq	Male Adj Pval	Female R Freq	Female S Freq	Female Adj Pval
REL										
11	0.01	0.02	0.00		0.00	0.00		0.06	0.00	
12	0.05	0.04	0.06		0.03	0.03		0.06	0.09	
21	0.16	0.20	0.10	0.114	0.19	0.08	0.194	0.21	0.13	0.518
22	0.78	0.74	0.84	0.735	0.78	0.89	0.704	0.67	0.78	0.743
Count	276	138	118		86	62		52	54	
Line W										
21	0.71	0.76	0.67	0.695	0.77	0.67	0.7944	0.73	0.67	1.191
22	0.29	0.24	0.33	0.275	0.23	0.33	0.3614	0.27	0.33	0.802
Count	364	184	180		102	76		82	104	
Line Y										
11	0.02	0.02	0.02		0.03	0.03		0.02	0.03	
12	0.04	0.04	0.04		0.03	0.04		0.05	0.04	
21	0.42	0.42	0.42	1.939	0.43	0.45	1.484	0.43	0.40	1.505
22	0.51	0.52	0.51	1.761	0.55	0.49	1.309	0.50	0.53	1.592
Count	364	218	146		118	74		100	70	
Line Z										
11	0.06	0.05	0.08		0.05	0.08		0.05	0.08	
12	0.24	0.22	0.27	1.401	0.26	0.31	0.955	0.15	0.26	0.727
21	0.39	0.39	0.38	2.642	0.31	0.28	0.289	0.53	0.42	0.538
22	0.31	0.34	0.27	1.055	0.38	0.33	0.757	0.28	0.24	1.048
Count	208	128	78		84	36		40	38	

Table 2.12. Haplotype data combining UTS2Dp1 and s13.193 genotypes for the REL and commercial lines. Haplotypes were imputed where possible, as described in materials and methods. Column and row designations are as for Tables 2.3 and 2.4.

Haplotypes	All Freq	All R Freq	All S Freq	Adj Pval	Male R Freq	Male S Freq	Male Adj Pval	Female R Freq	Female S Freq	Female Adj Pval
REL										
11	0.01	0.02	0.00		0.03	0.00		0.06	0.00	
12	0.05	0.04	0.06		0.03	0.07		0.06	0.09	
21	0.16	0.20	0.10	0.114	0.14	0.10	0.601	0.21	0.13	0.518
22	0.78	0.74	0.84	0.735	0.80	0.83	1.363	0.67	0.78	0.743
Count	276	138	118		69	58		52	54	
Line W										
21	0.62	0.62	0.61	1.781	0.62	0.59	1.489	0.63	0.63	1.688
22	0.38	0.38	0.39	1.724	0.38	0.41	1.359	0.37	0.37	1.606
Count	344	172	172		94	70		78	102	
Line Y										
11	0.06	0.05	0.07		0.06	0.04		0.04	0.10	
12	0.02	0.02	0.02		0.02	0.04		0.02	0.00	
21	0.44	0.43	0.47	1.140	0.48	0.50	0.701	0.36	0.44	0.462
22	0.48	0.50	0.45	0.848	0.45	0.42	0.721	0.58	0.46	0.354
Count	360	214	146		122	72		92	72	
Line Z										
11	0.23	0.19	0.29	0.293	0.20	0.31	0.522	0.20	0.30	0.827
12	0.09	0.10	0.08		0.13	0.08		0.04	0.07	
21	0.47	0.48	0.43	1.687	0.49	0.38	0.946	0.46	0.44	2.398
22	0.21	0.22	0.20	1.960	0.18	0.23	1.382	0.30	0.19	0.477
Count	278	170	106		110	48		50	54	

Table 2.13. Haplotype data combining UTS2Di3 and s13.193 genotypes for the REL and commercial lines. Haplotypes were imputed where possible, as described in materials and methods. Column and row designations are as for Tables 2.3 and 2.4.

Haplotypes	All Freq	All R Freq	All S Freq	Adj Pval	Male R Freq	Male S Freq	Male Adj Pval	Female R Freq	Female S Freq	Female Adj Pval
REL										
11	0.06	0.08	0.03		0.07	0.02		0.09	0.03	
12	0.40	0.43	0.39	1.136	0.47	0.46	0.339	0.36	0.27	0.172
21	0.05	0.06	0.04		0.02	0.01		0.11	0.08	
22	0.49	0.44	0.55	0.345	0.44	0.50	0.855	0.44	0.62	0.220
Count	344	162	150		96	82		66	66	
Line W										
11	0.47	0.50	0.45	2.003	0.51	0.40	1.320	0.48	0.48	3.535
12	0.26	0.27	0.25	3.122	0.28	0.30	2.031	0.26	0.22	1.854
21	0.16	0.14	0.19	1.182	0.10	0.18	0.662	0.18	0.19	1.834
22	0.10	0.09	0.11	2.259	0.10	0.12	2.924	0.08	0.11	1.870
Count	302	152	150		86	60		66	90	
Line Y										
11	0.27	0.25	0.31	1.364	0.27	0.32	1.824	0.23	0.30	1.376
12	0.17	0.20	0.14	0.853	0.18	0.15	2.266	0.22	0.12	0.611
21	0.23	0.23	0.23	3.892	0.27	0.23	1.382	0.17	0.24	1.023
22	0.32	0.32	0.32	3.967	0.28	0.31	1.506	0.38	0.33	1.294
Count	344	214	130		122	62		92	66	
Line Z										
11	0.43	0.42	0.42	2.734	0.38	0.32	0.644	0.50	0.45	1.170
12	0.03	0.05	0.00		0.04	0.00		0.07	0.00	
21	0.32	0.31	0.35	2.033	0.34	0.44	0.576	0.26	0.30	1.195
22	0.22	0.21	0.23	2.340	0.24	0.24	1.767	0.17	0.25	1.085
Count	258	178	78		122	34		54	40	

Table 2.14. Estimation of linkage disequilibrium between pairs of VNTR loci (PHS009 and PHS010) and SNP loci (AGTR1i1, UTS2Dp1, UTS2Di3 and s13.193) in experimental REL line, and commercial lines Y and Z. The reported values are Lewontin's normalized disequilibrium co-efficient *D'* (Lewontin, 1967) modified for multiple alleles (Hedrick, 1987). Values were calculated for the alleles in each of VNTR and SNP locus pair in the three lines. N = sample size in each population; n = number of VNTR alleles in that line.

			D'			
Line	N	n	AGTR1i1	UTS2Dp1	UTS2Di3	s13.193
PHS009						
Line Y	187	3	0.66	0.79	0.60	0.12
Line Z	191	2	0.72	0.69	0.66	0.24
REL	271	4	0.71	0.81	0.62	0.19
PHS010						
Line Y	187	4	0.68	0.65	0.63	0.26
Line Z	191	3	0.73	0.71	0.61	0.46
REL	275	3	0.68	0.79	0.65	0.54

Table 2.15. SNP haplotype data for PHS009-PHS010 (BB genotype) in commercial line Y. Haplotypes were imputed where possible, as described in materials and methods. Column and row designations are as for Tables 2.3 and 2.4.

	PHS009 (BB Genotype)				PHS010 (BB Genotype)			
	All Freq	All R Freq	All S Freq	Adj Pval	All Freq	All R Freq	All S Freq	Adj Pval
AGTR1i1+UTS2Dp1								
12	0.96	1.00	0.86	0.64	0.91	1.00	0.55	0.212
22	0.04	0.00	0.14		0.09	0.00	0.44	
Count	54	40	14		44	35	9	
AGTR1i1+ UTS2Di3								
11	0.41	0.40	0.42	1.873	0.38	0.42	0.22	0.768
12	0.58	0.60	0.50	1.378	0.55	0.58	0.44	1.274
22	0.02	0.00	0.08		0.07	0.00	0.33	
Count	52	40	12		42	33	9	
AGTR1i1+ s13.193								
11	0.62	0.63	0.58	0.853	0.49	0.58	0.27	0.423
12	0.34	0.37	0.25	0.540	0.31	0.31	0.33	2.615
21	0.02	0.00	0.08		0.00	0.00	0.00	
22	0.02	0.00	0.08		0.19	0.11	0.4	0.101
Count	50	38	12		51	36	15	
UTS2Dp1+UTS2Di3								
21	0.41	0.40	0.43	1.77	0.43	0.44	0.33	1.407
22	0.59	0.60	0.57	1.81	0.57	0.55	0.66	1.484
Count	54	40	14		49	43	6	
UTS2Dp1+ s13.193								
21	0.64	0.63	0.67	1.789	0.55	0.55	0.54	1.986
22	0.36	0.37	0.33	1.719	0.45	0.45	0.45	1.985
Count	50	38	12		53	42	11	
UTS2Di3+ s13.193								
11	0.30	0.29	0.33	2.426	0.23	0.21	0.27	2.868
12	0.08	0.08	0.08		0.15	0.19	0.00	0.591
21	0.34	0.34	0.33	2.891	0.34	0.36	0.27	2.676
22	0.28	0.29	0.25	2.465	0.28	0.24	0.45	0.919

Table 2.16. SNP haplotype data for PHS009 (FF genotype) and PHS010 (BB genotype) in commercial line Z. Haplotypes were imputed, where possible, as described in materials and methods. Column and row designations are as for Tables 2.3 and 2.4.

	PHS009 (FF Genotype)				PHS010 (BB Genotype)			
	All Freq	All R Freq	All S Freq	Adj Pval	All Freq	All R Freq	All S Freq	Adj Pval
AGTR1i1+UTS2Dp1								
11	0.25	0.19	0.66	0.06	0.18	0.13	0.36	0.208
12	0.65	0.69	0.33	0.603	0.72	0.74	0.64	1.422
21	0.08	0.09	0.00		0.06	0.08	0.00	
22	0.02	0.02	0.00		0.04	0.05	0.00	
Count	52	46	6		50	39	11	
AGTR1i1+ UTS2Di3								
11	0.25	0.24	0.33	1.444	0.19	0.16	0.33	0.559
12	0.67	0.66	0.67	1.769	0.62	0.61	0.67	1.666
21	0.02	0.03	0.00		0.06	0.08	0.00	
22	0.06	0.08	0.00		0.13	0.16	0.00	
Count	48	39	9		47	38	9	
AGTR1i1+ s13.193								
11	0.56	0.49	0.87	0.363	0.38	0.38	0.40	2.766
12	0.31	0.35	0.12	0.596	0.49	0.46	0.60	1.719
21	0.00	0.00	0.00		0.00	0.00	0.00	
22	0.13	0.16	0.00	0.509	0.13	0.16	0.00	0.609
Count	45	37	8		47	37	10	
UTS2Dp1+UTS2Di3								
11	0.09	0.06	0.18		0.00	0.00	0.00	
12	0.24	0.20	0.36	0.666	0.15	0.14	0.17	2.561
21	0.07	0.09	0.00		0.13	0.09	0.25	0.508
22	0.61	0.66	0.45	0.905	0.72	0.77	0.58	1.526
Count	46	35	11		47	35	12	
UTS2Dp1+ s13.193								
11	0.13	0.06	0.10	0.500	0.05	0.03	0.12	
12	0.26	0.21	0.50	0.204	0.10	0.09	0.12	
21	0.30	0.32	0.30	1.567	0.40	0.41	0.37	1.766
22	0.32	0.41	0.10	0.239	0.45	0.47	0.37	1.435
Count	47	34	10		42	34	8	
UTS2Di3+ s13.193								
11	0.14	0.13	0.18	1.351	0.08	0.08	0.11	
12	0.12	0.15	0.00	0.395	0.08	0.10	0.00	
21	0.27	0.26	0.36	1.041	0.32	0.31	0.33	1.866
22	0.47	0.46	0.45	1.838	0.51	0.50	0.55	1.667
Count	49	39	11		47	38	9	

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CHAPTER 3

Multi-generational genome wide association studies identify chromosomal regions associated with ascites phenotype

ABSTRACT

Ascites is a multi-faceted disease found commonly in fast growing broilers, which is initiated when the body is insufficiently oxygenated. A series of events follow, including an increase in pulmonary artery pressure, right ventricle hypertrophy, and accumulation of fluid in the abdominal cavity and pericardium. Advances in management practices along with improved selection programs have decreased ascites incidence in modern broilers. However, ascites syndrome remains an economically important disease throughout the world, causing estimated losses of $100 million/year. In this study, a 60K Illumina SNP BeadChip was used to perform a series of GWAS (genome wide association study) on the 16th and 18th generation of our relaxed (REL) line descended from a commercial elite broiler line beginning in 1995. Regions significantly associated with ascites incidence were identified on chromosome 2 around 70 megabase pairs (Mbp) and on chromosome Z around 60 Mbp. Five candidate single nucleotide polymorphisms (SNP) were evaluated as indicators for these two regions in order to identify association with ascites and right ventricle-to-total ventricle weight (RVTV) ratios. Chromosome 2 SNPs showed an association with RVTV ratios in males phenotyped as ascites resistant and ascites susceptible (P = 0.02 and P= 0.03 respectively). The chromosome Z region also indicates an association with resistant female RVTV values (P = 0.02). Regions of significance identified on chromosomes 2 and Z described in this study will be used as proposed candidate regions for further investigation into the genetics of ascites. This information will lead to a better understanding of the underlying genetics and gene networks contributing to ascites, and thus advances in ascites reduction through commercial breeding schemes.

