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Elucidating the genetic cause to ascites syndrome in broiler chickens utilizing multi-generational genome wide association studies

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Elucidating the Genetic Cause to Ascites Syndrome in Broiler Chickens Utilizing Multi-Generational Genome Wide Association Studies

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ABSTRACT

Ascites syndrome in broiler chickens has developed into a source of economic loss in the last three decades. Intensive selective pressure, and implementation of flock management practices, has successfully reduced ascites frequency, but has not eliminated its occurrence. For this reason, it is imperative to better understand the genetic cause to ascites in broiler chickens. Previous studies of this magnitude have been attempted, but, thus far, a consensus of genomic associations have not been made. This collection of studies was aimed at identifying and interpreting genomic and genetic associations to ascites phenotype specific to a broiler line representative of a 1990s elite male line. A next generation sequencing technique, termed genome wide association studies, was initially implemented to identify chromosomal regions experiencing correlations with ascetic events in broilers. Individual loci were then evaluated for their impact on resistance and susceptibility, with particular interest in sex effects and parental genotypes. Finally, statistical models were evaluated for their potential use in predicting ascites incidence. Models represent a less time consuming and more cost effective method aimed at conserving genetic accuracy in selected breeding programs. Together, these studies represent gains in the current knowledge of ascites genetics and serve as a possible source for novel selective breeding practices in an industry setting.

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LIST OF ABBREVIATIONS

AUC	area under the curve
Cdh13	cadherin 13
Cdh6	cadherin 6
GWAS	genome wide association study
HTR1A	5-hydroxytryptamine (serotonin) receptor 1A
LOX	lysyl oxidase
MAD	mean absolute deviation
MAS	marker assisted selection
Mbp	megabase pairs
MC4R	melanocortin 4 receptor
MCR	misclassification rate
Mef2	myocyte enhancer factor-2
Mef2c	myocyte enhancer factor 2C
MG	moderate growth and livability
MH	medial hypertrophy
PHS	Pulmonary hypertension syndrome
QTL	quantitative trait loci
REL	relaxed selected line
RG	feed efficiency
RMSE	root mean square error
ROC	receiver operative characteristic
RVTV	right ventricle-to-total ventricle weight
SNP	single nucleotide polymorphism
WOG	whole bird without giblets
YD	growth rate and breast yield

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CHAPTER 1

Literature Review

Domestication

Molecular and archeological evidence suggests the chicken was domesticated from a subspecies of jungle fowl found throughout Southeast Asia, the red jungle fowl (*Gallus gallus*), around 7,000-10,000 years ago (West and Zhou, 1988; Fumihito et al., 1994; Xiang et al., 2014). Domestication events occurred independently throughout this region, including China, Indonesia, Japan, and India (Liu, et al., 2006; Kanginakudru, et al., 2008). Diverse use of the chicken, including meat and egg production, religious activities, cockfighting, and ornamentation, all contribute to the chicken having the most extensive range of all domestic species (Serjeantson, 2009). Prior to WWII, chicken meat production was a secondary market for the egg industry (National Chicken Council). With the increased accessibility of feed ingredients amplifying poultry availability, and an increase in consumer consumption of poultry meat at a time of red meat rationing, both led to the early development of a primitive poultry industry.

Modernization of the Broiler

Beginning in the early 20th century, and continuing today, improving management techniques, nutritional evaluation, and implementing breeding schemes act as the groundwork for improvement in the poultry production industry (Titus, 1941; Hutt, 1949; Griffin and Goddard, 1994; Havenstein et al., 2003; Bessei, 2006). Initial emphasis on mass selection resulted in a positive response to economically important traits, because these traits maintained high heritabilities. In commercial broilers this genetic influence led to a rapid positive response in pedigreed selection systems (Hunton, 2006). At the beginning of the development of what is considered the precursor to the modern day poultry industry it was realized that a negative correlation exists between growth-related traits and reproduction-related traits (Siegel and Dunnington, 1985). For this reason, breeds originally selected as dual-purpose breeds (selection

emphasis on meat yield and egg production) were replaced by breeds with specialized selection. As a result, in North America today, the Cornish Cross represents a majority of the broiler market, whose selection has created a bird that reaches market weight at d 42.

Consumption of chicken meat has steadily increased since commercially available products have been monitored beginning in the mid-1900s. Chicken meat surpassed beef and pork in per capita consumption in pounds per year in the mid 1990's (MacDonald, 2008). Since 1929, the pounds of poultry produced in the U.S. has increased almost five-fold (Figure 1-1). A reason for the change in eating habits in the U.S. is partially due to the difference in price. In December of 2015 the price of beef per pound averaged $5.50, while the price of chicken averaged $1.94 per pound (Hahn, 2016).

As a result of an increase in demand for chicken meat during the 1940's selective breeding in chickens, through progeny testing, for the purpose of altering quantitative traits, began (Hunton, 2006). Commercial genetic companies formed and began focusing on selection efforts for traits affecting disease resistance, meat yield, feed efficiency, egg production, meat quality, among others. Massive gains in economically important traits, specifically breast filet yield, have been achieved. Evidence of this can be seen in feed conversion decline of 4.42 in a 1957 broiler population to 1.47 in 2003 (Havenstein et al., 2003) and the reduction of time it takes for a broiler to reach 1.5kg of live weight in 1925 versus 2005: 120 days to 30 days, respectively (Albers, 1998). In summary, massive changes in management and production schemes has led to the commercial broiler of today.

Modern Breeding Schemes

Modern broiler breeding companies have developed a broiler breeding and production system that can be visually represented in a pyramid scheme (Figure 1-2). At the top are pure-

bred pedigreed elite lines. In the Cobb-Vantress Inc. (Siloam Springs, AR) breeding program over 50 performance, production, and general health traits are evaluated from each pedigreed offspring (Katanbaf and Hardiman, 2010). Pedigreed birds will provide parental generations for the pure line great grandparent stock, which provides parental generations for the grandparent stock (Figure 1-3). Pure lines at the grandparent level experience the first line crosses in the breeding scheme. What results is a two-way cross representing individuals whose pure line heritage are under selection pressure for either growth, yield, and feed conversion ratio (termed male lines), or growth, yield, and reproductive traits (termed female lines). The parent generation is the first instance of ownership by production companies. Finally, commercial broilers represent a four-way cross of the male and female two-way cross. On a world-wide basis, it has been estimated that 400,000 pedigreed individuals representing 35-40 pure bred lines from various companies at the pedigree level will be the progenitors for approximately 400 billion commercial broilers (Pollock, 1999).

Domestication Effects

Ultimately, dramatic changes experienced in the domestic chicken selected for growth did not occur without negatively resulting consequences to the physiology of the birds. In tandem with selection for performance traits, undesirable qualities developed. Selection for rapid growth increases carcass fat, which directly influences leg lameness seen in broilers (Soller and Eitan, 1984), fluctuation in muscle characteristic consistency due to alterations in age of slaughter and development (Gous, 1986), and increases in disease accumulation, like sudden death syndrome (Gardiner, et al., 1988), pulmonary hypertension (Julian, 1993), and negatively influences reproductive performance (Anthony, 1998; Emmerson, 1997). These domestication

effects have been the subject of several review articles (Anthony, 1998; Petracci and Cavani, 2012).

Heart and Lung Function

The avian heart is a four chambered system consisting of right and left atria and ventricles. The right ventricle works at a low pressure sending blood through to the lungs for oxygenation, while the left ventricle works at a higher pressure as it perfuses blood for systemic circulation. The thickness of the left ventricle is approximately two to three times the thickness of the right ventricle (Olkowski et al., 1998; Tekeli, 2014). The size differential is owed to the variation in systolic pressures maintained by the right and left ventricle (Speer, 2016). As a result, the left ventricle is capable of producing a systolic pressure four to five times greater than in the right ventricle under normal conditions (King and McLelland, 1984).

From approximately 40 g at hatch, a broiler chick has the ability to reach up to 4,000 g at the processing age of eight weeks (Wideman et al., 2013). To sustain the physiological needs of the bird during the growth phase heart and lung functions must cope with rapid gain. The cardiac output, and subsequent stroke volume, are proportional to body mass in all avian species, but these correlations are higher than what is seen in mammals (Grubb, 1983). Cardiac output can be calculated as a function of the bird's heart rate, and the preload and afterload, or the stroke volume. A broiler heart at hatch has the ability of pumping 8 mL/min, defined as the bird's cardiac output, which averages 200 mL/min of cardiac output per kilogram (Wideman, 1999). By eight weeks of age a 4,000 g broiler now must pump 800 mL/min of oxygenated blood from the left ventricle into the body.

Upon re-entry of circulating blood into the right atrium, for the process of re-oxygenating the blood, the right ventricle acts to drive the blood from the heart through the lungs via the

pulmonary artery through the cardio-pulmonary system. It is the blood flow through the pulmonary vasculature that is used to calculate the pulmonary arterial pressure. The pulmonary artery pressure of a clinically healthy bird is measured around 20 mmHg (Chapman and Wideman, 2001). At this rate, blood travels from the pulmonary artery into the inter- and intra-parabronchial arterioles. Unilateral gas exchange takes place through diffusion of oxygen between parabronchi networks extending from the parabronchus lumen to the blood capillaries filled with pulmonary arterial blood.

Pulmonary Hypertension Syndrome

Pulmonary hypertension syndrome (**PHS**) is one such domestication effect experienced by the cardio-pulmonary system that affects high-yield broiler lines. Symptoms linked to PHS has been described consistently in North American since the 1950s (Sanger et al., 1958; Schmittle et al., 1958), although, early mentions of flocks of broilers being managed at high altitude conditions in Bolivia (Hall and Machicao, 1968) and Peru (Cueva et al., 1974) have also occured. Initially, PHS was most common in high elevation environments, but became a frequent occurrence in low altitude conditions in the 1980s (Julian, 1993).

The collection of manifestations that encompass PHS has been termed hydropericardium, ascites, water belly, alimentary toxemia, myocarditis, altitude disease, and congestive heart failure, among others (Sanger et al., 1958; Hall and Machicao, 1968; Huchzermeyer, 1984; Wilson et al., 1988). Though the terminology used to describe the disease was initially variable, PHS, or ascites, is currently the accepted name and will be used interchangeably when described here.

Development of PHS

Pulmonary hypertension syndrome is a cascade of adverse changes in a broiler resulting from selection for rapid growth and high oxygen demands that is a direct result of selective pressures placed on muscle gain through increased growth rate (Julian, 1993; Julian, 2000). Affected individuals can be phenotyped as ascites susceptible externally by evaluating the bird for lethargy or noting the bird as being cyanotic, or low in oxygen. Additionally, ascites phenotype can be determined internally by presence of fluid in body cavity (Figure 1-4), a flaccid right ventricle, and enlarged liver (Olkowski, et al., 1999).

Broilers diagnosed as pre-ascitic experience an increase in pulmonary artery pressure to ≥ 45 mmHg (Chapman and Wideman, 2001). This spike in pressure will result in the pulmonary vascular channels experiencing vasodilation. In mammals, an increase pulmonary artery pressure will increase the diameter of the pulmonary capillary by over 100% (Sobin et al., 1972; Mazzone, 1980). In avian species, the pulmonary capillaries are more rigid in structure, and are only able to accommodate and increase in capillary diameter of approximately 13% (Watson et al., 2008). Additionally, increase in pressure is associated with abnormalities forming within the inter-parabronchial pulmonary artery walls, termed medial hypertrophy (**MH**; Wagenvoort and Wagenvoort, 1970). The thickening of the artery walls results in the reduction in the amount of room available for the blood to flow. As a reduction in the inside radius of the vessel occurs due to MH, Poiseuille's law describes a simultaneous increase in blood pressure and decrease in flow rate. Therefore, as the vessel lumen thickens, the pressure required to push deoxygenated blood through the gas exchange system also increases. The need to push blood faster through the systems directly influences negative adverse side effects. Initially, the right ventricle's load increases as it is forced to increase its cardiac output to compensate for the lack of oxygen being

delivered throughout the body. Subsequently, an increase in flow rate dictates the blood will be pushed faster through the blood capillaries (Wideman et al., 2013). Full diffusion of O_2 takes place in the first 20-30% length of the capillary. At an increased flow rate through the capillaries, the blood must travel over a longer distance for full gas diffusion to take place. These steps indicate the beginning of denying the body of adequate oxygenation, which is termed as a hypoxia.

Chickens in chronic hypoxic environments will experience cardiac hypertrophy of the right ventricle, and these birds will have larger hearts than those raised in normal conditions (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 (Figure 1- 5). In addition, it has been previously shown that the RVTV ratio and the pulmonary arterial blood pressure are positively correlated further indicating association between heart morphology and cardiac health (Burton, 1968; Chapman and Wideman, 2001).

In the process of selective breeding broilers while emphasizing economically important traits, the growth rate of the heart has decreased in modern broilers when compared to a heritage line representative of the 1940s (Schmidt et al., 2009). Post-hatch to d 14 in both lines show a similar heart size to body size ratio. After d 14 the relative size of the heart to the body remains consistent in the heritage line, but a decline in this ratio is seen in modern broilers. It is probable that this decrease in relative size lends itself to a decrease in the cardiac capacity of the broiler, which would lead to a higher incidence in heart related ailments, like PHS.

The growth rate of the heart slows as the bird ages, such that the proportion of the heart to the total body weight decreases from hatch to d 42 despite large gains in body mass (Forman and Wideman, 2000; Tickle et al., 2014). Interestingly, increases in blood pressure due to

hypoxic conditions is noted in the chicken, but not in naturally occurring avian species, which can be contributed to a high level of selection pressures (Faraci, 1986).

It is estimated today that in broilers raised under conditions to achieve maximum growth approximately 3% will be afflicted with pulmonary arterial hypertension (Wideman and Hamal, 2011). The underlying mechanisms necessary for the development of PHS are present in modern broiler flocks under standard management techniques.

Methods for inducing PHS for study include both invasive and noninvasive techniques. Chronic evaluations include cold temperatures (Lubritz and McPherson, 1994; Acar et al., 1995; Wideman et al., 1998; Sato et al., 2002), long photoperiods (Hassanzadeh et al., 2000; Julian, 1990), and high elevation (Balog et al., 2000a) are chronic events seen in a commercial setting that impact the incidence of PHS.

In cold temperature environments blood viscosity and hematocrit increase in chickens (Vogel and Sturkie, 1963; Shlosberg et al., 1996; Stammers et al., 2003). Rapidly growing broilers require a high oxygen demand due to a high metabolic rate, which is further amplified by cold temperatures. Broilers grown at a cooler temperature have a lower body weight (Blahova et al., 2007), but ultimately consume more feed than their warm-environment counterparts, in order to maintain thermogenesis. An increase in cardiac output is observed in cold-stressed birds to meet increase oxygen demands, but manipulating the delicate cardiovascular system can lead to PHS (Julian et al., 1989).

Light restriction during growth reduces feed consumption in broilers, which slows the growth rate (Downs et al., 2006). Oxygen saturation is known to be higher in lighter broilers than in heavier broilers (Julian and Mirsalimi, 1992). Predisposition to low percent oxygen

saturation in the blood dictates that birds whose feed intake is at a maximum level while experiencing a long photoperiod are more likely to develop ascites (Lott et al., 1996).

To replicate elevated conditions, a hypobaric chamber is used to simulate a set elevation above sea level through creating a hypoxic environment. The chamber operates by reducing atmospheric pressure, which in turn, reduces the partial pressure of oxygen by approximately 2.5% for every 1,000 m increase in elevation (Brosnan et al., 2000). Previously published studies have utilized these noninvasive methods as a way to increase the frequency of PHS that replicate events that may occur in a normal broiler operation (Owen, et al., 1990; Mirsalimi, et al., 1993; Balog et al., 2000a; Balog et al., 2000b; los Santos et al., 2005; Pavlidis et al., 2007; Krishnamoorthy et al., 2014). Out of the techniques previously listed, high elevation is superior to other methods when inducing PHS because it does not pose an unideal environment which would prevent optimum growth in the broiler. High elevation also causes PHS at a frequency high enough for genetic study as compared to long day lengths.

