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Validation of Whole Genome Resequencing for Mapping the Genetics of Ascites in Broilers and Viral Susceptibility in Layers

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

by

Katherine Pepper Lee
Auburn University
Bachelor of Science in Biosystems Engineering, 2018

August 2022
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This dissertation is approved for recommendation to the Graduate Council.

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ABSTRACT

This dissertation focused on the efficacy and validity of whole genome resequencing (WGR) for fine mapping genetic determinants of particular traits in a given organism. Previously, our research group used WGR to identify haplotype blocks of single nucleotide polymorphisms associated with ascites resistance with some as strong candidates for use in marker-assisted selection (MAS). Chapter 2 discusses the completion of a MAS project through evaluation of ascites incidence as well as production traits of economic value to poultry producers. Thus, the MAS project also covered viability of this methodology in the industry. The MAS significantly reduced ascites incidence in broilers with no negative impact on bird growth while some growth characteristics even improved in the MAS genetic line. With the success of the MAS project and improvements in the bioinformatic pipeline, a new WGR project was conducted and is discussed in Chapter 3. The goal of the project was to assess whether WGR could map genetic determinants of resistance to infectious bronchitis virus in white leghorn chickens. The results of this project revealed a genetic region with strong potential for MAS in white leghorn chickens which could increase innate resistance in those birds. As a whole, this work supports the effectiveness of WGR as a powerful tool in the detection of genetic variability associated with disease resistance.

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DEDICATION

Trevor –

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TABLE OF CONTENTS

CHAPTER 1: Literature Review 1

1.1 Chicken as a Food Source and Poultry Breeding 2

1.2 Chicken Genetics 4

1.2.1 Genome Assemblies 4

1.2.2 Single Nucleotide Polymorphisms 5

1.2.3 Marker-Assisted Selection 6

1.3 Ascites 8

1.3.1 Etiology 8

1.3.2 Treatments and Mitigation Techniques 12

1.3.3 Impacts of Ascites 15

1.3.4 Induction Methods 15

1.4 Foundational Ascites Work 18

1.5 Infectious Bronchitis Virus 21

1.6 Figures 24

1.7 References 29

CHAPTER 2: SNP-based breeding for broiler resistance to ascites and evaluation of correlated production traits 41

2.1 Abstract 42

2.2 Background 43

2.3 Methods 45

2.3.1 Bleeding, Genotyping, and Husbandry of Breeder Stock 45

2.3.2 Hatchery Protocol 46

2.3.3 Hypobaric Trials 46

2.3.4 Floor Trials 46

2.3.5 Processing 47

2.3.6 Statistical Methods 48

2.4 Results 49

2.4.1 First-Generation Breeding 49

2.4.2 Second-Generation Breeding 49

2.4.3 Hypobaric Challenges 50

2.4.4 Floor Trials ... 51
2.4.5 Processing ... 52
2.5 Discussion ... 54
2.6 Conclusions ... 58
2.7 Tables and Figures ... 59
2.8 References ... 70
CHAPTER 3: Whole genome resequencing suggests limited genetic basis for wide variation in infectious bronchitis viral load in challenged naïve white leghorn chickens ... 74
3.1 Abstract ... 75
3.2 Introduction ... 75
3.3 Methods ... 76
3.3.1 Chickens ... 76
3.3.2 Experimental Design ... 76
3.3.3 Viral Load ... 77
3.3.4 Histomorphometry ... 77
3.3.5 Chicken DNA Isolation and Purification ... 78
3.3.6 Sequence Analysis for Identification of Gene Regions ... 79
3.4 Results ... 80
3.5 Discussion ... 82
3.6 Conclusions ... 83
3.7 Tables and Figures ... 85
3.8 References ... 89
CHAPTER 4: Conclusions ... 91
4.1 References ... 96
CHAPTER 5: Appendix ... 98

LIST OF ABBREVIATIONS

BW	bodyweight
BWG	bodyweight gain
CPQ	carboxypeptidase Q
EID50	embryo infectious doses 50%
FCR	feed conversion ratio
FI	feed intake
GWAS	genome-wide association study
IB	infectious bronchitis
IBV	infectious bronchitis virus
IPAH	idiopathic pulmonary arterial hypertension
LRRTM4	leucine rich repeat transmembrane neuronal 4
MAS	marker-assisted selection
MHC	major histocompatibility complex
MWCO	molecular weight cut-off
PHS	pulmonary hypertension syndrome
QTL	quantitative trait locus
REL	ascites relaxed (unselected) line
RES	ascites resistant line
RFLP	restriction fragment length polymorphism
ROS	reactive oxygen species
RVTV	right ventricle to total ventricle
SNP	single nucleotide polymorphism
SPF	specific pathogen free
SUS	ascites susceptible line
TD	tibial dyschondroplasia
VNTR	variable number tandem repeats
WGR	whole genome resequencing

LIST OF PUBLISHED PAPERS

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Lee, K.P., Breedlove, C., Khalid, Z., Sheikhsamani, E., Alrubaye, A., Dridi, S., Pummil, J., Toro, H., Rhoads, D.D. Whole genome resequencing suggests limited genetic basis for wide variation in infectious bronchitis viral load in challenged naïve white leghorn chickens. (*Submitted to Poultry Science*)

CHAPTER 1: LITERATURE REVIEW

1.1 Chicken as a Food Source and Poultry Breeding

Chicken is a staple in the diets of most people over any other meat type, representing 41% of meat consumed by humans each year (1). As such, the successful and efficient rearing of broilers, or meat-type chickens, is paramount to the continued supply of chicken meat. The consumption of chicken has been relatively steady or increasing for many decades despite fluctuation in consumption of other meat sources. As of 2020, the average person in the United States consumes 97.6 pounds of chicken per year (2).

To maintain sufficient supply of high-quality, affordable chicken meat, it is up to the poultry industry to produce birds that can remain healthy and grow as efficiently as possible. This task has traditionally been accomplished through the use of bird phenotyping for specific production-related traits as the primary driver of selection for many breeding schemes, which is both logical and useful. In a comparative study by Zuidhof *et al*. (3), it was found that broilers from 1957 to 2005 have seen a 3.3% increase each year in day 42 body weight and a 50% reduction in feed conversion ratio at a rate of 2.55% each year. Between those two time points they also found a 30% and 37% increase in tenders weight and 79% and 85% increase in breast weight in males and females, respectively. In spite of this progress, new problems are given the opportunity to arise without consideration of the potential genetic relationship between production traits and other less desirable traits. These include but are not limited to greater fat deposition (4–6), tendency for eating beyond metabolic requirements (5,7), dampened immune response (8), and greater overall mortality (9). Issues have also arisen with the musculoskeletal system of the bird. For example, tibial dyschondroplasia (TD) has been shown to have a moderate to high heritability but low correlation with body weight (10,11). Since TD is not a direct consequence of body weight, Hocking (12) posits that the genes leading to susceptibility to TD must have

been present in broiler founder populations and were linked to the genes that led to highly efficient growth. As these genes for growth were fixed into early populations of broilers through phenotypic selection, so were the genes for TD. One issue that can arise directly as a consequence of rapid growth is a metabolic disorder called ascites around which the work in Chapter 2 is centered.

In more modern breeding schemes, poultry geneticists have evolved from selection based solely on bodyweight (BW) to selecting for a number of interconnected factors impacting growth and bird health (13) (Figure 1-1). This has resulted in a 300-400% improvement in broiler growth rate over the last 60 years (3,14). The most common breeding scheme utilized today is the four-way cross, named this way because of the four genetic pools from which the broiler is produced. Four separate pure pedigree elite lines make up those four genetic pools, two each denoted as male and female lines. These lines are strictly maintained to ensure the unique genetic makeup of each. At the point of beginning to produce a broiler, each of the grandparent birds will come from one of the four unique pedigree elite lines, always using the same gender from each line. The parent flock called broiler breeders, then, is the product of two of the pedigree elite line flocks being interbred with the specific male-female flock combination being maintained each time a broiler-breeder parent flock is produced. Each of these two resulting broiler-breeder parent flocks are maintained as all-male or all-female and each are a unique genetic hybrid produced from the pedigree elite lines. Finally, the broiler stock is produced by crossing the two broiler-breeder flocks, with each resultant bird being a double hybrid (15). Figure 1-2 is a schematic from Tarrant (16) of this breeding scheme, showing the inverted pyramid structure that is created through the successive generations.

1.2 Chicken Genetics

1.2.1 Genome Assemblies

The chicken genome was the first livestock animal to be fully sequenced, with the first draft of the genome being published in 2004 (17). Hillier *et al*. accomplished this through several genomic methods amassing 6.6× coverage of the genome based on the DNA of a single female from an inbred red jungle fowl line. At the same time, the chromosomal repertoire was determined to be 38 autosomal chromosomes plus the Z and W sex chromosomes, with chromosomes 1-10, Z, and W defined as macrochromosomes, 11-16 defined as large microchromosomes, 17-32 defined as small microchromosomes, and 33-38 defined as the smallest microchromosomes (18). Micro- and macrochromosomes are debated in the ways that they differ, but the consistent differences are in size, GC content, gene density, and repetitive element density (17,19). Subsequent to the first draft genome, more precise genome maps based on single nucleotide polymorphisms (SNPs), bacterial artificial chromosomes, and fosmids were produced spanning an estimated 1.09 gigabases (19). The finalized first version of the genome was released in 2006, denoted Gallus_gallus-2.1 or galGal3 (20). As sequencing methods progressed, updated assemblies of the genome were produced and released. Rather than only using Sanger sequencing, next-generation sequencing technology led to the Gallus_gallus-4.0 (galGal4) assembly in 2011 (19,21). Improvements in this assembly over galGal3 included the removal of approximately 10 megabases of duplicate sequence, an increase in N50 contig (representing the contiguity of the assembly; 22) and scaffold size, a more than 2-fold increase each in the size of the Z and W chromosomes, and the addition of 15 megabases of sequence (19,23). Warren *et al*. (19) sought to improve upon this assembly through the use of deep coverage long single molecule sequencing technology. This assembly, termed Gallus_gallus_5.0

(24), added 183 megabases of sequence including 16.4 megabases added to previously sequenced chromosomes. This release included sequence from three microchromosomes (30, 31, and 33) for the first time, and increased the number of annotated genes by more than 4,000. Finally, GRCg6a (galGal6) (25) was released between 2018 and 2019, showing updated lengths of chromosomes 16 and 30, fewer unplaced scaffolds, and improved N50 (26). Two versions of GRCg6a were released: a maternal broiler and a paternal layer.

1.2.2 Single Nucleotide Polymorphisms

Although the majority of the genome is shared between two individuals of a given species, many small differences exist which account for the phenotypic variation between each individual. The first of these variations to be detected were restriction fragment length polymorphism or RFLP (27). Through restriction enzymes isolated from bacteria, a DNA sample could be digested in order to cleave the DNA at specific target sequences throughout the genome. Fragment length differences between two samples were identified via gel electrophoresis followed by Southern blotting. RFLP differences were often based on point mutations in some of the target sites of the restriction enzyme (28,29). Importantly, RFLP are co-dominantly inherited and presented the first method for the determination of the parental source of an allele (30). RFLPs as markers were replaced by variable number tandem repeats (VNTRs), which are repetitive elements of more than 6 nucleotides repeated in the genome immediately next to one another and presented the opportunity to identify genetic linkage in families (31,32). More than half of VNTR sites identified in humans are closely affiliated with genes and therefore are likely to impact gene expression (33). Problematically at the time, the detection of RFLP and VNTRs required a large amount of high-quality DNA and Southern blots or sequencing gels, making the detection of both marker types labor-intensive (34).

As detection methods progressed, single nucleotide polymorphisms (SNPs) quickly became the preferred method for mapping genetic variability. SNPs are point mutations found throughout the genome. Though similar to substitution mutations, they differ in that SNPs must be found in more than 1% of the population to be classified as such and occur at a much higher frequency than other polymorphisms making them preferable for the detection of variations associated with disease (35,36). In 2004, Wong *et al*. (37) published the first SNP map for the chicken genome, finding 2.8 million SNPs in total. Comparisons were made between wild (Red Jungle Fowl) and domestic lines (broiler, layer, or Silkie), between domestic lines (broiler-layer, broiler-Silkie, and layer-Silkie), and within domestic lines (broiler-broiler, layer-layer, and Silkie-Silkie). The majority of these comparisons revealed an average rate of 5 SNPs per kilobase, whereas in broiler-broiler and layer-layer the rate was 4 SNPs per kilobase. This difference was postulated to be due to the closed breeding population systems utilized to produce broilers. In the time since the publication of this SNP map, SNPs have become easier to genotype and have been utilized as a means to recognize alleles that are associated with resistance or susceptibility to a disease (35,38). Given the abundance and frequency of SNPs in the chicken genome, they function as excellent targets for marker-assisted selection programs.

1.2.3 Marker-Assisted Selection

Marker-assisted selection (MAS) refers to the process by which highly specific genetic selections can be made in order to elicit a targeted change in a chosen phenotype. Using SNPs mapped in regions of influence on the phenotype in question, geneticists can select for traits that exist in a subset of the existing population so that all individuals in future generations will express the desired phenotype. Collard *et al*. (39) explains that the linkage of these markers to alleles responsible for certain traits allows for their detection and usage in breeding systems.

MAS is a technique commonly used in modern agricultural breeding systems, especially in plants. Xu and Crouch (40) provide several rationales for the use of MAS in plant breeding. One of particular interest is the ability of MAS to allow for “pyramiding” or “gene stacking.” This is the accumulation of genes from multiple sources that codes for the same trait, commonly used to increase the tolerance of plants to certain stressors (41). In cereal crops, MAS has been used for the assessment of the suitability of certain cultivars for propagation, the incorporation of specific genes into offspring generations, the pyramiding of genes from a specific phenotype, and to eliminate unpreferred genes early on in the breeding program (39). Other plants on which MAS has been performed include beans, cassava, cotton, ryegrass, and tomato (42–45). MAS is especially important in crop breeding for disease resistance. The review by Farokhzadeh and ali Fakheri (46) discusses specific cases where MAS could or needs to be swiftly implemented to reduce disease incidence due to the availability of existing quantitative trait loci (QTL) maps for the particular disease or for the severity of the disease in those crops. These include tan spot in wheat, late blight in tomato, leaf rust in barley, and white mold in the common bean. There is also great potential for this type of targeted breeding to increase the success of crops that are grown under the restrictions to be certified organic, however there is still ongoing debate as to whether this would be appropriate for organic systems (47).

MAS has also been utilized in livestock, albeit not yet to the same extent as it has in plants. At this time, a large portion of the literature has been focused on the identification of QTLs relating to important traits and that could be utilized in livestock. A few of these traits in cattle include growth characteristics, meat quality, milk production in dairy cattle, reproductive traits, disease resistance, and temperature tolerance, many of which encompass several other traits that can be individually studied and utilized for selection (48–53). Common applications of MAS in

livestock breeding programs include marker-assisted introgression of desired alleles into a breed where that allele is not commonly found while still maintaining the overall genetic makeup of the recipient breed or to produce new hybrid populations whose genetic makeup is simply the most desirable alleles from each cross (45,54). A study by Pedersen *et al*. found that utilizing MAS in a dairy breeding scheme allowed for the reduction of inbreeding compared to more traditional selection methods (55). In swine, the simulated study of MAS utilization by Hayes and Goddard (56) showed that MAS would not be an economically feasible option for commercial breeding until markers more closely linked to QTLs were discovered.

More specifically in poultry, MAS has been feasible for a longer time than in other livestock species due to the more extensive knowledge of the chicken genome. Our understanding of the chicken genome has only expanded in the years since it was first assembled (17), revealing new opportunities to successfully complete highly targeted breeding. While selection programs are of great importance to the industry, the greatest roadblock arises from the multigenic nature of many traits that need to be addressed by these programs. A complex trait such as ascites resistance or susceptibility had not yet been successfully addressed by MAS until the completion of the work discussed in Chapter 2, indicating that ascites and other complex traits may be headed for dramatic improvements in commercial operations in the coming years.

1.3 Ascites

1.3.1 Etiology

Ascites, or “water belly” as it is sometimes known in the industry is an often-terminal disease culminating with the filling of the body cavity with fluid and, commonly, heart failure. The most recent and substantial review of ascites from the standpoints of pathogenesis, physiology, immunological response, and genetic influences was compiled in 2013 by Wideman *et al.* (57).