INTRODUCTION

Ascites, or pulmonary hypertension syndrome, encompasses a cascade of adverse effects that begins with the impaired ability to adequately oxygenate tissues throughout the body of a fast growing-broiler and ultimately leads to death (Wideman, 1999; Balog et al., 2000; Decuypere et al., 2005; Wideman et al., 2013). The development of ascites is credited to both the genetics of the broiler and external environmental factors (Owen et al., 1990; Lubritz et al., 1995; Wideman and French, 2000; Balog et al., 2003). The response of the body to the increase in oxygen demand is increased blood flow, which leads to overloading of the heart and lungs (Julian et al., 1986). Chickens in chronic hypoxic environments will experience cardiac hypertrophy of the right ventricle (Burton and Smith, 1967). Calculation of the subsequent right ventricle to total ventricle (RVTV) weight ratio indicates an increase in this value associated with cardiac hypertrophy. Amplified pressure in the cardiovascular system will advance to eventual right ventricle hypertrophy and concludes with right ventricle failure (Huchzermeyer and Deruyck, 1986). Subsequent failure of the liver due to inadequate portal blood flow leads to plasma transduction into the body cavity (Wideman et al., 2013). Death of the bird follows soon after. Selection schemes and management techniques have been implemented to reduce the overall incidence of ascites; however, it remains an economically important disease causing an estimated economic loss of $100 million/year as recently as 2015 (M. Cooper and S. Gustin, personal communication, Cobb-Vantress, Inc.).

Inducing ascites in an experimental setting can be achieved by altering the environment's temperature (Wideman et al., 1998; Sato et al., 2002), air quality (Chineme et al., 1995), and altitude (Balog et al., 2000). The first documentation of ascites occurred in La Paz, Bolivia where birds were being raised at an altitude of 3300 m above sea level (Hall and Machicao,

1968). An inverse correlation exists between elevation and the partial pressure of O_2. Increasing elevation leads to hypoxia, or the reduction of O_2 inspired and transferred to the tissues. In broiler chickens the depletion of oxygen in this manner leads to ascites syndrome (Ruiz-Feria and Wideman, 2001). At the University of Arkansas we have used a hypobaric chamber to simulate a high altitude environment as a non-invasive technique to reliably induce ascites (Owen et al.,1990; Balog et al., 2000).

Wideman et al. (2013) proposed that the moderate to high heritabilites of ascites reported from multiple studies (Lubritz et al., 1995; Wideman and French, 2000; de Greef, et al., 2001; Moghadam et al., 2001; Druyan et al., 2007) are likely due to multiple genes. Recently, a genome wide association study (GWAS) using a 3.4K SNPChip (Muira, et al., 2008) was conducted to scan the genome for candidate single nucleotide polymorphisms (SNPs) associated with ascites in a reciprocal cross between divergently selected ascites resistant and ascites susceptible lines developed at the University of Arkansas (Krishnamoorthy et al., 2014). Identification of potential genes relevant to sex biased ascites incidence were identified on chromosome 9. With advances in high throughput SNP genotying assays, followed by the development of a moderate density 60K Illumina SNP BeadChip (Groenen et al., 2011), GWAS can be used to more comprehensibly evaluate the broiler genome for ascites associated regions. Here, we report two GWAS for ascites phenotype conducted on two different generations of a pedigreed research line derived from a commercial elite broiler line and maintained at the University of Arkansas. Single regions were identified on two chromosomes that were significantly associated with phenotype for both generations. SNPs for these regions were then used for additional genotyping.

METHODS

Genome Data

All chromosomal positions are relative to the November 2011 ICGSC Gallus-gallus-4.0/galGal4 (GCA_000002315.2) assembly.

Bird Stocks and Hypobaric Chamber Trials

All animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee under protocol 12039. Within the hypobaric chamber are four batteries that house 40 identical cages measuring 0.6 x 0.6 x 0.3 m. Each cage has access to nipple waterers and trough feeders. The chamber is designed to control simulated altitude, ventilation, and temperature. For the duration of the trial the elevation was set to simulate approximately 2,900 m above sea level, or 533 mm of Hg. Daily, the elevation was observed with any adjustments being made to maintain the set altitude. Chamber airflow was set at 17 m^3/min and air filters were changed daily. The chamber was warmed to 92° C prior to introducing the chicks and the temperature was decreased weekly. The birds used for this study are from two different years spanning three generations. The 15^{th} and 17^{th} generation were used from a pedigreed elite broiler line that has remained under relaxed (REL) selection since 1995 (Pavlidis, et al., 2007). The parents were artificially inseminated using pooled semen and the eggs were pedigreed according to the hen. We will refer to these two hatches as the 16^{th} and 18^{th} generation of the REL line. Chicks were hatched at the University of Arkansas hatchery, wing banded, and immediately transferred randomly to cages in the hypobaric chamber. For the next six weeks mortality was recorded and necropsies were completed to record: probable cause of death, overt visual signs of ascites symptoms, total body weight, heart shape, right and total ventricle weight, and gender. At the end of the six week trial all remaining birds were euthanized by cervical dislocation and

scored as above. Final decision of ascites phenotype was based on the presence or absence of water belly, which represents the final stage of ascites progression prior to death. Additional evaluation of heart morphology was measured as the proportion of the right ventricle weight in grams to the total weight of both ventricles in grams.

DNA Isolation

At four days of age 10 µl of blood was extracted from all birds via a lancet puncture between the toes. A rapid DNA isolation method was used to isolate the DNA (Bailes, et al., 2007). For GWAS submission crude genomic DNA was purified further using Mackery-Nagel Plasmid prep plates for gDNA cleanup kit and quantified using a DyNA Quant from Hoefer and Hoechst 33258 fluorescent stain (Thermo Fisher Scientific, Waltham, MA).

Genome Wide Association Study

A female gender bias was observed in data from a previously published GWAS on chromosome 9 (Krishnamoorthy et al., 2014). For this reason, we decided to focus on elucidating possible male bias in the current study. A total of two GWAS was completed on 15 resistant and 22 susceptible males from the 16^{th} generation, and 39 resistant and 22 susceptible males from generation 18 REL line birds that were phenotyped as described above in a 6-week hypobaric chamber challenge. Variation in sample size was due to bird availability, although preference was made for birds phenotyped as ascites susceptibility early in the trial. The GWAS was conducted by DNA Landmarks (Qubec, Canada) using Illumina 60K SNPChip on REL line males.

SNP allele frequencies were calculated independently for resistant and susceptible individuals using Microsoft Excel (Microsoft Corp., Redmond, Wa). Loci with a minor allele frequency of

less than 0.05 were excluded. Allele frequencies were used to calculate expected genotype counts. Deviations from Hardy-Weinberg were computed for each locus based on observed vs expected genotype counts. Loci with a P-value less than 0.05 were excluded. Genotypic frequencies were calculated for resistant and susceptible subpopulations following Krishnamoorthy et al. (2014). Actual frequencies were then used to calculate expected frequencies. A chi-square test was performed comparing the actual and expected frequencies for genotypes independently for resistant and susceptible phenotype groups. The P-values obtained from this chi-square test were log transformed plotted as $1\text{-}\log_{10}(P)$ for visualization. For each locus an average $1\text{-}\text{Log}_{10}(P)$ was calculated for a sliding window of 10 flanking SNP, which covers approximately 375,000 bp. The purpose of the window is to account for linkage disequilibrium in closely positioned SNP, while the use of 10 SNPs is directly related to an increase in power of ascites association detection for a small sample size as seen in this study (Gao et al., 2012). We then completed further investigation into regions that surpassed a threshold of $1\text{-}\log_{10}(P) > 2.5$ in both generations due to a lack of largely significant peaks normally seen in GWAS completed on other livestock species.

Real-Time PCR

Specific SNPs were used to develop exonuclease (Taqman® probe, Thermo Fisher Scientific, Waltham, MA) assays for quantitative real-time PCR genotyping. PCR primers and probes, along with optimized annealing temperatures are presented in Table 3.1. Genotyping was competed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Richmond, CA). Reaction volume totaled 20 µL including 1x Taq-Buffer (50 mM Tris-Cl pH 8.3, 1 mM $MgCl_2$, 30 µg/mL of 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 of Taq polymerase, and 2 µL of DNA. A two-

step PCR procedure was used as follows: 90°C for 30 seconds, 10 cycles of 90°C for 15 seconds and SNP-specific annealing temperature for 30 seconds, followed by 90°C for 15 seconds, SNP-specific annealing temperature for 30 seconds, and a plate read for a total of 30 cycles. Verification of each primer set was completed on controls prior to genotyping birds used in this study.

Statistical Methods

Individuals genotyped for all SNP include 145 resistant and 123 susceptible males, and 98 resistant and 115 susceptible females. Birds were evaluated by ascites phenotype and RVTV ratio. Genotype frequency was calculated for ascites resistant and susceptible individuals by gender. A chi-square test was performed on expected versus observed counts, with a P-value of <0.05 indicating significance.

The right ventricle-to-total ventricle ratio was calculated based on associated weights recorded during necropsy. For each SNP locus a Student's t-test was used to compare RVTV ratios for each corresponding genotype, in which resistant and susceptible individuals were compared independently. Male and female ratios were calculated independently of each other, and RVTV ratios were considered significant with a P-value of < 0.05.

RESULTS

After application of quality control filtering the 60K Illumina SNP BeadChip analysis resulted in a total of 37,109 informative SNPs. Using $1\text{-}\mathrm{Log}_{10}P$ threshold of greater than 2.5, informative regions on chromosomes 2 and Z were identified as candidates for investigation into the genetic causes of ascites in broilers ($P \leq 0.0316$). Out of a total of 4779 SNPs on chromosome 2, 4215

SNP are polymorphic in the REL line (Figure 3.1). A region around 70 megabase pairs (Mbp) appears to show significant association in ascites resistant individuals in both generations by meeting the chosen threshold of 2.5. Interestingly, the GWAS of susceptible individuals did not indicate any such significance. Similarly, 1178 of 1385 SNP are informative on the Z chromosome, for which a region of significance is observed around 60 Mbp is detectable in both generations in susceptible individuals, but does not surpass the threshold in ascites resistant individuals (Figure 3.2).

Significant SNP regions were first identified by visualization of regions associated with ascites phenotype using $1\text{-}\log_{10}(P)$ values completed after application of a sliding SNP window. Two to three SNP per significant region were identified to be used as candidate SNP for these regions for further evaluation into association with ascites incidence. Two SNP were selected from chromosome 2 and 3 SNP were selected from chromosome Z as indicators for regions associated with ascites resistance and susceptibility, respectively. SNP were selected that passed quality control, had an individual $1\text{-}\log_{10}(P)$ at the threshold of 2.5, and whose genotypes were segregating in both generations. SNP were used for further genotype assays on a larger collection of DNA that had been isolated from hypobaric chamber trials completed with the offspring from the 18^{th} generation of the REL line (Table 3.2). For both SNP on chromosome 2 in male individuals there were no significant differences detected between frequencies of the resistant and susceptible individuals. In the susceptible males on SNP 2.708 the TT (0.44) and CC (0.44) genotypes both have RVTV averages higher than heterozygous susceptible individuals (0.41; P= 0.03). The greater RVTV values in susceptible males equate to a higher ascites incidence seen in both the TT genotyped males and in the lower frequency CC genotyped susceptible males. The average RVTV for SNP 2.713 for resistant males varied significantly among genotypes (P=

0.02). Homozygous AA genotyped birds sustain a significantly lower RVTV ratio (0.28) when compared to AG (0.30) individuals. The largest RVTV value in the GG genotype was not detected as being significantly different from AA or AG genotypes, likely because of the small frequency in the population. Differences detected in the RVTV of resistant birds evaluated on the 2,713 SNP indicate ascites percent was negligible, except for the GG genotype (AA-44% vs. AG-45%, GG-30%). While the lowest ascites percentage is evaluated on the GG SNP, the frequency of this SNP is low, and may not be adequately represented in the population to draw definitive conclusions as to the RVTV and ascites incidence relationship.

Right ventricle-to-total ventricle ratios did not vary significantly between genotypes on 2,708 in susceptible females (Table 3.3). Interestingly, the heterozygous genotype associated with the lowest RVTV in males has the highest RVTV values in females, which indicates the source of ascites genetics may vary based on sex. On SNP 2,718 the difference in resistant and susceptible genotypic frequency calculation approaches significance in females for the AA (P= 0.07) and AG (P= 0.09) genotypes. Further, the genotype with the highest ascites incidence. AA, is higher in frequency in susceptible females (0.38resistant vs. 0.55 susceptible), while the lowest ascites incidence genotype, AG, is present at a higher frequency in resistant females (0.59 resistant vs. 0.42 susceptible).

For all three chromosome Z SNPs, females (the homogametic sex) have two possible genotypes, versus the three possible genotypes in males. All Z chromosome SNPs in males appear to be statistically similar in RVTV ratio averages across all genotypes. Yet, the TT genotype from the Z.600 SNP approaches a significantly higher RVTV value in resistant males compared to TC and the TT genotypes (P= 0.06). This scenario is also seen in the TT genotype in susceptible males at Z.611 (P= 0.09). In both cases, the larger RVTV values are associated with higher percentages of

ascites incidence within each SNP. In the case of SNP Z.600 resistant females, the CW genotyped individuals have a significantly higher RVTV value (0.31) than TW genotype individuals (0.27). For the Z.611 locus, susceptible females have higher RVTV values in the TW genotype compared to CW females if the threshold of significance is evaluated at P=0.07. The variation seen in the susceptible female RVTV values on Z.611 is inversely related to the ascites incidence among the genotypic populations. No significant differences were detected in chi-square analyses of observed vs. expected counts for any genotype from males and females of all 5 SNP.