In addition to these chronic stressors, acute techniques have been used for assessment of ascites resistance. Acute techniques include micro-particle injections where micro-particles of cellulose are injected into the systemic circulatory system (Wideman et al., 2002; Wideman and Erf, 2002). Micro-particles become transplanted into the vasculature of the cardio-pulmonary system, which induces systemic hypoxia. In a second method, users clamp the left pulmonary artery (Wideman and Kirby, 1995). This method is used to increase cardiac output and elicit pulmonary hypertension, but proves to be more invasive than micro-particle injections. Such techniques evaluate ascites outcome at a specific point in time in the bird's development. Considering ascites frequency during a snapshot in the growth phase negates consideration of

ascites manifestation at all possible times of occurrence when the purpose of assessing ascites is to create better selections at the pedigreed level.

Ultimately, pulmonary hypertension syndrome frequency is reduced in commercial flocks through breeding schemes and management techniques like feed restriction (Bolukbasi et al., 2004) and shorter photoperiods (Lott et al., 1996). In pedigreed flocks, ascites incidence is evaluated, and this information is used to create breeding schemes that act to reduce the overall incidence being seen at the commercial broiler level of the production system. Use of environmental management techniques and of genetic breeding programs have gone a long way in reducing overall flock incidence. In 2007, economic loss due to ascites incidence in the United States was estimated at $100 million/year (Pavlidis et al.). The profound economic cost is due to the tendency of ascites frequency to be highest in the largest, fastest growing birds, for which the greatest amount of feed and the largest time investment have been made. An updated estimation in 2015 indicated economic loss has remained consistent between these two years; however, the USDA reported an increase in broiler production of 3.8 billion pounds between years 2007 and 2015 (Figure 1-1). Ultimately, while financial loss due to this disease remains economically relevant, it is clear that methods used to reduce ascites incidence have been marginally successful.

Genetics of PHS

Although shown to be influenced by environmental factors (Julian, 2000), PHS is also influenced through genetic parameters (Lubritz and McPherson, 1994; de Greef et al., 2001; Wideman and French, 2000). Incidence has corresponded with increased genetic selection on growth rate, live weight, and muscle yield. The genetic influence on PHS has been illustrated in the low to moderate heritability estimates (Moghadam et al., 2001; Lubritz et al., 1995; Druyan

et al., 2007), which has led to success in the creation of divergently selected ascites resistant and susceptible lines (Pavlidis et al. 2007; Druyan et al., 2009). In three ascites-induced selected male broiler lines, characterized as selections for growth rate and feed efficiency (**RG**), moderate growth and livability (**MG**), and growth rate and breast yield (**YD**), Lubritz et al. showed the frequency of ascites incidence of the YD line to be approximately 0.15 higher than that of RG and MG lines (1995). Additionally, heritabilities for ascites incidence in these cold-challenged lines indicate lines with selection pressures focused on increased yield and growth have higher heritabilities, .36 ± .10, .11 ± .08, and .44 ± .09, for RG, MG, and YD, respectively. The exact genetic cause behind ascites incidence has been debated. While some groups argue for the occurrence of one or a few dominant genes (Druyan and Cahaner, 2007; Wideman and French, 2000), other studies indicate cause for evaluating ascites incidence as a multi-genic disease (Rabie et al., 2005; Hamal et al., 2010).

Krishnamoorthy et al. (2014) used a genome wide association analysis to identify regions on chromosome 9 from an F2 generation from a cross of an ascites-selected resistant line and an ascites-selected susceptible line cross. Data indicate a gender-specific effect in relation to the line analyzed, and the authors went on to propose two candidate genes responsible for a portion of ascites incidence. 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 causal source. Additionally, Rabie et al. (2005) found an association with RVTV ratio and a region on chromosome 2. Gene expression has also been used as an evaluation tool in selectively bred resistant and susceptible lines to determine variations in expression of vasoactive mediators using micro-particle challenged birds exhibiting PHS (Hamal et al., 2010). A divergently selected ascites susceptible line exhibited an increase in the

expression of vasoconstriction receptors, while an ascites resistant line demonstrated higher expression of vasodilators. These studies indicate advances in the detection of genetic causation to PHS.

Marker Assisted Selection History

The process of artificial selection in domestic species has been practiced since initiation of domestication. At the beginning of the poultry industry's development initial success in trait selection was due to economically relevant traits having high heritabilities in the case of body weight (Le Bihan-Duval et al., 1998; Sanda et al., 2014; Venturini et al., 2014), breast yield (Le Bihan-Duval et al., 1998), and feed conversion (Leenstra and Pit, 1988). Breeding companies were able to place traditional quantitative genetic selection emphasis on these traits by maintaining breeding populations that were somewhat closed (Ewart, 1993). Markers in DNA, or marker assisted selection (**MAS**), used for the purpose of trait selection has been implemented in the poultry industry over the last 25 years (Soller, 1994). Reduced costs associated with DNA sequencing and SNP genotyping has made MAS a reliable and attainable approach for industry and researchers alike. Sequencing of 1.1 billion bases in the red jungle fowl genome in 2004 made application of molecular biology research more readily available (Hillier et al., 2004). Identification of 2.8 million single nucleotide polymorphism (**SNP**) furthered the ability to easily conduct molecular based research (Wong et al., 2004). The publicly available assembly released in 2006 (Gallus_gallus-2.1), and subsequent revised assembly in 2011 (Gallus_gallus-4.0) have aided development of new scientific techniques useful for selection purposes. To better illustrate that point, a publication search performed using Web of Science™ (Thomas Reuters © 2016) using title search terms poultry, chicken, and hen, with subject matter terms consisting of SNP

and genome, found a marked increase in the number of publications, consistent with the timeline of the publication of the chicken genome (Figure 1-6).

Marker Assisted Selection in Broilers

Variations in DNA, such as variations in alleles at a single locus among closely related individuals (SNPs) and short DNA sequences that are either inserted or deleted in the genome (INDELs) are common tools used in MAS. Not all DNA variants are considered functional. Functional variations are utilized as presumptive quantitative trait loci (**QTL**) to understand the relationship between molecular markers and phenotypic trait data (Kearsey, 1998). Quantitative trait loci alter the coding sequence of the DNA that can affect production, performance, and health-related phenotypes. Therefore, selection programs are based on influencing the frequency of advantageous or disadvantageous alleles (Siegel et al., 2006). In poultry species, the identification of novel QTL, in respect to economically important traits, have gone a long way to developing new areas of selection to be emphasized in breeding programs (Wolc et al., 2011; Godoy et al., 2015; Wolc et al., 2016). Addition of genotypic information increases the accuracy up to 50% over traditional BLUP analyses solely based on phenotypic information (Chen et al., 2011). Therefore, genomic selection pressures being applied at the pedigreed level in poultry production further accentuates the possibilities of traditional quantitative genetics in the pyramid breeding scheme. It is no coincidence that more gain in broiler market weight has been experienced in the industry over the last 25 years when MAS techniques have been available, than over the 60 years prior to the 1990s when selection was based solely on classical genetics (Figure 1-6).

Synopsis

Ascites incidence in broiler populations can be altered through environmental effects, such as increased elevation and temperature stress (Owen et al., 1990; Balog et al., 2003). In addition, ascites is influenced through genetic components (Lubritz et al., 1995; Wideman and French, 2000). Adjustments in management and selection practices have been set in place to reduce the estimated $100 million per year economic loss seen since 2007 (Pavlidis et al., 2007). Still, ascites presents itself as a relevant and economically important disease internationally. Use of MAS in detecting ascites frequency in flocks will provide additional resources in the development of fast growing broiler lines that are resistant to health defects. Our purpose here is first, to identify genetic causation to ascites incidence in broiler chicken lines maintained at the University of Arkansas since the 1990s, and second, to use prediction based models to effectively predict ascites outcome in broilers using a minimally invasive technique.

Figure 1-1 Yearly ready - to - cook broiler production with projections for 2016. Information as reported by Economic Research Service/USDA.

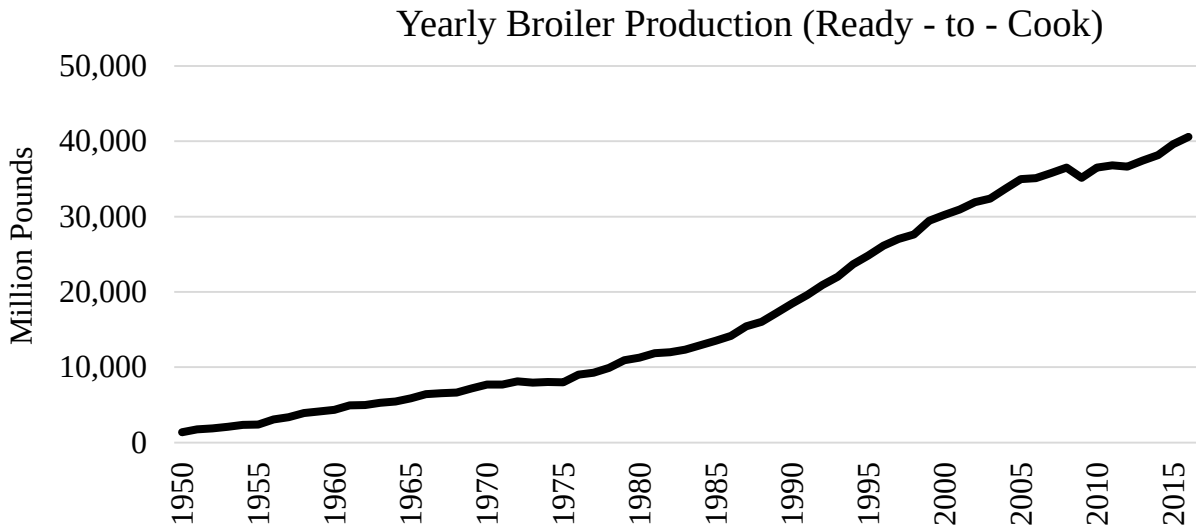


Figure 1-2. Breeding schemes in modern broiler genetics companies. Adapted from Pollock (1999).

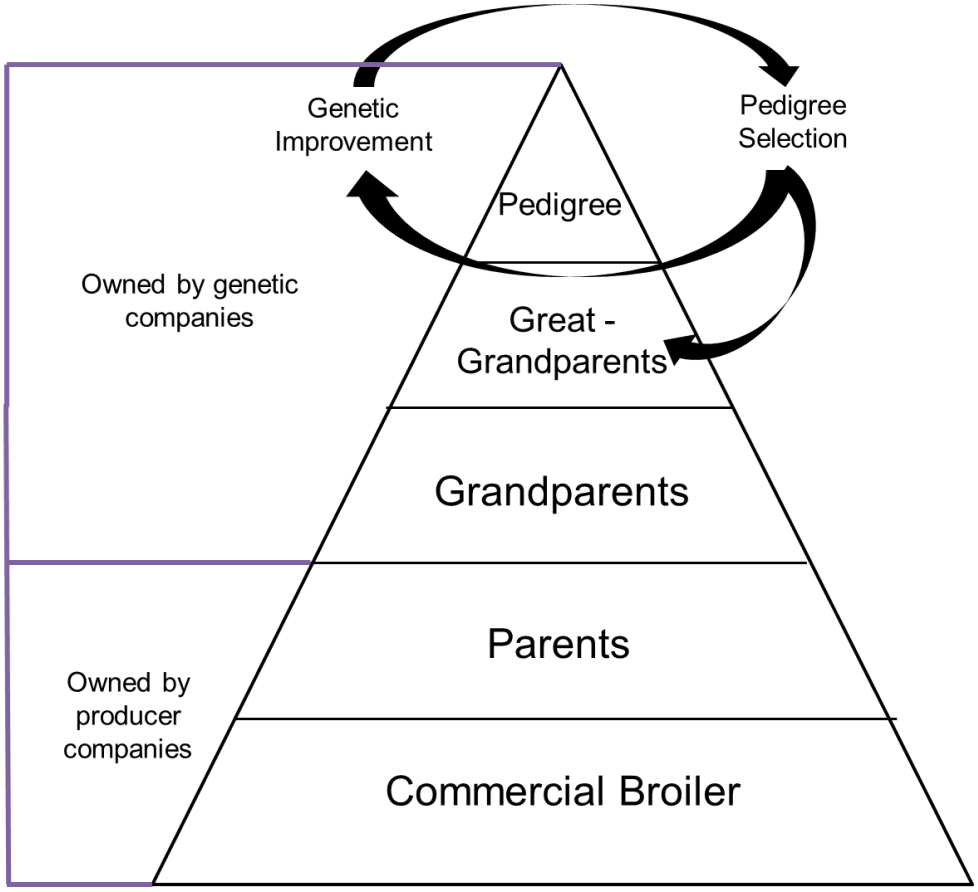


Figure 1-3. Line crosses experienced at each level in the pyramid breeding scheme.

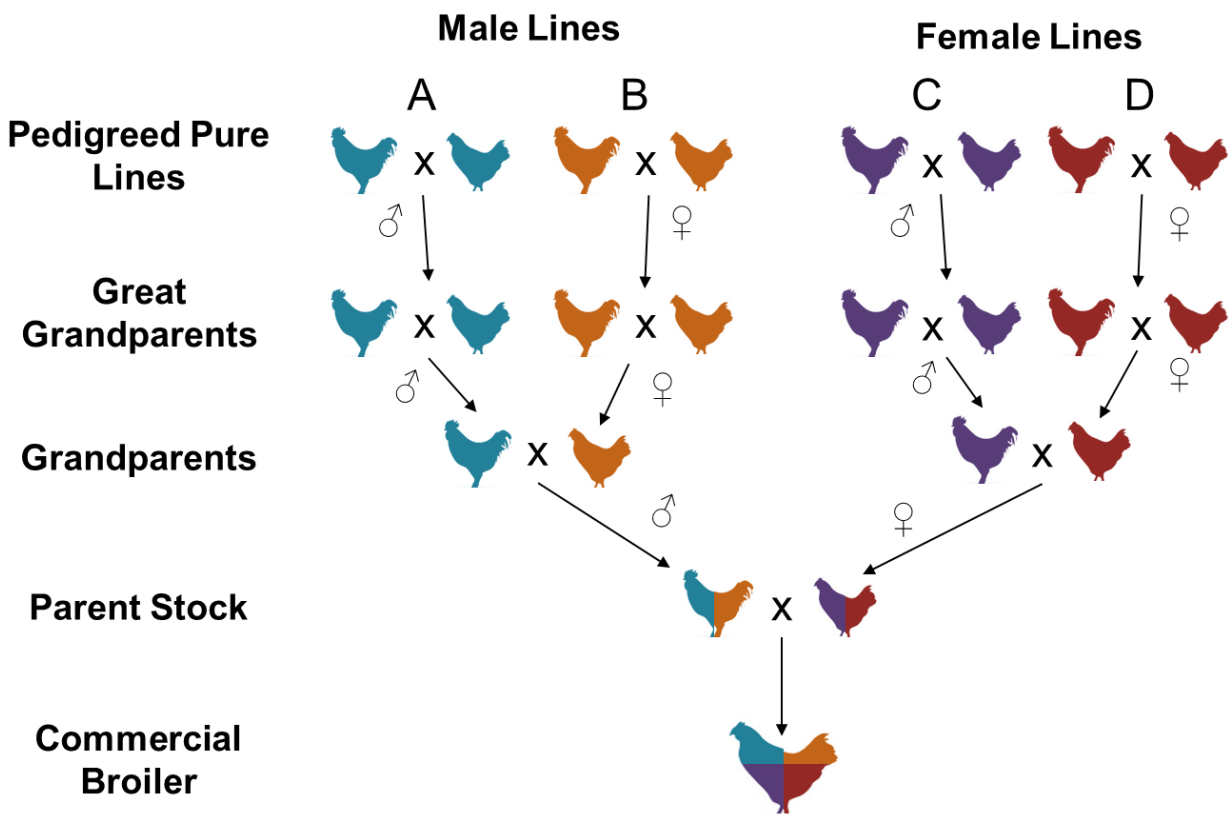


Figure 1-4. The bird on the left is a healthy two-week old broiler. The body cavity of the broiler on the right is distended due to the accumulation of fluid, which indicates the bird has ascites.