Ascites is a disease that is driven by metabolic stress. The earliest cause of ascites was identified to be associated with the rearing of birds at higher elevations, first reported in Bolivia (58). Higher elevations have decreased atmospheric pressure and therefore decreased partial pressure of oxygen, causing lower oxygen content per breath taken by the bird and, in turn, pulmonary arteriole vasoconstriction and pulmonary hypertension (59). After its initial discovery, ascites was then reported in areas of lower elevation and became more prominent in the early 1980s which led to our understanding of other sources of metabolic stress leading to pulmonary hypertension and ascites (60,61). One potential cause is the need of the bird to thermoregulate in cold temperatures, where the body's need to maintain homeostasis increases blood flow and oxygen demand (62). Another critical and significant source of metabolic stress in poultry is rapid growth. Targeted genetic selection has created a bird that can accrete impressive amounts of muscle in the shortest amount of time possible; however, this growth is highly metabolically taxing. In order to sufficiently oxygenate their tissues, birds would need to see a 100-fold increase in both cardiac output and also pulmonary vasculature in the 8-week grow-out period utilized in modern broiler operations (63). Unfortunately, with these changes in muscle deposition capability, the birds have not acquired an increased cardiopulmonary capacity to supply oxygenated blood to the rapidly growing body. Schmidt *et al.* (64) found that a commonly used modern broiler line even experienced a decreased relative heart size after day 14 of growth. Due to this insufficiency, there is very little reserve capacity of the vasculature that is not actively being utilized under optimal growth conditions, indicating a severe lack of ability to cope with any given metabolic stressor (65–72). This condition is further exacerbated by the predisposition of broilers to decreased red blood cell deformability which on its own results in pulmonary hypertension (73). In order to overcome this ever-increasing metabolic demand,

blood pressure is decreased by the dilation of the arteries throughout the body, thereby decreasing the time taken to get oxygen to the tissues but also initiating other physiological conditions subsequent to arterial hypotension (63). The increased velocity of blood moving through the pulmonary system leads to the new problem of incomplete gas exchange; blood must reside in the pulmonary alveoli for an amount of time such that carbon dioxide can be removed from the blood and replaced with oxygen. When blood pressure, and thereby blood velocity, is increased in the pulmonary system as a result of decreased arterial blood pressure, the blood does not spend sufficient time in the alveoli and does not fully exchange gases before sending this semi-oxygenated blood out to the tissues, a condition known as hypoxemia (74,75). Arterial hypotension metabolically leads to fluid retention in the kidneys while the liver is deprived of oxygen and subsequently damaged. In combination with elevated venous pressure, this leads to the release of the accumulated fluid from the liver surface into and filling the body cavity, thus resulting in clinical symptom of ascites (63,76,77).

Though the fluid-filled body cavity may be the most recognizable symptom of late-stage ascites, another key player leading to bird mortality is the heart. This requires an understanding of the avian cardiovascular anatomy. While the majority of the avian heart is comparable to the mammalian heart, the valve separating the right atrium and ventricle is different. In mammalian hearts, the right atrioventricular valve is a tricuspid valve but the avian version is monocuspid, consisting of a single muscle flap protruding from the wall of the right ventricle (71). Increased pulmonary arterial pressure has been demonstrated to cause thickening of the wall of the right ventricle by the addition of sarcomeres, impeding its ability to pump blood as effectively as normal (78,79). This hypertrophy is the basis for the elevated right ventricle to total ventricle (RVTV) ratio used as a quantitative metric for ascites development. Additionally, the valve

separating the right atrium and ventricle is also a part of the right ventricular wall, meaning that it experiences hypertrophy in the same way as the ventricle, resulting in leakage back into the right atrium (79). There is also evidence that this valve plays a role in the pump function of the right ventricle, making its damage a multi-faceted problem for the cardiovascular system of the bird (80). This insufficient performance of and damage to the right atrioventricular valve causes a volume overload in the right ventricle by increasing the blood volume in the right atrium that must be pumped by the ventricle, leading to dilation of that portion of the heart (81,82). Prolonged pulmonary hypertension compromises the muscle tone of the heart, specifically in the right ventricle as this is the section of the heart responsible for propelling blood into the pulmonary system for reoxygenation. Over time, the heart, especially the right ventricle, tend to show decreased muscle tone (flaccidity) from overexertion (59,83–88). As the hypertension progresses over time, the right atrioventricular valve fails causing high venous pressure and hypoxemia which damages the heart and leads to right ventricular failure (79). Additional changes commonly seen in the hearts of ascitic birds also include enlargement and a rounded shape to the apex related to right ventricular failure (81). Figure 1-3 shows a flow chart of the stages of ascites detailing the progression from metabolic stressor to fluid accumulation. After the exposure to the stressor, thereby increasing oxygen demand inducing physiological hypoxia, a cascade is initiated which increases cardiac output and overexerts pressure on the wall of the right ventricle. The right ventricle thickens to overcome the high pressure, rendering the right atrioventricular valve ineffective, overloading the volume in the right ventricle and leading to failure of the right heart. Following right ventricular failure, the building pressure in the veins leads to the liver’s release of fluid into the body cavity (89). While pulmonary hypertension leading to ascites is a problem needing attention for the sake of the poultry industry, it also has

clear parallels to human idiopathic pulmonary arterial hypertension (IPAH) which makes it even more beneficial for us to study and understand this disorder.

1.3.2 Treatments and Mitigation Techniques

Currently, there are several commonly utilized techniques shown to reduce ascites incidence. Many relate to the nutrition of the bird and are discussed in the reviews by Baghbanzadeh and Decuypere and by Khajali and Wideman (89,90) including growth rate management, physical form of the diet, and the supplementation of omega-3 fatty acids, L-carnitine, or antioxidants.

The management of growth rate is a logical way to also manage ascites incidence, given the previously established tenet that rapid growth is a substantial metabolic stressor leading to ascites. Feed restriction allows bird growth rate to be strategically slowed, especially during the first weeks of life when metabolic rates and thereby oxygen demand are extremely high (91–93). Problematically, this can reduce bird intake of anticoccidial feed additives which increases flock susceptibility to coccidiosis (89). Another method for managing growth rate is the reduction in nutrient density of the feed which has been shown to reduce ascites morbidity and mortality as long as feed energy content is maintained (94). An additional issue arises from the current trend to reduce overall crude protein content in feed through precise amino acid supplementation as a method to reduce feed cost. As shown by Behrooj *et al.* (95), reduced crude protein diets significantly increased ascites incidence in broilers experiencing another metabolic stressor such as hypoxia which they concluded to be contributed to reduced plasma nitric oxide as well as increased hematocrit and elevated RVTV. This is logical, given that physiological conditions of reduced nitric oxide have been found to lead to hypertension (96). These conclusions mean that birds predisposed to greater ascites incidence should not be fed the more cost-effective reduced crude protein diets, further increasing the economic toll on the grower. One final way that

growth rate can be limited is through the form of feed provided to the bird. Typically, broilers are provided with a crumble or pellet feed depending on age. Bölükbasi *et al*. (97) found that mash feed reduces ascites incidence compared to crumble or pellet forms and only slows birds from reaching market weight by 1 to 2 days, but it should be noted that this feed form comes with increased risk of pendulous or blocked crops so that risk must be considered before implementing this technique to reduce ascites incidence.

Feed additives have also been utilized to reduce ascites incidence. As previously described, decreased deformability of red blood cells in broilers and exacerbated by hypoxemia contributes to increased risk of ascites. Omega-3 fatty acids have been found to decrease blood viscosity and reduce right ventricular hypertrophy in rats and reduce growth rate in broilers, all of which indicate the potential for their use in feed formulation for genetic lines or growing locations with higher incidences of ascites (98,99). L-carnitine is vital in the metabolism of fatty acids and is vital to cardiac muscle which utilizes long-chain fatty acids for energy production (100). It has therefore been hypothesized that increased supplementation of L-carnitine in the diet may enable the bird to more effectively handle metabolic stressors that might lead to the development of ascites and has been shown to improve cardiovascular traits in rats which would be beneficial to birds facing the onset of ascites symptoms (101,102). Indeed, this has been demonstrated successfully by Khajali and Khajali (103) who showed that L-carnitine improved cardiopulmonary function of broilers reared at high altitude and by Yousefi *et al*. (104) who found that it reduced ascites mortality in broilers. Finally, antioxidants have been proposed as a means to reduce the effects of metabolic stress and ascites incidence. Cawthon *et al*. (105) found lower levels of certain endogenous antioxidants that would, under normal conditions, combat the production of reactive oxygen species (ROS) in the mitochondria of ascitic broilers. The

overproduction of ROS in pulmonary vascular tissues may cause damage which increases membrane permeability, potentially increasing pulmonary vascular resistance and eventually ascites incidence (90,106,107). It does appear that single antioxidant supplementation tends to be ineffective in reducing ascites mortality (108–111), however one study countered these results by providing an increased concentration of vitamin C and reducing ascites incidence by 65% compared to the control group (112). Additionally, vitamin E implants may have the potential to increase resistance to ascites-causing metabolic stressors by increasing protection from ROS (106). This method could potentially be utilized in flocks or growing locations where higher ascites incidence is anticipated. Others have found that the supplementation of several antioxidants does have strong potential to reduce ascites incidence, as Bautista-Ortega and Ruiz-Feria (113) showed that supplementing a combination of arginine, vitamin E, and vitamin C improved cardiopulmonary function and decreased damage caused by ROS. There is also promising data from researchers who supplemented both L-carnitine and coenzyme Q_{10} which decreased the incidence of pulmonary hypertension (114). The problem with each of these methods is the associated increase in the cost of feed with their supplementation.

Genetic selection is an excellent option for reducing ascites incidence by increasing innate resistance in the bird and possibly alleviating the need for any other mitigation techniques. However, it must be completed in a very targeted manner to avoid costly production losses as the largest, highest-yielding birds tend to be the most susceptible (15,79,115–117). This means that using solely phenotypic selection to increase innate resistance will inadvertently select for decreased yield. A highly specific method of selection is necessary to avoid the economic losses of phenotypic selection as well as the time wasted to backtrack over years of selection progress, an option for which is the topic discussed in Chapter 2.

1.3.3 Impacts of Ascites

As ascites causes both bird death prior to the time of processing and bird condemnations at the processing plant, it has impacts on mortality as well as economic impacts. Older statistics show that ascites has been the cause of 8% or more of bird mortality and had an economic impact of $100 million annually (118,119). One specific reason for greater losses from ascites is based on its progressive development in the bird over the course of days or weeks; this means that more feed and bird care labor have been invested in birds that eventually succumb to complications from ascites and the company loses the money invested with the death of the bird (15). However, it should be noted that these estimates are based on data nearing two to three decades old and updated evaluations of the impacts of ascites are warranted as ascites has been reduced in lower elevation regions through the efforts of genetics companies to cull out individuals from pedigree lines who are found by pulse oximetry to be hypoxemic (70,71).

1.3.4 Induction Methods

In order to effectively study ascites, researchers must be able to consistently and concisely induce the disease. Currently, there are five models most commonly used to accomplish this: salt inclusion in the feed or drinking water, arterial occlusion, micro-particle injection, cold stress, and hypoxia by hypobaric challenge.

The salt induction model utilizes the addition of sodium chloride in the feed or drinking water supplied to the birds. Salt individually is not the culprit, but rather the sodium ion portion of the molecule. Julian (78) posited that high levels of sodium compounds several mechanisms to eventually cause ascites. One of these mechanisms is an increase of sodium in the body causing fluid retention, which in turn increases blood volume known as hypervolemia which then lends to the damage of the right heart. Relatedly, high dietary sodium incites pulmonary hypertension,

increases hydraulic pressure in the blood vessels, increases red blood cell rigidity, and causes right ventricular dilation and hypertrophy (73,81,120). However, it should be noted that this method tends to yield lower ascites incidence than other induction methods; higher salt concentrations would likely increase ascites incidence but would also be lethal, especially in young broilers (121).

The arterial occlusion method is the most invasive method used to induce ascites, as it requires the surgical clamping of the left pulmonary artery. This is completed by opening the left thoracic air sac during the surgery and physically clamping the artery, thereby reducing blood flow to one lung (57). Wideman *et al.* (122) found that the increased venous pressure caused by an even seemingly minor applied pressure through unilateral arterial occlusion led to substantial venous resistance to flow which is one of the two mechanisms responsible for the fluid released from the surface of the liver (63). This induction method, however, runs the risk of health complications for the bird post-surgery.

Another ascites induction model involves the injection of cellulose microparticles. This method has seen such success that it has been patented (US patent no. 6,720,473) for use in the industry as a diagnostic tool for susceptibility in broiler populations. The microparticles are suspended in saline with heparin to prevent catastrophic development of pulmonary blood clots (123). They are circulated until they reach the pulmonary precapillary arterioles where they become lodged and impede blood flow, inducing pulmonary hypertension (124). Wideman and Erf used intravenous injections of microparticles into one wing and one leg vein for systemic and renal portal injection, respectively, which led to responses including pulmonary hypertension and systemic hypoxemia similar to the responses seen in the arterial occlusion method. Wideman *et*

al. (123) found that this microparticle injection method elicits sufficient results to serve as a viable replacement for the unilateral arterial occlusion method.

Cold stress is a model that most directly replicates one of the growing environments where birds are likely to develop ascites. Due to the metabolic needs of the body during a cold stress response, blood pressure is increased, surplus red blood cells are produced to increase oxygen-carrying capacity and consequently increases blood viscosity, and ascites ensues (115). The increased metabolic rate and greater oxygen demand occurs in order to maintain body temperature when exposed to cold environments (125,126). Pakdel *et al*. (127) utilized this model to examine the impact on ascites incidence in broilers. Both the control and experimental groups were reared under standard commercial conditions aside from the temperature gradient throughout the experimental period. While the control group experienced a temperature gradient starting from $34^{o}C$ on day of hatch and was incrementally reduced to $17^{o}C$ by day 35, the experimental group started from $30^{o}C$ on day of hatch, incrementally reduced to $10^{o}C$ by day 22, and maintained at $10^{o}C$ through day 35. The results from the cold stress group showed an average body weight reduction of over 400 grams, a 25% increase in hematocrit not contributable to natural physiological differences due to the age, a 35% increase in RVTV, and 16% ascites mortality while there was no ascites mortality in the control group.

All ascites induction for this dissertation research utilized the hypobaric challenge model through use of the hypobaric chamber at the University of Arkansas Poultry Research Farm. This method allows for birds to be placed in battery cages in a simulated high-elevation environment, accomplished by reducing the pressure in the chamber and therefore the partial pressure of oxygen. This induction method is reliable, non-invasive, and efficiently induces ascites regardless of genetic line or other factors and has been extensively utilized in ascites research

(63). Further, the hypobaric effect can be exacerbated by providing chick feed formulated for rapid growth for the duration of the trial.

1.4 Foundational Ascites Work

Ascites research lines have been developed at the University of Arkansas. Pavlidis *et al.* (128) used sibling selection in a commercial elite line to create three sublines: ascites susceptible (SUS), ascites resistant (RES), and a randomly mated subline to represent the founder population (Relaxed, REL). Sibling selection is the process of assessing the phenotype of one group of birds, and then using that data to select siblings of the phenotyped birds to be used for producing the next generation. Specifically, birds were reared in the hypobaric chamber at simulated 9,500 feet elevation while the siblings were reared in a similar chamber at the ambient pressure of northwest Arkansas, approximately 1,500 feet. Ascites phenotype data was collected throughout the trial. From this, the most susceptible birds were selected to produce the SUS line and the most resistant to produce the RES line. Based on the rapid divergence of the RES and SUS line with each successive generation of selection, it was concluded that a limited number of genes must be involved with resistance or susceptibility to ascites. Figure 1-4 shows this rapid divergence through the ascites mortality for each of the three experimental lines in the first 10 generations of selection. The founder population had an average ascites mortality around 75% when reared under hypoxic conditions. After selection began, the RES and SUS lines quickly began to be driven toward the extremes of ascites mortality. By generation 10, the RES line experienced ascites mortality of less than 10% while the SUS neared 90%. Figure 1-5 then shows the trend in cumulative ascites mortality through day 42 for each line based on a single generation during a hypobaric challenge. The SUS line not only reached nearly 100% ascites mortality by the end of the challenge, but also saw onset of morbidity and mortality earlier than

the other lines, starting between days 5 and 7 while the other lines did not see onset until 11 to 13 days. The RES line conversely reached ascites mortality of under 30% while the control population of REL birds was near 70%. Additionally, trends in average bodyweight of birds from the ascites lines as generations progressed were reported. Comparing the day 42 BW during a hypobaric challenge, the RES and SUS lines both showed a negative linear trend with similar day 42 BW by generation 10, however the RES exhibited the lowest BW. The 10-generation trends in day 42 BW after being reared at ambient pressure also had a negative linear trend. Between the lines in the tenth generation, the SUS outweighed the RES line by 163 grams, providing further evidence of smaller birds tending to be more resistant to ascites. In terms of heart condition when reared in the hypobaric chamber, generation 10 of selection exhibited the lowest RVTV ratio in the RES line, the highest in the SUS line, with REL being intermediate.

Later, Krishnamoorthy *et al*. (129) completed a genome-wide SNP panel with the goal to identify regions of the genome in linkage disequilibrium for susceptibility to ascites. The most significant regions from that study were found on chromosome 9 and included the genes angiotensin II type 1 receptor (AGTR1), urotensin 2 domain containing (UTS2D), and serotonin receptor/transporter type 2B (HTR2B). A subsequent study revealed that AGTR1 and UTS2D were not significantly associated with ascites in other populations (130).

Alzahrani (131) further studied the relationship between HTR2B and the variability in ascites phenotype. First, SNPs in the HTR2B region were detected by PCR amplification and sequencing, with 19 SNPs being found. Two were chosen: SNP586 which was associated with the promoter region for HTR2B and SNP9093 which is located in exon 3. TaqMan exonuclease assay primers and probes were designed to allow for genotype determination of DNAs of birds that had been reared under hypobaric conditions for ascites phenotype from the ascites research

lines and three commercial lines. After completing this analysis, it was found that HTR2B was unlikely to be a universal QTL for ascites phenotype.

Tarrant *et al*. (132) utilized multi-generational genome-wide association studies (GWAS) to identify new candidate regions associated with ascites. Generations 16 and 18 of the REL line were assessed and regions significantly associated with ascites phenotype were identified on chromosomes 2 and Z. Candidate genes coding for the melanocortin 4 receptor (MC4R) and cadherin 6 (CDH6) were identified on chromosome 2, while myocyte enhancer factor 2c (MEF2c) was identified on the Z chromosome. However, marker-assisted selection using these regions indicated only minor contributions to ascites phenotype.