DISCUSSION

Multiple GWAS have been conducted, spanning two generations in a randomly mated control broiler line (REL), to detect loci that showed association with ascites phenotype in both generations to identify loci that were robust and consistent in association with ascites. Evaluation of ascites resistant and susceptible individuals occured on 29 chromosomes using a 60K SNP chip. Evaluation of P-values at each SNP locus as an averaged sliding window reduced the overall significance seen in SNP associations. For this reason, genotyping on candidate regions to evaluated associations with ascites outcome was completed at 5 loci. It is important to note that the regions previously identified on chromosome 9 in REL line broilers were not significant in these analyses focused on association in male individuals (Krishnamoorthy et al., 2014). The prior GWAS used an F2 cross of the resistant and susceptible lines, which were divergently selected from the predecessor of the REL line. This suggests that epistasis can play a major role in ascites genetics since the F2 cross GWAS identified regions different from a GWAS in the REL line. Genome-wide association studies provide a powerful

insight into the genetic basis for complex diseases; however, this genotyping technology is subject to Type 1 and Type 2 errors, depending on correction techniques used (Johnson et al., 2010). Through use of a sliding window, GWAS P-values are corrected to account for data sets with high levels of linkage disequilibrium in a method less labor intensive than permutation corrections (Gao 2011). Ultimately, GWAS information from multiple generations provides a better understanding of the chromosomal regions that are influencing disease occurrence, rather than focusing on generation-specific loci whose associations are merely an artifact of chance in a relaxed-selected line.

Utilizing the sliding window analysis method, two GWAS conducted on two generations of the relaxed selection REL line indicated regions on an autosomal chromosome (2) and a sex chromosome (Z) associated with ascites phenotype or cardiac hypertrophy. Although these regions were initially identified as indicators for ascites, their influence on RVTV values is equally informative. The region of significance on chromosome 2 in male and female broilers indicates that a variation exists in the RVTV ratio among genotypes of candidate SNP. When the oxygen demand of the body increases in a fast growing broiler the right ventricle experiences an increase in workload as the cardiac output being transferred to the lungs for future oxygenation increases (Peacock et al., 1989). This results in morphologic changes to the right ventricle that leads to ventricle hypertrophy (Burton et al., 1968). Right ventricle hypertrophy serves as a precursor for the development of ascites (Julian et al., 1986). Single nucleotide polymorphisms whose RVTV values are positively correlated to ascites incidence in susceptible individuals may play a larger role in a bird's ascites phenotype, relative to SNPs that do not show such a trend. Right ventricle to total ventricle values calculated for female genotyped at SNP

2.708 are not directly correlated to ascites susceptibility. Rather, these loci, and their associated RVTV ratios, may be artifacts of linkage disequilibrium.

The region of significance identified on chromosome 2 contains two candidate genes, MC4R and Cdh6. MC4R encodes the melanocortin-4 receptor that acts as a key regulator in appetite and body size (Huszar et al., 1997). Mouse knockouts for MC4R have elevated food intake and maturity-onset obesity (Huszar et al., 1997; Chen et al., 2000). Additionally, despite being associated with obesity, MC4R deficient mice have lower mean arterial pressure and are not hypertensive (Tallam et al., 2005; Tallam et al., 2006). Further, chronic hypothalamic stimulation of MC4R in rats increased arterial pressure regardless of food intake and weight gain (Kuo et al., 2003). Therefore, MC4R could play an integral role in the regulation of arterial pressure associated with ascites in broilers. CDH6 encodes cadherin 6, critical for the development of the renal vesicle and proximal tubule through promotion of mesenchymal to epithelial transition during embryogenesis (Cho et al., 1998). Cdh6 is also found as a surface receptor protein on platelets (Elrod et al., 2007) and can function in regulating platelet aggregation (Edwards et al., 2007). Inhibition of Cdh6 results in a reduction in thrombus formation (Dunne et al., 2012). Therefore dysregulation of Cdh6 could contribute to abnormalities in clotting or vascular lesions observed in the lungs during ascites progression in broilers (Wideman et al., 2011). Extremes in ascites incidence evaluated from female-resistant and susceptible frequencies on SNP 2.713 may provide insight into genotype-based incidence in females.

Within the Z chromosome region that we identified in the GWAS is the gene for myocyte enhancer factor 2C (Mef2c) a member of the family of MADS-box transcription factors involved in myogenesis and morphogenesis of skeletal, smooth, and cardiac muscle cells (Black and

Olson, 1998). Mef2c is the earliest of the Mef2 family to be expressed in the chick, which occurs at the beginning of cardiac and skeletal muscle differentiation during embryogenesis (Edmondson et al., 1994). Embryonic inactivation of Mef2c in mice inhibits formation of the right ventricle, and leads to embryonic lethality (Lin et al., 1997). MEF2c is a key regulator for reprogramming fibroblasts to the myocte lineage (Song et al., 2012) and is known to up regulate other genes known for cardiac formation, GATA4 and NKX2.5 (Dodou et al., 2004; Skerjanc et al., 1998). While significance did not reach the P-value standard set in this study, resistant and susceptible males in this region identified in both GWAS indicate this location may be critical to the development of ascites. Preliminary data suggests that SNP Z.611 homozygous T male individuals phenotyped as ascites susceptible approach statistically significance for higher RVTV ratios compared to other genotypes ($P = 0.07$), while also retaining a higher ascites incidence. Overall, the variation in genotypic frequency, coupled with the variable incidence of ascites, exhibited by the SNP 2.713 AA and AG genotyped females, and TT susceptible males exhibiting high RVTV values and high ascites incidence on SNP 2.708, indicate these regions may be useful for further investigation into ascites incidence.

Previously, Krishnamoorthy et al. (2014) used a genome wide association analysis to identify weakly associated regions on chromosome 9 from an F2 generation from a cross of an ascites-selected resistant line and an ascites-selected susceptible line cross. Importantly, a female sex-effect was detected in Krishnamoorthy et al. (2014). The same region evaluated in Krishnamoorthy et al. (2014) showed nothing of interest in the multi-generational GWAS completed in this study. Rabie et al. (2005) cited many chromosomal regions as responsible for ascites incidence including chromosomes 2, 5, 8, 10, 27, and 28. Notably, chromosome 9 was not implicated as a casual source. Rabie et al. (2005) did find an association with RVTV ratio and a

region on chromosome 2, but this region was located at position 105.8- 126.9 Mb, compared to the region at 70 Mb and 71 Mb detected through this study. Combined with data analyzed by Krishnamoorthy et al. (2014) and Rabie et al. (2005), the current study has introduced additional information as to potential regions associated with ascites phenotype, and has evaluated the usefulness in utilizing an RVTV parameter in evaluation of ascites outcome for future studies.

Ascites is the manifestation of multiple symptoms (Olkowski et al., 1999), and thus, is a complex disease, whose occurrence is subject to many genetic factors. In order to aid commercial selection programs in the reduction of ascites, and increase overall heart health, information from studies such as the one presented here will elucidate genetic causes to adverse attributes evaluated in fast-growing broilers.

Table 3.1. Location of SNP identified from GWAS. Annealing temperature, forward and reverse primers, and probes also included for each SNP.

SNP ID	Chr	SNP Position	Reference SNP	Reference/ Alternative Allele (Strand)	Annealing Temp (°C)		Primer		Probe
2.708	2	70835627	rs14203518	T/C (Fwd)	56.4	F	CTCAGCTGGTCCTGCTAACAT	Probe 1	CTAAAGTATGAGTAtCCAAGTCTT[1]
						R	TCTGAGGGAGGGAAAAAGGT	Probe 2	CTAAAGTATGAGTAcCCAAGTC
2.713	2	71320330	rs14203691	A/G (Fwd)	52	F	TAATGGAAACAACCTCTGTGCTCTGGA	Probe 1	TCCTAtCCTGAAGAAAGAGCAAATAAAT
						R	GCCTCCCATGTCTTTGGCTTGGA	Probe 2	TCCTAcCCTGAAGAAAGAGCAAATA
Z.591	Z	59169596	rs10723172	C/T (Fwd)	67	F	GGGGGATAGAGGAGGCTGGTGT	Probe 1	TAcGACACAATAGGCTTTTCCATAAG
						R	TCACCCTGTCATCGTTTTTGAAACATG	Probe 2	TAtGACACAATAGGCTTTTCCATAAGT
Z.600	Z	60058344	rs14748694	T/C (Rev)	68	F	GTCCGGCTCTGTGTCTGCCCTGA	Probe 1	ACaAAGAGTGGAAATATGGATTTCCAGCATC
						R	TCCAACAGAACTCCCTGGTGTTTCACC	Probe 2	ACgAAGAGTGGAAATATGGATTTCCAGCAT
Z.611	Z	61154772	rs16774018	C/T (Rev)	59	F	AGGCATTGCTTCCTTCTGGGAGAAC	Probe 1	TGcTTGGATATTCATAAAGTTCTCCC
						R	CAGCTGTTAGTTTGGTGGGGGCTTT	Probe 2	TGtTTGGATATTCATAAAGTTCTCCCA

[1]Lower case letters indicate loci specific for SNP.

Table 3.2. Data collected from single nucleotide polymorphisms from 145 resistant and 123 susceptible male individuals on chromosomes 2 and Z. Included are SNP identification names, location, individual counts, percent incidence of ascites-susceptible birds, observed genotypic frequencies, and corresponding P-values calculated for Chi-squared tests. Additionally, RVTV averaged ratios for resistant and susceptible individuals are included. Information for males and females presented separately.

SNP ID	SNP Location (Chr:Mbp)	Genotype	Ascites (%)	R* Freq	S* Freq	Pval	R RVTV Avg	S RVTV Avg
2.708	Gga2:70.83	TT	46%	0.55	0.58	0.78	0.29	0.44[a]
		TC	42%	0.36	0.33	0.69	0.31	0.41[b]
		CC	45%	0.09	0.09	0.93	0.30	0.44[a, b]
2.713	Gga2:71.32	AA	44%	0.48	0.48	0.98	0.28[b]	0.45
		AG	45%	0.48	0.49	0.82	0.30[a, b]	0.43
		GG	30%	0.04	0.03	0.37	0.34[a]	0.44
Z.591	GgaZ:59.169	CC	50%	0.26	0.31	0.43	0.28	0.44
		CT	46%	0.45	0.45	0.94	0.30	0.43
		TT	40%	0.29	0.24	0.36	0.29	0.44
Z.600	GgaZ:60.058	TT	50%	0.15	0.17	0.63	0.32	0.43
		TC	46%	0.45	0.46	0.99	0.30	0.43
		CC	45%	0.40	0.37	0.75	0.29	0.44
Z.611	GgaZ:61.154	CC	33%	0.26	0.21	0.46	0.29	0.43
		CT	38%	0.32	0.33	0.90	0.30	0.43
		TT	40%	0.42	0.46	0.66	0.20	0.45

* R indicates birds that were phenotyped as ascites resistant following a high-altitude challenged hypobaric chamber trial; S indicates birds that were phenotyped as ascites susceptible following a high-altitude challenged hypobaric chamber trial.
[a, b] Means within the same column and with no common superscript differ significantly ($P<0.05$).

Table 3.3. Data collected from single nucleotide polymorphisms from 98 resistant and 115 susceptible female individuals on chromosomes 2 and Z. Included are SNP identification names, location, individual counts, percent incidence of ascites-susceptible birds, observed genotypic frequencies, and corresponding P-values calculated for Chi-squared tests. Additionally, RVTV averaged ratios for resistant and susceptible individuals are included. Information for males and females presented separately.

SNP ID	SNP Location (Chr:Mbp)	Genotype	Ascites (%)	R* Freq	S* Freq	Pval	R RVTV Avg	S RVTV Avg
2.708	Gga2:70.83	TT	55%	0.52	0.63	0.36	0.30	0.43 [b]
		TC	41%	0.40	0.27	0.13	0.30	0.46 [a]
		CC	56%	0.08	0.10	0.65	0.28	0.44 [a, b]
2.713	Gga2:71.32	AA	62%	0.38	0.55	0.07	0.29	0.43
		AG	44%	0.59	0.42	0.09	0.29	0.44
		GG	50%	0.03	0.03	0.89	0.32	0.44
Z.591	GgaZ:59.169	CW	50%	0.44	0.55	0.92	0.30	0.43
		TW	51%	0.56	0.45	0.92	0.29	0.43
Z.600	GgaZ:60.058	TW	47%	0.40	0.33	0.36	0.27[b]	0.43
		CW	55%	0.60	0.60	0.49	0.31[a]	0.44
Z.611	GgaZ:61.154	CW	48%	0.63	0.55	0.46	0.29	0.45
		TW	57%	0.37	0.45	0.37	0.31	0.43

* R indicates birds that were phenotyped as ascites resistant following a high-altitude challenged hypobaric chamber trial; S indicates birds that were phenotyped as ascites susceptible following a high-altitude challenged hypobaric chamber trial.

[a, b]Means within the same column and with no common superscript differ significantly ($P<0.05$).