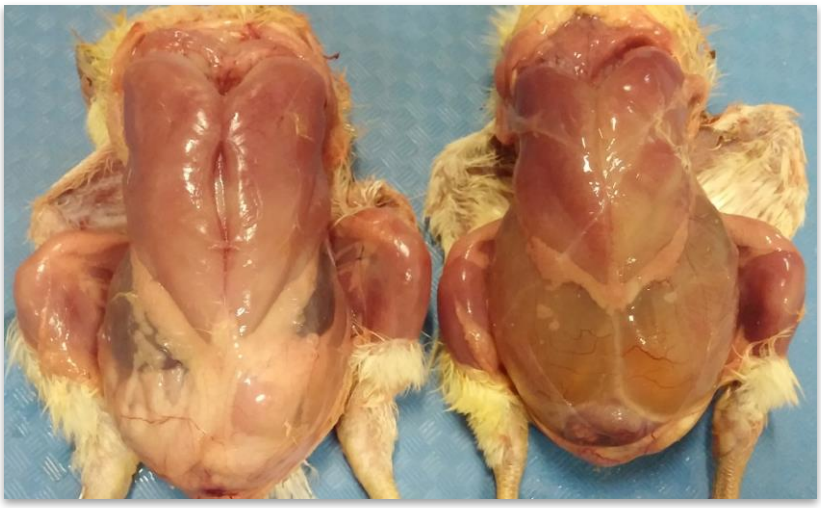


Figure 1-5. The heart on the left is from a healthy broiler. The heart on the right is that of a boiler affected by ascites. Hypertrophy of the right ventricle has led a flaccid ventricle and rounded heart shape. The heart on the right has a higher RVTV ratio than that of the left heart because of the increase in size of the right ventricle.

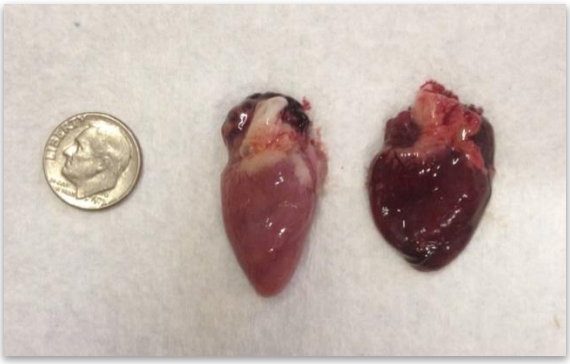
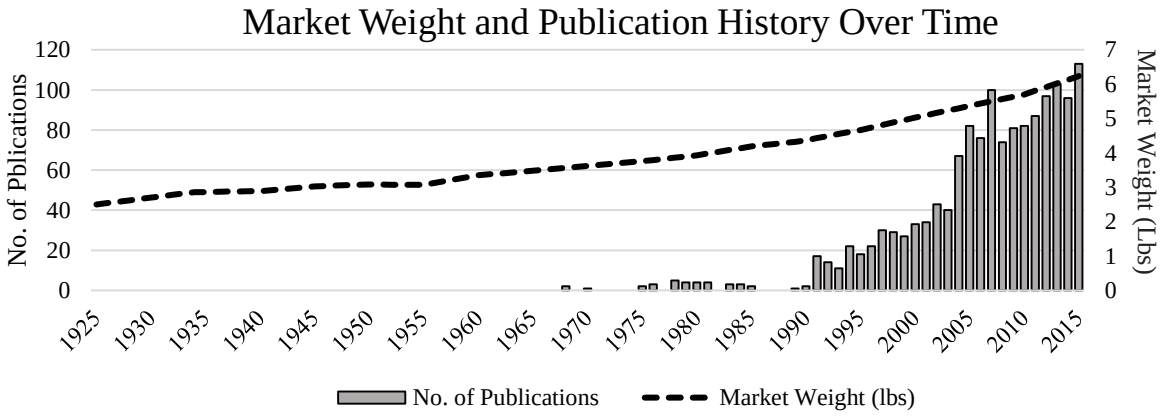


Figure 1-6. Molecular genetics relevant publications plotted against the reported market weight in lbs). The number of publications per year found using Web of Science search engine. Search terms included chicken, broiler, hen, SNP, and genome. Projection for 2016 is included. Pounds of the average broiler live weight at market age reported since 1925. Adapted from the U.S. Broiler Performance reported by the National Chicken Council.



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CHAPTER 2

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

ABSTRACT

Ascites is a multi-faceted disease commonly observed 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 $100 million/year. In this study, a 60K Illumina SNP BeadChip was used to perform a series of GWAS (genome wide association study) on the 16th and 18th generation of our relaxed (REL) line descended from a commercial elite broiler line beginning in 1995. Regions significantly associated with ascites incidence were identified on chromosome 2 around 70 megabase pairs (Mbp) and on chromosome Z around 60 Mbp. Five candidate single nucleotide polymorphism (SNP) were evaluated as indicators for these two regions in order to identify association with ascites and right ventricle-to-total ventricle weight (RVTV) ratios. Chromosome 2 SNPs showed an association with RVTV ratios in males phenotyped as ascites resistant and ascites susceptible ($P < 0.04$). The chromosome Z region also indicates an association to resistant female RVTV values ($P < 0.01$) and susceptible female RVTV values ($P < 0.03$). Data also indicate a possible male-specific effect occurring in regards to ascites incidence for the Z chromosome region. 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 affects 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). Amplified pressure in the cardiovascular system will advance to eventual right ventricle hypertrophy and concludes with right ventricle failure (Huchzermeyer and Deruyck, 1986). 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 (Cooper and Gustin, 2015 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 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 genotyping 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. Single nucleotide polymorphisms 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

Within the hypobaric chamber 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 2900 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 16^{th} and 18^{th} generation were used from a pedigreed elite broiler line that has remained under relaxed (**REL**) selection since 1995 (Pavlidis, et al., 2007). 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.

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).

Genome Wide Association Study

A total of two GWAS were completed on two generations of REL line birds that were phenotyped as described above in a six-week hypobaric chamber challenge. The GWAS was conducted by DNA Landmarks (Quebec, Canada) using an Illumina 60K SNPChip on REL line males. Thirty-seven males and 47 females representing REL line generation 16, and 68 males from generation 18, were used for this study.

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. 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 $1\text{-}\mathrm{Log}_{10}(P)$ was calculated for a sliding window of 10 flanking SNPs.

Real-Time PCR

Specific SNPs were used to develop exonuclease (Taqman® probe) assays for quantitative real-time PCR genotyping. PCR primers and probes, along with annealing temperatures are presented in Table 2-1. 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 second, and a plate read for a total of 30 cycles.

Statistical Methods

Genotyped individuals were evaluated by ascites phenotype and right ventricle-to-total ventricle ratio (**RVTV**). 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 RVTV 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, were resistant and susceptible individuals were compared independently. Male and female RVTV 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. Of these, 30,650 are mapped to chromosomes 1-28, and the Z chromosome. Using 1-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 were polymorphic in the REL line (Figure 2-1). A region around 70 megabase pairs (**Mbp**) appeared to show significant association in ascites resistant individuals in both generations. Similarly, 1178 of 1385 SNP were informative on the Z chromosome, for which a region of significance

was observed around 60 Mbp and detectable in both generations in susceptible individuals (Figure 2-2).

Two representative SNPs from the chromosome 2 region, and 3 from the chromosome Z region, were used for further genotype assays on a larger collection of DNAs from the REL line (Table 2-2). For SNP 2.708 in the susceptible males the TT and CC genotype both have higher RVTV averages than the heterozygous individuals. While greater RVTV value equates to a higher ascites incidence in TT susceptible males, this trend is not replicated in the lower frequency CC genotyped susceptible males. Right ventricle-to-total ventricle ratios did not vary significantly between genotypes on chromosome 2 in females (Table 2-3). The average RVTV for SNP 2.713 for resistant males varied significantly between genotypes ($P < 0.05$, Table 2-2). The GG genotype males have the highest RVTV average ratio for the ascites resistant males, and this genotype has the lowest frequency of ascites (GG-29% vs AG-55%, AA-58%; Table 2-2). Similarly, homozygous AA genotype susceptible males have a significantly higher RVTV ratio (0.47) when compared to AG (0.43) or GG (0.43) individuals. Overall, the higher RVTV ratio for a genotype is associated with the highest percent ascites incidence between all 2.713 genotypes. The GG ascites susceptible males maintain an RVTV average approximately equal to that of AG genotypes; however, due to the low number of GG susceptible individuals ($n = 4$), it is not significantly different from the RVTV ratios representative of the other genotypes.

For all three chromosome Z SNPs, females (the heterogametic sex) have only two possible genotypes, versus the three possible genotypes found in males. All Z chromosome SNPs in males appear to be statistically similar in RVTV ratio averages across all genotypes. In the case of SNP Z.600 resistant females, the C genotype individuals have significantly higher RVTV values (0.32) than T genotype individuals (0.29; Table 2-3). For the Z.611 locus,

susceptible females have higher RVTV values in T genotype compared to C females. Interestingly, the variation seen in the RVTV values of both susceptible males and females does not correspond to an expected positive correlation in ascites incidence calculations for genotypes at every SNP loci.

No significant differences were detected in Chi-square analyses of observed versus expected counts for any genotype from males and females of all five SNP, although, significance was approached for GG male genotype on SNP 2.713 and the TT genotype on SNP Z.611 (P-value = 0.06). These males also represent the lowest ascites percent incidence compared to all other genotypes in both sexes.

DISCUSSION

Multiple GWAS were 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. Ascites resistant and susceptible individuals were evaluated on 29 chromosomes using a 60K SNP chip. Evaluation of P-values at each SNP loci as an averaged sliding window reduced the overall significance seen in a previously completed GWAS focusing on ascites incidence in REL line broilers (Krishnamoorthy et al., 2014). It is important to note that the regions previously identified on chromosome 9 were not significant in these analyses. The prior GWAS used a F_2 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 F_2 cross GWAS identified different regions than 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 indicates that a variation exists in the RVTV ratio between 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. RVTV values of susceptible individuals that do not follow this trend are not as likely to be directly correlated to ascites phenotype. Rather, these loci, and their associated RVTV ratios may be an artifact of linkage disequilibrium. High RVTV values, coupled with the high incidence of ascites exhibited

by CC genotyped individuals for both chromosome 2 SNP in susceptible males, dictate that this region can be used as an indicator for the potential of the broiler to develop ascites.

The region of significance identified on chromosome 2 contains two candidate genes, MC4R and CDH6. MC4R encodes 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 regulation of arterial pressures 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).

Within the Z chromosome region 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 myocyte lineage (Song et al., 2012) and is known to up regulate other genes known for cardiocyte 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 statistical significance for lower RVTV ratios compared to other genotypes ($P = 0.074$).

Ascites occurs due to 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 2-1. 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 (rs#)	Reference/ Alternative Allele (Strand)	Annealing Temp (°C)		Primer		Probe
2.708	2	70835627	14203518	T/C (Fwd)	56.4	F	CTCAGCTGGTCCTGCTAACAT	Probe 1	CTAAAGTATGAGTAtCCAAGTCTT[1]
						R	TCTGAGGGAGGGAAAAAGGT	Probe 2	CTAAAGTATGAGTAcCCAAGTC
2.713	2	71320330	14203691	A/G (Fwd)	52	F	TAATGGAAACAACCTCTGTGCTCTGGA	Probe 1	TCCTAtCCTGAAGAAAGAGCAAATAAAT
						R	GCCTCCCATGTCTTTGGCTTGGA	Probe 2	TCCTAcCCTGAAGAAAGAGCAAATA
Z.591	Z	59169596	10723172	C/T (Fwd)	67	F	GGGGGATAGAGGAGGCTGGTGT	Probe 1	TAcGACACAATAGGCTTTTCCATAAG
						R	TCACCCTGTCATCGTTTTTGAAACATG	Probe 2	TAtGACACAATAGGCTTTTCCATAAGT
Z.600	Z	60058344	14748694	T/C (Rev)	68	F	GTCCGGCTCTGTGTCTGCCCTGA	Probe 1	ACaAAGAGTGGAAATATGGATTTCCAGCATC
						R	TCCAACAGAACTCCCTGGTGTTTCACC	Probe 2	ACgAAGAGTGGAAATATGGATTTCCAGCAT
Z.611	Z	61154772	16774018	C/T (Rev)	59	F	AGGCATTGCTTCCTTCTGGGAGAAC	Probe 1	TGcTTGGATATTCATAAAGTTCTCCC
						R	CAGCTGTTAGTTTGGTGGGGGCTTT	Probe 2	TGtTTGGATATTCATAAAGTTCTCCCA

[1]Lower case letters indicate loci specific for SNP

Table 2-2. Data collected from single nucleotide polymorphisms from 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.

REL Line Males

SNP ID	SNP Location (Chr:Mbp)	Genotype	Ascites (%)	R Count (N)	S Count (N)	R[a] Freq	S[b] Freq	Pval	R RVTV Avg	S RVTV Avg
		TT	62%			0.35	0.52	0.12	0.31	0.46[a]
2.708	Gga2:70.83	TC	44%	74	81	0.47	0.34	0.21	0.32	0.42[b]
		CC	46%			0.18	0.14	0.53	0.32	0.49[a]
		AA	58%			0.42	0.49	0.52	0.30[b]	0.47[a]
2.713	Gga2:71.32	AG	55%	79	93	0.45	0.47	0.87	0.30[b]	0.44[b]
		GG	29%			0.13	0.04	0.06	0.35[a]	0.44[ab]
		CC	37%			0.28	0.22	0.38	0.29	0.44
Z.591	GgaZ:59.169	CT	43%	116	86	0.42	0.44	0.91	0.31	0.44
		TT	47%			0.28	0.34	0.50	0.29	0.44
		TT	46%			0.18	0.19	0.80	0.32	0.45
Z.600	GgaZ:60.058	TC	46%	189	151	0.44	0.48	0.70	0.31	0.44
		CC	42%			0.37	0.33	0.53	0.30	0.45
		CC	39%			0.50	0.59	0.41	0.30	0.45
Z.611	GgaZ:61.154	CT	36%	115	61	0.30	0.33	0.79	0.30	0.45
		TT	18%			0.20	0.08	0.06	0.29	0.43

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

Table 2-3. Data collected from single nucleotide polymorphisms from 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.

REL Line Females

SNP ID	SNP Location (Chr:Mbp)	Genotype	Ascites (%)	R Count (N)	S Count (N)	R[a] Freq	S[b] Freq	Pval	R RVTV Avg	S RVTV Avg
2.708	Gga2:70.83	TT	58%	78	104	0.51	0.54	0.81	0.32	0.46
		TC	57%			0.33	0.34	0.97	0.33	0.47
		CC	52%			0.16	0.12	0.60	0.32	0.48
2.713	Gga2:71.32	AA	62%	86	119	0.45	0.53	0.45	0.44	0.44
		AG	52%			0.50	0.39	0.26	0.46	0.46
		GG	69%			0.05	0.08	0.41	0.45	0.45
Z.591	GgaZ:59.169	C	46%	72	69	0.46	0.41	0.64	0.28	0.43
		T	51%			0.54	0.59	0.68	0.30	0.43
Z.600	GgaZ:60.058	T	45%	153	143	0.42	0.36	0.45	0.29[b]	0.45
		C	51%			0.58	0.64	0.55	0.32[a]	0.45
Z.611	GgaZ:61.154	C	52%	73	79	0.71	0.71	0.98	0.31	0.43[b]
		T	52%			0.29	0.29	0.97	0.29	0.46[a]

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

Figure 2-1. Genome wide association study results indicate a region of interest around 70 Mbp on chromosome 2 in resistant males comparing two 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.