Dey *et al*. (133) identified the carboxypeptidase Q (CPQ) gene as a determinant of ascites phenotype in broilers via whole genome resequencing (WGR). Birds from the eighteenth generation of the REL line as well as birds from two commercial elite lines (one male line and one female line) were reared in the hypobaric chamber for 6 weeks at simulated 9,000 feet elevation. DNA was extracted from blood samples using a rapid method followed by organic extraction and redissolution in Te (10 mM TrisCl pH 7.5, 0.1 mM EDTA) (134). After DNA concentration was measured in each sample, 8 equal-weight pools of 10 DNAs each were created with 2 replicates of each pool: susceptible males, resistant males, susceptible females, and resistant females. These pools were then sequenced, giving 65 Gb of genomic data per pool. After aligning the sequences to Galgal5 separately for each gender, the SNP data was then exported for filtering; low coverage depth or missing SNP frequencies were filtered out. Finally, a calculation of the difference in average SNP frequencies of the two pools of each phenotype combination provided data that was plotted by chromosome position. This SNP frequency plot was searched for clusters of SNPs with opposing SNP frequencies between the susceptible and

resistant pools. The work initially focused on chromosome 2 based on the earlier data from GWAS but whereas GWAS identified MC4R and CDH6, the WGR identified a different region encompassing the 3’ end of the CPQ gene. Further genotyping based on SNPs in the CPQ gene confirmed the association of this regions with ascites.

Following the work done by Dey *et al.*, Parveen *et al*. (135) analyzed the WGR data for the rest of the genome. This led to the detection of 28 regions on 13 chromosomes which encompassed 64 genes including CPQ on chromosome 2 and leucine-rich repeat transmembrane neuronal 4 (LRRTM4) on chromosome 22. This region was identified in both males and females during WGR, making it a more promising candidate for genetic selection. LRRTM4 was found to be associated with many human blood and metabolic traits based on information sourced from the NCBI Phenotype-Genotype Integrator (136). Additionally, LRRTM4 was found to have an epistatic interaction with CPQ and the two in combination showed even greater promise for successful selection for ascites resistance.

Based on the available evidence from these studies, a MAS project was proposed that would take the logical next step: starting from REL breeder stock and using the two previously described validated genetic regions, breed two generations of MAS birds such that the F_2 generation would have only non-reference SNPs in those regions. Then, assess the impact of the genetic selection on ascites phenotype and on broiler production traits.

1.5 Infectious Bronchitis Virus

Infectious bronchitis (IB) is a respiratory illness caused by a gamma-coronavirus, infectious bronchitis virus (IBV), which was originally identified in North Dakota in 1931 and is now considered the second most threatening poultry disease (137–139). IBV has the capability to cause economic losses in several ways, including bird death, decreased growth rate, and

processing plant condemnations as well as the immense drop in egg production when IBV infects a flock of layer hens (140). The severity of infection is unique between flocks as it is influenced by the variant of the virus, environmental factors including but not limited to climate, ventilation, or ammonia levels, and bird age, type, and immune status (141,142). Symptoms are also understandably variable but can include coughing, sneezing, rales (clicking or rattling lung sounds), conjunctivitis, facial swelling, depressed activity and huddling behavior, reduced feed intake, and stunted weight gain (143). Initial infection through the respiratory tract causes destruction to the cilia found in the nasal passageways and tracheal epithelium with the largest viral load being found in the trachea three days after inoculation (140,144). In the cell, the virus drives the production of proteins which either inhibit or induce apoptosis depending on the tissue; in tissues directly related to symptomology, apoptosis is induced, while it is inhibited in other nearby tissues (144,145). Some IBV strains also cause nephritis, damaging the renal system after the initial respiratory symptomology including ruffled feathers, wet droppings, and increased water intake (143,146). While some birds die from IBV directly, secondary bacterial infections are the greater cause of overall mortality (140,141).

Due to the virus' inherent rapid mutation rate producing numerous variants, IBV is difficult to prevent using a vaccine and therefore the current prevention strategy involves one, or a combination of vaccine strains, to hopefully protect the birds from the variants the birds are most likely to encounter during their lifespan (137). Currently, live attenuated and inactivated IB vaccines are the most commonly used in the commercial setting. Both options have associated strengths and weaknesses. Live attenuated vaccines were the first successful IB prevention utilized commercially and are administered through the drinking water or by spraying chicks during the first 7 days (147). These vaccines, however, provide short-term immunity and require

a booster in the weeks following the initial administration (140). Inactivated IB vaccines are favorable in that they provide greater duration of protection when administered following a live vaccine (148). The largest drawback to inactivate vaccines is their route of administration being intramuscular injection which is less feasible in large-scale production operations. Problematically, the immune response measured in vaccinated flocks has been found to be extremely variable due to differences between flocks in bird type, flock size, and the specific housing, lighting, and ventilation (149). This is in addition to the necessity and potential for failure in finding the appropriate cocktail of vaccines to combat the variants most likely to be encountered by a given flock. Due to this and the continual emergence of new local variants, outbreaks continue to occur even in vaccinated flocks (150).

Given the challenges associated with preventing IBV using vaccines, it would be preferred to have a more consistent option to reduce the occurrence of IB in flocks. An additional point of interest warranting the study of IBV is its relationship with SARS-CoV-2. As previously stated, IBV is a gamma-coronavirus while SARS-CoV-2 is a beta-coronavirus (151). Though the organ system affected varies among coronaviruses, both gamma- and beta-coronaviruses cause respiratory illnesses (152). With the success of the ascites MAS project, continued improvements in the WGR pipeline, and the inherent connections to the COVID-19 pandemic, a project was proposed that would elucidate the genetic determinants of resistance or susceptibility to IBV in white leghorn chickens. This project would begin with an IBV challenge of young birds. Two weeks post-challenge the birds would be euthanized and sampled for assessment of the level of infection. Birds with high and low viral burdens could then be compared by WGR to identify genetic regions of influence. Once found, these regions could be used in the breeding schemes of layer hens in order to increase innate resistance to IBV.

1.6 Figures

Figure 1-1: Driving factors for traditional versus modern phenotypic selection in commercial breeding schemes (13).

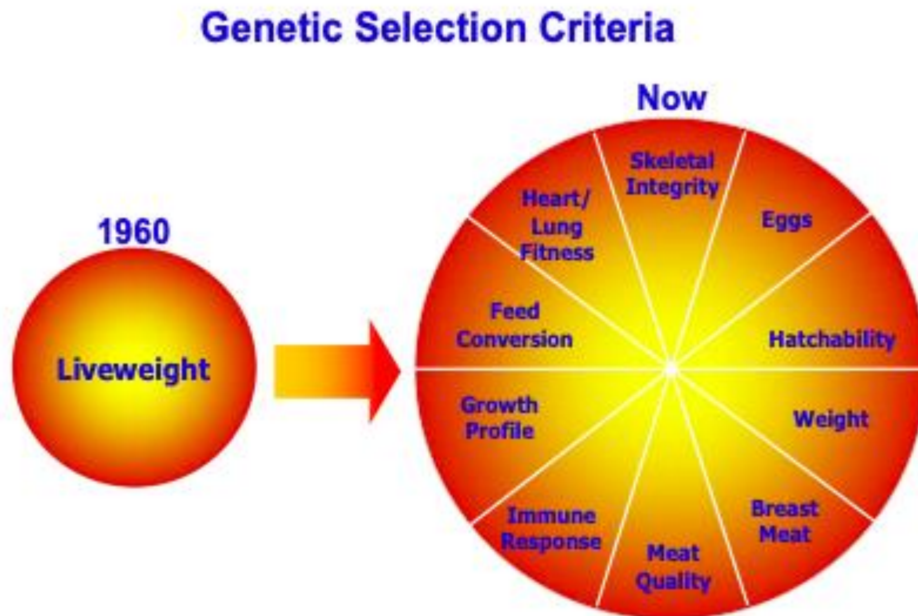


Figure 1-2: Breakdown of commercial broiler four-way breeding scheme (16).

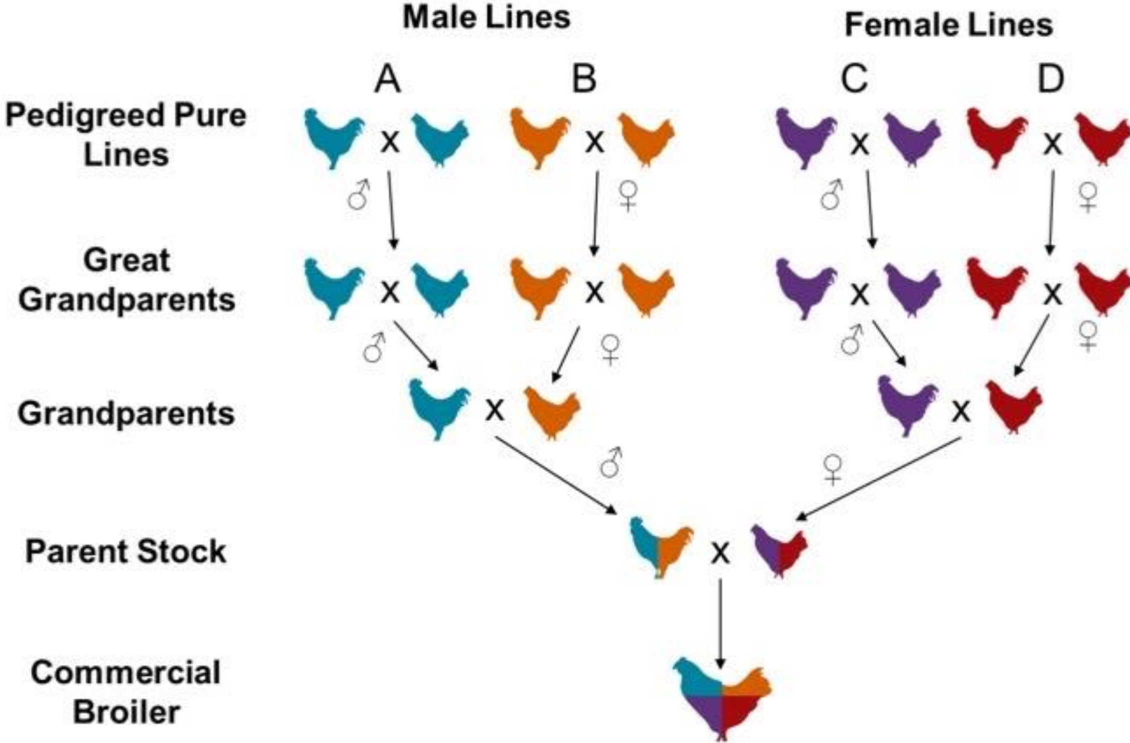


Figure 1-3: Physiological and pathophysiological flow of ascites development (89).

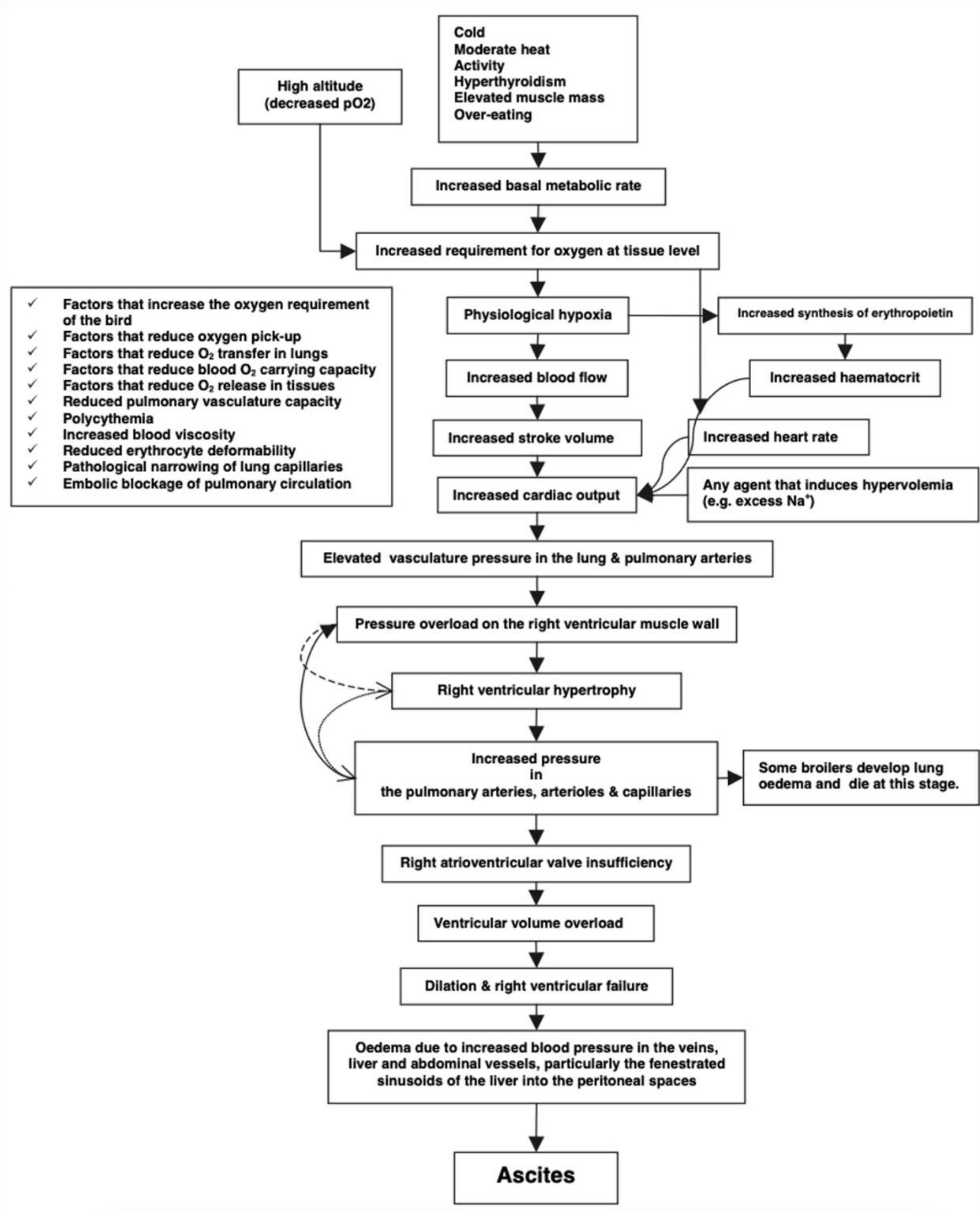


Figure 1-4: Trend in ascites mortality of divergently selected ascites experimental lines over 20 generations of selection (128).

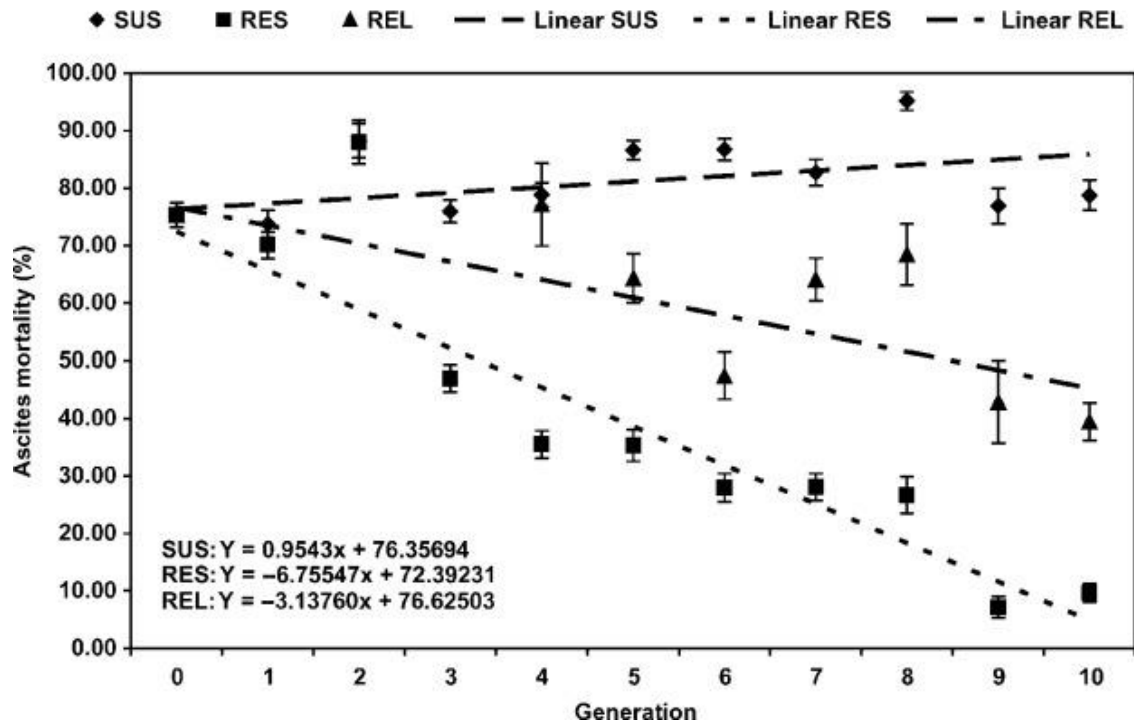
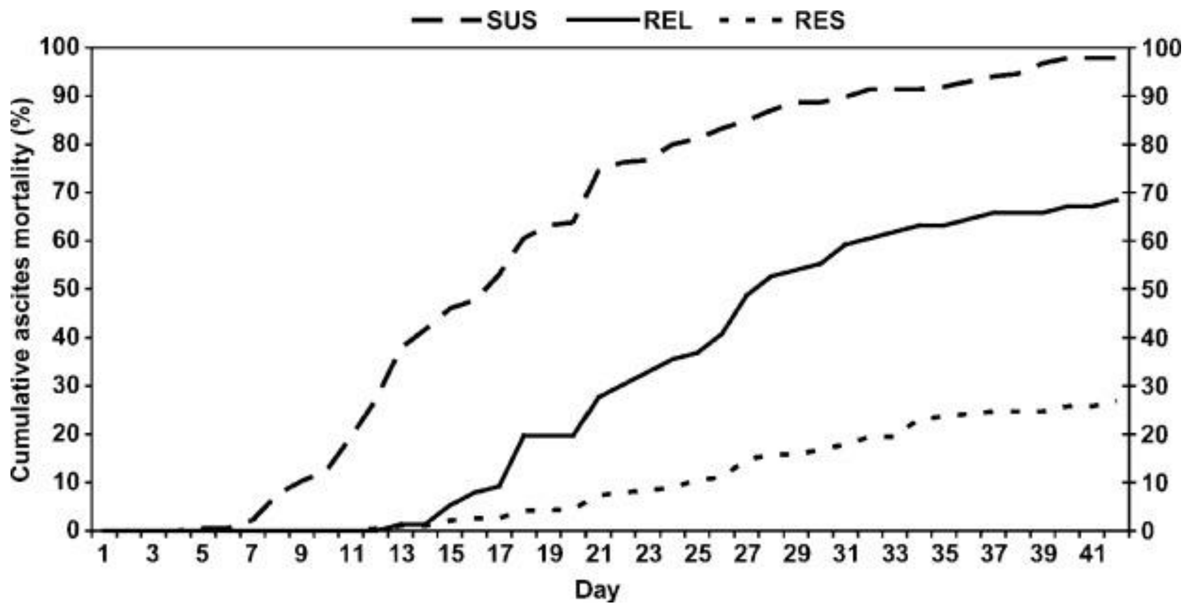


Figure 1-5: Trend in ascites mortality of divergently selected ascites experimental lines through the hypobaric challenge of one generation of birds (128).