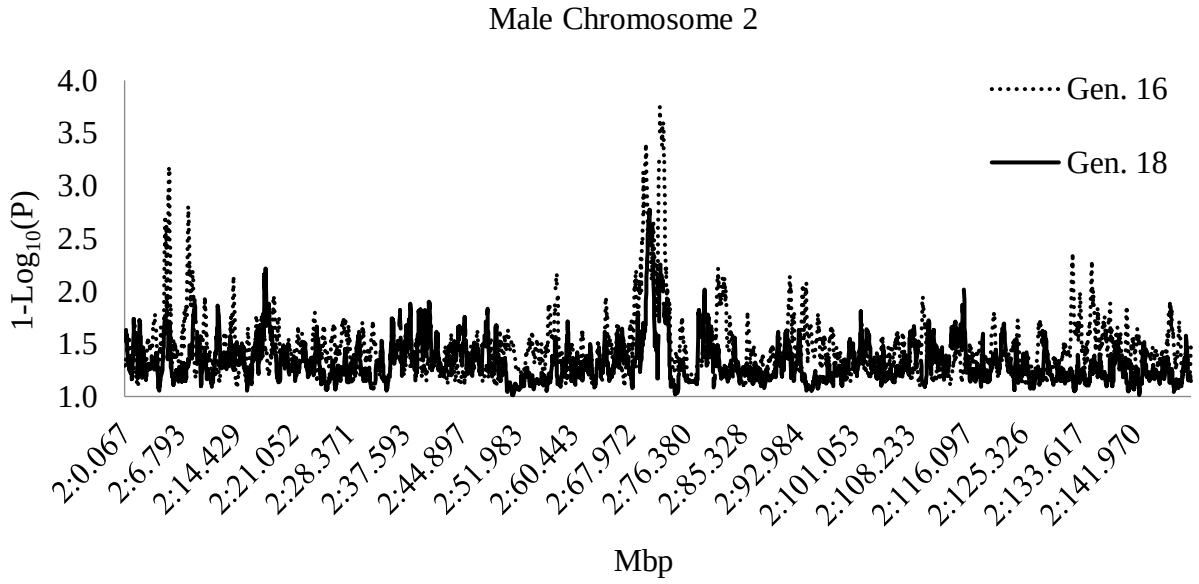

Figure 3.1. Genome wide association study results indicate a region of interest around 70 Mbp on chromosome 2 in resistant males comparing 2 generations of REL line individuals. Single nucleotide polymorphism loci are identified as the corresponding Mbp along the chromosome 2. Association of SNP loci to ascites resistance is visualized as a 1-LOGP value.

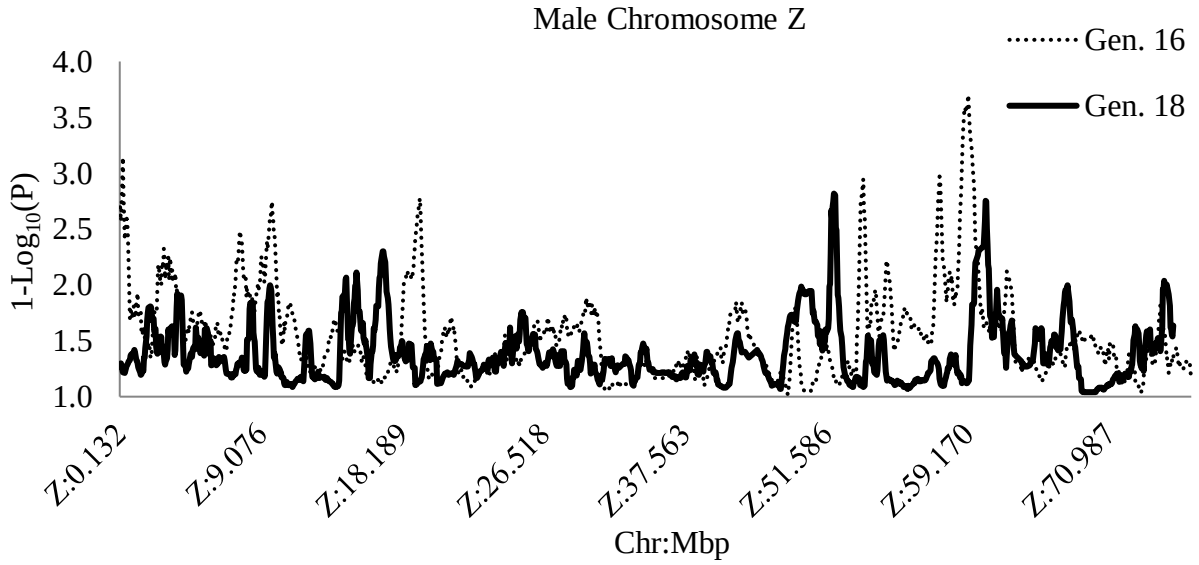

Figure 3.2. Genome wide association results indicating a region of interest around 60 Mbp on chromosome Z in susceptible males comparing 2 generations of REL line individuals. Single nucleotide polymorphism loci are identified as the corresponding Mbp along the Z chromosome. Association of SNP loci to ascites susceptibility is visualized as a 1-LOGP value.

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CHAPTER 4

Whole genome resequencing identifies the *CPQ* gene as a determinant of ascites in broilers

ABSTRACT

Pulmonary hypertension syndrome leading to ascites in broilers is a disease that can be attributed to increased body weight in fast growing broilers resulting in increased demand for oxygen in the cells and tissues to cope with the increased metabolic load. Although there are environmental components, the disease exhibits moderate to high heritability. The current study uses high throughput whole genome resequencing (WGR) to identify genes and chromosomal regions associated with ascites. The WGR data identified the carboxypeptidase Q (CPQ) gene on chromosome 2. The association was confirmed by genotyping a large collection of DNAs from phenotyped birds from three distinct broiler lines using SNPs in intron 6 and exon 8 of the CPQ gene. By combining the genotype data for these two SNP loci we identified three different alleles segregating in the three broiler lines. Particular genotypes could be associated with resistance to ascites. We further determine that particular genotypes most associated with resistance overexpress CPQ mRNA in three tissues which might explain the role of these alleles in contributing to resistance. Our findings indicate CPQ is an important determinant of pulmonary hypertension syndrome leading to ascites in broilers. We identified particular SNPs that can be used for marker-assisted selection of broilers for resistance to the disease. The WGR approach to map determinants contributing to complex traits may have advantages over other GWAS approaches. The CPQ gene has been associated with pulmonary hypertension in genome-wide association studies in humans. Therefore, ascites investigations in broilers are likely to provide insights into some forms of hypertension in humans.

INTRODUCTION

Idiopathic pulmonary arteriole hypertension (IPAH), also known as ascites in poultry, is a metabolic disorder attributed to rapid growth in modern broilers. Broilers (meat-type chickens) are selected for rapid growth and increased muscle mass. Since the 1950s, this has yielded an improved growth rate of about 5% per year (Julian, 2000). Fast-growing broilers, with enhanced metabolic rate and muscle mass, have higher demands for oxygen. However, the size or capacity of the vital organs, such as the heart and lungs, is not increased proportionately for adequate oxygen circulation in these broilers (Julian, 2000; Decuypere et al., 2000). The failure of the pulmonary vasculature system to cope with the increasing oxygen requirements in fast growing broilers leads to constriction of pulmonary arterioles and lack of oxygen in the tissues starting even at the embryonic stages (Decuypere et al., 2000; Closter et al., 2009; Wideman et al., 2013). Tissue hypoxia triggers a cascade of events including an increase in vascular pressure in the lungs and pulmonary arteries, right ventricular hypertrophy (RVH; elevated right ventricular to total ventricular ratios- RV: TV) and valvular insufficiency leading to a drop in cardiac output and hypoxemia (Wideman, 2001; Balog, 2003; Pakdel et al., 2005b; Wideman et al., 2013). This triggers proliferation of red blood cells, which in turn increases the hematocrit value and blood viscosity leading to the manifestation of pulmonary edema, variable changes in the liver, accumulation of serous fluid in the abdominal cavity, and right ventricular failure resulting in premature death of the birds (Pakdel et al., 2005b; Druyan et al., 2007; Wideman et al., 2007; Shenoy et al., 2011).

Ascites is one of the health traits that is of concern in breeding and management for broilers. The goals are to minimize economic losses from ascites-induced mortality, reduce hypoxemic affects on meat quality, and improve overall animal well-being (Closter et al., 2012). Evaluation

of ascites-indicator traits such as cardiac hypertrophy (right ventricular to total ventricular ratios - RV: TV), abdominal fluid, hematocrit value, pulse oximetry has shown to have moderate to high heritabilities (Lubritz et al., 1995; Maxwell and Robertson, 1996). For example, heritability of RV: TV ranges from 0.25-0.54, whereas that of abdominal fluid range from 0.36-0.44 (Lubritz et al., 1995; De Greef et al., 2001; Tavárez et al., 2016). This is indicative of the importance of the genetic components that play a key role in disease occurrence (Lubritz et al., 1995; Shlosberg et al., 1996; Wideman and French, 1999; De Greef et al., 2001; Pakdel et al., 2002; Pavlidis et al., 2007; Closter et al., 2012; Tavárez et al., 2016; Ahmadpanah et al., 2017). Ascites incidence in modern broiler lines can be reduced by effective genetic selection of the birds against susceptibility for the disease. However, because most of the ascites-indicator traits, such as RV-TV ratio, ascitic fluid, can best be measured *post-mortem*, selection of broilers against ascites susceptibility is difficult. Hence, in current breeding programs, information from siblings and relatives are used for genetic selection of the broiler lines against ascites (Pakdel et al., 2005b).

Development of genetic markers for resistance towards ascites is essential for more effective selection schemes in broiler breeding programs. Although several independent studies have indicated ascites to be a polygenic trait (De Greef et al., 2001; Moghadam et al., 2001; Pakdel et al., 2005b; Rabie et al., 2005; Druyan et al., 2007; Hamal et al., 2010 a, b; Hassanzadeh et al., 2014; Liu et al., 2017; Li et al., 2017), little is known about the genes contributing to the disease. We have previously used SNP-based genome-wide association studies to attempt to map determinants for the disease (Krishnamoorthy et al., 2014; Dey et al., 2017; Tarrant et al., 2017). We have now utilized high throughput next-generation sequencing (NGS) for whole-genome resequencing (WGR) to identify regions with multiple SNPs showing association with ascites. In this study, we applied WGR analyses for chromosomes 2 and 9, which identified one of the most

promising markers for ascites phenotype. The data identifies alleles of the carboxypeptidase Q (CPQ) gene specifically to resistance to ascites in a gender-biased manner.

MATERIALS AND METHODS

Bird Stocks and Selection Based on Hypobaric Chamber Trials

All animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee under protocol 12039. Hypobaric chambers used in this study consisted of four identical stainless steel batteries that formed 40 identical cages each measuring 0.6 x 0.6 x 0.3 m. The cages were connected with trough feeders and nipple waterers. Environmental conditions such as temperature, humidity, ventilation rate and altitude were maintained constant within the chambers. Airflow within the chambers was set at $17 m^3$/min, with a daily change of air filters. Elevation within the hypobaric chambers was maintained at 2,744 m (9000 ft; 543 mm of Hg) above sea level for the duration of this study. Birds were initially brooded in a warm room (92°C), with a subsequent weekly decrease in temperature. The birds used in this study represent one research line (REL) and two commercial elite lines (Lines Y and Z). Birds from the 18^{th} generation of the REL line were used for the WGR data. The females in the 17^{th} generation were artificially inseminated using pooled semen from same generation males, and the eggs were pedigreed according to the hen. Therefore, the chicks hatched at the University of Arkansas hatchery belonged to the 18^{th} generation REL line, and were immediately wing-banded and transferred to cages in the hypobaric chambers. The birds were monitored for the next six weeks for mortality and necropsies with records of the cause of death, visual symptoms of ascites, heart flaccidity and morphology including right ventricle and total ventricle weights (in

grams), gender, fluid accumulation in the abdomen if any. After six weeks, the remaining birds were euthanized by cervical dislocation and scored as above. Additional observations were made, especially for the presence abdominal fluid accumulation, which would mark the final progression of the disease phenotype. Finally, evaluating all the observations, the birds were phenotyped as susceptible or resistant towards ascites. Line Y is an elite male line selected over several generations for growth, yield and efficient feed-conversion rates, while Line Z is a female elite line selected for growth traits as well as reproductive performance. These birds were also challenged in our hypobaric chamber as above.

DNA Isolation from Blood Samples

At four days of age, 10 µl of blood was extracted birds via wing vein lancet puncture. DNA was isolated from blood samples using our rapid protocol (Bailes et al., 2007). Selected DNAs were then further purified by extraction with phenol: chloroform: isoamyl alcohol (50:48:2), and then chloroform: isoamyl alcohol (24:1). Ethanol precipitated DNAs were dissolved in 10 mM TrisCl 0.1 mM EDTA pH 7.5. DNAs were quantified using a Nanovue (GE Healthcare, Piscataway, NJ).

DNA Preparation for NGS

DNAs submitted for library construction consisted of pools of equal weights of 10 DNAs. Eight pools were generated representing both genders and both phenotypes, with two different pools for each gender-phenotype. Pools (100µl) of 40 ng/µl DNA were submitted for bar-coded library construction followed by 2*125 paired-end reads on Illumina HiSeq 2500 to generate at least 65 Gigabases (Gb) per pool. Library construction and sequencing were performed by the Research Technology Support Facility at Michigan State University.

NGS Data Analysis and Bioinformatics

The NGS raw sequences were obtained as FASTQ files containing about 25 million raw 100bp sequences. The templated assembly for WGR was performed per chromosome was conducted for each gender separately using Arraystar14 software (DNASTAR Lasergene Suite 14) for SNP identification and quantification. SNP data were exported to Excel for further filtering and analysis. During variant calling, several filters to screen SNPs with low depth of coverage (<9), low genotype quality score, missing data from a replicate, false indel detection were applied to each pool in order to yield good quality SNPs. Two datasets were generated for males and females, each consisting of the variant calls for the two replicates of the resistant and susceptible samples. A 25% threshold was applied to exclude false discovery rate of variants among the resistant and susceptible data. The output variant calling contained the data for all variants, the individual genotypes (homozygous, heterozygous, or hemizygous for a variant) and related information. Average of the two replicates for SNP variation was plotted by chromosome position to identify SNP clusters for causative candidate genes for the disease.

Genome Annotations

The WGR assembly was templated on the reference sequence of *Gallus_gallus-5* assembly downloaded from NCBI (*https://www.ncbi.nlm.nih.gov/genome/?term=gallus%20gallus*). All genome positions with RefID correspond to the RefID from *Gallus_gallus-5* assembly. All genome positions also correspond to the *Gallus_gallus-5* assembly (Accession ID: GCF_000002315.4) downloaded from NCBI.