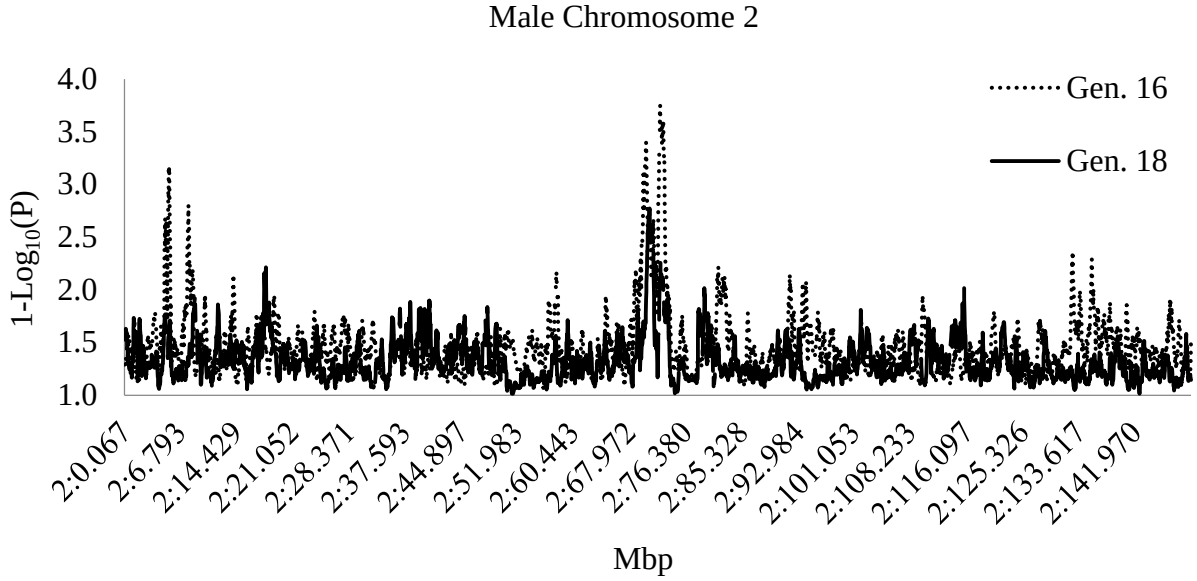
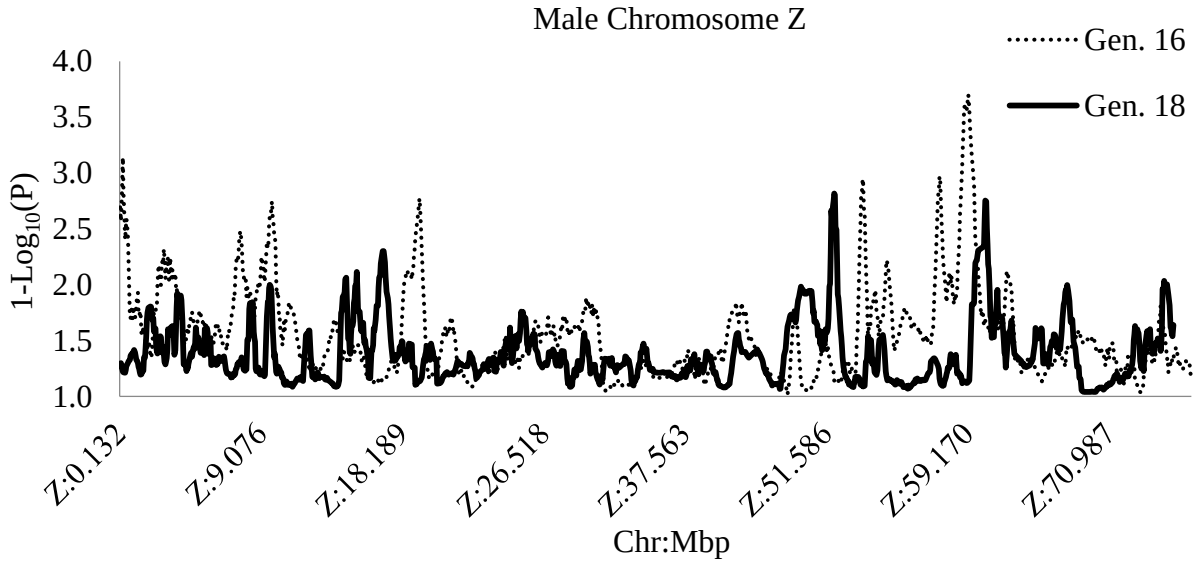


Figure 2-2. Genome wide association results indicating a region of interest around 60 Mbp on chromosome Z in susceptible males comparing two generations of REL line individuals. Single nucleotide polymorphism loci are identified as the corresponding Mbp along the Z chromosome. Association of SNP loci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



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CHAPTER 3

Marker assisted selection for ascites resistance in broilers using a chromosome Z locus

ABSTRACT

Ascites syndrome, or pulmonary hypertension, in broiler chickens remains an economically relevant disease in the poultry industry despite continuous efforts in management practices and genetic selection schemes to circumvent adverse effects in the broiler. Understanding ascites genetics will help continue the progress made in reducing ascites frequency that has already been achieved through utilizing selection techniques at the pedigree level. Here, we have assessed a single locus in a region of significance evaluated through multi-generational genome wide association studies in ascites susceptible individuals. Initially, a C/T SNP located at 60.058 Mbp on chromosome Z indicated association with ascites incidence in male broilers from. This SNP was used to genotype 576 male and female REL line broilers that were phenotyped for ascites in a six week trial in a hypobaric chamber. In both males and females there were no significant associations of ascites phenotype with genotype. However, detectable differences were present in the percent of phenotyped ascites resistant male offspring through evaluation of the parent genotypes. There were four possible parental crosses which result in a heterozygote male. Interestingly, the CT x CW and TT x CW crosses produce CT male individuals for which 63% and 36% of individuals are resistant, respectively. This data is consistent with a parental allele effect (imprinting) influencing ascites outcome; an affect which has never before been found in poultry.

INTRODUCTION

Generations of intensive genetic selection for fast growth and high yield has produced the modern broiler. Broilers of today are more efficient, produce more muscle mass, and less fat than the broilers of previous generations (Havenstein et al., 2003; Zuidhof et al., 2014). Rigorous selection practices have resulted in changes in the physiology of the broiler compared to its ancestor, which lead to novel diseases and disorders (Olkowski et al., 2007; Collins et al., 2014). Ascites syndrome in broiler chickens is a disease that is linked to selection pressures for economically important traits in meat-type chickens (Julian, 1998). Ascites is the manifestation of multiple unfavorable effects, which culminates in death (Julian, 2000). Selection is traditionally placed on economically important traits. These selective pressures do not emphasize non-economically relevant traits that are essential to the proper physiological function of the body, which has led to lung and heart being proportionally smaller to the body in the modern broiler (Schmidt et al., 2009).

Genetic selection against ascites incidence, along with implementation of management techniques such as reduced day length (Hassanzadeh et al., 2003) and feed restriction (Acar et al., 1995), have aided in the reduction of ascites occurrence over the last two decades. However, ascites remains an economically relevant disease in the poultry industry. The estimated cost due to ascites syndrome was estimated at $100 million per year in North America in 2015 (Tarrant et al., in review).

Genetic selection for resistant broilers includes challenging birds with invasive and non-invasive techniques to induce ascites (Wideman and Erf, 2002; Pavlidis et al., 2007; Wideman, 2014). Birds that prove to be resistant to ascites by displaying resilience to such methods as micro-particle injections are used as breeders. Environmental or overly-invasive challenge

techniques which result in ascites phenotyping through necropsy are used to determine family data. Siblings of the birds that do not succumb will become breeders for the next generation, which will hopefully provide an increased resistance to the offspring. These methods for selectively breeding resistant broilers are effective because of the genetic component to ascites incidence. Previous studies have indicated specific chromosomal regions associated with ascites frequency (Krishnamoorthy, et al., 2014). Unfortunately, selection for resistance can only go so far since resistance may be negatively correlated with production traits. Pulmonary hypertension and right ventricle failure have been positively correlated with body weight (Moghadam et al., 2001). Further, feed restriction can reduce ascites incidence, but will result in decreased breast yield (Acar et al., 1995). There are many suggestions for the chromosomal regions associated with ascites incidence, but there has yet to be a consensus on such a region in genetically independent lines (Rabie et al., 2005; Krishnamoorthy et al., 2014). The purpose of this study was to use a closed population of unselected, relaxed-mated (**REL**) line birds originating with a population of broilers from an elite male line of the 1990s to evaluate a SNP identified from a locus on the Z chromosome which has indicated an association with ascites incidence in male broilers.

METHODS

Genome data

All chromosomal positions are relative to the ICGSC Gallus-gallus-4.0/galGal4 (GCA_000002315.2) assembly published in November 2011 (http://genome.ucsc.edu).

Bird stock

Relaxed (**REL**) line birds used in this study represent a pedigreed elite male boiler line representative of the 1990s (Pavlidis et al., 2007). The REL line is maintained under unselected conditions at the University of Arkansas under IACUC Protocol 15039.

Hypobaric Chamber Trials

A total of 481 male and female REL line broilers were raised in a total of three hypobaric chamber trials for a duration of six weeks under IACUC protocol 15040. The chamber houses four stainless steel batteries consisting of 10 pens in each battery measuring 0.6 x 0.6 x 0.3 m. The pressure was set to simulate approximately 2900 m above sea level. Temperature and ventilation were monitored and adjusted daily in the chamber, in accordance to industry standards. Birds were provided ad libidum feed and water through access to waters and trough feeders. At the end of the six-week trial all birds were necropsied to be phenotyped as ascites resistant or susceptible based on cardiac morphometrics and abdominal fluid accumulation.

Floor Trial and Processing

Siblings representing most of the parent genotypic crosses were raised at sea level and processed at eight weeks of age. Birds were allowed ad libidum feed and water. Weights were recorded at d 0, d 14, d28, d35, and d42. Absolute weights were recorded for whole bird without giblets (**WOG**), ab fat, chilled weight, rack, breast fillet, tenders, wings, and legs. Percent weights were also calculated for breast, dark, fat, and WOG. Finally, pH and color values (L*, a*, and b*) were measured on the right breast fillet. Each measurement was taken three times, and the results were averaged for each individual.

Blood Extraction, DNA Isolation, and Genotyping

Blood was collected from the wing vein in breeders, or from between the toes of chicks at four days of age. DNA was isolated using methods described in Bailes et al. (2007). A previously conducted genome wide association study completed was completed on generation 16 and 18 indicated an association with ascites incidence in male individuals (Tarrant et al., in review). Through this study a C/T SNP was identified on the Z chromosome in a region indicated significance around 60 Mbp (rs14748694). To further investigate this locus an exonuclease assay, using TaqMan® probes, was developed for genotyping this SNP using real-time PCR as described in Tarrant et al. (in review). Assay mix included 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, for a total volume of 20 μL. Real time PCR followed two steps of 90°C for 30 seconds, 10 cycles of 90°C for 15 seconds 68ºC for 30 seconds, followed by 30 cycles of 90°C for 15 seconds, 68°C for 30 seconds with a plate read.

Statistical Analysis

A Chi-squared analysis was used to determine the statistical variation in the raw count totals for ascites phenotyped individuals. Processing values were evaluated using Tukey HSD with an alpha value of 0.05.

RESULTS

The SNP selected from the previous GWAS studies (Tarrant et al., in review) was employed as a representative for the Z chromosomal region around 60 Mbp. The locus was identified as being sex-specific to males. The two GWAS represent two generations, separated by two years, of the REL line birds phenotyped for ascites phenotype. According to the ICGSC

Gallus_gallus-4.0/galGal4 assembly the SNP is approximately 80 kbp upstream of the Ensembl gene myocyte enhancement factor – 2 (**Mef2c**). Mef2c falls within the MADS-box transcription factor family (Black and Olson, 1998). It is required in the process of myogenesis, specifically in the differentiation of ventricular cardiomyocytes (Vong et al., 2006). The importance of the Mef2c gene to healthy heart development can be evaluated in knock out mice, whose loss of the Mef2c gene not only reduces the size of the left ventricle, but prevents right ventricle development altogether (Lin et al., 1997).

REL line breeders were genotyped for the SNP and assigned to breeding groups based on pre-determined genotypes. Pooled semen was used to inseminate hens within each group. Male and female breeders within each group were selected to produce offspring of every possible genotypic combination for hypobaric trials. Raw counts of genotypes and their phenotypes from the hypobaric challenge are presented in Table 3-1. Females are the heterogametic sex, therefore, they are presented as having only two genotypes, CW and TW. In male offspring, the percent of individuals that were phenotyped as ascites resistant was highest in homozygous C individuals, while heterozygous individuals had the lowest percent resistant. CW genotype females exhibit a higher present resistance. A Chi-squared test was applied to individual counts in males and females separately. Although the percent of resistant individuals for each genotype is variable, there are no significant differences in either sex, or between allele frequencies when comparing the sexes. It is likely the relatively low sample size in males and females exhibiting C alleles contributes to the lack of significant variation. It was determined that the lowest frequency genotypes in the parent generation were the CC sires and the CW dams. Compared to the CT genotype (0.41) and the TT genotype (0.54) the frequency of CC sires was 0.05. Further,

the CW dam genotype frequency was at 0.26 compared to CW females compared to 0.74 for TW dams.

The region selected for intensive study initially indicted an association with ascites in both REL line generations 16 and 18. Using selected breedings to produce offspring genotyped and raised in the hypobaric chamber did not relay findings determined through the GWAS. To understand why regions of significance were detected from the initial GWAS it was then decided to examine the parent genotypes. Table 3-2 shows the parental genotype crosses associated with each male genotype outcome. Also shown are the raw counts for all offspring in each cross group, raw counts for assignments resistant phenotype offspring, and raw counts for same susceptible phenotype offspring. CC genotype male offspring that are the result of CC x CW cross were excluded from further analysis because there were so few representatives. The CT x CW crosses produce male offspring that are genotyped as either CC or CT. This cross produced the individuals that were the most resistant to high altitude challenge (62% and 63%). Four parental crosses result in heterozygote offspring, but crosses are significantly variable in their resistance to ascites. Specifically, the highly resistant CT x CW cross (63%) and the TT x CW cross (36%). Both crosses resulting in TT genotyped male offspring did not vary significantly. Female offspring percent resistance was not variable between crosses resulting in CW genotypes, or within TW genotypes (Table 3-3). Though the TW resistant individuals are approximately 10% lower calculated resistance column of each cross when compared to CW genotyped females, these values are not significantly different.

Appraisal of processing data revealed male genotypes showed no significant differences in parts evaluated for absolute weight (Table 3-4), percent of carcass and meat weights (Table 3-5), or in breast fillet pH and color (Table 3-6). At d 0 and d 14 significant differences existed in

body weight, but these differences are not detectable at d 21, 28, 35 or 42 (Figure 3-1A). Processing data was also evaluated as parent genotypic crosses. CC sires were not included in mating combinations used for this study. Mean male wing and leg weights of the TT x CW cross was significantly larger than that of the CT x TW cross (Table 3-7). The difference of WOG weight of TT x CW approaches significance in comparison to CT x TW at a P-value of 0.12. TT x CW males have significantly larger mean body weights at d 0, d 14, and at processing d 42 (Figure 3-1B). As would be expected, the TT x CW crosses exhibiting larger mean values are associated with a reduced percent of ascites resistance (36%) compared to that of CT x TW (57%). Despite these distinctions, breast and tender weights, percent weights (Table 3-8), and breast traits (Table 3-9) showed no significant differences. Homozygous T males which were processed that had resulted from a CT x TW or TT x TW cross showed no significant differences in any trait measured (Tables 3-10, 11, and 12).

Female body weights differed at d 0 and d 14 by genotype (Figure 3-2A), but significant differences between CW and TW females did not exist in processing trait evaluations (Tables 13, 14, 15). Absolute weight values (Table 3-16) and percent weight values (Table 3-17) showed no differences in crosses resulting in CW female offspring. The b* value was greater in CT x CW crosses versus CT x TW crosses, but all other breast traits showed no differences (Table 3-18). Four crosses were evaluated that are associated with TW female offspring. For each of the traits measured, or calculated, differences did not arise (Tables 3-19, 20, and 21). Crosses creating both CW and TW offspring exhibit significant differences in body weight at d 0, but this variation did not exist in any of the other time points measured (Figure 3-2B, C).