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CHAPTER 2: SNP-BASED BREEDING FOR BROILER RESISTANCE TO ASCITES AND EVALUATION OF CORRELATED PRODUCTION TRAITS

2.1 Abstract

The goal of this study was to evaluate marker-assisted selection (MAS) in broiler chickens using previously mapped gene regions associated with ascites syndrome incidence. The second-generation MAS products were assessed for impact on ascites phenotype and whether there were associated changes in important production traits. Previously, we used whole genome resequencing (WGR) to fine-map 28 chromosomal regions as associated with ascites phenotype in our experimental ascites broiler line (Relaxed, REL) based on a hypobaric chamber challenge. Genotypes for single nucleotide polymorphisms (SNPs) in mapped regions on chromosomes 2 and 22, were used for MAS in our REL line. After two generations, birds homozygous for the genotypes associated with resistance for both chromosomal regions were established. The MAS F_2 generation was then compared to the REL line for ascites susceptibility and 25 production traits. Selection based on SNPs in the carboxypeptidase Q (CPQ, Gga2) and leucine rich repeat transmembrane neuronal 4 (LRRTM4, Gga22) gene regions resulted in a sex- and simulated altitude- dependent reduction of ascites incidence in two F_2 cohorts of the MAS line. Comparisons of the F_2 MAS and REL lines for production traits when reared at ambient pressure found no significant negative impacts for feed intake (FI), feed conversion ratio (FCR), or deboned part yields for either sex for two F_2 cohorts. There were, however, improvements in the MAS for full-trial body weight gain (BWG), FCR, absolute and relative tender weights, and relative drumstick weight. These results validate the mapping of the 28 chromosomal regions and demonstrate that fine mapping by WGR is an effective strategy for addressing a complex trait; it also stands as the first successful SNP-based selection program against a complex disease trait, such as ascites. The MAS line is comparable and, in some instances, superior, in growth

performance to the REL control while being more resistant to ascites. This study indicates that MAS based on WGR can provide significant breeding potential in agricultural systems.

2.2 Background

Since the 1950s, poultry breeding programs have selected for the increased ability of broilers to rapidly accrete muscle tissue, thus minimizing grow-out time and increasing profits. There have been, however, some negative results of this genetic progress, one of which is pulmonary hypertension syndrome (PHS), or ascites. Traditionally, ascites has been associated with rearing birds at higher elevations where partial pressures of oxygen are lower (1-11)(59,153,154), or in colder rearing environments (12, 13). Ascites syndrome is the terminal result of prolonged pulmonary hypertension, as liver damage releases ascitic fluid into the body cavity (9, 14, 15). Prolonged hypertension is driven by increased oxygen demands of a rapidly growing body supplied by an inadequate cardiopulmonary system (16-18). The incidence can be amplified as a result of an oxygen-reduced environment(79), or increased thermoregulation in cold environments (9, 15). The bird's body increases blood flow, increasing O_2 delivery, and subsequently increases pulmonary blood pressure in an attempt to respond to metabolic demands, which leads to incomplete gas exchange in the lungs (15, 19-21). Semi-oxygenated blood is then sent to the organs with substantial detrimental effect on the liver, causing the accumulation of abdominal fluid. It has been estimated that ascites is the cause of up to 8% of broiler mortality and accounts for up to $100,000,000 in economic losses annually, making it both a significant animal welfare and economic concern (6, 14, 15, 21-25).

Mitigation of ascites incidence has achieved varying degrees of success typically employing i) feed restriction (1, 24, 26-31) , ii) nutrient density modification to reduce protein (29, 31-33), or iii) feed additives such as arginine or antioxidants (29, 30, 34-37). Several of the methods for

reducing ascites simply slow growth and negatively affect flock production performance. Variability of efficacy found in these mitigation methods can result from genetic differences between commercial lines, environmental variations due to geography, and flock management. As ascites is estimated to have a relatively high heritability with reports ranging from 0.22 to 0.41, it is logical that increased ascites resistance through genetic selection could have significant advantages and increase production potential (4, 11, 38-40).

Previous research at the University of Arkansas on the genetic basis of ascites involved the development of three research lines from a commercial elite line through divergent selection for ascites resistance when exposed to simulated high elevation conditions (11). The base population (Relaxed, REL) for the selection study was derived from a commercial elite line in the 1990s and maintained through random mating without selection. Sib-selection based on ascites phenotype assessed through a 6-week hypobaric chamber challenge produced ascites resistant (RES) and ascites susceptible (SUS) lines. Rapid response in divergent selection with successive generations suggested a limited number of major genes. A series of genome-wide association studies (GWAS) using SNP panels identified a few candidate SNPs as associated with ascites phenotype, but subsequent MAS-based breeding projects were unsuccessful in validating these few loci (41-44). More recently, whole genome resequencing (WGR) identified 28 genomic regions where SNP clusters (100s to 1000s of SNPs) showed frequency bias with respect to ascites phenotype (45, 46). Two of these regions were validated by further genotyping of additional DNA samples and found to have potential epistatic interaction. One region spanned more than 120 kbp on chromosome 2 including the 3’ end of the gene for carboxypeptidase Q (CPQ). The second was an approximately 50 kbp region on chromosome 22 spanning the 3’ end of the gene for leucine-rich repeat transmembrane neuronal 4 (LRRTM4). Both these genes have

been associated in human GWAS with blood traits, heart rate, and blood pressure consistent with factors contributing to ascites incidence in poultry.

Therefore, the current study reports on whether MAS based on SNP genotypes for the regions of both CPQ and LRRTM4 can produce offspring with greater innate ascites resistance. Since ascites susceptibility could potentially be linked to important production traits, we also assessed the impact of selection on important broiler production traits.

2.3 Methods

All breeding, hatching, grow-out, and processing took place at the University of Arkansas Poultry Research farm. All animal procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (Approval Numbers 18083 and 18088) and performed in accordance with relevant guidelines and regulations. This study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

2.3.1 Bleeding, Genotyping, and Husbandry of Breeder Stock

Birds used for breeding were genotyped by collecting 10 µl of blood from the brachial vein which was further processed using a rapid DNA extraction method (47). These DNAs were then genotyped using exonuclease assays run in triplicate for both CPQ and LRRTM4 genes, as described (45, 46). Selected breeders were then moved into individual breeder cages for insemination and production of MAS offspring. At 18 wk, the birds were put on a lighting schedule to induce egg production: a starting schedule of 12 hours light:12 hours dark that progressed for 4 weeks to 16 hours light:8 hours dark, which was maintained through the insemination/egg collection period. Insemination occurred two times weekly, and eggs were collected daily and were labeled by hen. All eggs were stored at 18°C and 60% relative humidity until sufficient numbers were reached to begin hatching the next generation.

2.3.2 Hatchery Protocol

Sets of eggs were placed in a setting incubator (Jamesway Incubator Co., Cambridge, Ontario, Canada) at 99.6°F and 85% relative humidity for 18 days. On d 18, the eggs were candled, infertile eggs removed, fertile eggs placed into hatch baskets, and transferred by mating combination to a hatching incubator (Jamesway Incubator Co.) at 98.0°F and 88% relative humidity. On d 21, hatched chicks were wing-banded using different band colors for each sub-population, and individual band numbers recorded.

2.3.3 Hypobaric Trials

For both hypobaric cohorts, birds of each line (MAS and REL) were mixed in one of 40 battery cages (measuring 0.6 × 0.6 × 0.3 m) with appropriate numbers from each line to maintain even distribution throughout all the cages. No mortality data other than wing band number was collected for the first week so that final mortality rates would not be confounded by chicks that failed to start. For the remaining four weeks of the trial, the husbandry and necropsy methods followed those described previously (11, 41, 44). As the birds grew, bird densities were reduced in cages to maintain compliance with animal welfare requirements. Birds were initially chosen for removal due to observation of clinical ascites phenotype through palpation of the abdomen. All birds removed were euthanized and subsequently necropsied for ascites phenotype. Once all birds showing ascites phenotype had been removed, additional birds were chosen at random to meet welfare requirements and maintain consistent numbers for both lines. All birds remaining at the end of 5 weeks were euthanized and necropsied to determine ascites phenotype.

2.3.4 Floor Trials

Hatches were placed in floor pens that were top-dressed with fresh pine shavings. Similar numbers for each line were placed at similar densities. Surplus chicks were placed in a separate

pen. Cardboard trays for feed were placed and remained in the pens for the first 7 days of the trial. Feed and water were provided *ad libitum* throughout the trial. One continuous water line per row of pens was adjusted as needed for bird height. Health inspections occurred twice daily at a minimum. All mortalities were removed upon discovery and wing band number, pen of origin, body weight, and any clinical observations including ascites incidence were recorded. If available, the bird was replaced with another of the same sex and genetic line from the surplus birds. Feed was formulated to Cobb-Vantress, Inc., industry recommended standards (formulations can be found in Supplemental Table 1) and was added as needed throughout the trial. Feeding phases were as follows: starter from placement to d 14, finisher from d 14 to d 35, and withdrawal from d 35 to d 55. Pen weights were collected at time of placement, d 14, d 28, d 42, and d 49. Feed intake and feed conversion ratio (FCR) were recorded from d 49 until processing.

2.3.5 Processing

The day prior to processing, a subset of each cohort consisting of 25 males and 25 females per genetic line (n=100 per cohort) were randomly selected. These birds were removed from the pen first on the morning of processing and were removed from the processing line prior to evisceration to be necropsied for organ weights of liver, lungs (set), spleen, and heart, as well as RVTV. These bird carcasses were not chilled or deboned. Also on the day prior to processing, a second subset from each cohort were randomly chosen, consisting of 50 males and 50 females per genetic line (n=200 per cohort). These birds were wing-banded to be assessed for MAS impact on meat quality measurements of the breast fillet. Feed was removed 10 hours prior to processing to ensure feed passage. All birds were collected the morning of processing and transported to the University of Arkansas Poultry Pilot Processing Plant. At the processing plant,

back dock live weight was collected prior to the birds being electrically stunned and exsanguinated, followed by a scalding water bath to loosen feathers, then feather, head, and paw removal. Carcasses were then eviscerated, and the hot carcass and fat pad weights were collected. Carcasses were chilled for 3 hours and deboned thereafter for the determination of absolute weight and relative (to back dock live weight) yield of wings, breasts, tenders, thighs, and drumsticks. The 200 carcasses marked for further evaluation were processed as described but also evaluated for muscle quality traits including breast fillet weight, color, and pH at 4- and 24-hours chill time. The deviation between the 4- and 24-hour fillet weights were used to calculate drip loss. Color and pH were measured using a Minolta CR-400 handheld model with PC-linked SpectramagicX software and Testo model 205 handheld spear-tip probe, respectively. Color readings were taken on the dorsal surface of the breast, while pH was measured in the cranial region of the breast. The breasts were then frozen until the completion of both trials for cooking and shear force measurements. Shear requirements were calculated based on four measurements in the cranial region using a TA.XT*Plus* equipped with a Meullenet-Owens Razor Shear head attachment.

2.3.6 Statistical Methods

All statistical analyses other than the survival model were conducted in R with statistical significance denoted by a P-value ≤ 0.05.

Hypobaric mortality data were analyzed using a generalized linear model of final ascites mortality as well as a survival model which showed the effect of genetic line and sex on the probability of survival over time.

Live performance data were analyzed using two-way ANOVA between the main effects of trial and genetic line. Parts weights, organ weights, heart characteristic, and meat quality

characteristic data from processing were analyzed using three-way ANOVA between the main effects of trial, genetic line, and sex. All means were separated by Tukey's Honest Significant Difference (HSD) test.

2.4 Results

2.4.1 First-Generation Breeding

Breeders for the first parent generation were selected from REL breeder stock in July of 2018. After bleeding and genotyping, two P_0 crosses were then created: one consisting of birds with all non-reference SNPs (compared to galGal6; 46) for each gene region, designated P_0-1, and the other consisting of birds with non-reference SNPs for CPQ and heterozygous SNPs for LRRTM4, designated P_0-2. P_0-1 consisted of 10 males and 13 females; P_0-2 consisted of 12 males and 24 females. Separately, ungenotyped REL birds were used to breed generation 1 of the control population. Semen was collected from all the males of each P_0 group, pooled, and used to artificially inseminate each of the females from the same P_0 group.

2.4.2 Second-Generation Breeding

After hatching, the F_1 progeny produced from P_0-1 and P_0-2 were kept in floor pens and managed as breeders until they reached 18 weeks of age, at which point they were then bled and genotyped. Breeders for each F1 population were placed in individual breeder cages and photo stimulated. In May of 2019, breeders from the F_1 were selected based on being homozygous for the non-reference SNPs for both genes; though all breeder SNP genotypes from this point forward were the same, the F1 populations were kept separate in order to complete reciprocal matings between them. Breeders from F_1-1 (from P_0-1) consisted of 12 males and 31 females; F_1-2 (from P_0-2) consisted of 12 males and 37 females. To produce the control group, 24 males and 72 females from the REL line were also utilized. Insemination began at the same time, when the

MAS breeders were 22 weeks of age and the REL control breeders were 20 weeks of age. For the REL, pooled semen from all 24 males were used to artificially inseminate all 72 REL females. For the MAS, pooled semen from the F_1-1 males was used to artificially inseminate the F_1-2 females and similarly the F_1-2 males were used to inseminate the F_1-1 females. This reciprocal mating scheme produced the F2 generation of birds which would possess only the non-reference SNPs for the CPQ and LRRTM4 genes. Sets of eggs for hatching included at least 250 eggs each from REL and the F_2 of the MAS. At transfer, all eggs were candled and infertile or eggs with embryonic mortality were removed and stored for breakout on hatch day along with eggs that did not hatch; no significant difference ($P>0.05$) in hatchery breakout was found between the two lines. After hatch, birds received a wing band that represented their genetic line (either MAS or REL). Sets of eggs were produced every two weeks for four total hatches. The first and fourth hatches were subjected to 5-week hypobaric challenges. The second and third hatches were placed for 8-week floor pen trials to evaluate change in production traits associated with MAS.

2.4.3 Hypobaric Challenges

F2 chicks for challenge in the hypobaric (Hypo) chamber were placed on November 28, 2019 (Hypo1) and January 8, 2020 (Hypo2). Hypo1 was maintained at 9,000 ft simulated altitude while Hypo2 was initially set at 9,000 ft simulated altitude, then after 2 weeks increased to 11,000 ft simulated elevation to induce a higher incidence of ascites. In Hypo1, all hatched birds were placed in the chamber (n=578) whereas in Hypo2, similar numbers of birds were placed from each line and fewer total birds were placed (n=433) which reduced the number of birds that would need to be culled for compliance with bird density regulations.

The hypobaric challenge results indicate a sex- and elevation-dependent reduction in ascites incidence in both cohorts. Hypo1 saw an overall decrease (P=0.041) in ascites mortality between the MAS and REL birds, with a 27.3% reduction for ascites in MAS males and a 39.8% in MAS females [Table 1]. For Hypo2, there was an overall numerical, although not statistically significant (P=0.162), decrease in ascites mortality between the lines, with reductions of 23.4% in males (P=0.126) and only 5.2% reduction in females [Table 2]. There was no significant difference (P>0.05) between the lines for the right ventricle to total ventricle (RVTV) ratio or body weight. The Kaplan-Meier survival model curve visually echoes these trends, however the analyzed P-values from this model are only numerically different (P>0.05) [Figure 1].