Designing PCR, Quantitative RT-PCR (qRT-PCR) Primers and TaqMan Probes

Forward and reverse primer sets were designed for the genomic DNA amplification of the targeted regions on intron-6 and exon-8 regions. Other sets of primers were designed for amplification of only the cDNA for the exon 8 region and the TBP (Tata-Binding Protein) reference gene. Chicken CPQ sequence downloaded from UCSC genome browser in FASTA format were manipulated using SeqBuilder software (DNASTAR Lasergene Suite 14) to design the primers and probes. Similarly, chicken TBP gene sequence was used for designing the primers for the housekeeping cDNA amplification. Primers were designed using Primer3 (version 0.4.0) tool (*http://bioinfo.ut.ee/primer3-0.4.0/primer3/*) using custom primer size (19-30bp), GC content (40-60%) and Tm range (60-65°C). The selected primers were further edited and optimized before a BLAT search (*https://genome.ucsc.edu/cgi-bin/hgBlat?hgsid=579965045_NRGjtJZFAlbgonB1tCxWIJrk0Wjs&command=start*) to check for any non-specific binding. All primer and probe sets used in this study had unique binding sites with a 100% match in the targeted region. Probes for the 5'exonuclease (TaqMan) assays were designed in a similar manner using the CPQ template and marking the polymorphic sites using the editing tools in Seqbuilder followed by optimization of probe conditions and a BLAT search. Primers for reverse transcriptase PCR primers for gene expression assays targeted two exons separated by a large intron (>20,000bp) so as to amplify only cDNAs and eliminate any genomic DNA. The primers and probes were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Probes incorporated Zen modifications quenched with Iowa Black. Primer and probe sequences designed and used in this study are listed in Table 4.1.

5'-Exonuclease Allelic Discrimination Assay (TaqMan Genotyping) and DNA Sequencing

Targeted SNPs were genotyped by TaqMan 5'-exonuclease assays using qRT-PCR in 96 well plates using a CFX96 real-time thermocycler (Bio-Rad Laboratories, Inc., Hercules, California). Primers and probes for these assays are shown in Table 4.1. Exonuclease assays were optimized for soak temperature for genotype discrimination. Each 20µL reaction consisted of 1X Taq Buffer (50mM Tris-Cl, pH 8.3, 30µg/mL of BSA), 0.25mM $MgCl_2$, 0.2mM dNTP, 0.5µM each of the specific forward and reverse primers, 0.25 µM each of the two probes, 4 units of Taq polymerase, and 2µL of genomic DNA (50-100ng). Cycle parameters were 90°C for 30s, 10 cycles of 90°C for 15s, and probe-specific soak for 30s (Table 4.1), followed by 30 cycles of 90°C for 15s, probe-specific soak for 30s (Table 4.1) with plate read. Genotype calls were highly repeatable, and were confirmed by product sequencing.

Genotype calls were verified for select samples by purifying the product using RapidTips® functional pipette (Chiral Technologies; West Chester, PA) and then quantified by Hoechst 33258 fluorescence (Promega Glomax MultiJr Single-Tube Multimode Reader, Thermo Fisher Scientific). Cleaned samples were submitted for capillary sequencing (Eurofins Genomics, Louisville, KY). Sequence ab1 files were aligned using SeqMan Pro 14 (DNASTAR Lasergene Suite 14) for editing and scoring sequence data.

RNA Extraction and Gene Expression Assay

Total RNA from tissue homogenates were extracted using Trizol Reagent (Ambion, Thermo Fisher Scientific) according to the manufacturer's instructions. RNAs were quantified using a Nanovue (GE Healthcare, Life Sciences, Pittsburgh, Pennsylvania, USA) and RNA integrity was assessed by agarose gel electrophoresis. Expression levels were determined using single-step

qRT-PCR employing Pyrophage® Exo-minus (Lucigen, Middleton, Wisconsin, USA). Total RNA (500 ng) was denatured at 65°C for 10 mins, and then added to a mastermix consisting of 1x Pyrophage® 3173 PCR buffer, 1x Evagreen dye (Biotium, USA), 0.5 µM dNTPs, 2 µM each of specific forward and reverse primers (Table 4.1), 0.5 µl of Pyrophage® 3137 DNA polymerase Exo- (Lucigen, Middleton, Wisconsin, USA). The final reaction volume was 20 µl. Negative controls consisted of reactions without RNA. The PCR cycling was initial denaturation at 90ºC for 3 mins, 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 reactions were run in triplicate. TBP was used as the reference control, and all Ct values were normalized to TBP. Fold change was calculated using the ΔΔCt method.

Statistical Analyses

Genotype and haplotype frequencies were calculated for ascites-resistant and ascites-susceptible phenotypic groups in each line. Observed genotype counts for each phenotype were compared to expected counts computed from the frequency of that genotype in the entire populations using the chitest function in Microsoft Excel to compute P-values. Genotype data for birds with inconclusive necropsy for ascites were included in the frequency for the entire population but excluded from the phenotypic groups. Two-tailed student t-test with unequal variances was performed to detect differences in gene expression for the alleles. Data has been shown as Mean ± SD. The threshold level of significance throughout the study was $P<0.05$.

RESULTS

Identification of CPQ as a Candidate Gene from WGR Data

Eight pools of equal DNA quantities were constructed for two replicates for each ascites phenotype (resistant vs. susceptible) and each gender. Illumina libraries were constructed with bar-coding, and paired-end sequenced using Illumina Hiseq 2500 to generate FASTQ 2x125 datasets of ≥67 Gb per DNA pool. The reads were mapped to the *Gallus_gallus-5* reference genome using ArrayStar 14 software. SNP frequencies per replicate, SNP Q score, and read depths for each gender, phenotype, and were output as text files, parsed into chromosome or chromosome segment using a text editor (TheGun; *http://www.movsd.com/thegun.htm*) then imported into MS Excel. The data were filtered to remove SNPs with no reads for one or more pools, or low SNP Q scores. The difference in SNP frequencies of the two replicates for each phenotype (resistant minus susceptible) was plotted for each gender by chromosome position to generate scatterplots to identify clusters of SNPs skewed from nearly equal frequencies in both phenotypes. Owing to our earlier interest in chromosomes 2 and 9 (Krishnamoorthy et al., 2014; Dey et al., 2017; Tarrant et al., 2017), we first analyzed these chromosomes. The WGR data identified 287,663 SNPs in males and 281,576 SNPs in females for chromosome 9, but we did not find any regions with noticeable skew in the SNP frequencies, even for the two regions we had identified earlier (Krishnamoorthy et al., 2014; Dey et al., 2017). For Gga2 there were 1,701,018 SNPs in males and 1,700,493 SNPs in females. We identified a single region in males from 127.62 to 127.75 Mbps where a cluster of SNPs with high frequency differences of up to about 78% among phenotypes (Figure 4.1A). We observed the same cluster for females, but the difference in SNP frequencies was less suggesting a male-bias for the association (Figures 4.1B and 4.1C). In males, the peak spans a 123.1 kbp region, where out of 910 SNPs (post-filtration)

there were 132 SNPs with frequency differences ≥ 20% and 56 of these had frequency differences ≥ 40%. The predominantly positive difference in SNP frequencies indicates that the non-reference SNP pattern is associated with resistance (Figure 4.1A-B). In females, a peak from 127.62 to 127.75 Mbps encompassed a 121.2 Kbp region, comprising of 368 filtered SNPs, out of which 94 SNPs had a frequency difference of ≥20%. We identified five other regions on chromosome 2 in males with less obvious SNP frequency skew (Table 4.2). However, none of the other five regions showed as great a difference in SNP frequencies and most showed less significant deviation with respect to phenotype. For each region, we investigated the gene underlying the SNP peak for function and association with ascites related traits in humans using the NCBI Phenotype-Genotype Integrator (*https://www.ncbi.nlm.nih.gov/gap/phegeni*). The peak from 127.62-127.75 Mbp appeared to be associated with the most relevant gene. The peak in males and females encompasses the 3' end of the gene for carboxypeptidase Q (CPQ; also called plasma glutamate carboxypeptidase- PGCP). Most importantly the CPQ gene has been associated with hypertension, blood pressure, and heart rate, in human GWAS studies. The peak we observed in males begins in intron 4 and extends 43 kbp downstream of the final exon, exon 8.

We therefore developed exonuclease assays for further genotyping for this region. Majority of the SNPs within the CPQ gene are intronic. We identified five SNPs affecting the coding sequence including three missense SNPs, and four SNPs in the 3'-UTR (Table 4.3). For the exonuclease assay we chose to target a region with two SNPs in intron 6 which showed one of the highest SNP frequency differences of all the filtered SNPs in males and females (Table 4.4), and a missense SNP in exon 8 affecting a conserved lysine residue (Table 4.3). The intron 6 SNPs were a T/C SNP at 127,670,527 bp (ID: rs80617053)and a A/C SNP at 127,670,534 bp

(ID: rs80617053) (Table 4.4). From resequencing data we verified that three other nearby SNPs in intron 6 were in linkage with the targeted SNPs (Table 4.4). The exon 8 SNP is a G/C SNP at 127708214 bp (ID: rs738850243; Table 4.3).

CPQ Genotype Analysis in Three Broiler Lines

The exonuclease assays were applied to a collection of DNAs from three different broiler lines phenotyped for ascites in hypobaric challenges; the REL line, and two commercial broiler lines, Line Y and Line Z. For the intron-6 SNPs, we genotyped 964 DNAs for REL line birds, 188 DNAs for commercial elite line Y, and 185 DNAs for commercial elite line Z. In the REL line, 66% of all birds and 72% of males homozygous for the non-reference SNP (C/C) were resistant (P= 0.003 and 0.043 respectively; Table 4.5). Although 62% of the females homozygous for the non-reference SNP were resistant in this line, the difference did not reach the threshold significance of $P<0.05$. In line Y, 74% of all birds and 81% of males homozygous for the non-reference SNP (C/C) were resistant (P= 0.0005 and 0.0116, Table 4.5). Homozygous non-reference females were 65% resistant but this did not reach the significance threshold, likely owing to a low count (n=29). In Line Z, 82% of all birds and 86% of all males homozygous for the non-reference SNP were resistant (P= 0.0020 and 0.0553; Table 4.5). Again, the homozygous females for the non-reference SNP did not reach the significance threshold. Therefore, our data indicated that the two SNPs provide a marker for selection for male-specific resistance to ascites in broiler lines. The homozygous reference genotype for the two SNPs in REL and line Z for both the entire population, and for males in both the lines showed nearly equal (50:50) frequencies of phenotypes (REL: P= 0.0006, 0.0063 respectively; Line Z: P= 0.0054, 0.0007 respectively; Table 4.5). This further implicates the homozygous non-reference genotype for enhanced resistance in the population. However, in Line Y, 64% of the birds in the population

and 63% males with the homozygous reference allele were susceptible birds (P= 0.0174, and 0.0302 respectively), indicating that the homozygous reference genotype favors susceptibility in this line. No significant deviation was observed for the heterozygous genotype in any of the three lines.

Similar to that observed for the intron-6 SNPs, analysis of the distribution of the exon 8 (rs738850243) SNP genotypes indicated the homozygous non-reference genotype (C/C) to be significantly associated with the resistant phenotype in all the three lines of REL, Y and Z. Considering all birds with the non-reference genotype (C/C), 69% individuals in REL, 89% in line Y and 90% in line Z were resistant (P= 0.009, 0.00003, 0.0013 respectively; Table 4.6). Birds with the homozygous reference genotype (G/G) had about an equal distribution among resistant and susceptibles at the significance threshold value. In line Y, 60% of the birds with the reference genotype were susceptible while 40% were resistant at P=0.057 (Table 4.6). Therefore, the observations for the exon-8 reference genotype distribution was also similar to that of intron-6, where the genotype favored susceptibility only in Line Y. However, for the rs738850243 SNP, we were unable to positively conclude if the SNP had a gender-specific effect because the ascites-phenotype distribution did not reach the threshold P-value for any of the genotypes for either gender in REL. However, in lines Y and Z, 90% and 93% of the birds in males and 84% and 87% of the birds in females respectively, with homozygous non-reference genotype were resistant at significant threshold (Table 4.6). We did not observe a significant difference between resistant and susceptible birds with the heterozygous genotype in any of the three tested lines.

Assessment of CPQ Haplotype and Genotype Patterns in Association with Pulmonary Hypertension

Given that the homozygous non-reference SNPs in intron 6 and exon 8 were individually associated with ascites resistance, we examined the association of the haplotype/genotype patterns formed with these SNPs in the three broiler lines.

Based on the genotype data we constructed haplotypes for the two intron-6 SNPs (rs80617053 and rs80618855) and the exon-8 SNP (rs738850243). We identified three haplotypes: T-A-G (reference haplotype), C-C-C (non-reference haplotype), and C-C-G (recombinant haplotype), with potentially six different genotypes: genotype G1 (homozygous reference): T-A-G/ T-A-G; genotype G2 (homozygous non-reference): C-C-C/ C-C-C; genotype G3 (homozygous recombinant): C-C-G/ C-C-G; genotype G4 (heterozygous recombinant): C-C-G/ C-C-C; genotype G5 (heterozygous): T-A-G/ C-C-C; and genotype G6 (heterozygous recombinant): T-A-G/ C-C-G.