CONCLUSIONS

Major changes in the physiology of the chicken have resulted in modern broilers afflicted with ascites incidence at an increased rate compared to broilers from 20 years ago (Figure 3-3). Broiler mortality due to high altitude challenge in the hypobaric chamber is reported between individuals representing the unselected REL line from the 1990s and individuals from two modern broiler lines. By d 34 30% of the REL line birds had succumbed to ascites, while 50% of the modern line birds died under high altitude conditions. This is likely a direct result of selective breeding with emphasis on the increase of body weight exhibiting the associated influx in ascites incidence due to this selection practice (Krishnamoorthy et al., 2016, unpublished data).

Ultimately, phenotype is a direct result of genotype and the environment. Of course environment plays a pivotal role in the manifestation of disease, as in ascites syndrome in broilers. Induction of ascites through environmental manipulation has been used for the past three decades to further explore details in ascites etiology (Julian and Wilson, 1992; Shlosber et al., 1996; Ipek and Sahan, 2006; Shi et al., 2014; Tekeli, 2014).

Genome wide association studies identify regions in the genome that indicate significant associations to economically important traits in valuable livestock species (Guo et al., 2012; Wang et al., 2014; Reyer et al., 2015; Zhang et al., 2015). Identification of QTL have indicated few complex traits or diseases explored through genome wide association studies are explained solely by genetic variation (Altmüller et al., 2001; Manolio et al., 2009). For this reason there is cause to suggest additional sources genetic correlation to traits be explored.

In concept, paternal and maternal genetic material that is passed to an offspring have an equal chance of being active (Barlow and Bartolomei, 2014). Epigenetics challenges traditional

ideas of gene activation. Epigenetic processes lead to alterations in gene activity, with no corresponding changes in the DNA sequence (Weinhold, 2006).

Maternal effects are defined as the influence on the offspring phenotype due to maternal phenotype or genotype (Wolf and Wade, 2009). Maternal effects for ascites related traits have been documented in cold stressed broilers (Pakdel et al., 2005) and in broilers raised in normal rearing conditions (Navarro, et al., 2006). Parent – of – origin – dependent effects indicate that gene function is directly related to chromosomal inheritance from the father or the mother (Reik and Walter, 2001). Genomic imprinting is the disparity of genotypic expression owing to the source of inheritance of genetic material (Hall, 1997). Specifically, gene expression of the male or female offspring is limited to the chromosome of either parent in diploid organisms if that gene is imprinted. This epigenetic mechanism has been identified in arthropods (Anaka et al., 2009), marsupials (Renfree et al., 2009), human disorders (Nicholls, 2000; Dong et al., 2005), and in economically important livestock species (Thomsen et al., 2004; O'Doherty et al., 2015). These effects are caused by processes such as DNA methylation and chromatid modification (Weinhold, 2006).

Conflicting reports of monoallelic (Koski et al., 2000) versus biallelic (O'Neill et al., 2000) gene expression in chickens indicates that orthologs of known imprinted genes in mammals may be expressed epigenetically. Although epigenetic mechanisms are not well described in avian species (Fresard et al., 2013) it is possible that the discrepancy detected in ascites resistance between parental crosses for this study are due to underlying epigenetic factors. Interestingly, the respective parental crosses for the two ascites – resistant – extreme heterozygote male offspring, CT x CW (63% resistance) and TT x CW (36% resistance), do not fall into traditional parent – of – origin effects. Presumably, the CT heterozygous male offspring

obtains the T allele from the sire, and the C allele from the dam, in both the CT x CW and TT x CW crosses. This negates the hypothesis that obtaining an allele from the sire or dam influences ascites resistance based on the data presented here, but does not completely forgo the conclusion that imprinting is at work. Further, results from processing data obtained from this study indicate that the elimination of the most ascites-susceptible cross (TT x CW) would do little to impact the economic value of the male bird being produced from the REL line.

Table 3-1. Data collected after the completion of the hypobaric chamber trials. Individuals were genotyped for a C/T SNP on the Z chromosome and phenotyped as ascites resistant or susceptible. A calculated percent of resistant individuals for each genotype is also included.

	Genotype	Total Count (N)	Ascites Resistant (N)	Ascites Susceptible (N)	% Resistant
Males	CC	40	25	15	63%
	CT	142	74	68	52%
	TT	88	49	39	56%
Females	CW	63	35	28	56%
	TW	148	68	80	46%

Table 3-2. Counts of ascites resistant and susceptible males with associated genotype and parental genotypic cross.

Genotype	Parental Crosses (Sire x Dam)	Total Count (N)	Ascites Resistant (N)	Ascites Susceptible (N)	% Resistant
CC	CT x CW	37	23	14	62%[a]
CT	CT x CW	52	33	19	63%[a]
CT	CT x TW	40	20	20	50%[ab]
CT	CC x TW	14	8	6	57%[ab]
CT	TT x CW	36	13	23	36%[b]
TT	CT x TW	35	19	16	54%[ab]
TT	TT x TW	53	30	23	57%[ab]

[1]Different letter superscripts indicate significant differences ($P \leq 0.05$).

Table 3-3. Counts of ascites resistant and susceptible females with associated genotype and parental genotypic cross.

Genotype	Parental Crosses (Sire x Dam)	Total Count (N)	Ascites Resistant (N)	Ascites Susceptible (N)	% Resistant
CW	CC x TW	9	5	4	56%
CW	CT x CW	54	30	24	56%
CW	CT x TW	43	23	20	53%
TW	CT x CW	36	19	17	53%
TW	CT x TW	50	23	27	46%
TW	TT x CW	32	15	17	47%
TW	TT x TW	66	30	36	45%

Table 3-4. Absolute weight means[1] for male offspring.

		Male Genotypes		
		CC	CT	TT
	% Resistance[2]	63	52	56
	N	36	87	57
Absolute Weights (g)	WOG[3]	2018 ± 38	2003 ± 24	1946 ± 29
	Ab Fat	96 ± 3	91 ± 2	89 ± 2
	Chilled	2079 ± 40	2059 ± 25	2002 ± 30
	Breast	395 ± 10	391 ± 6	384 ± 8
	Tenders	102 ± 2	102 ± 1	101 ± 2
	Wings	221 ± 3	219 ± 2	214 ± 2
	Legs	299 ± 6	297 ± 3	290 ± 4
	Thighs	396 ± 9	397 ± 5	385 ± 6
	Rack	648 ± 13	636 ± 8	610 ± 10

[1]Average means ± SE in grams; [2] Percentage mortality due to ascites; [3] Carcass weight without giblets.

*Means within the same row with superscripts that differ are significantly different ($p<0.05$)

Table 3-5. Percent weight means[1] for male offspring.

		Male Genotypes		
		CC	CT	TT
	% Resistance[2]	63	52	56
	N	36	87	57
Percent Weights	% Breast[3]	0.1721 ± 0.0025	0.1721 ± 0.0016	0.1728 ± 0.0019
	% WOG[4]	0.6996 ± 0.0024	0.6995 ± 0.0016	0.6981 ± 0.0019
	% Fat[5]	0.0335 ± 0.0012	0.0320 ± 0.0008	0.0317 ± 0.0299
	% Dark[6]	0.2412 ± 0.0026	0.2416 ± 0.0017	0.2410 ± 0.0020

[1]Average percent means ± SE in grams; [2]Percentage mortality due to ascites; [3]((Pectoralis Major + Pectoralis Minor)/Dock Weight) *100; [4]((WOG)/Dock Weight); [5](Abdominal Fat/Dock Weight)*100; [6]((Thigh + Leg)/Dock Weight)*100.

Table 3-6. Averaged breast fillet traits for male offspring.

		Male Genotypes		
		CC	CT	TT
	% Resistance[1]	63	52	56
	N	36	87	57
Breast Traits	pH	5.86 ± 0.11	5.78 ± 0.06	5.65 ± 0.07
	L*	49.29 ± 0.45	49.43 ± 0.29	49.99 ± 0.34
	a*	4.20 ± 0.16	4.28 ± 0.10	4.20 ± 0.12
	b*	1.19 ± 0.23	1.06 ± 0.15	1.44 ± 0.18

[1]Percentage mortality due to ascites.

Table 3-7. Absolute weight means[1] for parental crosses resulting in heterozygous male offspring.

		CT Offspring		
	Parental Cross	CTxCW	CTxTW	TTxCW
	% Resistance[2]	50	57	36
	N	24	28	24
Absolute Weights (g)	WOG[3]	1967 ± 37^{ab}	1960 ± 40^{b}	2087 ± 32^{a}
	Ab Fat	93 ± 3	88 ± 4	93 ± 3
	Chilled	2018 ± 38	2028 ± 41	2114 ± 37
	Breast	381 ± 10	390 ± 12	399 ± 10
	Tenders	97 ± 3^{ab}	102 ± 3^{b}	107 ± 3^{a}
	Wings	215 ± 3^{b}	213 ± 4^{b}	2279± 3^{a}
	Legs	291 ± 7^{ab}	286 ± 5^{b}	312 ± 5^{a}
	Thighs	390 ± 11^{ab}	383 ± 10^{b}	415 ± 9^{a}
	Rack	635 ± 14	625 ± 13	649 ± 13

[1]Average means ± SE in grams; [2] Percentage mortality due to ascites; [3] Carcass weight without giblets.

*Means within the same row with superscripts that differ are significantly different ($p<0.05$)

Table 3-8. Percent weight means[1] for parental crosses resulting in heterozygous male offspring.

			CT Offspring	
	Parental Cross	CTxCW	CTxCW	CTxCW
	% Resistance[2]	50	57	36
	N	24	28	24
Percent Weights	% Breast[3]	0.1706 ± 0.0031	0.1752 ± 0.0030	0.1704 ± 0.0023
	% WOG[4]	0.7023 ± 0.0029	0.6993 ± 0.0033	0.6972 ± 0.0025
	% Fat[5]	0.0333 ± 0.0012	0.0317 ± 0.0016	0.0317 ± 0.0292
	% Dark[6]	0.2404 ± 0.0036	0.2391 ± 0.0027	0.2441 ± 0.0028

[1]Average percent means ± SE in grams; [2]Percentage mortality due to ascites; [3]((Pectoralis Major + Pectoralis Minor)/Dock Weight) *100; [4]((WOG)/Dock Weight); [5](Abdominal Fat/Dock Weight)*100; [6]((Thigh + Leg)/Dock Weight)*100.

Table 3-9. Averaged breast fillet traits for parental crosses resulting in heterozygous male offspring.

			CT Offspring	
	Parental Cross	CTxCW	CTxCW	CTxCW
	% Resistance[1]	50	57	36
	N	24	28	24
Breast Traits	pH	5.84 ± 0.09	5.72 ± 0.08	5.78 ± 0.08
	L*	49.09 ± 0.55	49.22 ± 0.54	49.85 ± 0.40
	a*	4.53 ± 0.18	4.23 ± 0.19	4.14 ± 0.17
	b*	1.22 ± 0.28	0.71 ± 0.27	1.31 ± 0.17

[1]Percentage mortality due to ascites.

Table 3-10. Absolute weight means[1] for parental crosses resulting in homozygous T male offspring.

		TT Offspring	
	Parental Cross	CTxTW	TTxTW
	% Resistance[2]	54	57
	N	22	35
Absolute Weights (g)	WOG[3]	1921 ± 71	1950 ± 43
	Ab Fat	86 ± 5	90 ± 4
	Chilled	1981 ± 75	2001 ± 45
	Breast	385 ± 17	381 ± 11
	Tenders	100 ± 4	101 ± 3
	Wings	210 ± 5	216 ± 4
	Legs	279 ± 10	294 ± 6
	Thighs	372 ± 17	390 ± 8
	Rack	615 ± 23	608 ± 15

[1]Average means ± SE in grams; [2] Percentage mortality due to ascites; [3] Carcass weight without giblets.

Table 3-11. Percent weight means[1] for parental crosses resulting in homozygous T male offspring.

		TT Offspring	
	Parental Cross	CTxTW	TTxTW
	% Resistance[2]	54	57
	N	22	35
Percent Weights	% Breast[3]	0.1752 ± 0.0031	0.1709 ± 0.0031
	% WOG[4]	0.7010 ± 0.0024	0.6955 ± 0.0028
	% Fat[5]	0.0308 ± 0.0014	0.0322 ± 0.0013
	% Dark[6]	0.2355 ± 0.0038	0.2431 ± 0.0020

[1]Average percent means ± SE in grams; [2]Percentage mortality due to ascites; [3]((Pectoralis Major + Pectoralis Minor)/Dock Weight) *100; [4]((WOG)/Dock Weight); [5](Abdominal Fat/Dock Weight)*100; [6]((Thigh + Leg)/Dock Weight)*100.

Table 3-12. Averaged breast fillet traits resulting in homozygous T male offspring.

		TT Offspring	
	Parental Cross	CTxTW	TTxTW
	% Resistance[1]	54	57
	N	22	35
Breast Traits	pH	5.58 ± 0.15	5.66 ± 0.10
	L*	50.45 ± 0.70	49.77 ± 0.51
	a*	4.13 ± 0.22	4.22 ± 0.19
	b*	1.37 ± 0.32	1.63 ± 0.28

[1]Percentage mortality due to ascites.

Table 3-13. Absolute weight means[1] for female offspring.

		Female Genotypes	
	Parental Cross	CW	TW
	% Resistance[2]	56	46
	N	46	147
Absolute Weights (g)	WOG[3]	1686 ± 19	1679 ± 16
	Ab Fat	90 ± 3	90 ± 1
	Chilled	1742 ± 20	1742 ± 17
	Breast	345 ± 6	338 ± 4
	Tenders	93 ± 1	94 ± 1
	Wings	183 ± 1	185 ± 1
	Legs	236 ± 2	238 ± 2
	Thighs	318 ± 5	321 ± 4
	Rack	541 ± 8	537 ± 5

[1]Average means ± SE in grams; [2] Percentage mortality due to ascites; [3] Carcass weight without giblets.

Table 3-14. Percent weight means[1] for female offspring.

		Female Genotypes	
	Parental Cross	CW	TW
	% Resistance[2]	56	46
	N	46	147
Percent Weights	% Breast[3]	0.1822 ± 0.0021	0.1786 ± 0.0013
	% WOG[4]	0.7001 ± 0.0027	0.6954 ± 0.0015
	% Fat[5]	0.0376 ± 0.0010	0.0372 ± 0.0007
	% Dark[6]	0.2302 ± 0.0017	0.2323 ± 0.0013

[1]Average percent means ± SE in grams; [2]Percentage mortality due to ascites; [3]((Pectoralis Major + Pectoralis Minor)/Dock Weight) *100; [4]((WOG)/Dock Weight); [5](Abdominal Fat/Dock Weight)*100; [6]((Thigh + Leg)/Dock Weight)*100.

Table 3-15. Averaged breast fillet traits for female offspring.

		Female Genotypes	
	Parental Cross	CW	TW
	% Resistance[1]	56	46
	N	46	147
Breast Traits	pH	5.62 ± 0.09	5.66 ± 0.05
	L*	51.36 ± 0.30	50.89 ± 0.22
	a*	3.75 ± 0.14	3.92 ± 0.07
	b*	2.42 ± 0.20	2.22 ± 0.11

[1]Percentage mortality due to ascites.

Table 3-16. Absolute weight means[1] for parental crosses resulting in hemizygous C female offspring.

		CW Offspring	
	Parental Cross	CTxCW	CTxTW
	% Resistance[2]	56	53
	N	25	21
Absolute Weights (g)	WOG[3]	1695 ± 28	1675 ± 27
	Ab Fat	94 ± 4	85 ± 3
	Chilled	1752 ± 29	1730 ± 29
	Breast	345 ± 9	346 ± 8
	Tenders	95 ± 2	91 ± 1
	Wings	183 ± 2	183 ± 2
	Legs	238 ± 3	233 ± 4
	Thighs	324 ± 6	311 ± 7
	Rack	540 ± 11	541 ± 12

[1]Average means ± SE in grams; [2]percent mortality due to ascites incidence for each genotype; [3]carcass without giblets.