2.4.4 Floor Trials

Birds reared for the floor trials were placed on December 12, 2019 (Floor1) and December 28, 2019 (Floor2). On the day of placement, the total number of hatched birds was counted for each line and the number of birds placed per pen determined based on the smallest group; for Floor1 this was 14 birds per pen (0.133 m^2/bird) and for Floor2 13 birds per pen (0.143 m^2/bird) with all excess birds placed in spare pens for mortality replacement up until the beginning of the FCR measurement period from d 49 to d 55. As both floor trials were run concurrently in the same barn, though offset by two weeks, we considered whether the live performance and processing data should be analyzed as completely separate trials with separate analyses or together by adding the main effect of trial. After consultation with a professional agricultural statistician and colleagues at the University of Arkansas, the latter option was chosen. However, the interaction between line and trial was assessed for each measurement, and any traits identified as having an interaction between line and trial were noted and are discussed.

Conclusions about the overall effect of MAS on that particular measurement were not drawn in those cases.

Live performance data from both cohorts is shown in Table 2. Significant differences were found between trials: d0 body weight (BW) ($P<0.001$), d42 BW ($P=0.038$), d0-42 body weight gain (BWG) ($P=0.045$), d49 BW ($P=0.006$), d0-49 BWG ($P=0.007$), d54 BW ($P=0.017$), and full-trial BWG ($P=0.019$). Additionally, the genetic lines were significantly different in d49-54 BWG ($P=0.036$) and FCR ($P<0.001$), both of which were improved in the MAS. Significant differences were found between trial and genetic line for d14 BW ($P=0.019$) and d0-14 BWG ($P=0.015$).

2.4.5 Processing

Total sample sizes and tabular representations of the data are as follows: Table 3, live weight and carcass characteristics (n=868); Table 4, deboned parts (n=868); Table 5, organ weights (n=295); Table 6, heart characteristics (n=295); Table 7, meat quality characteristics (n=395). A significant improvement was seen in the MAS birds for absolute and relative tender weights ($P<0.001$, $P<0.001$, respectively), relative drumstick weight ($P=0.016$), and significant differences were found for 24-hour L* color measurement ($P<0.001$), 24-hour a* color measurement ($P=0.037$), and 24-hour pH ($P=0.003$). Significant differences were found between sexes in relative hot carcass weight ($P<0.001$), wing weight ($P<0.001$), relative thigh weight ($P<0.001$), relative drumstick weight ($P<0.001$), absolute and relative heart weight ($P<0.001$, $P=0.018$, respectively), liver weight ($P<0.001$), lung weight ($P<0.001$), spleen weight ($P<0.001$), RV weight ($P<0.001$), TV weight ($P<0.001$), drip loss ($P<0.001$), 24-hour L* color measurement ($P<0.001$), 24-hour pH ($P<0.001$), and shearing peaks ($P=0.016$).

Significant differences were also found between the two floor trials for many characteristics, including wing weight (P<0.001), relative breast weight (P<0.001), relative thigh weight (P<0.001), relative drumstick weight (P=0.002), relative heart weight (P<0.001), liver weight (P=0.009), relative spleen weight (P=0.014), 24-hour a* color measurement (P=0.003), 24-hour b* color measurement (P=0.017), 24-hour pH (P<0.001), and shearing peaks (P=0.002). Due to this, there were also several significant interactions. Between trial and genetic line, significant differences were found for RV weight (P=0.046), RVTV (P=0.005), shear force requirement (P<0.001), and 1:3 shear area (P<0.001). While RV was found to be significantly different, Tukey's HSD test was unable to separate the means. RVTV was found to be the largest in MAS birds from both trials and the smallest in REL birds from both trials. The largest shear force measurements were found in the Floor2 REL breasts, with intermediate force requirements in the Floor1 MAS group, and the lowest requirements in the Floor1 REL and Floor2 MAS groups. The largest 1:3 shear area measurements were found in the Floor2 REL group, with all other groups having comparable lower measurements.

A number of significant differences were found between trial and sex. These were live weight (P=0.035), hot carcass weight (P=0.028), fat pad weight (P=0.019), chilled carcass weight (P=0.036), absolute and relative tender weight (P=0.010, P=0.014, respectively), thigh weight (P=0.025), drumstick weight (P=0.032), relative liver weight (P=0.032), shear force requirement (P=0.010), and 1:3 shear area (P=0.040). The live weight and weights of hot carcass, chilled carcass, and tenders were the largest for the Floor1 males, moderate for Floor2 males, and smallest for females in both trials. The largest fat pads were found in the Floor1 males, moderate for Floor1 females, small intermediate for Floor2 females, and smallest in Floor2 males. Relative tender weight was found to be the greatest in Floor2 females, moderate in Floor1 females, and

the lowest in males from both trials. The mean weights of thighs and drumsticks were separated into four distinct groups, from largest to smallest being Floor1 males, Floor2 males, Floor1 females, and Floor2 females. The relative weight of liver was found to be the largest in females from both trials and the smallest in males from both trials. For both the shear force requirement and the 1:3 shear area, the measurements from Floor1 females were found to be the largest, the Floor1 males were found to be the smallest, and both sexes in Floor2 were intermediate.

Finally, there were interactions found between genetic line and sex. These were live weight (P=0.011) hot carcass weight (P=0.012), chilled carcass weight (P=0.019), breast weight (P=0.050), thigh weight (P=0.006), drumstick weight (P=0.032), and 24-hour b* color measurement (P=0.025). For live weight and the weights of hot carcass, chilled carcass, thighs, and drumsticks, MAS males were found to be the largest, REL males were intermediate, and females from both the MAS and REL lines were found to be the smallest. Breast weight was the largest for males from both the MAS and REL lines, with the smallest weight in the females from both lines. The 24-hour b* color measurement was found to be the highest in MAS females, with the three other groups having comparable lower b* measurements.

There was also a single three-way interaction (P=0.039) found for the processing measurements, which was fat pad. In order from largest to smallest, the means were separated as follows: Floor1 MAS females, Floor1 REL females, Floor2 females from both lines, Floor1 REL males, Floor1 MAS males, Floor2 MAS males, and Floor2 REL males.

2.5 Discussion

Ascites has been reported to have a significant sex-dependent incidence in flocks (39, 48). In the REL line, females show an earlier onset and higher overall incidence of ascites (11). However, a reduction of ascites incidence was observed between MAS females of the first and

fourth cohorts for hypobaric challenges where the simulated altitude differed. At moderate elevations (9,000 ft) in Hypo1, ascites incidence was reduced by nearly 40% for the MAS females, while at higher simulated altitude (11,000 ft) in Hypo2, there was only a 5% reduction of female mortality from 61.9% in the REL line to 58.7% in the MAS line. For males, incidence was reduced in the MAS line by 27% in Hypo1, while being reduced by 23% in Hypo2 with the increased simulated altitude. This further demonstrates the sex-linked nature of ascites resistance or susceptibility and suggests more research into epistasis with genes on the sex chromosomes are warranted. Additional research into the impact of MAS on each sex is also warranted. The timeline of this study, and the capacity of the hypobaric chamber, did not allow for single-sex experiments to allow for greater sample sizes. However, even with relatively small cohort sizes, significant reduction of ascites for the MAS line was observed.

An additional point of success in these results comes from the lack of impact on production traits. If MAS negatively impacted economically important traits in any major way, then MAS would be much less appealing to the industry. From the live production data, a significant improvement of the MAS was found in body weight gain from the full trial and in FCR. A limited number of traits showed significant differences between overall MAS and REL averages, all of which were improved in the MAS birds; these were absolute and relative tender weights and relative drumstick weight. Additionally, there were some improvements in the MAS that were only seen in one sex. These were live weight, hot carcass, chilled carcass, thigh weight, and drumstick weight, all of which were larger in MAS males over REL males while the MAS and REL female measurements in each of these cases were statistically equivalent. Given these improvements, MAS appears to be not only capable of reducing ascites incidence, but to have a positive impact on some growth characteristics. Notably, there was a significant three-way

interaction between trial, genetic line, and sex for fat pad weight relative to back-dock live weight. From the trend in this characteristic, we observed that the females from each cohort had larger relative fat pad weights than the males, but as this did not have an impact on the trends of the deboned parts data, we are less concerned about this outcome.

We also identified differences between MAS and REL for breast color measurements. The measurement of each breast fillet was broken into three components: L* represents the lightness from 0 (black) to 100 (white); a* represents the color spectrum from -60 (green) to +60 (red); b* represents the color spectrum from -60 (blue) to +60 (yellow) (155). A significant difference was found in the L* and a* measurements between the two genetic lines, and in the L* measurement between the sexes. The difference in L* measurements between the two genetic lines is especially interesting as this means that MAS line breast fillets were consistently lighter in color than the REL. While the classification of Qiao et al. (50) considers all of the L* measurements from both cohorts except for MAS female to be "normal" in lightness (MAS female would be classified as "lighter than normal"). This color difference is difficult to visually discern, however fillets with higher L* values may have higher moisture contents. Thus, the fillets from MAS may have consistently greater moisture than the REL. The data also showed an interaction between genetic line and sex with the b* measurement. However, other changes occurred that were only numeric; some measurements increased nearly one unit between the two cohorts, others changed which group had a greater value for a particular measurement. These variations could trace back to minor differences in formulation of individual batches of feed used for the two trials or to other causes still unknown. While these are relatively small fluctuations, it is difficult to know exactly how much of an impact it might have on consumer perspective of the color of these fillets (51).

These data validate the WGR approach for identifying regions for MAS for improving multigene traits in commercial breeding programs. However, there are limitations. One limitation is that the regions we identified in REL by WGR may not be relevant in current elite lines. The REL is the unselected descendant from a commercial elite line from the 1990s (11). The genetics of modern elite broiler lines have undoubtedly changed in the ensuing two decades. WGR for ascites in two current commercial broiler crosses did not find associations with either the CPQ or LRRTM4 regions (unpublished). Current elite lines could be subjected to WGR using the hypobaric chamber challenge to identify line-specific regions associated with ascites phenotype. Despite the unique and proprietary nature of each commercial line, each could be assessed individually, candidate gene regions validated, and then informative regions included in selection programs. Alternatively, commercial broilers could be subjected to WGR to identify the regions to be selected for in the elite lines to produce the required genetics in the terminal cross for production of those broiler products. Further research and MAS projects for the major modern commercial crosses are needed to fully understand the efficacy of this method against ascites.

Though its prevalence in the US market varies by specific commercial products (unpublished data) and geographical region, ascites still remains problematic in the global market given worldwide variation in climates, elevations, and management strategies. Published data for mortality and economic impact are nearing or surpass two decades old (21, 25), warranting new assessments of the impact on the US and global markets. Based on existing statistics, significant reduction could potentially prevent millions of bird deaths, saving millions of dollars for the industry. Our results document that genetics can be used to significantly reduce ascites without compromising production.

2.6 Conclusions

These results represent the first documented success in fine-mapping and marker-assisted selection for a complex trait in a poultry species. WGR has the potential to not only identify other genetic regions for selection against ascites, but also for other complex traits. One problem with general selection against ascites is the tendency for smaller birds to be more resistant to the disease (32, 52), meaning strictly phenotype-based selection could negatively impact growth rate and feed efficiency. Given the specificity of the regions utilized in our MAS experiment, there is a strong potential that they could be easily integrated into the current breeding programs of poultry genetics companies. This could increase innate resistance to ascites without having to "back-track" over years of selection for growth traits. WGR and MAS hold great promise for targeted genetic selection in agricultural systems.

2.7 Tables and Figures

Figure 2-1 Survival plots for the two hypobaric chamber challenges comparing the MAS and REL lines according to sex.

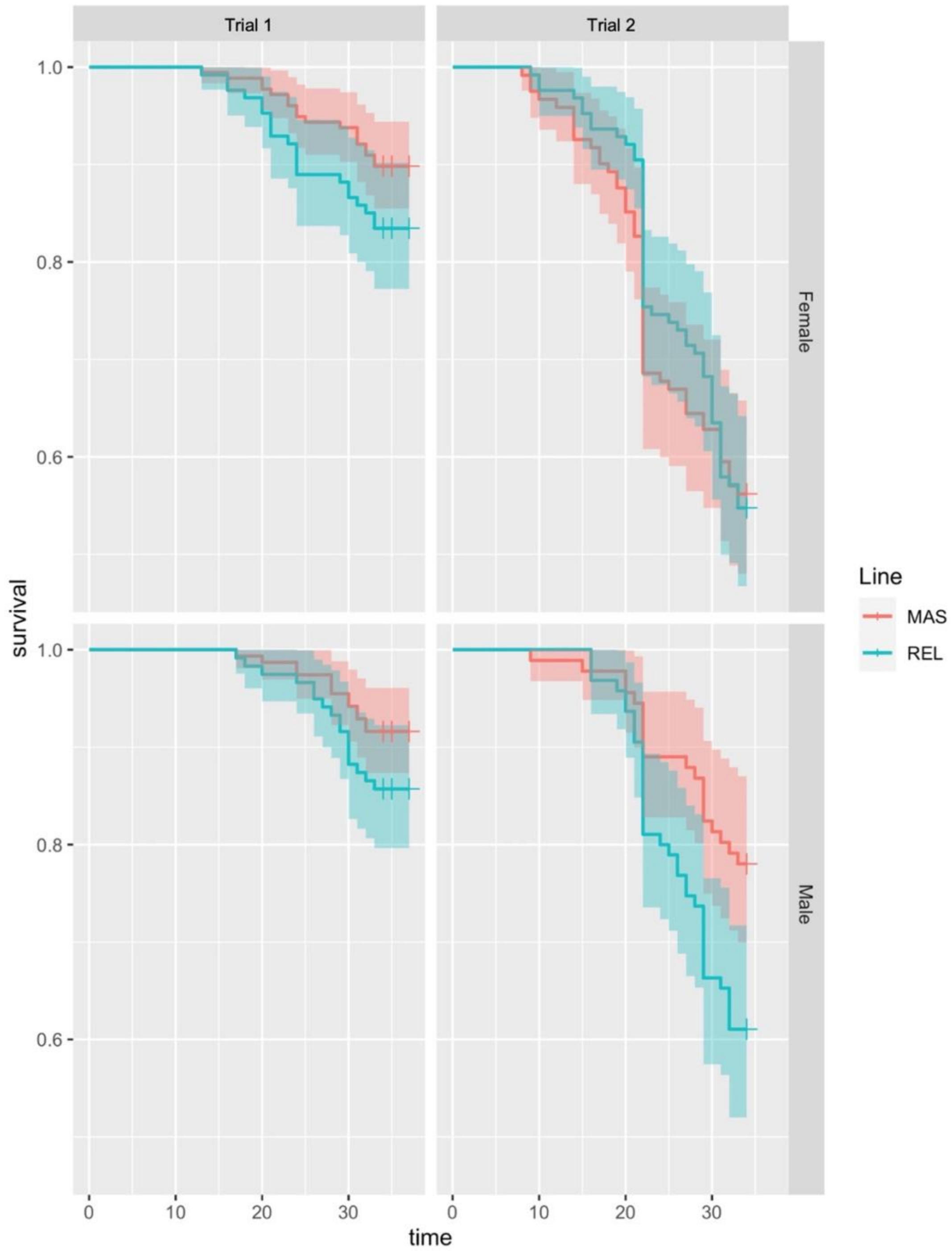

Table 2-1. Ascites incidence and cardiac hypertrophy (RVTV) for the two hypobaric trials comparing the MAS and REL lines overall and by gender.

			Ascites			
Item	n	RVTV	No	Yes	Percent, %	Difference, %
Hypobaric trial 1						
Sex						
Male	274	0.305	237	37	13.50	28.00
Female	304	0.321	247	57	18.75	
Line						
MAS	332	0.310	288	44	13.25	34.83
REL	246	0.318	196	50	20.33	
Interactions						
Male × MAS	155	0.299	137	18	11.61	27.27
Male × REL	119	0.314	100	19	15.97	
Female × MAS	177	0.319	151	26	14.69	39.82
Female × REL	127	0.323	96	31	24.41	
P-value						
Sex		0.009			0.047	
Genetic Line		0.160			0.041	
Sex × Line		0.367			0.742	
Hypobaric Trial 2						
Sex						
Male	186	0.387	108	78	41.94	30.47
Female	247	0.421	98	149	60.32	
Line						
MAS	212	0.403	108	104	49.06	11.86
REL	221	0.410	98	123	55.66	
Interactions						
Male × MAS	91	0.39	58	33	36.26	23.44
Male × REL	95	0.39	50	45	47.37	
Female × MAS	121	0.42	50	71	58.68	5.21
Female × REL	126	0.43	48	78	61.90	
P-value						
Sex		<0.001			<0.001	
Genetic Line		0.404			0.162	
Sex × Line		0.550			0.415	

Abbreviations: MAS = marker-assisted selection line; REL = Relaxed (control) line; RV = right ventricle; TV = total ventricle

Table 2-2. Live performance data from the replicate floor trials.