As suggested by the association of single SNPs, individuals homozygous for the non-reference G2 consisted of significantly higher frequency of resistant birds as compared to susceptibles in all the three REL, Y and Z lines. This observation was also true considering each gender in all the lines. In REL, 71% of all samples, 76% of males, and 69% of females with G2 were resistant ($P< 0.05$). In Line Y, 89% of all, 90% of males, and 87% of females with G2 were resistant ($P< 0.05$; Table 4.7). In Line Z, 90% of all, 93% of males, and 84% of males with G2 genotype were resistant ($P< 0.05$; Table 4.7). We further observed from our haplotype analysis that the reference haplotype (i.e genotype G1) strongly favors susceptibility in the commercial lines Y and Z, considering all birds and for both genders (Table 4.7). However, in REL, the distribution

of resistant and susceptible birds were nearly equal for genotype G1 considering the entire population and males, and slightly higher with 59% susceptibles in females (P< 0.055, Table 4.7). This further confirms the fact that the homozygous non-reference genotype (G2) favors resistance for ascites in broilers. For the other four genotypes, only G3 and G4 were present at more than 10% but neither showed any association with ascites (Table 4.7).

Tissue-specific Expression Assay

To further dissect the role of the gene polymorphisms in association with ascites, we assessed the relationship between the various genotypes/ haplotypes and the gene expression patterns in the disease-associated tissues of heart, lung, and liver. Our results indicate that the individuals with homozygous non-reference genotype/ haplotype (G2) exhibited the highest level of expression in all the three tissues with a six-fold increase in the lung (P= 0.001), 4.7 folds increase in each of heart (P= 0.001), and liver (P= 0.007) as compared to those with the reference genotype/ haplotype G1 (Figure 4.2). Individuals with G4 (non-reference intron-6 variants + heterozygous exon-8) had the next highest expression level, consistently in all the three tissues, with fold changes of 4.5 (P= 0.0005), 3.4 (P= 0.0007) and 4.04 (P= 0.018) in the lung, heart, and liver respectively. The fold changes in birds with the genotype/ haplotype G3 (non-reference intron-6 variants + reference exon-8) was much lower followed by that in the heterozygous genotype G5, and then those with the genotype G6 (heterozygous intron-6 variants + reference exon-8) where the expression was the least of all the genotypes/ haplotypes in all the tissues (Figure 4.2). However, the significance of the expression level did not quite reach the threshold for the latter three genotypes, except for the heterozygous genotype G5 in the liver where the expression was almost three folds higher as compared to G1 (P= 0.025; Figure 4.2).

DISCUSSION

The CPQ gene encodes a metallopeptidase belonging to the M28 peptidase family. The CPQ protein is thought to play a role in the hydrolysis of circulating peptides in human blood plasma (Gingras et al., 1995), including processing thyroglobulin (Tg) precursor to release thyroxine hormone (Uniprot; Ahmetov et al., 2016). In human GWAS the CPQ gene has shown association with hypertension, blood pressure, heart rate, and electrocardiography. The protein is conserved across many vertebrates and the predicted chicken mature protein is more than 70% identical with those from mouse, rat, monkeys, and human.

Our genotyping data on different broiler lines demonstrate consistent, strong association of the homozygous non-reference genotypes with resistance for both the intron-6 SNP locus (rs80617053 and rs80617055) and exon-8 SNP (rs738850243). The associations are most pronounced in males. Generally, increased ascites resistance is not seen in heterozygotes arguing that the reference allele is dominant over the non-reference allele. The exon 8 SNP would result in a lysine to asparagine substitution in residue 444 of the encoded mature protein. Based on structural data from related proteins in NCBI, the K444N substitution would be on a surface exposed region of the enzyme and not near the active site. Therefore, if this substitution is mediating the QTL effect, it would have to be through affecting some protein-protein interaction, perhaps affecting CPQ activity on a circulating peptide hormone involved in blood pressure homeostasis. Our gene expression data argue that the non-reference genotypes express higher levels of CPQ mRNA than the reference genotypes in the three tissues tested; heart, lung and liver (Figure 4.2). Therefore, our data suggest that the non-reference SNPs affect one or more regulatory sequences located within or downstream of the 3' region of the CPQ gene which affect relative mRNA expression levels.

The identification of the CPQ region is based on a collection of SNPs spanning approximately 134 kbps of Gga2, suggestive of a haplotype block; but our haplotype analysis shows that there is recombination between the intron 6 and exon 8 SNPs. In the REL line, out of 866 genotypes there are 354 genotypes (G3, G4, and G6) indicative of a recombinant allele pairing the non-reference intron 6 SNPs with the exon 8 reference SNP. From the combined genotype data (Table 4.7) we estimate this allele represents 30.7% of all alleles with the all reference allele representing 27.3% and the all non-reference allele representing 42%, in the REL samples. Interestingly we never detect the other recombinant allele of reference SNPs for intron 6 and non-reference SNP for exon 8. Thus, recombination in the intervening 37,679 bases between the intron 6 and exon 8 SNPs must be extremely rare. Further inspection of the phenotypes associated with G1, G4 and G2 for at least the REL and Line Z indicate that the missense exon 8 SNP cannot be the sole basis for the QTL effect since genotype G4 is heterozygous for the exon 8 SNP (Table 4.7). In both REL and Line Z the heterozygotes (G4) for the exon 8 SNP are nearly equivalent for the frequency of resistant phenotype birds. In Line Y, there were insufficient numbers of G4 birds for evaluation. Therefore, selection for resistance to ascites should not be based on the exon 8 SNP but is more effective using a combination of SNPs loci from this region.

Our genotype specific expression demonstrates that genotype G2 (homozygous non-reference) has the highest expression in three different tissues (Figure 4.2). Genotype G4 is expressed at nearly the same level. Genotype G4 is heterozygous for the non-reference allele and the recombinant allele and so it is homozygous for the non-reference SNPs in intron 6 and heterozygous for the missense SNP in exon 8. As genotype G4 is also associated with increased resistance to ascites this suggests that the level of expression of the mRNA, and perhaps the

protein, is more critical for contributing to resistance to ascites. This would also be consistent with the intron 6 SNPs being better markers for selection for resistance. Further testing of additional SNPs from this region may further elucidate additional genetic diversity (alleles) in this region. The use of only a single SNP locus may not be as informative as two or more SNP loci. Combining the intron 6 and exon 8 SNPs allowed us to establish that this region also shows an association with ascites phenotype even in females, whereas single SNP loci were generally only informative in males.

Prior to this study, we had used multiple 60k SNP panels for GWAS in the REL line, to identify a region on Gga2 around 70.8 Mbp as a candidate for association with ascites (Tarrant et al., 2017). However, that study failed to identify the association of the CPQ gene and our new WGR SNP data do not support the region around 70.8. There were only three SNPs (rs14250202, rs15154673, and rs14692385) in the 60k SNP panel that are located within the 134 kbp region we identified here. Two of these SNPs showed little to no difference in frequency between phenotypes and the other is monomorphic in the WGR data for the REL samples. Thus, the WGR approach is an unbiased, robust, efficient method to identify genomic regions contributing to complex traits, such as pulmonary hypertension or other diseases. In this context, it is worth noting that neither of the regions we had identified on Gga9 (Krishnamoorthy et al., 2014; Dey et al., 2017) or a region on GgaZ (Tarrant et al., 2017) were confirmed in our WGR analyses. Preliminary further analysis of additional chromosomes from our WGR data has identified an additional 30 regions which appear similar to the region we found for CPQ, where there is a region of skewed SNP frequencies between phenotypes (unpublished). This further validates WGR (NGS) in identifying QTL regions and reducing false discoveries from GWAS.

Pulmonary hypertension or ascites is a metabolic disorder, and is genetically correlated with body weight (Closter et al., 2012). Males, have higher body weight, and increased oxygen demand, so they are predisposed to ascites. This is consistent with greater SNP frequency skew in our WGR data for males.

Although CPQ has been associated with hypertension and blood pressure in humans, the precise role of the CPQ protein in mediating these bodily functions are still unclear. Our study indicates that the CPQ gene is also associated with pulmonary hypertension in broilers. Similarly, broilers have been show to form plexiform arteriopathies analogous to those in some forms of human pulmonary hypertension (Wideman et al., 2013). Our current findings of an association of CPQ with hypertension in chickens and the possible contribution of relative expression levels for some alleles may inform biomedical investigations on the role of CPQ in human hypertension. Fast growing broilers with an increased metabolic rate induce an increased secretion of thyroxine, which is deiodinated to triiodothyronine in the liver and kidney (Luger et al.,2001). Increased triiodothyronine is positively correlated with increased oxygen consumption. At low-temperature conditions, when increased oxygen is required, the circulating concentration of plasma triiodothyronine increases. The study demonstrated that the plasma thyroxine and triiodothyronine concentrations were significantly higher in healthy birds as compared to ascitic birds. Furthermore, when healthy birds were exposed to cold temperatures, the plasma thyroxin levels were reduced, but eventually recovered except in the ascitic birds (Luger et al.,2001). This indicates that the susceptible birds are unable to produce plasma thyroxine at a sufficient rate during increased oxygen demand. There is evidence that CPQ protein plays a role in the release of thyroxine. Over expression of the gene (genotypes G2 and G4) could lead to increased thyroxine and triiodothyronine production during oxygen demand and thus contribute to ascites

resistance, allowing the birds to cope with the higher metabolic rates and increased oxygen requirements.

The CPQ gene is the most prominent candidate gene for ascites identified to date, and could be employed in marker assisted selection for increased ascites resistance. The correlation of altered mRNA expression levels for some alleles with resistance to ascites could also relate to the association of CPQ with human hypertension and development of new therapies. Understanding the gene network and downstream protein-protein interactions of the gene is necessary to fully comprehend the importance of the gene in disease-associated phenotypes. Additionally, the WGR approach should be easily adapted to other complex traits and diseases with genetic components in different agricultural species.

Table 4.1. Primers, probes and conditions for qRT-PCR. For each CPQ locus: position is the base-pair position on chromosome 2 according to the *Gallus_gallus-5* assembly; Primers are 5'-3' for forward (F-) and reverse (R-); Probes are 5'-3' with allele 1 (P1) labeled with FAM and allele 2 (P2) labeled with HEX; and the soak temperature (°C) used in the qPCR assay.

Assay	Locus	Position (bp)	Primers/Probes[1]	Annealing/ Soak
CPQ intron 6 SNP genotyping	CPQ_127.7F	Gga2: 127670335-357	CTGCCTAATTGCACTGCCTTTGC	60
	CPQ_127.7R	Gga2: 127670568-591	CATGACTGATTCTGTGGCCTTCCT	
	CPQp1	Gga2: 127670526-552	C**T**ACAATA**A**AAAGAAGAGTTGATTCCC	
	CPQp2	Gga2: 127670526-550	C**C**ACAATA**C**AAAGAAGAGTTGATTC	
CPQ exon 8 SNP genotyping	CPQex8F	Gga2: 127708143-169	CGTGATGACCTCAGTAAATACTTCTGG	56
	CPQex8R	Gga2: 127708565- 591	CCTGCTGCAAAGGATAAGTTTGCATAC	
	CPQex8P1	Gga2: 127708196- 216	TG**C**TTTGGATCCTGAACTGTC	
	CPQex8P2	Gga2: 127708196- 216	TG**G**TTTGGATCCTGAACTGTC	
CPQ qRT-PCR	CPQ_cDNAex7F	Gga2: 127677758- 780	CAATTTCTGGATGAGGGACGGAG	55
	CPQ_cDNAex8R	Gga2: 127708263-283	CAGCATCTCCTCCATGTCAGC	
TBP qRT-PCR	TBPF	Gga3: 40848487:506	GAACCACGTACTACTGCGCT	55
	TBPR	Gga3: 40846933-952	CTGCTGAACTGCTGGTGTGT	

[1] Nucleotides in bold in the probes indicate the SNPs being assayed.

Table 4.2: Regions on chromosome 2 which showed skew for SNP frequency differences from WGR in males.

Gga 2 Locus (bp)	Maximum SNP frequency difference	P-value	Gene	Function
34069064-34089402	-0.50	0.000327	HACL1	Peroxizomal α-oxidation of 3-methyl branched and 2-hydroxy fatty acids [Foulon et al., 2005]; Refsum Disease (UniprotKB; Singh et al. 1993).
34105406-34212779	-0.59	1.265E-34	ANKRD28	Regulation of focal adhesion and cellular migration (Kiyokawa et al., 2009).
34217438-34325741	-0.37	1.695E-16	LOC101751380	Unknown.
38501592-38575922	+0.51	0.0006	NEK10	Serine/threonine protein kinase responsible for regulation of G2/M cell cycle phase transition (Moniz et al., 2011).
38581179-38714035	+0.55	0.00021	SLC4A7	Sodium bicarbonate co-transporter (UniprotKB).
127544099-127708625	+0.66	1.43E-29	CPQ	Hydrolysis of circulating peptides in blood plasma (Gingras et al.,1999); release of thyroxine from thyroglobulin precursor (UniprotKB, Ahmetoy et al., 2016); hypertension, heart rate, blood pressure, electrocardiography [NCBI Phenotype Genotype Integrator].

Table 4.3: CPQ coding and 3'-UTR SNPs.

Location	Reference ID	Position (bp)	Reference Allele	Variant Allele	Synonymous/ Non-synonymous	Amino acid substitution
Exon 2	rs314744227	127564588	A	G	Non-synonymous	Asn to Ser
Exon 2	rs312495364	127564640	G	A	Synonymous	
Exon 3	rs732164847	127576175	G	A	Non-synonymous	Val to Ile
Exon 4	rs732906703	127604368	G	A	Synonymous	
Exon 8*	rs738850243	127708214	G	C	Non-synonymous	Lys to Asn
3'-UTR	rs741020811	127708364	A	C		
3'-UTR	rs738375273	127708464	T	A		
3'-UTR	rs735740908	127708512	T	A		
3'-UTR	rs737738647	127708525	A	G		

* Indicates the target SNPs for the exonuclease assay.