Table 3-17. Percent weight means[1] for parental crosses resulting in hemizygous C female offspring.

		CW Offspring	
	Parental Cross	CTxCW	CTxTW
	% Resistance[2]	56	53
	N	25	21
Percent Weights	% Breast[3]	0.1811 ± 0.0031	0.1834 ± 0.0026
	% WOG[4]	0.6977 ± 0.0034	0.7029 ± 0.0044
	% Fat[5]	0.0392 ± 0.0018	0.0359 ± 0.0013
	% Dark[6]	0.2315 ± 0.0021	0.2288 ± 0.0027

[1]Average percent means ± SE in grams; [2]Percentage mortality due to ascites; [3]((Pectoralis Major + Pectoralis Minor)/Dock Weight) *100; [4]((WOG)/Dock Weight); [5](Abdominal Fat/Dock Weight)*100; [6]((Thigh + Leg)/Dock Weight)*100.

Table 3-18. Averaged breast fillet traits resulting in hemizygous C female offspring.

		CW Offspring	
	Parental Cross	CTxCW	CTxTW
	% Resistance[1]	56	53
	N	25	21
Breast Traits	pH	5.74 ± 0.09	5.45 ± 0.16
	L*	51.45 ± 0.34	51.24 ± 0.52
	a*	3.81 ± 0.23	3.68 ± 0.14
	b*	2.88 ± 0.26[a]	1.88 ± 0.27[b]

[1]Percentage mortality due to ascites.

Table 3-19. Absolute weight means[1] for parental crosses resulting in hemizygous T female offspring.

		TW Offspring			
	Parental Cross	CTxCW	CTxTW	TTxCW	TTxTW
	% Resistance[2]	53	46	47	45
	N	24	29	44	50
Absolute Weights (g)	WOG[3]	1700 ± 36	1655 ± 40	1674 ± 29	1687 ± 26
	Ab Fat	94 ± 3	86 ± 3	86 ± 2	94 ± 4
	Chilled	1762 ± 39	1715 ± 42	1747 ± 33	1742 ± 27
	Breast	342 ± 12	341 ± 11	334 ± 7	337 ± 7
	Tenders	94 ± 3	91 ± 2	94 ± 2	94 ± 2
	Wings	186 ± 3	184 ± 3	185 ± 2	185 ± 2
	Legs	241 ± 4	231 ± 6	240 ± 4	238 ± 3
	Thighs	323 ± 8	316 ± 9	326 ± 8	320 ± 6
	Rack	551 ± 12	532 ± 13	531 ± 10	540 ± 9

[1]Average means ± SE in grams; [2] Percentage mortality due to ascites; [3] Carcass weight without giblets.

Table 3-20. Percent weight means[1] for parental crosses resulting in hemizygous T female offspring.

		TW Offspring			
	Parental Cross	CTxCW	CTxTW	TTxCW	TTxTW
	% Resistance[2]	53	46	47	45
	N	24	29	44	50
Percent Weights	% Breast[3]	0.1789 ± 0.0033	0.1810 ± 0.0030	0.1776 ± 0.0019	0.1777 ± 0.0023
	% WOG[4]	0.7007 ± 0.0030	0.6960 ± 0.0033	0.6948 ± 0.0018	0.6929 ± 0.0033
	% Fat[5]	0.0388 ± 0.0012	0.0365 ± 0.0011	0.0356 ± 0.0338	0.0381 ± 0.0010
	% Dark[6]	0.2327 ± 0.0028	0.2306 ± 0.0030	0.2351 ± 0.0022	0.2304 ± 0.0022

[1]Average percent means ± SE in grams; [2]Percentage mortality due to ascites; [3]((Pectoralis Major + Pectoralis Minor)/Dock Weight) *100; [4]((WOG)/Dock Weight); [5](Abdominal Fat/Dock Weight)*100; [6]((Thigh + Leg)/Dock Weight)*100.

Table 3-21. Averaged breast fillet traits resulting in hemizygous T female offspring.

		TW Offspring			
	Parental Cross	CTxCW	CTxTW	TTxCW	TTxTW
	% Resistance[2]	53	46	47	45
	N	24	29	44	50
Breast Traits	pH	5.74 ± 0.15	5.67 ± 0.09	5.63 ± 0.09	5.62 ± 0.06
	L*	51.71 ± 0.64	50.93 ± 0.48	50.90 ± 0.37	50.41 ± 0.36
	a*	3.81 ± 0.18	3.84 ± 0.14	4.10 ± 0.11	3.85 ± 0.13
	b*	2.48 ± 0.32	2.33 ± 0.21	2.16 ± 0.20	2.07 ± 0.15

[1]Percentage mortality due to ascites.

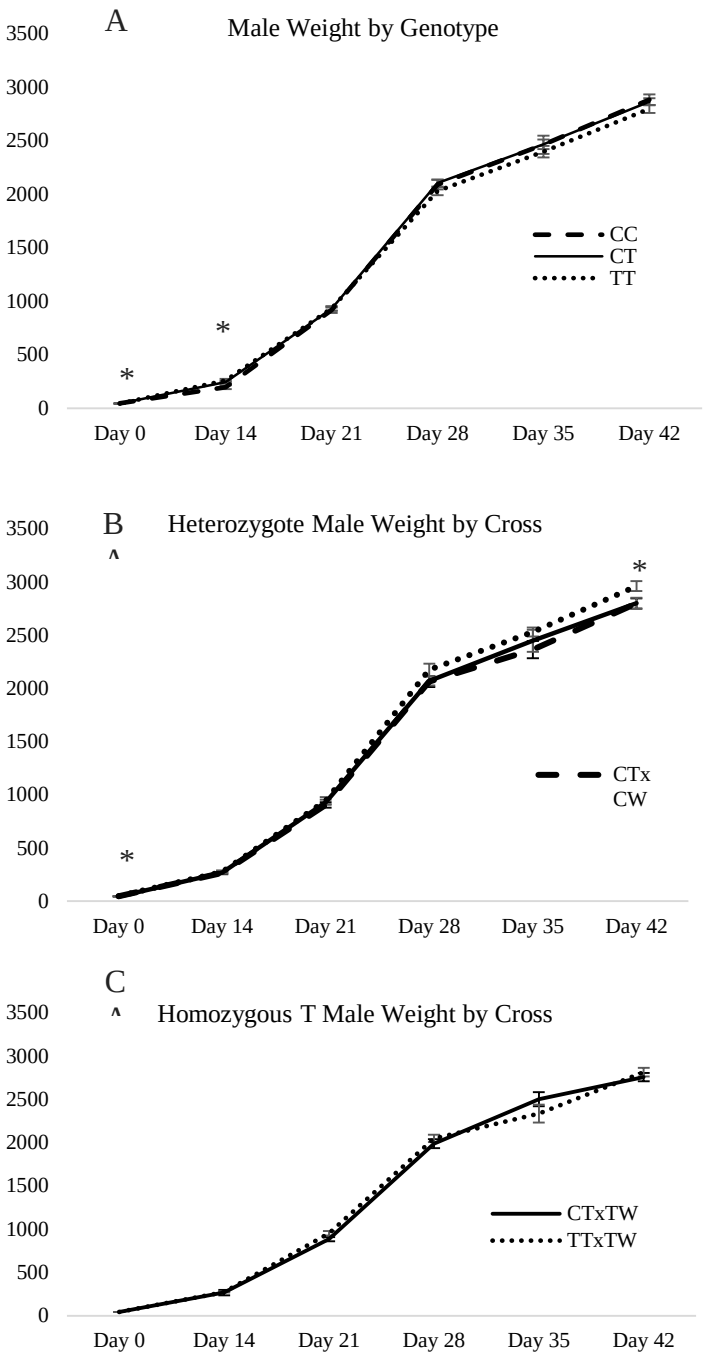

Figure 3-1. Growth of male broilers measured over 42 days ± SE. Birds are displayed by their SNP genotype (A). Additionally, heterozygote offspring (B) and homozygous T offspring (C) are shown with respect to their parent genotypic crosses.

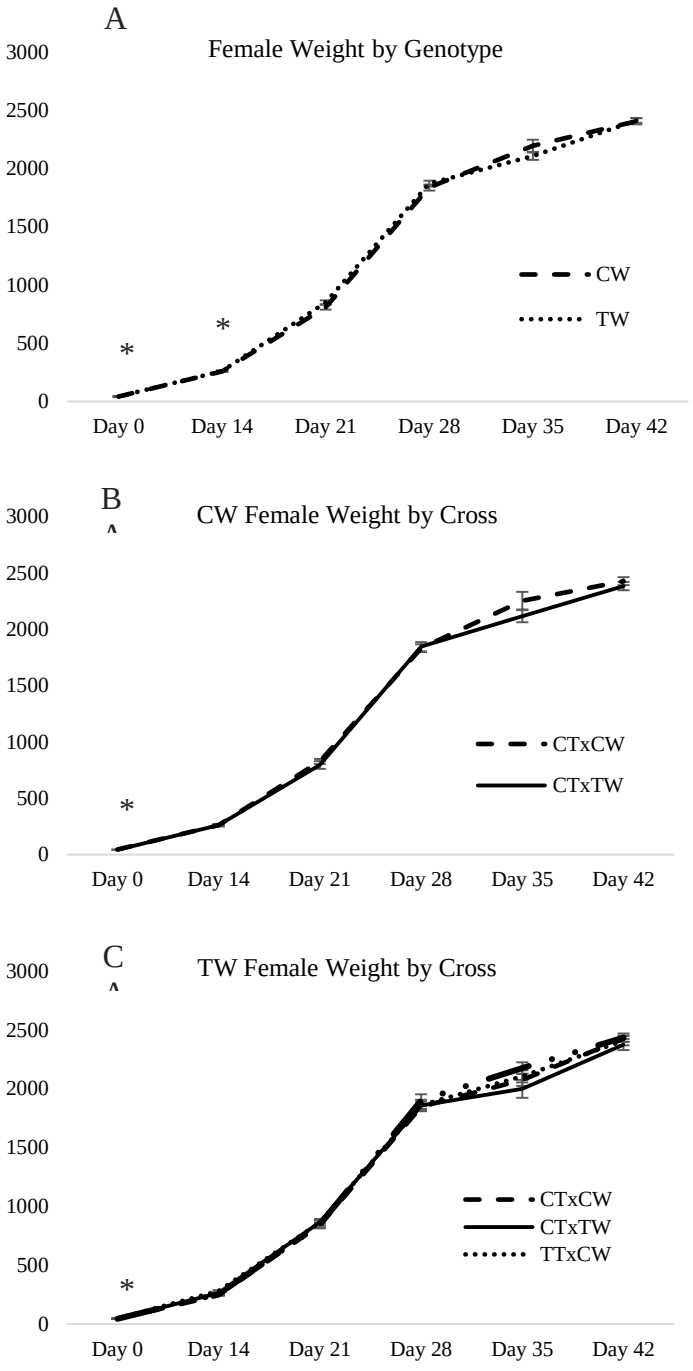

Figure 3-2. Growth of female broilers measured over 42 days ± SE. Birds are displayed by their SNP genotype (A). Additionally, heterozygote offspring (B) and homozygous T offspring (C) are shown with respect to their parent genotypic crosses.

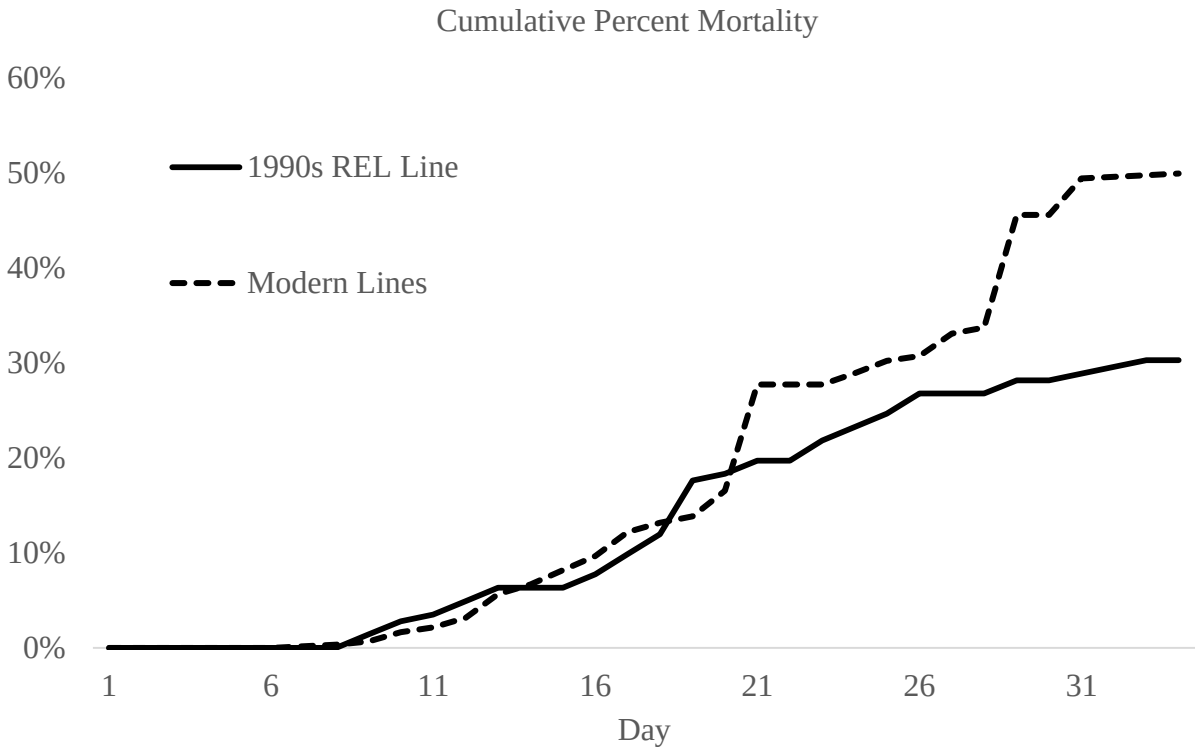

Figure 3-3. Mortality due to ascites of a 1990s unselected REL line and data combined from two modern genetic lines when challenged in a high-altitude simulated environment (unpublished data).

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CHAPTER 4

Predicting ascites incidence in simulated altitude-challenge using single nucleotide polymorphisms identified in multi-generational genome wide association studies

ABSTRACT

Assessing pedigreed broiler lines for ascites resistance in an industry setting is time consuming and reduces genetic accuracy with the implementation of sibling selection. The purpose of this study is to evaluate the effectiveness of developing prediction models produced with SNPs with the goal of predicting ascites incidence. Ascites is the manifestation of a series of adverse changes in a broiler which results in losses estimated at $100 million/year in the U.S. A multi-generational genome wide association study (GWAS) in an unselected REL-line maintained at the University of Arkansas since the 1990s identified chromosomal regions associated with ascites incidence in males when challenged at high altitude. From the identified regions of significance 8 SNPs were identified on chromosome 11, and 12 SNP were identified on chromosome Z. Ascites phenotype and genotype data were determined for 295 male and female individuals from lines originating with the REL line. Five regression modeling techniques were compared for their ascites predictive ability using a 70/30 validation. For both males and females the neural networking model was the best fit prediction model. In males the training and validation data set R^2 were 0.929 and 0.942, respectively. Reduction of the model to the 13 most important SNPs resulted in an increase in the prediction accuracy and fit of the model to R^2 values of 0.999 and 0.998. In females the training and validation data set R^2 were 0.944 and 0928, respectively; although, reduction in the number of SNP inputs into the model decreased the overall model robustness. These models indicate we have elucidated the genetic predictors to ascites outcome in male broilers from an elite line of the 1990s with a high level of accuracy.
Keywords: broiler, ascites, QTL, neural network

INTRODUCTION

Ascites, or pulmonary hypertension syndrome, is an accumulation of adverse physiological changes that occur in fast growing broilers (Julian and Wilson, 1992). At the beginning of onset, oxygenation of the body is maintained through an elevation in the pulmonary arterial pressure, followed by an increase in right ventricle work load (Julian, et al. 1987; Wideman and French, 1999). Eventually, this work load causes change in the right ventricle morphology that will result in hypertrophy and death. It is clear that in addition to environmental conditions, genetics plays a role in ascites incidence (Lubritz et al., 1995; Wideman and French, 1999; Wideman and French, 2000; Anthony et al., 2001; de Greef et al., 2001; Deeb et al., 2002; Pakdel et al., 2005; Pavlidis et al., 2007). Progress in selection for ascites resistance and susceptibility due to moderate to high heritabilities of ascites incidence and ascites-related traits like right ventricle-to-total ventricle ratio are reported in these studies.