Item	n	d0, g/bird	d14, g/bird	d28, g/bird	d42, g/bird	d49, g/bird	d54, g/bird	BWG, g/bird (d49-54)	FI, g/bird (d49-54)	FCR, g:g (d49-54)
Main effect of trial										
Floor1	40	38.00[a]	255.0[a]	934.8	1987[a]	2522[a]	2888[a]	365.5	0.932	2.566
Floor2	40	36.65[b]	236.7[b]	947.7	1949[b]	2458[b]	2822[b]	368.3	0.937	2.560
SEM		0.15	2.1	6.3	13	16	20	4.6	0.007	0.021
Main effect of genetic line										
MAS	40	37.30	246.4	940.3	1976	2507	2875	372.9[a]	0.935	2.512[b]
REL	40	37.35	245.3	942.2	1960	2474	2835	361.0[b]	0.933	2.613[a]
SEM		0.19	2.8	6.4	13	17	20	4.3	0.007	0.021
Trial × Line										
Floor1 × MAS	20	38.06	252.4[a]	927.9	1993	2541	2909	368.4	0.928	2.523
Floor1 × REL	20	37.94	257.6[a]	941.8	1981	2504	2867	362.5	0.935	2.609
Floor2 × MAS	20	36.54	240.4[b]	952.6	1960	2473	2842	377.7	0.943	2.500
Floor2 × REL	20	36.76	233.0[b]	942.7	1939	2444	2803	359.5	0.931	2.618
SEM		0.22	3.2	9.7	21	26	31	7.1	0.010	0.031
***P*-values**										
Trial		**<0.001**	**<0.001**	0.142	**0.038**	**0.006**	**0.017**	0.585	0.583	0.773
Genetic line		0.800	0.674	0.819	0.358	0.155	0.137	**0.036**	0.812	**<0.001**
Trial × Line		0.393	**0.019**	0.174	0.801	0.872	0.946	0.279	0.303	0.536

Abbreviations: Floor1 = Floor cohort 1; Floor2 = Floor cohort 2; MAS = marker-assisted selection line; REL = Relaxed (control) line; d = day; BWG = body weight gain; FI = feed intake; FCR = feed conversion ratio

Table 2-3. Live weight and carcass characteristics from the replicate floor trials.

Item	n	Live Weight, kg	Hot carcass Weight, kg	Hot carcass Yield[1], %	Fat pad Weight, kg	Fat pad Yield, %	Chilled carcass Weight, kg	Chilled carcass Yield, %
Main effect of trial								
Floor1	453	2814^{a}	1996^{a}	70.94	67.20^{a}	2.402^{a}	2058^{a}	73.13
Floor2	415	2730^{b}	1941^{b}	71.11	59.53^{b}	2.194^{b}	2002^{b}	73.35
SEM		19	14	0.07	0.88	0.030	14	0.07
Main effect of genetic line								
MAS	439	2795^{a}	1988^{a}	71.12	64.03	2.307	2050^{a}	73.33
REL	429	2752^{b}	1951^{b}	70.91	63.00	2.297	2013^{b}	73.14
SEM		19	14	0.07	0.91	0.031	14	0.07
Main effect of sex								
Male	395	3117^{a}	2223^{a}	71.33^{a}	65.56^{a}	2.104^{a}	2281^{a}	73.22
Female	473	2488^{b}	1759^{b}	70.75^{b}	61.81^{b}	2.469^{b}	1823^{b}	73.25
SEM		13	10	0.07	0.91	0.029	10	0.08
Trial × Line								
Floor1 × MAS	233	2826	2010	71.09	67.00	2.385	2072	73.29
Floor1 × REL	220	2803	1983	70.77	67.42	2.420	2045	72.97
Floor2 × MAS	206	2761	1964	71.15	60.67	2.218	2025	73.38
Floor2 × REL	209	2699	1918	71.06	58.41	2.170	1979	73.33
SEM		27	20	0.10	1.36	0.045	20	0.11
Trial × Sex								
Floor1 × Male	204	3174^{a}	2264^{a}	71.29	70.71^{a}	2.236	2322^{a}	73.13
Floor1 × Female	249	2513^{c}	1773^{c}	70.65	64.31^{b}	2.540	1838^{c}	73.14
Floor2 × Male	191	3055^{b}	2180^{b}	71.38	60.07^{c}	1.962	2238^{b}	73.32
Floor2 × Female	224	2460^{c}	1743^{c}	70.87	59.08^{bc}	2.391	1806^{c}	73.38
SEM		19	15	0.10	1.29	0.042	15	0.11
Line × Sex								
MAS × Male	209	3147^{a}	2248^{a}	71.42	66.98	2.128	2306^{a}	73.30
MAS × Female	230	2476^{c}	1753^{c}	70.84	61.33	2.469	1818^{c}	73.36
REL × Male	186	3085^{b}	2197^{b}	71.23	63.95	2.076	2255^{b}	73.13
REL × Female	243	2498^{c}	1764^{c}	70.67	62.27	2.468	1827^{c}	73.15
SEM		21	16	0.10	1.38	0.042	16	0.11
Trial × Line × Sex								
Floor1 x MAS x Male	114					2.201^{cd}		
Floor1 x MAS x Female	118					2.564^{a}		
Floor1 x REL x Male	90					2.281^{bcd}		
Floor1 x REL x Female	127					2.518^{ab}		
Floor2 x MAS x Male	95					2.042^{de}		
Floor2 x MAS x Female	111					2.369^{abc}		
Floor2 x REL x Male	96					1.883^{e}		
Floor2 x REL x Female	113					2.413^{abc}		
SEM						0.062		
***P*-values**								
Trial		**<0.001**	**<0.001**	0.078	**<0.001**	**<0.001**	**<0.001**	0.270
Genetic line		**0.008**	**0.003**	0.054	0.313	0.618	**0.003**	0.069
Sex		**<0.001**	**<0.001**	**<0.001**	**0.002**	**<0.001**	**<0.001**	0.640
Trial × Line		0.235	0.432	0.293	0.274	0.349	0.468	0.174

Table 2-3 (Cont.)

Trial × Sex	**0.035**	**0.028**	0.514	**0.019**	0.108	**0.036**	0.909
Line × Sex	**0.011**	**0.012**	0.942	0.200	0.628	**0.019**	0.915
Trial × Line × Sex	0.980	0.992	0.637	0.093	**0.039**	0.963	0.889

Abbreviations: Floor1 = Floor cohort 1; Floor2 = Floor cohort 2; MAS = marker-assisted selection line; REL = Relaxed (control) line
1: Yields calculated based on back-dock live weight

Table 2-4. Deboned parts characteristics from the replicate floor trials.

Item	n	Wings		Breast fillets		Tenders		Thighs		Drumsticks	
		Weight, kg	Yield[1], %	Weight, kg	Yield, %	Weight, kg	Yield, %	Weight, kg	Yield, %	Weight, kg	Yield, %
Main effect of trial											
Floor1	453	223.2[a]	7.947	431.6	15.33[b]	112.2	4.001[b]	380.4[a]	13.47[a]	271.9[a]	9.653[a]
Floor2	415	216.6[b]	7.936	432.4	15.79[a]	111.2	4.080[a]	362.7[b]	13.25[b]	260.3[b]	9.537[b]
SEM		1.5	0.020	3.7	0.07	0.8	0.020	3.3	0.05	2.1	0.027
Main effect of genetic line											
MAS	439	221.2	7.928	435.9	15.57	114.0[a]	4.091[a]	376.3[a]	13.41	269.8[a]	9.649[a]
REL	429	218.9	7.956	427.9	15.54	109.5[b]	3.985[b]	367.5[b]	13.31	262.9[b]	9.546[b]
SEM		1.5	0.020	3.7	0.07	0.8	0.018	3.3	0.05	2.1	0.027
Main effect of sex											
Male	395	247.5[a]	7.943	483.2[a]	15.49	121.8[a]	3.907[b]	427.6[a]	13.71[a]	302.7[a]	9.723[a]
Female	473	197.2[b]	7.940	389.1[b]	15.60	103.3[b]	4.149[a]	325.4[b]	13.07[b]	236.2[b]	9.494[b]
SEM		1.0	0.019	3.2	0.07	0.7	0.018	2.5	0.05	1.5	0.027
Trial × Line											
Floor1 × MAS	233	224.1	7.925	436.7	15.37	114.4	4.057	383.5	13.53	274.7	9.717
Floor1 × REL	220	222.2	7.970	426.1	15.29	110.0	3.941	377.4	13.39	269.1	9.586
Floor2 × MAS	206	217.9	7.932	435.0	15.79	113.5	4.130	368.2	13.27	264.1	9.571
Floor2 × REL	209	215.4	7.940	429.8	15.80	108.9	4.031	357.1	13.22	256.5	9.504
SEM		2.2	0.029	5.3	0.10	1.2	0.029	4.7	0.08	3.0	0.040
Trial × Sex											
Floor1 × Male	204	252.1	7.938	486.7	15.34	123.6[a]	3.899[c]	439.6[a]	13.85	310.2[a]	9.779
Floor1 × Female	249	199.4	7.954	386.2	15.33	102.7[c]	4.084[b]	330.9[c]	13.15	240.0[c]	9.550
Floor2 × Male	191	242.5	7.949	479.4	15.66	119.9[b]	3.915[c]	414.8[b]	13.57	294.7[b]	9.662
Floor2 × Female	224	194.7	7.925	392.3	15.90	104.0[c]	4.220[a]	319.3[d]	12.97	231.9[d]	9.433
SEM		1.3	0.029	4.7	0.10	1.1	0.027	3.6	0.08	2.0	0.038
Line × Sex											
MAS × Male	209	248.8	7.911	489.8[a]	15.53	124.2	3.944	434.2[a]	13.79	306.8[a]	9.765
MAS × Female	230	196.3	7.944	387.0[b]	15.60	104.7	4.225	323.7[c]	13.07	236.4[c]	9.544

Table 2-4 (Cont.)

REL × Male	186	246.0	7.980	475.8^{a}	15.45	119.4	3.865	420.6^{b}	13.63	298.5^{b}	9.675
REL × Female	243	198.0	7.937	391.0^{b}	15.61	101.8	4.076	326.7^{c}	13.06	235.7^{c}	9.447
SEM		1.7	0.028	5.0	0.11	1.1	0.026	3.7	0.08	2.3	0.041
***P*-values**											
Trial		**<0.001**	0.630	0.834	**<0.001**	0.296	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.002**
Genetic line		0.840	0.286	0.324	0.665	**<0.001**	**<0.001**	**0.003**	0.258	**<0.001**	**0.016**
Sex		**<0.001**	0.773	**<0.001**	0.180	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Trial × Line		0.085	0.393	0.698	0.532	0.924	0.631	0.404	0.897	0.564	0.563
Trial × Sex		0.053	0.558	0.079	0.210	**0.010**	**0.014**	**0.025**	0.481	**0.032**	0.903
Line × Sex		0.213	0.185	**0.050**	0.538	0.339	0.127	**0.006**	0.287	**0.032**	0.842
Trial × Line × Sex		0.245	0.073	0.568	0.441	0.725	0.668	0.607	0.251	0.906	0.827

Abbreviations: Floor1 = Floor cohort 1; Floor2 = Floor cohort 2; MAS = marker-assisted selection line; REL = Relaxed (control) line

1: Yields calculated based on back-dock live weight

Table 2-5. Organ weights from the replicate floor trials.

Item	n	Heart		Liver		Lungs		Spleen	
		Weight, g	Yield[1], %	Weight, g	Yield, %	Weight, g	Yield, %	Weight, g	Yield, %
Main effect of trial									
Floor1	95	14.95	0.626[b]	50.91[a]	2.108	15.78	0.663	2.878	0.122[b]
Floor2	100	15.17	0.666[a]	47.51[b]	2.077	15.47	0.680	3.083	0.135[a]
SEM		0.30	0.009	1.05	0.034	0.36	0.013	0.079	0.003
Main effect of genetic line									
MAS	98	15.07	0.643	49.78	2.108	15.43	0.663	3.001	0.129
REL	97	15.06	0.651	48.58	2.076	15.82	0.681	2.965	0.128
SEM		0.31	0.009	0.98	0.030	0.37	0.013	0.080	0.003
Main effect of sex									
Male	98	17.01[a]	0.660[a]	51.42[a]	1.983[b]	17.16[a]	0.664	3.176[a]	0.123
Female	97	13.10[b]	0.633[b]	46.88[b]	2.208[a]	14.09[b]	0.680	2.788[b]	0.134
SEM		0.20	0.008	1.05	0.032	0.34	0.013	0.083	0.003
Trial × Line									
Floor1 × MAS	48	15.02	0.624	51.40	2.111	15.78	0.661	2.808	0.118
Floor1 × REL	47	14.88	0.629	50.43	2.105	15.78	0.665	2.951	0.125
Floor2 × MAS	50	15.11	0.660	48.18	2.106	15.09	0.664	3.186	0.139
Floor2 × REL	50	15.24	0.671	46.84	2.048	15.85	0.696	2.978	0.130
SEM		0.49	0.013	1.57	0.050	0.57	0.019	0.114	0.004
Trial × Sex									
Floor1 × Male	48	17.00	0.638	52.54	1.960[b]	17.51	0.655	3.098	0.116
Floor1 × Female	47	12.88	0.615	49.26	2.269[a]	14.05	0.671	2.655	0.127
Floor2 × Male	50	17.02	0.681	50.34	2.006[b]	16.81	0.672	3.250	0.129
Floor2 × Female	50	13.31	0.650	44.61	2.151[a]	14.13	0.689	2.914	0.140
SEM		0.31	0.013	1.72	0.050	0.57	0.022	0.122	0.004
Line × Sex									
MAS × Male	48	17.19	0.663	52.28	1.999	17.01	0.654	3.231	0.124
MAS × Female	50	13.02	0.623	47.33	2.220	13.95	0.670	2.780	0.134
REL × Male	50	16.83	0.657	50.58	1.969	17.31	0.672	3.122	0.122
REL × Female	47	13.19	0.644	46.45	2.194	14.22	0.690	2.798	0.134
SEM		0.32	0.012	1.50	0.049	0.50	0.019	0.133	0.005
***P*-values**									
Trial		0.434	**<0.001**	**0.009**	0.348	0.470	0.332	0.052	**0.014**
Genetic Line		0.999	0.466	0.357	0.508	0.370	0.283	0.731	0.446
Sex		**<0.001**	**0.018**	**<0.001**	**<0.001**	**<0.001**	0.322	**<0.001**	0.556
Trial × Line		0.646	0.707	0.888	0.396	0.384	0.447	0.116	0.130
Trial × Sex		0.469	0.690	0.347	**0.032**	0.364	0.964	0.636	0.610
Line × Sex		0.363	0.284	0.752	0.948	0.971	0.986	0.580	0.978
Trial × Line × Sex		0.284	0.396	0.601	0.660	0.396	0.692	0.806	0.652

Abbreviations: Floor1 = Floor cohort 1; Floor2 = Floor cohort 2; MAS = marker-assisted selection line; REL = Relaxed (control) line

1: Yields calculated based on back-dock live weight

Table 2-6. Heart characteristics from the replicate floor trials.

Item	n	RV, g	TV, g	RVTV
Main effect of trial				
Floor1	95	2.306	10.31	0.225[a]
Floor2	100	2.270	10.43	0.217[b]
SEM		0.056	0.22	0.003
Main effect of genetic line				
MAS	98	2.284	10.40	0.220
REL	97	2.292	10.34	0.222
SEM		0.059	0.22	0.003
Main effect of sex				
Male	98	2.589[a]	11.85[a]	0.219
Female	97	1.986[b]	8.87[b]	0.223
SEM		0.044	0.14	0.003
Trial × Line				
Floor1 × MAS	48	2.354[a]	10.41	0.230[a]
Floor1 × REL	47	2.259[a]	10.20	0.220[ab]
Floor2 × MAS	50	2.218[a]	10.40	0.210[a]
Floor2 × REL	50	2.323[a]	10.46	0.224[ab]
SEM		0.091	0.36	0.005
Trial × Sex				
Floor1 × Male	48	2.618	11.96	0.221
Floor1 × Female	47	1.995	8.64	0.230
Floor2 × Male	50	2.562	11.76	0.218
Floor2 × Female	50	1.979	9.09	0.216
SEM		0.066	0.21	0.004
Line × Sex				
MAS × Male	48	2.609	12.04	0.218
MAS × Female	50	1.978	8.83	0.221
REL × Male	50	2.571	11.68	0.220
REL × Female	47	1.995	8.91	0.224
SEM		0.075	0.24	0.005
***P*-values**				
Trial		0.538	0.499	**0.049**
Genetic Line		0.802	0.712	0.517
Sex		**<0.001**	**<0.001**	0.481
Trial × Line		**0.046**	0.489	**0.005**
Trial × Sex		0.691	0.078	0.192
Line × Sex		0.630	0.237	0.862
Trial × Line × Sex		0.907	0.154	0.075

Abbreviations: Floor1 = Floor cohort 1; Floor2 = Floor cohort 2; MAS = marker-assisted selection line; REL = Relaxed (control) line; RV = right ventricle; TV = total ventricle.

Table 2.7. Meat quality characteristics from the replicate floor trials.