Table 4.4: Target and linked SNPs for TaqMan assay on intron-6. "Difference in variant allele frequency" columns are calculated by subtracting the average frequency of the non-reference SNP in susceptible from that in resistant from the GBS data.

SNP ID	Position (bp)	Reference Allele	Non-reference Allele	Difference in variant allele frequency- Males	Difference in variant allele frequency- Females
rs80607298	127670474	G	C	+0.50	+0.22
rs80629588	127670479	A	G	+0.43	+0.22
rs80565266	127670525	A	G	+0.55	+0.21
rs80617053*	127670527	T	C	+0.59	+0.23
rs80618855*	127670534	A	C	+0.51	+0.24

* Indicates the target SNPs for the exonuclease assay.

Table 4.5. Genotype data for CPQ intron-6 SNPs (NCBI SNP ID: rs80617053 and rs80618855)in broiler lines. The REL, Y and Z lines were genotyped using 5'-exonuclease assay where TA is homozygous reference allele, YM (TA/CC) is heterozygous, and CC is homozygous non-reference allele. Genotype frequencies (Freq) were determined for the entire line (All) or for the ascites resistant (R) or susceptible (S) subpopulations based on phenotype in a hypobaric challenge. The samples were also analyzed by gender. P-values for a simple Bonferroni correction (Adj Pval) of chi square test of observed vs. expected are presented for genotypes with frequency ≥0.10.

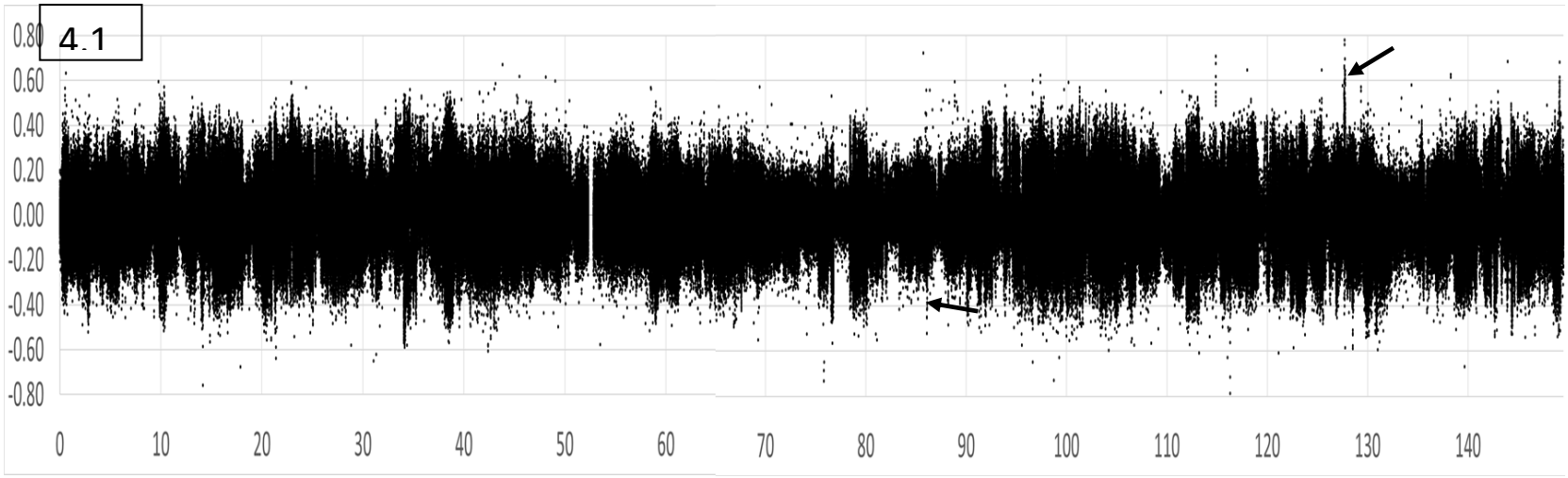
		All				Male				Female			
Line	Geno-type	Count	R Freq	S Freq	Adj Pval	Count	R Freq	S Freq	Adj Pval	Count	R Freq	S Freq	Adj Pval
REL	TA	198	0.46	0.54	0.0006	89	0.49	0.51	0.0063	84	0.46	0.54	0.1868
	YM	151	0.50	0.50	0.0641	71	0.56	0.44	0.3296	70	0.45	0.55	0.2625
	CC	615	0.66	0.34	0.0035	291	0.72	0.28	0.0439	255	0.62	0.38	0.1538
Line Y	TA	86	0.36	0.64	0.0174	38	0.37	0.63	0.0302	48	0.35	0.65	0.5745
	YM	36	0.44	0.56	1.2534	17	0.53	0.47	2.2127	19	0.37	0.63	1.4578
	CC	66	0.74	0.26	0.0005	37	0.81	0.19	0.0116	29	0.65	0.35	0.0744
Line Z	TA	54	0.45	0.55	0.0054	21	0.38	0.62	0.0007	31	0.48	0.52	1.5799
	YM	27	0.44	0.56	0.0577	14	0.64	0.36	1.2900	12	0.18	0.82	0.0509
	CC	104	0.82	0.18	0.0017	71	0.86	0.14	0.0553	32	0.72	0.28	0.1293

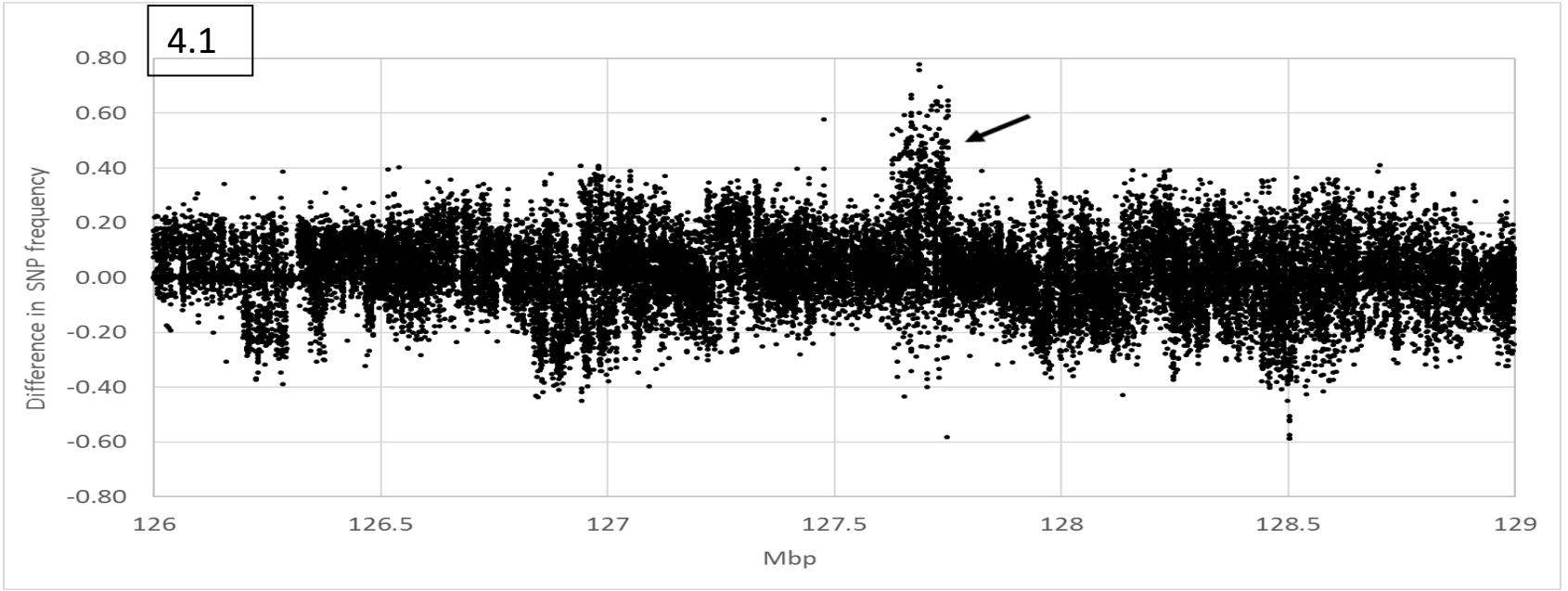
Table 4.6. Genotype data for CPQ exon-8 SNP (NCBI SNP ID: rs738850243) in broiler lines. The REL, Y and Z lines were genotyped using 5'-exonuclease allelic discrimination assay where G is homozygous reference allele, S (G/C) is heterozygous, and C is homozygous non-reference allele.

		All				Male				Female			
Line	Geno-type	Count	R Freq	S Freq	Adj Pval	Count	R Freq	S Freq	Adj Pval	Count	R Freq	S Freq	Adj Pval
REL	G	574	0.55	0.45	0.0064	269	0.59	0.41	0.1116	230	0.52	0.48	0.0931
	S	244	0.65	0.35	0.8667	110	0.70	0.30	0.9183	109	0.60	0.40	2.1702
	C	373	0.69	0.31	0.0092	167	0.72	0.28	0.2078	167	0.68	0.32	0.0595
Line Y	G	103	0.40	0.60	0.0566	45	0.47	0.53	0.2925	58	0.35	0.65	0.3350
	S	8	0.37	0.63	1.2975	3	0.33	0.67	1.1090	5	0.40	0.60	2.4799
	C	37	0.89	0.11	0.00003	20	0.90	0.10	0.0138	15	0.87	0.13	0.0034
Line Z	G	91	0.50	0.50	0.006	46	0.52	0.48	0.0052	41	0.44	0.56	0.5460
	S	33	0.73	0.27	1.2233	23	0.87	0.13	0.3764	10	0.40	0.60	1.0935
	C	49	0.90	0.10	0.0013	30	0.93	0.07	0.0338	19	0.84	0.16	0.0265

Table 4.7: Genotype data combining CPQ intron-6 (NCBI SNP ID: rs80617053 and rs80618855) and exon-8 (NCBI SNP ID: rs738850243) variants for the REL and commercial lines Y and Z.

Combined Genotypes	All Freq	All R Freq	All S Freq	Adj Pval	Male All Count	Male R Freq	Male S Freq	Male Adj Pval	Female All Count	Female R Freq	Female S Freq	Female Adj Pval
REL												
G1	166	0.45	0.55	0.0007	76	0.51	0.49	0.036	65	0.41	0.59	0.050
G6	82	0.53	0.47		42	0.55	0.45		32	0.52	0.48	
G5	59	0.51	0.49		20	0.65	0.35		34	0.44	0.56	
G3	178	0.56	0.44	1.040	83	0.63	0.34	2.611	76	0.50	0.50	0.972
G4	94	0.73	0.27	0.061	49	0.73	0.27	0.610	36	0.71	0.29	0.270
G2	287	0.71	0.29	0.001	137	0.76	0.24	0.051	115	0.69	0.31	0.027
Line Y												
G1	75	0.32	0.68	0.002	32	0.37	0.63	0.049	43	0.28	0.72	0.050
G6	4	0.50	0.50		2	0.50	0.50		2	0.50	0.50	
G5	5	0.40	0.60		2	0.00	1.00		3	0.67	0.33	
G3	23	0.61	0.39	1.089	11	0.73	0.27	1.036	12	0.50	0.50	2.141
G4	2	0.50	0.50		1	1.00	0.00		1	0.00	1.00	
G2	35	0.89	0.11	0.00003	20	0.90	0.10	0.004	15	0.87	0.13	0.007
Line Z												
G1	45	0.38	0.62	0.0004	19	0.37	0.63	0.004	25	0.36	0.64	0.033
G6	11	0.46	0.54		5	0.40	0.60		5	0.40	0.60	
G5	12	0.50	0.50		8	0.75	0.25		4	0.00	1.00	
G3	32	0.66	0.34	3.865	22	0.68	0.32	1.546	9	0.56	0.44	1.283
G4	18	0.89	0.11	0.141	15	0.93	0.07	0.049	3	0.67	0.33	0.486
G2	49	0.90	0.10	0.001	30	0.93	0.07	0.039	19	0.84	0.16	0.022


4.1
0.80
0.60
0.40
0.20
0.00
-0.20
-0.40
-0.60
-0.80
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140


4.1
0.80
0.60
0.40
0.20
0.00
-0.20
-0.40
-0.60
-0.80
Difference in SNP frequency
126
126.5
127
127.5
128
128.5
129
Mbp