Ascites incidence has been successfully reduced as a result of selection practices and through maintaining ideal flock management practices; however, it remains an economically important disease estimated to cause loss of $100 million annually (as reported in Tarrant et al., in review). For this reason commercial genetic companies consistently rely on methods to induce ascites to evaluate disease incidence in genetic lines so ascites susceptible individuals can be identified and removed from the breeding population.

Current methods for assessing ascites incidence in commercial lines includes assessing ascites development during chronic high altitude challenge in a hypobaric chamber (Pavlidis et al., 2007) and through acute challenged micro-particle injections (Wideman et al., 2002). While chronic evaluations methods require the bird to be terminated after ascites susceptibility or resistance is appraised, acute evaluations consider ascites frequency at a single time point in the

broiler's growth. To incorporate findings from chronic evaluations into broiler lines sibling selection is be used to integrate the genetics of ascites-resistant families into breeding schemes. Further, this method necessitates that birds are raised to several weeks of age, which is both costly and time consuming.

The development of a successful prediction model that allows ascites susceptibility to be evaluated immediately post-hatch would provide a time- and cost-efficient solution useful throughout the broiler's growth phase compared to current techniques being used to appraise ascites potential. This method of ascites screening would also result in the smallest genetic impact to the selected line by eliminating the requirement of sacrificing the bird, and the requisite for implementing sibling selection demanded in chronic evaluations. In this study, we consider several regression modeling types to estimate the outcome of ascites resistance and susceptibility in pedigreed broiler lines maintained since the 1990s.

METHODS

Bird Handling

The three hundred fourteen broilers used for this study were from two divergently selected ascites lines and an unselected control line, which all originate with an elite broiler line of the 1990s that is maintained at the University of Arkansas under IACUC Protocol 15040. To determine ascites phenotype, broilers were challenged in a high – altitude environment during a six weeks trial simulating 2900 m above sea level. Birds were raised with ad libitum feed in four batteries measuring 2.4 x 3.7 x 2.4 m. Each battery contains 10 cages and is equipped with trough feeders and nipple waters. Temperature, ventilation, altitude, and humidity were monitored and regulated throughout the duration of the trial.

Genome Data

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

DNA Isolation

A total of 297 male and female individuals were lanceted and 20 µl of blood was collected into 20 µl of citrate solution. Samples were re-suspended in lysis buffer (1 M Tris-HCl, pH 8.0, 5 M NaCl, 0.4 M disodium EDTA) in round bottomed centrifuge tubes. A 10% solution of SDS was added and inverted, followed by addition of 20 mg/ml of proteinase K. Samples were stored in an orbital shaker at 37 °C overnight. Sodium chloride (5M) was added and the samples were hand shaken until a foam appearance was observed, and spun at 5000 rpm for 15 minutes. Supernatant was pipetted into 15 mL centrifuge tubes with 100% ethanol and inverted. A hook made from melting the tip of a Pasteur pipette into a hook shape was used to collect the DNA, which was then rinsed in 70% ethanol. The ethanol was allowed to evaporate, and the DNA was transferred to a screw top tube containing 1 ml of TE pH 8.0. Samples were placed in an orbital shaker overnight at 37 °C until the DNA dissolved.

Genotyping

Twenty SNP were identified as regions of interest in ascites phenotype based on genome wide association studies previously conducted (Tarrant et al., in review; Table 4-1). Twelve SNP reside in three regions on the Z chromosome, and the remaining eight are from a single region on chromosome 11. Genotyping for SNPs were completed using KASP™ chemistry in 16x well format. The PCR conditions for all SNP were 94 °C for 15 minutes, 94 °C for 20 seconds, 65 °C for 1 minute nine times (-0.8 °C per cycle), followed by up to 35 cycles of 94 °C for 20 seconds, 55 °C for 1 minute.

Statistical Analysis

All analyses were completed in the latest version of JMP® Pro (v. 12.1.0; SAS Institute Inc., 2015). Models were created in order to predict the ascites outcome in the individuals sampled. In order to determine the most effective prediction model, five models were initially created to evaluate three subsets of data: SNPs only on chromosome 11, SNPs only on chromosome Z, and a combination of all SNPs. Sexes were evaluated independently of each other. Models evaluated included a logistic regression, two gradient boosting tree methods, decision tree and boosted tree, and two black box models, bootstrap forest and neural network. To avoid overfitting of the data for each model, 70% of the birds were used for training the models, and the remaining 30% were used to validate the models. Model outputs were then compared to determine the most robust and well-fitting model when considering chromosome 11 SNPs, chromosome Z SNPs, and SNPs on both chromosomes independently. Comparisons were made on evaluation of the R^2, root mean square error (**RMSE**), mean absolute deviation (**MAD**), misclassification rate (**MCR**), and area under the curve (**AUC**) values. A Chi-squared test was then performed on the AUC for each model to determine if the variation seen in the AUC values reported for each model was statistically different. Additionally, the most impactful SNPs for each model were determined through the main effect value that describes the variance of each SNP in respect to the distribution of other SNPs.

RESULTS AND CONCLUSIONS

Initial selection of SNPs in this study was based on regions that indicate association with ascites outcome phenotype through genome wide association studies conducted on REL line broilers (Tarrant et al., Chapter 2). Single nucleotide polymorphisms selected from chromosome 11 lie in, and around, cadherin 13 (**Cdh13**; Table 4-1). The Cdh13 gene encodes T-cadherin,

which acts as an adiponectin receptor (Hug et al., 2004). Adiponectin is a blood circulating protein, whose levels are associated with atherosclerosis, insulin resistance, and cardiovascular disease (Hotta et al., 2000; Yamauchi et al., 2003; Hashimoto et al., 2006). Three SNPs were selected from the 19 Mbp region on chromosome Z. Within this region is the 5-hydroxytryptamine (serotonin) receptor 1A (**HTR1A**) gene. Mice with HTR1A receptor knockouts experience an increase in heart rate and a vulnerability to cardiac death (Carnevali et al., 2012). The SNP identified around 60 Mbp on chromosome Z is in a region that appears to have several possible contributing factors to cardiac health in an individual. The coding sequence of a member of the MADS-Box transcription factors, myocyte enhancer factor 2C (**Mef2c**), is located around 60.25 Mbp. Mef2c is essential to the development of the right ventricle (Lin et al., 1997). Further, varied expression in Mef2c results in attenuation of cardiac hypertrophy in mice (Pereira et al., 2009). MicroRNA 9-2, located on chromosome Z at 60.29 Mbp, has the capability of targeting the myocardin pathway (Wang et al., 2010). Consequently, this pathway induces cardiac hyptertrophy in response to hypertrophic stress signals (Xing et al., 2006). Finally, SNPs chosen in the area of 80 Mbp on chromosome Z were selected in their relative location to lysyl oxidase (**LOX**). The LOX gene contributes to the structuring of collagen and elastin extracellular matrices during development, for which, LOX abnormalities contribute to the deterioration of the cardiovascular development (Mäki et al., 2002).

A model comparison was initially completed on five model types developed for each SNP input: chromosome 11 SNP, chromosome Z SNP, and SNP from both chromosomes. Upon evaluating all SNP, Chr 11, and Chr Z input combinations, the neural networking model was identified as the most robust model for predicting ascites incidence in male individuals in each case (Table 4-2). Within each input the largest R^2 value, coupled with the lowest RMSE, was

associated with the neural networks. Further, statistically significant variation between the modeling techniques were detected in the evaluation of AUC values. The receiver operative characteristic (**ROC**) curve is used as an indicator for decision performance in neural networking models (Woods and Bowyer, 1997). Specifically, the ROC curve can be used to judge the predictive ability of statistical methods by quantifying the area under the ROC curve, otherwise known as AUC (Hanley and McNeil, 1982). In the male ALL SNP input, the neural networking AUC is significantly larger than all models, aside from the boosted tree model. Ultimately, a largely superior R^2 value dictate that the neural network is the best fitting prediction model. A similar pattern is seen in the Chr 11 SNP inputs, with the neural networking model AUC remaining significantly larger than all other models, except the boosted tree. The Chr Z AUC inputs show AUC values of the boosted tree, nominal logistic, and neural networking model to be statistically similar. The MCR of the neural network indicates this model has a lower predictive error rate than other models using only chromosome Z SNPs as inputs. The modeling statistics indicate that Chr Z SNP inputs are better ascites incidence predictors than Chr 11 inputs; however, the neural networking model that incorporates SNPs from both chromosomes appears to be the best fitting model with a undoubtedly larger R^2 value, and lower MCR. Descriptive statistics on female neural network prediction models show greater robustness compared to other modeling techniques as seen in male neural networking models for all SNP inputs (Table 4-3). Additionally, the neural networking model created with SNPs from both chromosomes demonstrates a superior model when compared to neural networking models created with SNPs from single chromosomes. Interestingly, Chr 11 inputs create a better prediction model than Chr Z inputs, contrary to the patter seen in male inputs.

Neural networks are a black box computational method modeled off of neurological connections present in the brain (Amari, 1990). Neural networks act in a similar manner to use a series of weighted connections to connect the variables being input into the system to potential outcomes through connective nodes (Spining et al., 1994; Dayhoff and DeLeo, 2001). The effectiveness of neural networks is attributed to the superior fit of the data in a non-linear fashion and the ability of the model to learn and adapt the internal workings of the system to a fluctuating environment (Basheer and Hajmeer, 2000).

Because the neural network considers all variables when constructing a predictive model, an effort was made to reduce the number of SNPs used in the model, while attempting to maintain the level of predictability. This process was completed by determining the main effect value attributed to each SNP, and removing the SNP contributing the least to the model. In males, seven SNP from both chromosome 11 and Z were removed; however, in females, removal of the lowest contributing SNP decreased the model's accuracy. It was determined that the most robust model for females includes all 20 SNPs.

A reduction in the number of SNPs used for male individuals, from 20 total to 13 total SNPs spanning both chromosomes, increased the training and validation R^2 value from 0.929 and 0.942 to 0.999 and 0.998 (Table 4-4). Additionally, the neural network model that uses fewer SNPs as inputs has lower RSME, MAD, and MCR values. It is therefore concluded that the robustness of the model increases when fewer SNP are used. Additionally, due to the similarity seen in the training and validation R^2 values for the model using the most informative SNPs, it was determined that this is a well fit model. The 13 most informative SNPs used for the male neural networking model are denoted in Table 4-5, along with their respective total effect contributions to the model. The initial neural network created included 20 SNP from four

regions (one region on chromosome 11 and three regions on chromosome Z). Interestingly, when the number of SNP inputs were reduced to create a more accurate predictive model, SNPs from each of the four regions remained. Yet, the top four highest contributing SNPs reside on the Z chromosome: 19,850,532, 19,853,553, 60,189,777, and 80,805,286. While all SNPs were originally selected for this study with the knowledge of their potential association with ascites incidence the high level of contribution, seen specifically in two SNPs from 19 Mbp, indicates the impact of the Z chromosome on male ascites incidence.

Descriptive statistics for the training and validation data sets of the best fitting model for predicting female ascites phenotype, which includes all 20 SNPs evaluated for this study, is shown in Table 4-6. While the statistics reported are inferior to that of the model developed for males, the training and validation R^2 values of 0.944 and 0.928 still indicate a moderately high level in the goodness-of-fit for this model. Because more SNPs were used to construct this model than that for males, the contribution of the total effect of individual SNPs is not as high as seen in males (Table 4-7). Furthermore, the highest contributing SNPs are located on chromosome 11 (15,617,716 and 15,846,469), indicating that the influence of the Z chromosome on ascites incidence in males is not replicated in females. This information demonstrates the conclusion that the genetic component behind ascites phenotype in male and female broilers is variable.

When considering either i) SNPs located on chromosome 11, ii) SNPs located on chromosome Z, or iii) a combination of SNPs from both locations, the neural network model is the best-fit and most robust model for either sex. After determining the effectiveness of the neural networking models when using SNPs from both chromosomes, an attempt was made to determine the fewest SNPs required to maintain the prediction ability of the models, which

increased the model robustness in males, but not in females. This data indicates that the SNPs selected for this study were particularly important in ascites association in male broilers. The accuracy for prediction of disease outcome for males was greater because initial SNP selection was completed by locating regions of significance in male broilers identified from two genome wide association studies completed on REL line individuals phenotyped for ascites outcome (Tarrant et al., in review). Though not as predictive, the SNP inputs used in this study create an effective female prognostic model.

In commercial flocks over the last two decades' efforts have been placed in reducing ascites through genetic selection and through regulation of environmental conditions. It is clear these methods have aided in the reduction of overall incidence, but using these tactics alone is not enough to eradicate the disease. For this reason, it is important to determine methods that can bring selection practices even closer to eliminating ascites presence in modern broilers. The use of SNP panels in predicting future ascites outcome will be useful in retaining genetic accuracy lost through sibling selection, thus, contributing to a decline in ascites frequency that is currently being experienced in the poultry industry.

Table 4-1. SNPs identified from chromosomes 11 and Z used to develop predictive models.

Chr.	Position	Reference SNP	Reference Allele/ Alternative Allele	SNP Location
11	15,398,867	rs14027234	A/G	Intergenic Region
11	15,481,212	rs14027310	A/G	Cdh13 Intron
11	15,501,981	rs312593326	A/G	Cdh13 Intron
11	15,617,716	rs14027422	T/C	Cdh13 Intron
11	15,677,381	rs14966647	A/G	Cdh13 Intron
11	15,810,516	rs14966714	T/C	Cdh13 Intron
11	15,810,521	rs14966715	G/A	Cdh13 Intron
11	15,846,469	rs14027623	C/T	Intergenic Region
Z	19,850,532	rs14753903	G/T	Intergenic Region
Z	19,853,553	rs16761496	T/G	HTR1A Exon
Z	19,855,351	rs316810252	G/C	Intergenic Region
Z	60,058,344	rs14748694	A/G	Intergenic Region
Z	60,076,934	rs14748688	C/T	Intergenic Region
Z	60,189,777	rs317821780	G/A	Intergenic Region
Z	60,287,175	rs14747886	A/G	Intergenic Region
Z	60,441,865	rs14690172	A/G	Intergenic Region
Z	61,301,140	rs14774275	A/C	Intergenic Region
Z	80,794,843	rs14684720	T/C	Intergenic Region
Z	80,805,286	rs735134779	A/G	LOX Intron
Z	80,838,161	rs15990713	T/C	Intergenic Region

Table 4-2. Comparisons between regression modeling techniques for male broilers. Three sets of SNPs were used as inputs for models: SNPs from both chromosomes, SNPs from chromosome 11, and SNPs from chromosome Z.

SNP Input	Model	R^2	RMSE [1]	MAD [2]	MCR [3]	AUC [4]
All SNP	Boosted Tree	0.831	0.23	0.138	0.053	0.976^{ab}
	Bootstrap Forest	0.569	0.349	0.323	0.093	0.954^{b}
	Nominal Logistic	0.756	0.281	0.167	0.098	0.955^{c}
	Neural Networking	0.940	0.136	0.049	0.016	0.993^{a}
	Partition	0.656	0.325	0.214	0.139	0.916^{cd}
		R^2	RMSE	MAD	MCR	AUC
Chr 11	Boosted Tree	0.591	0.349	0.254	0.159	0.904^{ab}
	Bootstrap Forest	0.475	0.384	0.354	0.185	0.906^{b}
	Nominal Logistic	0.583	0.351	0.226	0.179	0.891^{bc}
	Neural Networking	0.684	0.321	0.197	0.162	0.923^{a}
	Partition	0.553	0.362	0.243	0.179	0.872^{c}
		R^2	RMSE	MAD	MCR	AUC
Chr Z	Boosted Tree	0.661	0.33	0.252	0.179	0.932^{a}
	Bootstrap Forest	0.492	0.376	0.262	0.172	0.849^{b}
	Nominal Logistic	0.697	0.326	0.213	0.157	0.923^{a}
	Neural Networking	0.758	0.296	0.194	0.128	0.956^{a}
	Partition	0.386	0.412	0.355	0.219	0.792^{b}

[1] RMSE = root mean squared error, [2] MAD = mean absolute deviation; [3] MCR = misclassification rate; [4] AUC = area under the curve. [*]Means within the same column and with no common superscript differ significantly ($P<0.05$).