Item	n	Drip loss[1], g	24-hr Color L*(D65)	a*(D65)	b*(D65)	24-hr pH	Shear Force (N)	Area 1:3	Peaks
Main effect of trial									
Floor1	198	2.052	52.34	2.050[a]	8.932[a]	5.946[a]	14.04	187.7	8.823[b]
Floor2	197	2.165	52.72	1.776[b]	8.607[b]	5.857[b]	14.26	191.9	9.545[a]
SEM		0.116	0.19	0.071	0.108	0.013	0.23	2.7	0.182
Main effect of genetic line									
MAS	196	2.161	53.03[a]	1.805[b]	8.956[a]	5.875[b]	13.85[b]	184.0[b]	8.981
REL	199	2.047	52.04[b]	2.011[a]	8.582[b]	5.926[a]	14.45[a]	195.6[a]	9.384
SEM		0.114	0.19	0.069	0.107	0.013	0.22	2.6	0.179
Main effect of sex									
Male	198	1.612[b]	51.50[b]	1.923	8.384[b]	5.935[a]	13.76[b]	186.0[b]	8.859[b]
Female	197	2.617[a]	53.56[a]	1.893	9.157[a]	5.866[b]	14.55[a]	193.7[a]	9.512[a]
SEM		0.125	0.18	0.071	0.106	0.013	0.22	2.7	0.187
Trial × Line									
Floor1 × MAS	97	2.117	52.89	1.905	9.093	5.925	14.32[ab]	189.1[b]	8.521
Floor1 × REL	101	1.990	51.79	2.190	8.774	5.966	13.78[b]	186.5[b]	9.113
Floor2 × MAS	99	2.204	53.16	1.706	8.821	5.825	13.39[b]	179.0[b]	9.427
Floor2 × REL	98	2.106	52.30	1.824	8.389	5.885	15.14[a]	204.9[a]	9.667
SEM		0.166	0.27	0.103	0.155	0.018	0.33	3.9	0.268
Trial × Sex									
Floor1 × Male	101	1.460	51.12	2.152	8.469	5.980	13.28[b]	180.4[b]	8.441
Floor1 × Female	97	2.696	53.56	1.945	9.414	5.910	14.83[a]	195.3[a]	9.229
Floor2 × Male	97	1.771	51.89	1.682	8.295	5.888	14.26[ab]	191.9[ab]	9.299
Floor2 × Female	100	2.542	53.56	1.842	8.908	5.822	14.28[ab]	192.1[ab]	9.783
SEM		0.179	0.27	0.113	0.149	0.019	0.33	3.9	0.290
Line × Sex									
MAS × Male	98	1.670	51.80	1.855	8.411[b]	5.916	13.62	180.7	8.699
MAS × Female	98	2.663	54.25	1.754	9.506[a]	5.833	14.09	187.3	9.265
REL × Male	100	1.556	51.20	1.992	8.356[b]	5.954	13.91	191.3	9.018

Table 2-7 (Cont.)

REL × Female	99	2.570	52.88	2.029	8.811[b]	5.898	15.01	200.0	9.758
SEM		0.178	0.26	0.104	0.156	0.019	0.32	4.0	0.264
***P*-values**									
Trial		0.629	0.113	**0.003**	**0.017**	**<0.001**	0.457	0.239	**0.002**
Genetic Line		0.515	**<0.001**	**0.037**	**0.009**	**0.003**	**0.045**	**0.001**	0.060
Sex		**<0.001**	**<0.001**	0.803	**<0.001**	**<0.001**	**0.009**	**0.031**	**0.016**
Trial × Line		0.908	0.611	0.367	0.757	0.622	**<0.001**	**<0.001**	0.378
Trial × Sex		0.128	0.112	0.052	0.236	0.848	**0.010**	**0.040**	0.681
Line × Sex		0.967	0.112	0.456	**0.025**	0.473	0.287	0.778	0.555
Trial × Line × Sex		0.096	0.794	0.290	0.694	0.766	0.803	0.956	0.667

Abbreviations: Floor1 = Floor cohort 1; Floor2 = Floor cohort 2; MAS = marker-assisted selection line; REL = Relaxed (control) line

1: Drip loss calculated as the difference in breast weight before and after 24-hour chill

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CHAPTER 3: WHOLE GENOME RESEQUENCING SUGGESTS LIMITED GENETIC BASIS FOR WIDE VARIATION IN INFECTIOUS BRONCHITIS VIRAL LOAD IN CHALLENGED NAÏVE WHITE LEGHORN CHICKENS

3.1 Abstract

Infectious bronchitis virus (IBV) is a gamma-coronavirus which causes respiratory illness and mortality in poultry species. The goal of this study was to complete whole genome resequencing (WGR) on a population of IBV-challenged birds to map genomic regions associated with IBV severity. Specific pathogen-free white leghorn chickens were challenged at 18 days of age with an Arkansas-type virulent IBV strain, then 5 days later sampled to quantify viral load in the trachea. DNA from the 48 lowest and highest viral titers were used for the WGR analysis. Three pipelines were utilized to process the data varying on removal of PCR artifacts and pool size for comparisons. Although each pipeline identified regions only a single region on chromosome 18 containing two genes (ARHGAP44 and CDRT1) was found in multiple pipelines. In humans, ARHGAP44 has been associated with viral pathogenesis and immune function. Further investigation of this gene in non-naïve populations is warranted to further assess this regions contribution to IBV susceptibility in poultry.

3.2 Introduction

Infectious bronchitis (IB) is a highly contagious respiratory illness causing major economic losses to the global poultry industry. Chickens of all ages are susceptible, but younger birds commonly suffer a more severe disease (1). IB virus (IBV), a gamma-coronavirus, is known to infect all members of the *Gallus gallus* species. Various genetic factors underlying the chicken immune response have been associated with susceptibility and resistance to IB. The main considerations have been in relation to different major histocompatibility complex (MHC) (or B complex) haplotypes (2,3). Nevertheless, upregulation of proinflammatory responses by non-MHC genes have also been associated with susceptibility to IBV (3,4).

While a larger scope of research has been directed to understanding differences in resistance to IBV between B complex haplotypes, limited information is available on other genetic determinants that could be associated with resistance to IBV. We have previously demonstrated that Whole Genome Resequencing (WGR) can fine map genetic determinants of complex traits in poultry (5,6). WGR identifies contiguous regions of hundreds to thousands of single nucleotide polymorphisms (SNPs) spanning 10-1000 kbp differentially represented in the phenotypic tails of a trait. The approach has been validated using marker assisted selection to breed for resistance to ascites (7). Given the recent pandemic-related interest in highly infectious coronaviruses, and the significant costs of IBV in the poultry industry, we have investigated whether WGR can be used to identify gene regions associated with IBV resistance.

3.3 Methods

3.3.1 Chickens

Four hundred specific pathogen-free (SPF) white leghorn chickens (Charles River Laboratories, Norwich, CT) were hatched, individually wing-banded, and maintained in floor pens in biosafety level 2 facilities, with water and food *ad libitum*. All procedures and animal care followed federal and institutional standards at the Auburn University College of Veterinary Medicine and approved by the Auburn University Institutional Animal Care and Use Committee (Approval #2020-3754) and performed in accordance with relevant guidelines and regulations.

3.3.2 Experimental Design

At 18 days of age, all chickens were challenged with an Arkansas (Ark)-type virulent IBV (GenBank accession No. JN861120) previously characterized (8). Challenge was performed ocularly with 50µl of viral suspension containing 10^4 embryo infectious doses 50% (EID50) per chicken. All chickens were euthanized 5 days after challenge for sampling and gender

determination. Males were not investigated in the current study. Viral load (IBV RNA) in the tracheas was measured in all female chickens by quantitative real time polymerase chain reaction (qRT-PCR). Tracheal samples were also collected from all birds for histomorphometry. From a total of 198 female chickens, 48 chickens showing the lowest and 48 chickens showing the highest relative viral RNA loads in the trachea were selected for WGR. In addition, tracheal mucosal thickness of both groups, i.e., high and low IBV RNA, was determined by histomorphometry.

3.3.3 Viral Load

Tracheal samples were homogenized with TriReagent (Molecular Research Center, Cincinnati, OH) and total RNA was extracted using the Zymo Research Direct-zol RNA Miniprep kit (Zymo Research Corporation, Irvine, CA). With the exception of omitting the DNase I treatment, the manufacturer's protocol was followed. Total RNA was quantified using NanoDrop spectroscopy (Thermo Scientific, Waltham, MA) and diluted below 170 ng/µl prior to qRT-PCR. Viral loads (IBV RNA) were determined using TaqMan qRT-PCR as described (9). The relative viral copy number from qRT-PCR was divided by the total RNA concentration input.

3.3.4 Histomorphometry

Tracheae were fixed in 10% neutral buffered formalin and sectioned along the median axis. Sections of trachea were then routinely processed, embedded in paraffin, sectioned at 4 to 6 µm, and stained with hematoxylin and eosin for histomorphometry. Five repeated measures were individually taken for mucosal thickness utilizing ImageJ software (https://imagej.nih.gov/ij/download.html) as previously described (10). The averaged measures of mucosal thickness (arbitrary units using ImageJ) by individual were used in further analyses.

3.3.5 Chicken DNA Isolation and Purification

Chicks were sampled for DNA on day 17 prior to viral challenge. Crude DNA lysates were prepared by the method of Bailes et al. (11). Briefly, 10 µl of whole chicken blood was pipetted from a 28-gauge lancet puncture of a wing vein and triturated into 400 µl of cold 64 mM Sucrose, 20 mM TrisCl pH 7.5, 10 mM $MgCl_2$, 0.5% Triton X-100 in a 96 well assay block on ice. After all samples were collected, the block was sealed with a silicone mat lid, inverted several times, then centrifuged at 1000 × g for 5 minutes. The plate was inverted to decant off the supernate. The nuclear pellets were suspended by trituration into TEN+Pronase (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 10 mM NaCl, 100 µg/ml Pronase E; Sigma-Aldrich, St. Louis, MO). The sealed block was incubated at 37°C for 60 minutes while shaking at an approximate 45-degree angle, then incubated in a 65°C water bath for 15 minutes. Assay blocks were stored at -20°C in a non-frost-free freezer. Crude DNA samples from birds selected for high and low viral load phenotypic groups were identified. For these samples, 100 µl of the crude DNA lysate was mixed with 100 µl TEN+Pronase, then incubated at 37°C for 60 min with shaking at an approximate 45-degree angle. The block was then incubated at 65°C in a water bath for 10 min.

A stack of assay blocks and filter plates was prepared for centrifugal filtration consisting of a 1.2 µm filter plate (Pall #8040, Pall Corporation, Westborough, MA) on top of a 100 MWCO filter plate (Pall #8036), on top of a 0.5 ml assay block to collect the final flow through. The pronase-treated, heat-inactivated DNA solutions were pipetted into the corresponding wells. The stack was centrifuged at 1500 × g for 10 min (or longer until the upper wells were empty) at 5°C. Te (200 µl; 10 mM TrisCl pH 7.5, 0.1 mM EDTA) was added to each well in the top plate, and the stack centrifuged as above. The Te rinse was performed two times. The 1.2 µm plate was discarded and 100 µl Te was added to the wells of the 100 MWCO filter plate sitting on the

emptied waste collection block. The 100 MWCO filter plate was covered and the stack placed on a platform orbital shaker at 500 rpm for 10 minutes at room temperature. The suspended DNA was transferred to a 96-well raised-lip PCR plate. DNAs were quantified by Hoechst 33258 fluorescence using a GloMax Jr (Promega Corp. Madison, WI). The PCR plate was sealed with silicone mat lid and stored frozen at -20°C.

3.3.6 Sequence Analysis for Identification of Gene Regions

Barcoded libraries from each DNA (50-200 ng) were produced using the RIPTIDE kit (iGenomX, Carlsbad, CA). Pooled libraries were fractionated for insert size of ~375 bp. Pooled libraries were submitted to NovoGene (Davis, CA) for QC and sequenced in one lane on an Illumina NovaSeq. Sequence data was demultiplexed using FGBio (v 0.8.1). Reads for each library were mapped onto the bGalgal1 (GRCg7b; maternal broiler) using Bowtie2 v2.4.4 (12) and converted to indexed bam files using SAMtools v1.14 (13). We used two groupings of bam files for analyses: Method 1) bam files were randomly grouped into one of two pools 24 susceptible and 24 resistant, or Method 2) all 96 bam files were analyzed as one pool of 48 susceptible and 48 resistant. Each pool was then merged and filtered in a single vcf (variant call file) using NGSEP (14). Filtering in NGSEP included: a) only biallelic SNPs, b) minimum mapping quality ≥20, c) exclude SNPs in 3’ 3 bases, d) minimum genotype Qscore: 20, e) maximum alignments per start position: 2, f) minimum read depth for genotype call: 4, and g) minimum Minor Allele Frequency (MAF): 0.05. Filtering for Method 1 was for a minimum of 20 genotypes per position, while for Method 2 filtering was for a minimum of 40 genotype calls per position. We also employed a control, Method A, for filtering for the 2-pool analysis in Method 1. Method A allowed a maximum alignment per start position of 5 (filter setting e) which is more tolerant of PCR duplications in library construction and amplification.

The final vcf was processed with SNPtest v2.5.4-beta3 (15), to impute genotype calls and calculate Minor Allele Frequencies (MAF). Differences in MAF for Control (High)-Case (Low) for each filtered biallelic SNPs were then visualized in Integrative Genomics Viewer (IGV) (16) to identify regions where clusters of hundreds to thousands of successive SNPs show a skewed difference in frequencies with respect to phenotype. MAF). For Method 2, SNPtest was also used to calculate P-values using models of association for Additive, Dominant, Recessive, General, or Heterozygote.

3.4 Results

Five days after inoculation, most chickens exhibited typical IB respiratory signs varying from unapparent, mild, moderate, to severe. Consistent with this observation, viral loads varied from almost undetectable to considerably high amounts of viral RNA in the trachea of individual chickens. The median tracheal viral load for the females was 1835 (log10 relative IBV RNA); details on pathology and viral load have been published elsewhere (17). We selected the 48 females with the lowest IBV tracheal RNA to represent the low group ranging from 0.196 to 404.0 with a mean of 180.7 and standard error of 18.57. Conversely, the high group had viral loads ranging from 553,300 to 28,390,000 with a mean of 918,851 and standard error of 70,211. As seen in Figure 1A, there was a significant difference ($P < 0.0001$) between the high and low group of females. The histomorphometry analysis of tracheal mucosal thickness of each of 48 individual chickens in the high and low groups is shown in Figure 1B. The mucosal thickness of each individual bird was consistent with the viral loads, i.e., increased mucosal thickness in chickens with higher viral load. Indeed, the 48 females showing high viral RNA showed significantly higher mucosal thickness ($P = 0.0004$) than the 48 females showing the lowest IBV RNA levels.

DNAs from each of the 48 birds from the high and the low group were processed to produce individually barcoded NGS libraries, which were pooled and sequenced on a single lane of an Illumina NovaSeq. The demultiplexed reads yielded a range of 2.0-8.2 Gb per DNA with an average of 7.56±0.44 (SEM). Reads were aligned to the most recent broiler assembly bGalgal1 (GRCg7b; maternal broiler) rather than the paternal layer because the broiler assembly is female and contains marginally more assembled bases; 6.5 Mbp for the autosomes and chromosome Z. The pipeline we employed using Bowtie2, together with SAMtools, has been found to be as effective as other aligner-caller combinations for identification of SNPs when filtering (we used NGSEP) is employed (18). We used three alternative methods to process the reads during the alignment (described in Methods section). Method A tolerates PCR duplicates from the library production while Methods 1 and 2 filter for reduction of duplicates. Method A and Method 1 processed the reads from each of the phenotype groups in pools of 24, which allowed replicate comparisons of MAF from the SNP genotype calls between pools from each phenotype group (i.e., two comparisons of 24 high vs 24 low). For Method 2, the comparison was between all 48 low vs all 48 high.

We identified 16 regions using Method A which tolerates significant numbers of PCR duplications. Restricting the level of PCR duplication in Method 1 reduced the number of regions identified from 16 to 13. Significantly, none of the regions from Method A were present in those from Method 1. When we combined all the data with reduction of PCR duplicates in Method 2, the number of regions identified decreased to 6, but most importantly, one of the regions on chromosome 18 was retained between Method 1 and Method 2. We considered the regions other than this one to be likely false positives. As the region on Gga18 from 2.850-2.867 Mbp was identified in Methods 1 and 2, this region is much more likely to be a QTL for IBV

viral load. The region contains all or part of the genes for ARHGAP44 (Rho GTPase activating protein 44) and CDRT1 (CMT1A duplicated region transcript 1). ARHGAP44 in humans is predicted to be involved in modification of dendritic spine, negative regulation of Rac protein signal transduction, and regulation of plasma membrane bounded cell projection organization in leading edge membranes. It is expressed broadly and at high levels in the lung, colon and brain. The CDRT1 homolog in humans is FBXW10B (F-box and WD repeat domain containing 10B), one of many F-box containing protein-ubiquitin ligases. It is expressed in most tissues but at the highest levels in testis. There is no indication in the databases for Online Mendelian Inheritance in Man, or NCBI PheGenI, that would connect either ARHGAP44 or CDRT1 to viral infection or viral load.

3.5 Discussion

We performed whole genome mapping of naïve chickens showing high and low susceptibility to a challenge with a virulent Ark-type IBV. Measurements of viral load and tracheal damage were used as continuous quantitative traits to analyze host resistance to IBV. Possible variation in susceptibility to viral infection was minimized by using white leghorn chickens, i.e., pure breed chickens instead of crossbred broiler lines, only females, and challenging at the same age. Furthermore, the use of SPF chickens ensured absence of both maternal IBV-specific antibodies and other avian pathogens that could alter the outcome of IBV infection. Rigorous efforts were made to administer the exact same dose to all individuals during challenge. Challenge was performed at 18 days of age to avoid suboptimal responses associated with an immature immune system at an earlier age (19-21).