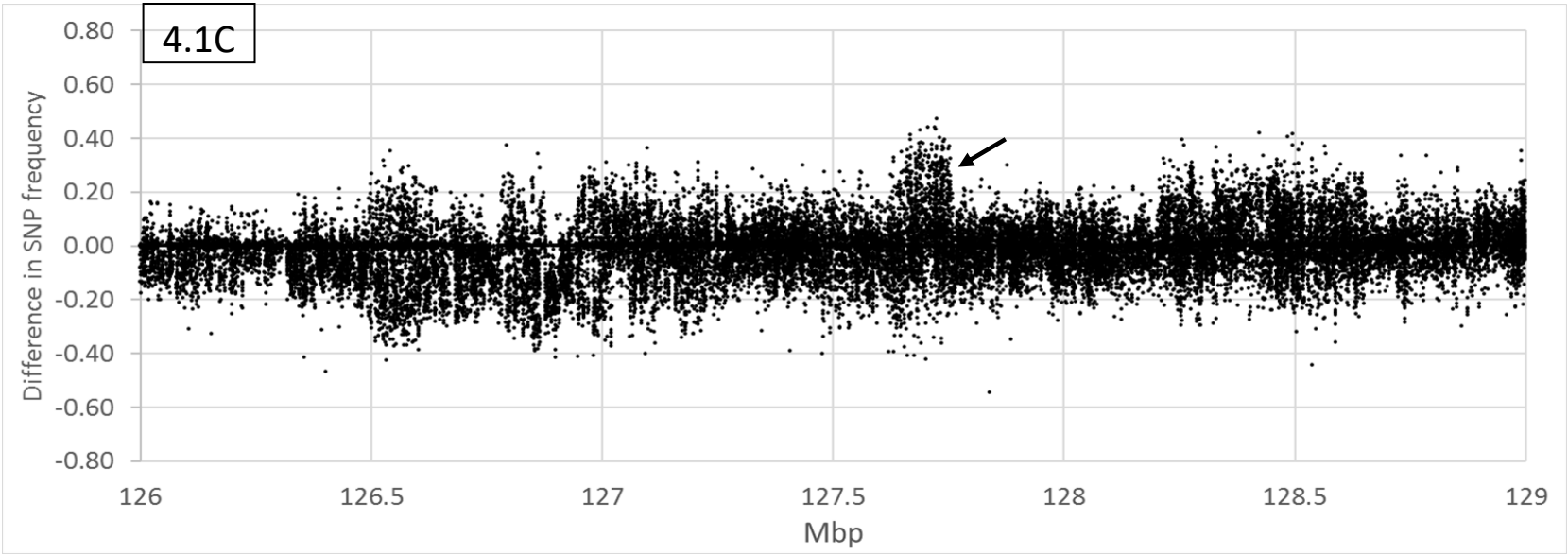


Figure 4.1: **Scatterplot representing GBS data on Chromosome 2.** Variation in SNP frequencies has been plotted as a difference of the average non-reference SNP frequencies of the two replicates of resistant and susceptible sample reads along Y-axis. Therefore, each dot represents the variation in SNP frequencies among resistant and susceptible reads. Thus, a positive value for the SNPs at the loci along Y-axis indicates that those SNPs favor resistance, whereas a negative Y value indicates that the SNP loci favor susceptibility for ascites. X axis represents locus along chromosome 2. **(A):** Scatterplot representing Difference in SNP frequency (Y-axis) along chromosome 2 loci (in Mbp, X-axis) in males. The black arrow indicates the CPQ gene locus. **(B):** An enlarged version of the scatterplot showing a highly variant SNP cluster along positive Y axis within the CPQ gene (black arrow) indicating the variant SNPs to favoring resistance for the disease in males. **(C):** Scatterplot indicating the highly polymorphic CPQ gene locus (black arrow) in females favoring resistance for the disease.

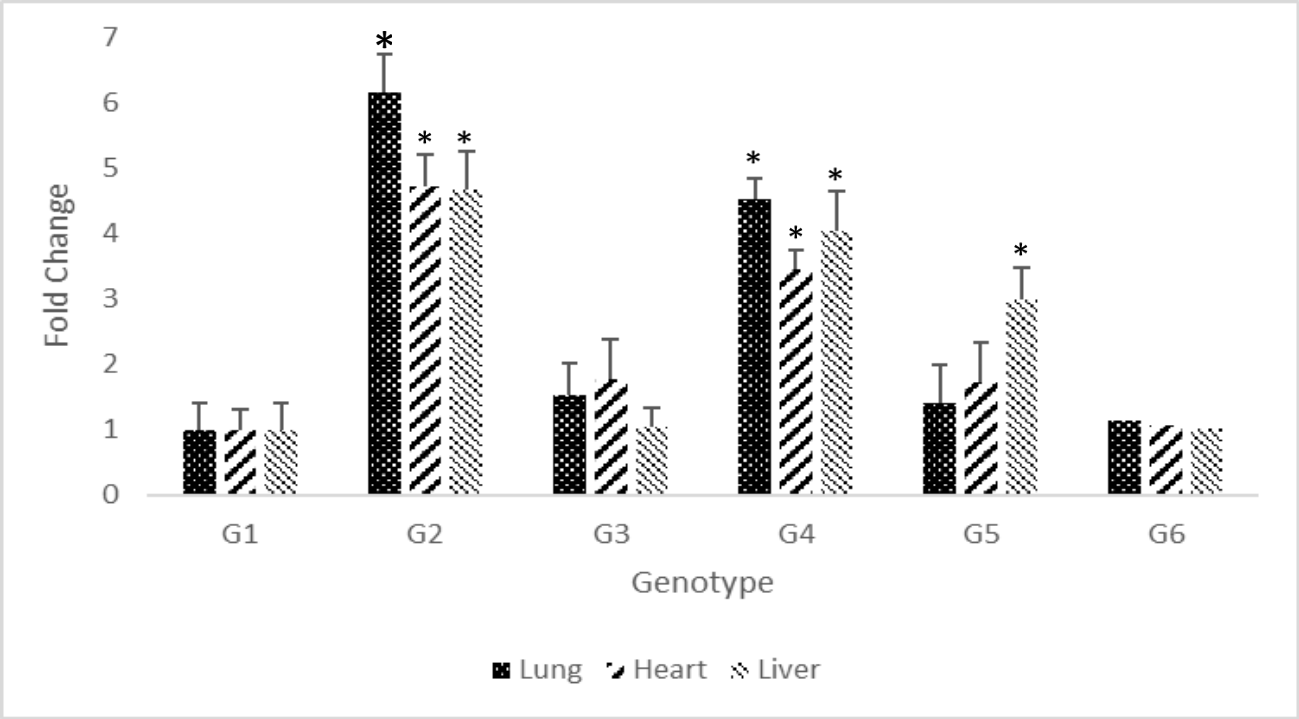


Figure 4.2: CPQ mRNA expression levels for CPQ genotypes in lung, heart and liver. The specific Genotypes (G1-G6) are described in the text._Fold change is computed from the mean of the ΔΔCt values with error bars indicating the standard deviation. Sample numbers were: G1, N=12; G2, N= 14; G3, N= 9; G4, N= 8; G5, N= 11; G6, N=1. * indicates genotypes significantly different from G1 (homozygous reference haplotype) as determined by two-tailed student's t-test with unequal variance ($p < 0.02$).

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CHAPTER 5

Conclusions

Global meat consumption has increased over the past decade. While the per capita consumption of beef and pork meat has reduced or remained constant from 1965 to 2017, consumption of broiler and turkey meat have increased steadily during the same time in the United States (Table 5.1). Poultry meat is also the preferred to red meat (beef, pork, lamb) from a health-related point of view. The fat content of poultry meat is lower than that of red meat. Moreover, poultry meat comprises mainly monounsaturated fatty acids that are beneficial for health. Poultry meat is also rich in polyunsaturated fatty acids including omega-6 or n-6 linoleic acid (18:2 n-6) and arachidonic acid (20:4 n-6), which are mainly derived from the vegetarian diet of the birds copious with alpha-linolenic acid (Marangoni et al., 2015). In contrast, red meat is richer in saturated fats and cholesterol that lead to heart diseases. Higher intake of red meat has also been positively correlated with diseases such as age-related macular degeneration (Chong et al., 2009). In addition to beneficial lipids, poultry meat serves as a good source of highly digestible protein with minimal collagen content, group B vitamins (such as thiamin, vitamin B6, pantothenic acid) and minerals including iron and zinc (Marangoni et al., 2015). Poultry meat has also been correlated with reduced risk of obesity (Te et al., 2012; Astrup et al., 2014), cardiovascular diseases (Hu et al., 2000), type-2 diabetes mellitus (Feskens et al., 2013; Esposito et al., 2014) and types of cancer (Bingham et al., 1999; Bosetti et al., 2000; Yang et al., 2012; Saheli et al., 2013).

To meet the increasing demands for broiler meat, intense genetic selection has been performed for heritable and economically beneficial traits such as increased growth rate, higher feed-conversion rate and higher white meat yield (Julian, 1993, 2000; Decuypere et al., 2000; Balog, 2003; Havenstein et al., 2003). This has brought about dramatic improvements in the size and performance of the current broilers compared to those in the 1950s (Havenstein et al., 2003;

Schmidt et al., 2009). However, these changes are accompanied by an increased incidence of various metabolic disorders including ascites. Ascites, in most cases, occurs as a result of insufficiency of the pulmonary vasculature to accommodate the enhanced cardiac output. Due to this disproportion, there are increased oxygen requirements in fast growing broilers (Wideman et al., 2007; Druyan et al., 2008; Closter et al., 2009). Although proper housing and management techniques can considerably reduce the incidence of ascites, the key to the prevention of IPAH or ascites lies in understanding and manipulating the genetic components of the disease. Therefore, we attempted to investigate and elucidate the genetic correlates of the disease to gain a better insight into the disease and identify major QTL markers to reduce ascites.

Based on the findings from GWAS and microsatellite studies (Krishnamoorthy et al., 2014), our re-analysis of a region at 11.8 to 13.6 Mbp (Gal gal 4 assembly) on chromosome 9 indicates a limited association of the region with ascites. This region encompasses the AGTR1 and UTS2D genes, which we considered to be potential candidates for contributing to the disease due to their functional relevance with cardiovascular disorders. However, our SNP and haplotype-based analyses for these genes indicate they have no significant association with the disease. Interestingly, although we chose some common SNPs within and flanking the genes, and the SNPs and VNTR loci were within 1.4 Mbp distance, we observed relatively low linkage disequilibrium between SNP and VNTR loci This is indicative of a high rate of recombination taking place in these gene regions. Nonetheless, our data supported exclusion of these genes as QTL markers for ascites. We thereafter performed multi-generational GWAS using two different generations of REL birds for a more comprehensive understanding of the genetic associations of the disease in this line. Comparison of the data from the two generations identified two regions- a region around 70 Mbp on chromosome 2 and one around 60 Mbp on chromosome Z (Gal gal 4

assembly). Further investigations indicated the association of the chromosome 2 loci with RVTV ratios in males, consistent with a previous finding relating broiler heart morphology with the chromosome (Rabie et al., 2005). The region identified on chromosome Z also showed an association with RVTV ratios but in ascites-resistant females. These findings suggest that the SNPs in these regions individually or through epistatic interactions may play a role in regulating the development of right ventricular hypertrophy in broiler populations. The SNPs tested in these two regions may serve as markers for ascites phenotype in a gender-specific manner. Although we detected two possible candidate genes on chromosome 2 (MC4R and CDH6) and one on chromosome Z (MEF2c) that are proximal to the SNPs identified in this study, there was little direct association of these gene regions with ascites. Consistent with our previous analyses, we did not find any evidence of the association of chromosome 9 from these GWAS data. Next, we used next-generation sequencing to identify regions of interest related to ascites phenotype. The most prominent region identified in this study spanned from 127.62Mbp to 127.75 Mbp (Gal gal 5 assembly) on chromosome 2 in both genders. This region encompasses part of the CPQ gene. Subsequent SNP-based analyses on different broiler breeder stocks demonstrated a strong association of the gene with ascites in three independent populations. We were able to demonstrate alleles and haplotype patterns within the gene associated with ascites resistance in both genders. These alleles can thus be used as genetic markers for the disease. We further determined that some of these alleles upregulate the CPQ gene expression. Upregulation of the gene, therefore, is likely to favor resistance for ascites. Future studies directed towards the understanding of the CPQ gene networks and protein pathways will fully elucidate the importance of the gene in disease-associated phenotypes. The CPQ gene has also shown significant association with human cardiovascular traits including hypertension. The whole

genome resequencing data detected no association of chromosome 9 regions, further validating our previous SNP mapping experiments and GWAS analyses.

The findings described in this dissertation provide important tools to discern the genetics of IPAH and ascites in broilers. The markers detected in these studies will be useful for application in large-scale broiler breeding programs in the poultry industry to improve resistance for ascites. Our findings also implicate that selection against ascites is possible. In addition, our studies open new avenues for investigating the functional contributions of the indicated genes. With the growth in the world population and a continually expanding global broiler market, efficient poultry production will be possible by minimizing the losses afflicted by disease-related traits and improving animal welfare. Ascites, being an important trait in terms of economic losses especially in developing countries, understanding the genetic regulation of the trait would be essential for controlling the incidence of the disease. The results from these studies not only unravel portions of ascites genomics in broilers but may also have some usefulness in pulmonary hypertension in humans.

Table 5.1. Per capita consumption of poultry and red meat (in pounds)- 1965 to 2017. For each year, the consumption is listed for each meat type. "Total Red Meat" includes Beef, pork and other sources of red meat. "Total Chicken" includes broiler and other meat-chickens. "Total Poultry" includes chicken and turkey. (Adapted from National Chicken Council, June 2017: *http://www.nationalchickencouncil.org*).

Year	Beef	Pork	Total Red Meat	Broilers	Total Chicken	Total Poultry
1960	63.3	59.1	131.6	23.6	28.0	34.3
1966	78.1	50.3	135.8	32.1	35.8	43.7
1972	85.3	54.7	144.8	38.1	41.6	50.7
1978	87.3	47.0	138.1	42.5	44.8	53.6
1984	78.4	51.5	133.2	49.2	51.3	62.6
1990	67.7	49.7	119.9	59.5	66.2	79.1
1996	67.1	48.3	117.7	69.2	70.0	88.2
2002	67.8	51.6	121.1	80.7	82.2	99.9
2008	62.5	49.5	113.4	83.5	84.9	102.5
2014	54.1	46.4	101.7	83.3	84.6	100.3
2015	53.9	49.7	104.8	88.9	90.0	106.0
2016 (Estimated)	55.6	50.1	106.9	89.8	91.0	107.7
2017 (Projected)	57.1	50.8	109.2	90.4	91.6	108.6

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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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Fax: 479-575-3846 • http://vpred.uark.edu/199

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.

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