Table 4-3. Comparisons between regression modeling techniques for female broilers. Three sets of SNPs were used as inputs for models: SNPs from both chromosomes, SNPs from chromosome 11, and SNPs from chromosome Z.

SNP Input	Model	R^2	RMSE [1]	MAD [2]	MCR [3]	AUC [4]
All SNP	Boosted Tree	0.786	0.268	0.181	0.090	0.968^{b}
	Bootstrap Forest	0.497	0.378	0.351	0.139	0.921^{cd}
	Nominal Logistic	0.722	0.292	0.181	0.137	0.950^{bc}
	Neural Networking	0.927	0.127	0.071	0.030	0.994^{a}
	Partition	0.608	0.352	0.256	0.174	0.907^{d}
		R^2	RMSE	MAD	MCR	AUC
Chr 11	Boosted Tree	0.565	0.376	0.279	0.201	0.914^{b}
	Bootstrap Forest	0.262	0.444	0.432	0.257	0.865^{c}
	Nominal Logistic	0.563	0.377	0.279	0.210	0.912^{b}
	Neural Networking	0.729	0.321	0.206	0.162	0.957^{a}
	Partition	0.459	0.401	0.325	0.264	0.887^{c}
		R^2	RMSE	MAD	MCR	AUC
Chr Z	Boosted Tree	0.549	0.371	0.294	0.201	0.883^{b}
	Bootstrap Forest	0.283	0.439	0.421	0.299	0.823^{cd}
	Nominal Logistic	0.543	0.376	0.285	0.217	0.875^{bc}
	Neural Networking	0.654	0.337	0.229	0.168	0.921^{a}
	Partition	0.441	0.403	0.330	0.236	0.833^{d}

[1] RMSE = root mean squared error, [2] MAD = mean absolute deviation; [3] MCR = misclassification rate; [4] AUC = area under the curve. [*]Means within the same column and with no common superscript differ significantly ($P<0.05$).

Table 4-4. Training and validation statistics on two neural networking models developed from SNPs on chromosomes 11 and Z in males. The All SNPs model includes 8 SNP inputs on chromosome 11 and 12 SNP inputs on chromosome Z. The 13 SNPs model displays descriptive statistics for a neural network completed using seven fewer SNPs to complete the analysis.

	All SNPs		13 SNPs	
	Training	Validation	Training	Validation
R^2	0.929	0.942	0.999	0.998
RMSE [1]	0.156	0.129	0.002	0.008
MAD [2]	0.071	0.068	<0.001	0.002
MCR [3]	0.022	0.000	0.000	0.000
AUC [4]	0.992	0.997	1.000	1.000

[1] RMSE = root mean squared error, [2] MAD = mean absolute deviation; [3] MCR = misclassification rate; [4] AUC = area under the curve.

Table 4-5. Contributions of individual SNPs to the neural network model developed to predict male ascites incidence using the fewest number of SNPs. Contributions are evaluated by the calculated total effect value ± standard error.

Chromosome	Position	Total Effect
11	15,398,867	0.020 ± 0.001
11	15,481,212	0.174 ± 0.003
11	15,501,981	0.106 ± 0.002
11	15,617,716	0.212 ± 0.003
11	15,846,469	0.069 ± 0.002
Z	19,850,532	0.457 ± 0.005
Z	19,853,553	0.493 ± 0.005
Z	19,855,351	0.041 ± 0.001
Z	60,058,344	0.146 ± 0.003
Z	60,189,777	0.235 ± 0.004
Z	61,301,140	0.076 ± 0.002
Z	80,794,843	0.054 ± 0.002
Z	80,805,286	0.239 ± 0.004

Table 4-6. Training and validation statistics on a neural networking model developed from SNPs on chromosomes 11 and Z in female individuals.

	All SNPs	
	Training	Validation
R^2	0.944	0.928
RMSE [1]	0.158	0.177
MAD [2]	0.052	0.071
MCR [3]	0.043	0.073
AUC [4]	0.995	0.992

[1] RMSE = root mean squared error, [2] MAD = mean absolute deviation; [3] MCR = misclassification rate; [4] AUC = area under the curve.

Table 4-7. Contributions of individual SNPs to the neural network model developed to predict female ascites incidence using 20 SNPs. Contributions are evaluated by the calculated total effect value ± standard error.

Chromosome	Position	Total Effect
11	15,398,867	0.055 ± 0.002
11	15,481,212	0.046 ± 0.001
11	15,501,981	0.187 ± 0.003
11	15,617,716	0.381 ± 0.005
11	15,677,381	0.013 ± 0.001
11	15,810,516	0.045 ± 0.002
11	15,810,521	<0.001 ± <0.001
11	15,846,469	0.249 ± 0.004
Z	19,850,532	0.192 ± 0.003
Z	19,853,553	0.144 ± 0.003
Z	19,855,351	0.009 ± 0.001
Z	60,058,344	0.021 ± 0.001
Z	60,076,934	0.050 ± 0.002
Z	60,189,777	0.032 ± 0.001
Z	60,287,175	0.022 ± 0.001
Z	60,441,865	0.044 ± 0.001
Z	61,301,140	0.121 ± 0.002
Z	80,794,843	0.020 ± 0.001
Z	80,805,286	0.020 ± 0.001
Z	80,838,161	0.015 ± 0.001

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CHAPTER 5

Discussion

The rise in chicken production has resulted from increased availability (Figure 4-1), improved pricing (Figure 2), and increasing health conscious behavior by consumers (Figure 4-3), which has resulted in chicken per capita consumption surpassing that of its red meat counterparts. Chicken meat is a healthier option to beef and pork. Trans-fats that are found in red meat are not present in chicken (Farrell, 2010). Additionally, poultry meat can also be used as a vector, termed enriched meat, to transport additional nutrients to the consumer like omega (n)-3 fatty acids and the antioxidant selenium whose absence can be fatal if left out of the diet (Yu et al., 2008). Further, the choice of chicken over red meat consumption is linked to a decreased risk of colorectal cancer (English et al., 2004) and is associated with a reduced risk for age-related macular degeneration when chosen as a protein source over beef or pork (Chong et al. 2009). The increasing importance of chicken products has led to intensive selection on traits relevant to the consumer market. Specifically, emphasis on highly heritable growth performance traits, including both rapid gain in body weight and high feed efficiency, has led to a dramatically different broiler than the broiler being marketed in previous decades (Havenstein et al., 2003). Jointly, selection for production traits has led to an increase in welfare related issues, like as lameness (Knowles et al., 2008), and pulmonary hypertension (Julian, 1998). Further, management practices to optimize broiler performance, such as increased day length (Schwean-Lardner et al., 2013) and ad libitum feeding schedules (Özkan et al., 2006) are further accentuating these issues. A direct result for these selection practices include an increase in cardiovascular disease (Julian, 1993). While management techniques can be used to manipulate ascites incidence genetic selection in broilers will be the answer to decreasing PHS incidence, and creating prevention parameters, in fast growing meat type chickens.

We began looking into the genetic causation of ascites incidence by evaluating a next generation sequencing technique: genome wide association studies. The GWAS were conducted to evaluate chromosomal regions associated with the syndrome. Identification of a region on chromosome 2 indicated an association with right ventricle to total ventricle ratio, which indicated an affiliation to heart hypertrophy detected in PHS susceptible individuals. These findings were consistent with previous studies on the correlation between heart morphology on chromosome 2 in broilers (Rabie et al., 2005). An additional region on chromosome Z consisting of three additional SNP indicated further association with heart morphology in ascites susceptible and resistant individuals. Further investigation into a single SNP in the chromosome Z region indicated that the genotype of the parents to male broilers, but not female, from a 1990s line are associated with the male broiler's ascites phenotype. Avian species do not operate under the same dosage effect that is seen in mammals. In species with X and Y sex chromosomes gene dosage dictates that in many genes seen, for example, on the X chromosome are evaluated at the same expression levels in females as in males (Lin et al., 2007). The absence of a dosage mechanism in organisms, like chickens, containing Z and W sex chromosomes, result in male bias for genes on the Z chromosome (Ellegren et al., 2007). This explanation may be the reasoning behind regions of significance evaluated solely on male individuals. Finally, it was shown that a collection of 20 SNPs located on two chromosomes were effective at predicting ascites incidence at a high level of accuracy using both traditional regression techniques, but to a greater degree, using a black box artificial neural networking model.

The findings from these studies represent further knowledge into the genetics behind ascites syndrome in rapidly-growing broilers. The broiler production market is a global-reaching market with continually increasing demand. The countries with the broiler production are

America, China, and Brazil, with America exporting 6.7 billion pounds, representing approximately 16.7% of the total production, in 2015 alone (FAO USDA). These values are expected to increase as a result of world population growth, and novel integration of large poultry production schemes into developing countries. As estimated in 2015 in the U.S. 0.05% of plant condemnations and carcass downgrading are contributed to ascites incidence (Cooper and Gustin, 2015, personal communication). If this approximated rate was applied to the total broiler production in pounds recorded for 2015 almost 2 billion pounds produced would be afflicted. Despite ascites continually decreasing in frequency, due to intensive selection and management practices, there remains the opportunity to retain millions of dollars of what is lost annually with the better understanding of ascites genetics, and application of this knowledge to large scale breeding schemes.

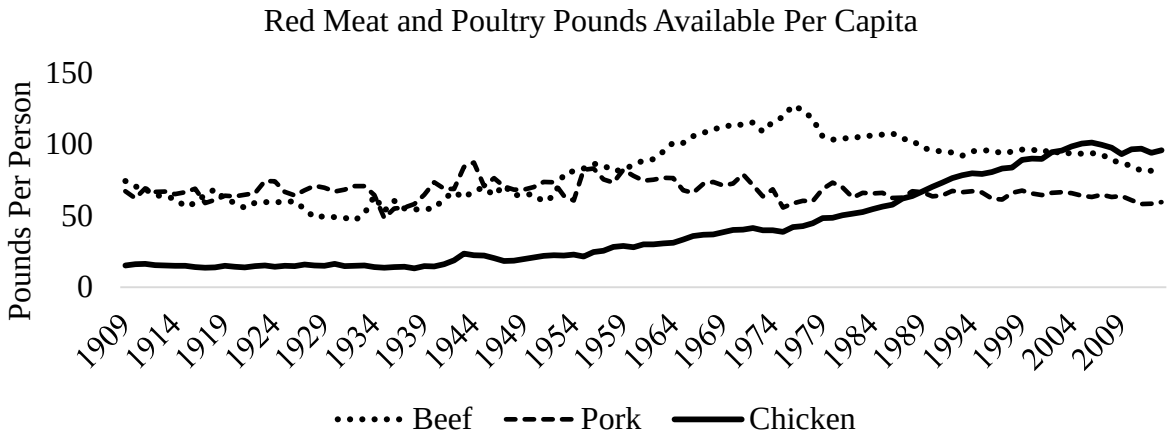

Figure 5-1. Pounds per person of produced beef, pork, and chicken in the U.S. Data available through USDA ERS.

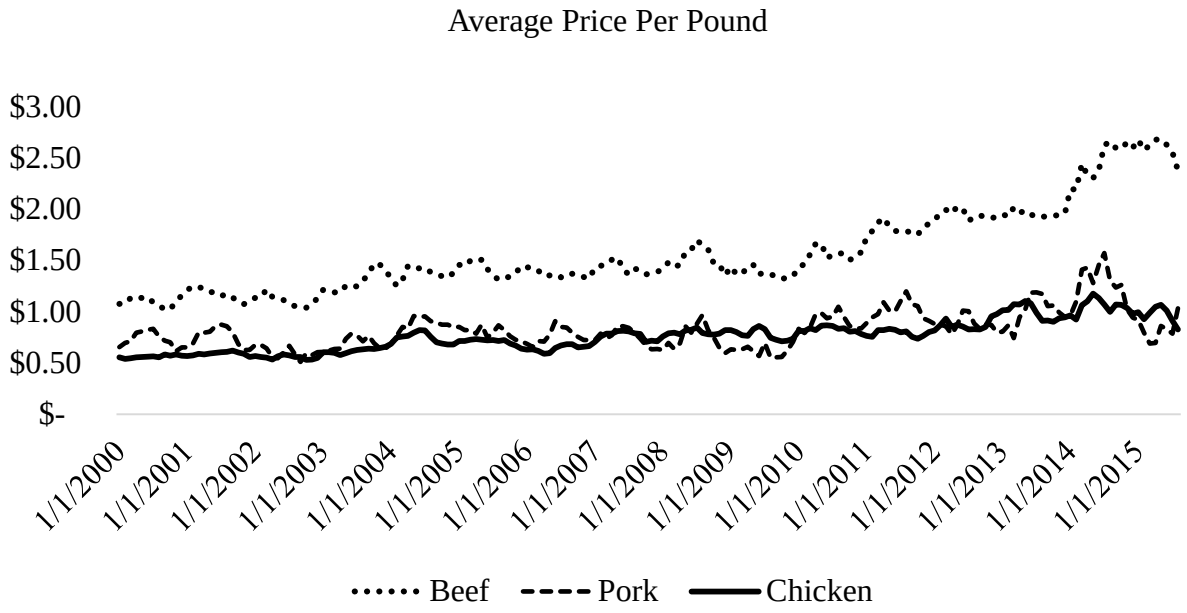

Figure 5-2. Price per pound of beef, pork, and chicken parts evaluated since 2000. Data available through USDA ERS.

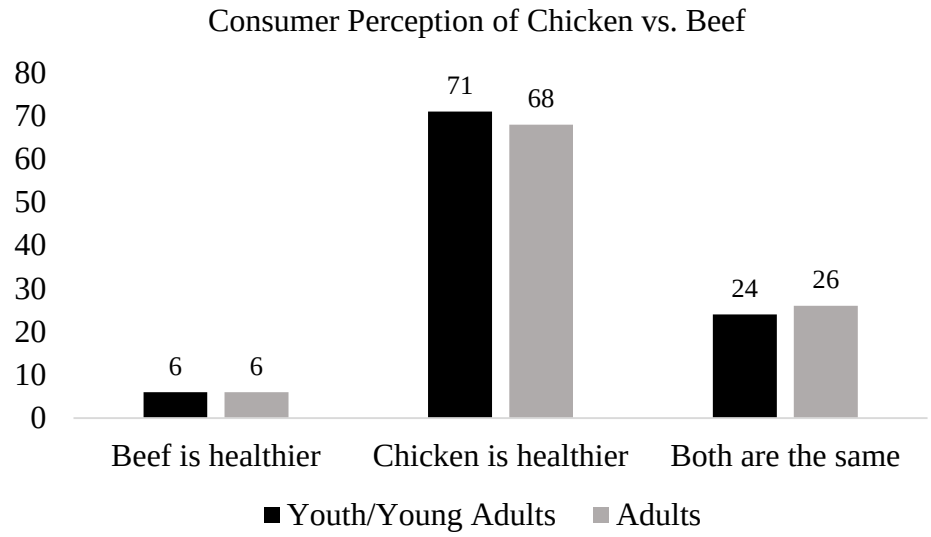

Figure 5-3. Consumer perception of healthfulness of beef versus chicken. Values presented as percent of 3000 individuals surveyed from a balanced representation of U.S. population. Data is as reported in Husted (2005).

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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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UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Nicholas Anthony

FROM: Craig N. Coon, Chairman

DATE: Apr 8, 2015

SUBJECT: IACUC Approval

Expiration Date: Apr 8, 2018

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

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

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

CNC/aem

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