We employed three different methods to process/filter the SNP data from the WGR. There was no filtering for PCR duplicates in Method A, while for Methods 1 and 2, we employed

filters for PCR duplicates. For both Method A and Method 1, we compared 24 low to 24 high, with two replicates of the comparison while Method 2 compared all 48 low to all 48 high. With Method A, we identified 16 potential regions as associated with IBV load, while with Method 1 there were 13 regions. Surprisingly, none of the regions were shared between the two methods. To further test the significance of removal of PCR duplicates, WGR data from a commercial broiler breeder male line, phenotyped for susceptibility to a bacterial chondronecrosis osteomyelitis (BCO) challenge, was analyzed using Method A and Method 1. The WGR data consisted of 24 susceptible and 24 resistant to a challenge with a BCO isolate (22). Method A identified 12 regions, while Method 1 identified 27, but the two methods shared 5 regions, whereas the same manipulation of the pipeline for IBV WGR changed all regions. This suggests that there are no major regions affecting IB resistance and the regions mapped are all false positives. To further examine this, Method 2 was performed where the data from all 48 low and all 48 high were compared and the number of samples was sufficient to compute P-values with SNPtest according to alternative genetic models. Method 2 identified 6 regions with no regions shared with Method A and one region shared with Method 1. Thus, the region on chromosome 18 from 2.850 to 2.865 Mbp may be relevant to reduced viral load. However, of the two genes with coding sequences in this region, ARHGAP44 and CDRT1, only one appears to function in pathways relevant to immune function or viral pathogenesis. ARHGAP44 is associated with cytoskeletal functions related to retroviral release (23) and modulates immunocytolysis by Natural Killer cells during carcinogenesis (24).

3.6 Conclusions

Overall, the WGR analyses are most consistent with little genetic basis for the wide variation in IB viral load, since the analysis pipelines we used resulted in only one region shared between

two of the methods. This is in contrast with WGR in ascites and BCO. The lack of genetic diversity could be from the use of SPF leghorns, pure breed chickens. In which case, WGR in commercial layers or broilers, which are out-crosses, would present higher genetic diversity.

3.7 Tables and Figures

Table 3-1. Regions mapped for IBV phenotype based on the three pipelines. For each region the chromosome (Chr) and approximate Start (Mbp), End (Mbp) and size (kbp) of the region. The Method in which the region was identified, the Magnitude of the affect (MAF Control-Case), the approximate maximum -logP for Method 2, and the genetic Models with significant P values (Additive, Dominant, Recessive, Heterozygous).

Chr	Start	End	Size	Method	Magnitude	-logP	Model ADRH
1	41.1	42.8	1700	A	-0.3444		
1	55.2	55.3	100	1	-0.429		
1	74.36	75	640	1	0.429		
1	78.45	78.52	70	1	-0.338		
1	99.53	99.57	40	2	0.3	3	ADRH
1	130.73	130.84	110	1	-0.344		
1	147.4	148.1	700	A	-0.4286		
1	153.45	153.5	50	1	-0.375		
2	82.7	82.77	70	A	-0.2167		
2	122.9	123.3	400	A	0.375		
2	139.8	139.9	100	A	-0.2857		
2	149.02	149.6	580	2	0.25	4	H
3	34.28	34.44	160	A	0.35		
3	57.6	58.2	600	A	0.4375		
3	70.63	70.67	40	A	-0.2738		
3	72.8	72.9	100	A	-0.3333		
4	76.93	77.09	160	1	0.4375		
5	11.9	12.03	130	1	-0.423		
6	3.16	3.26	100	A	0.4375		
8	27.12	27.2	80	A	0.2738		
8	28.88	29.08	200	A	-0.3571		
9	7.68	7.74	60	A	-0.3571		
10	18.26	18.46	200	1	-0.215		
11	18.65	18.9	250	A	0.3333		
12	0.647	0.729	82	1	-0.222		
12	1.35	1.55	200	2	-0.25	3.5	ADR
13	1	1.14	140	2	0.3	3.5	ADRH
13	17.45	17.65	200	A	-0.375		

Chr	Start	End	Size	Method	Magnitude	-logP	Model ADRH
15	6.928	6.95	22	2	0	3.8	H
18	2.85	2.865	15	1	0.3		
18	2.82	2.865	45	2	0.25	4.2	ARH
20	5.75	5.86	110	1	-0.26		
23	3.04	3.06	20	1	0.214		
25	1.15	1.19	40	A	-0.2286		
20	5.6	5.95	350	1	-0.23		

Figure 3-1 Viral load (IBV RNA by qRT-PCR) in the trachea and tracheal mucosal thickness in female SPF chickens 5 days after challenge with 104 EID50 of a virulent Ark-type IBV at 18 days of age. (A) Forty-six chickens with highest and lowest viral loads (log-transformed relative IBV RNA). (B) Mucosal thickness of chickens included in the higher and lower viral load groups. Boxes: 25th percentile, median, 75th percentile; whiskers: minimum and maximum). Significant differences determined by t-test at P < 0.05.

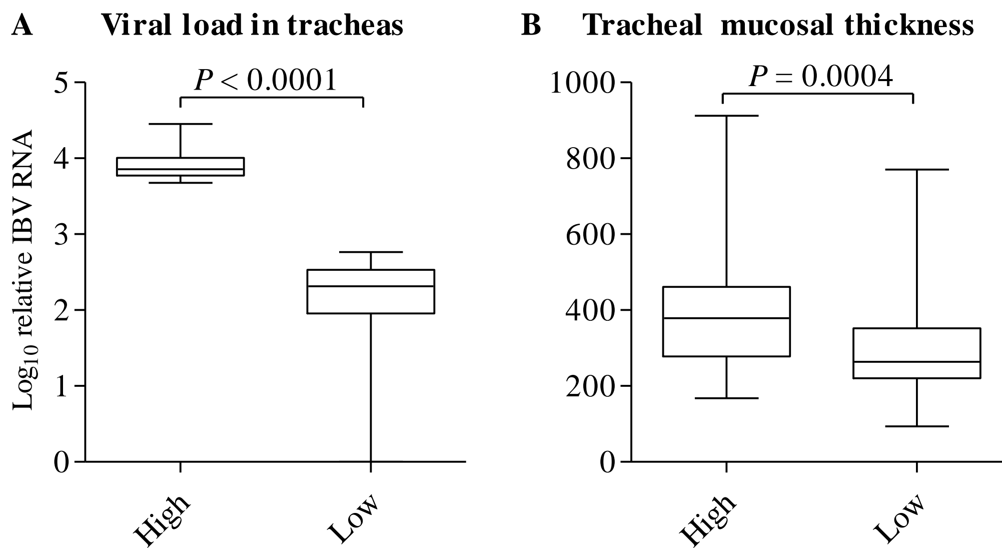
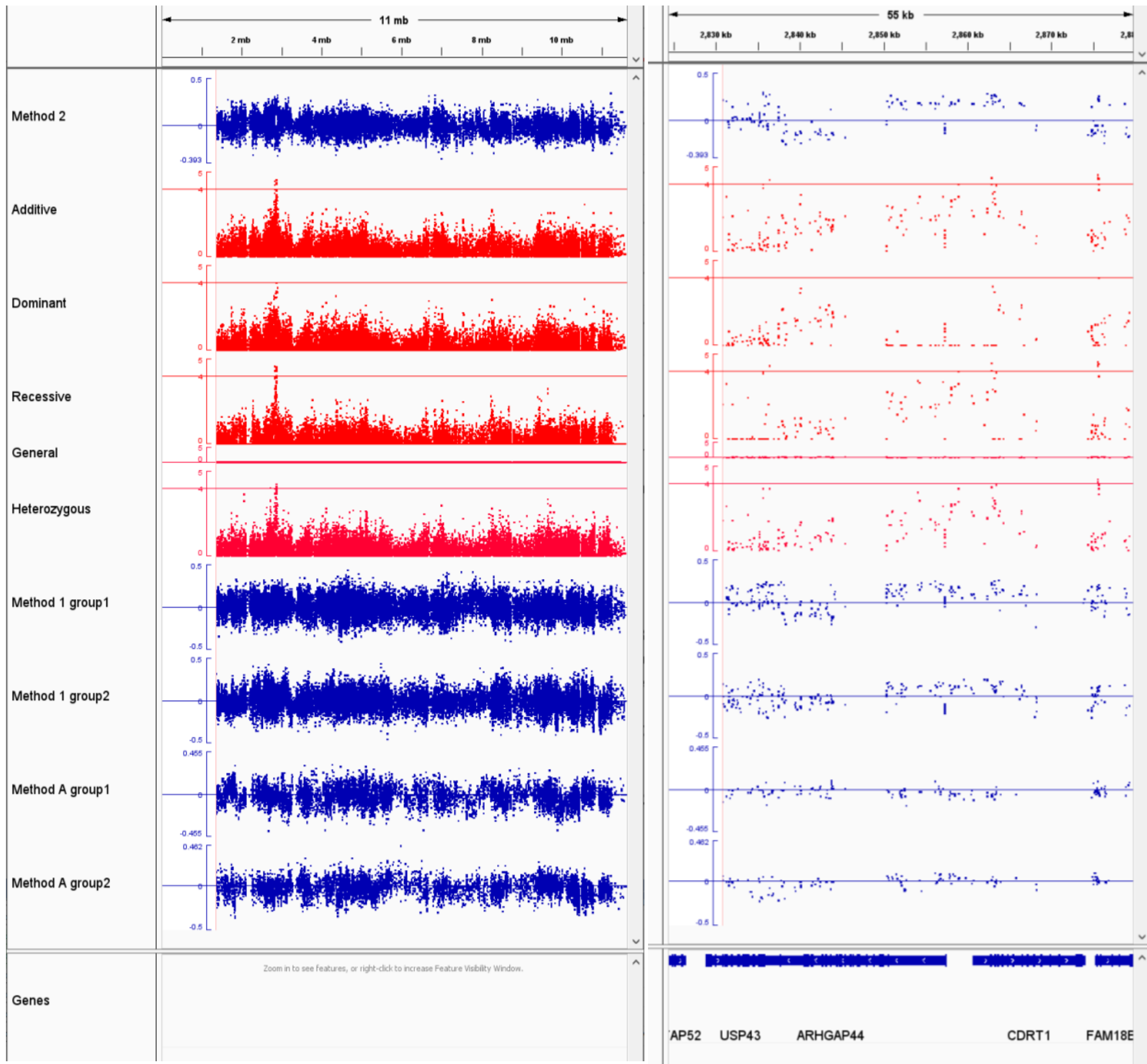


Figure 3-2 Integrative Genome Viewer for the region identified on chromosome 18. Left panel is the view of the entire chromosome and the right panel is zoomed in on the region around 2.8 Mbp. Blue plots are for the MAF and red plots are for the -logP values for the genetic models for Method 2.



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CHAPTER 4: CONCLUSIONS

Chicken is a crucial source of dietary animal protein for humans. With a global population that continues growing, there is increasing need for efficient and stable production of chicken products. One of the ways chicken production could be hindered or compromised is through disease which decreases supply and increases cost to the consumer.

Genetic progress toward highly efficient growth in broilers has been the pursuit of poultry geneticists for more than 60 years of breeding. In that span of time, broilers have seen a 400% increase in growth rate (1). While these changes have been an overall benefit to the industry, there have also been negative consequences of this selection, as well. Some critical issues include skeletal insufficiencies leading to lameness such as tibial dyschondroplasia and spondylolisthesis (commonly known as "kinky back"), greater fat deposition, Sudden Death Syndrome, and a decrease in meat quality characterized by myopathies such as woody breast, white striping, and spaghetti meat (2-5). Metabolic disorders have also risen with genetic improvement, one of which is ascites. Ascites is the terminal outcome of prolonged pulmonary hypertension, identifiable by the fluid-filled body cavity of afflicted birds (6). Ascites accounts for at least 8% of bird mortality and up to $100 million in economic losses making it a concern of both animal welfare and industry economic impact (7, 8).

While ascites represents an internal threat caused by increased growth efficiency in an individual animal, infectious bronchitis virus (IBV) represents an external threat to the health of the bird and the flock. Infectious bronchitis (IB), the illness caused by IBV, is a respiratory gamma-coronavirus with similarities to SARS-CoV-2 (9-11). IBV ranks as the second most problematic disease in poultry and is difficult to prevent due to the extensive number of distinct strains, environmental factors, and the variation in immune response from flock to flock (12, 13). Typically, birds are vaccinated with a viral vaccine cocktail to protect from variants that are

specific to that area, although with variable efficacy (14). An improved prevention method for this illness is severely needed to reduce its impact on bird morbidity and mortality as well as the economic impacts of such a problematic virus.

Whole genome resequencing (WGR) has allowed for new insights into the genetic determinants contributing to phenotypic variation. This bioinformatic analysis enables the assessment of genetic variation in the genome associated with a given disease by identifying single nucleotide polymorphisms (SNPs) that are differentially represented between the resistant and susceptible phenotypes. Given the importance of maintaining a supply of chicken products to meet the demand from the ever-growing global population and the threat of various avian diseases that could diminish that supply, this body of work applied WGR to ascites and IBV.

The project described in Chapter 2 is the culmination of WGR completed previously at the University of Arkansas. That work revealed two chromosomal regions, LRRTM4 and CPQ, contributing to genetic resistance to ascites in some chicken lines to the development of ascites. Resistance to ascites in hypobaric challenges was associated with homozygous non-reference SNPs in these two epistatic regions (15, 16). In order to demonstrate the efficacy and accuracy of the WGR mapping, we conducted a marker-assisted selection (MAS) project where the SNPs in those regions were driven to non-reference homozygosity over two generations of selection. In theory, this would produce a population of birds with a greater innate resistance to the development of ascites when presented with a metabolic stressor such as a hypobaric challenge. The concern for industry geneticists about selection for ascites resistance is that birds phenotypically selected to be more resistant also tend to have lower body weight (17, 18). This has long been observed in the study of ascites and was also a result found by Pavlidis *et al.* (19) when establishing the ascites susceptible and resistant research lines at the University of

Arkansas. The susceptible line showed decreasing body weight at market age after each successive generation of selection for ascites resistance. Therefore, the MAS project also required assessment of the impact of selection on broiler growth characteristics. The over-arching question was whether these regions would degrade highly efficient growth if they were integrated into an industry breeding scheme.

Two cohorts each were raised under the conditions of a hypobaric challenge to assess ascites phenotype or under standard broiler rearing practices to assess the impact on broiler growth characteristics. The hypobaric challenge cohorts (Hypo1 and Hypo2) were subjected to a simulated elevation of 9,000 feet and 11,000 feet, respectively. This change between cohorts was made in order to increase ascites incidence in the second cohort and assess the impact of selection under more severe metabolic stress. The two cohorts both saw at least a numerical reduction in ascites incidence in the MAS genetic line compared to the control line with the reduction being significant (P=0.041) in Hypo1. The floor trial cohorts reared under broiler-standard practices were treated as biological replicates. The results of the floor trials showed no significant impacts in the MAS line to any growth characteristic, processing measurement, organ weight, or meat quality metric. In fact, significant improvements were seen in the MAS line in several production traits. Overall, this project demonstrates the feasibility of utilizing identified genetic markers in modern broiler-breeding selection schemes in the industry, reducing the incidence of ascites while maintaining the more than 50 years of breeding progress.

Chapter 3 presented a new WGR project focused on identifying regions associated with resistance or susceptibility to severe IBV response phenotype in white leghorn chickens. Challenge of the birds with an Arkansas-type virulent strain of IBV allowed for the identification of the birds with the 48 highest viral loads and the 48 lowest viral loads. The DNAs collected

from those birds were then purified using a new method, separately barcoded, and the pooled libraries sequenced. The Illumina data was analyzed through three alternative WGR pipelines, denoted as Methods A, 1, and 2. Methods 1 and 2 are updated versions of Method A. The outcome of the Method 1 and 2 analyses pinpointed a single region identified using both methods. The regions found using Method A and the remaining regions found using Methods 1 and 2 are assumed to be false positives. The one region identified in multiple analyses, located on chromosome 18 between 2.850-2.867 Mbp, included two genes: ARHGAP44 and CDRT1. ARHGAP44 appears to be the more promising of the two, as it has documented involvement with the release of retroviruses and with a molecule preventing the destruction of tumor cells by Natural Killer cells (20, 21).

Taken together, this body of work expands upon the usefulness of WGR as a tool that can be used to help reduce the incidence of diseases in poultry. By successfully identifying regions with strong associations to disease phenotype, WGR results can then be integrated into breeding and genetic selection schemes, providing geneticists with the ability to reduce disease incidence through the increase of innate resistance in their populations.

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CHAPTER 5: APPENDIX

Office of Research Compliance

To:	Nicholas Anthony
Fr:	Craig Coon
Date:	February 9th, 2018
Subject:	IACUC Approval
Expiration Date:	February 1st, 2021

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18088:** *Utilizaton of Hypobaric Hypoxia to Induce Ascites in Broiler Chickens*.

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 February 1st, 2021 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 following individuals are approved to work on this study: Nicholas Anthony, Sara Orlowski, and Joseph Hiltz. Please submit personnel additions to this protocol via the modification form prior to their start of work.

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

CNC/tmp

18088

Office of Research Compliance

To:	Nicholas Anthony
Fr:	Craig Coon
Date:	February 9th, 2018
Subject:	IACUC Approval
Expiration Date:	February 1st, 2021

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18083:** *General Rearing of Selected chicken and Quail Populations*.

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 February 1st, 2021 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.

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