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Identification of Genomic, Proteomic, and Metabolomic Signatures Associated with Pulmonary Hypertension Syndrome in Broilers

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

by

Duaa Almansaf
King Faisal University
Bachelor of Science in Biology, 2011
University of Arkansas
Master of Science in Cell and Molecular Biology, 2016

August 2022
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

_____
Douglas D Rhoads, Ph.D.
Dissertation Director

_____
Ralph Henry, Ph.D.
Committee Member

_____
Charles Rosenkrans, Ph.D.
Committee Member

_____
David S McNabb, Ph.D.
Committee Member

ABSTRACT

The present dissertation contains a collection of studies that examine the genomic, proteomic, and metabolomic association to pulmonary hypertension or ascites phenotype in fast-growing broilers. Pulmonary hypertension is a multifactorial metabolic disease influenced by physiological, environmental, and nutritional factors. It is characterized by a number of structural changes including, thrombosis and adverse pulmonary vascular remodeling. Thus, the atrial pressure is increased, and the right ventricle becomes hypertrophied, resulting in heart failure and the death of the bird. Pulmonary hypertension or ascites is a global problem that has negatively impacted the economy. The increased mortality rate of broilers (25%) is estimated to cost $1 billion per year in economic losses. Even though molecular genetic techniques were used in the breeding and selection, they have significantly reduced the occurrence of ascites, but not fully eradicated it. Hence, the main objective of this dissertation was to: measure the expression of the CPQ gene in eight tissues (heart, liver, kidney, thigh, breast, spleen, lung, thymus) to investigate the possible effect of a specific genotype on the gene expression, develop a TaqMan assay for the 125 Kbp chromosomal deletion of CPQ gene to test the potential associations with ascites phenotype, and to identify noninvasive biomarkers for early ascites detection by examining the proteomic and metabolomic changes prior and post ascites development.

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DEDICATION

I would like to dedicate this dissertation to my beloved parents. A special dedication to the soul of my father, Abdullah Hussain Almansaf, who has planted the passion for learning and education in my soul and heart since I was in elementary school. My dad, who was a source of strength, hope, knowledge, and happiness, has been waiting for my success, yet destiny was faster than I had expected. May God bless him and grant his soul everlasting peace. I am sincerely thankful to my mother, Badriah Muhammed Almansaf, for her support, love, and help. May God bless her and confer good health upon her. Thanks, my mom and dad, for your heartfelt prayers. Without your prayers, I would not have reached this point and achieved my dreams.

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LIST OF SUBMITTED ARTICLES

Chapter 2:

Transcriptomic Analysis of Broiler Right Ventricle in Response to Chronic, Hypobaric Hypoxia and Hypertrophy and 125 Kilobase Deletion Affecting the Carboxy Peptidase Q Gene in Broilers. Almansaf, D. [1], Licknack, T.J.[1], Anthony, N. B[2], Schmidt, C[3]., Rhoads, D. D[1] (*Will be Submitted soon to Poultry Science*).

Chapter 3:

Identification of Proteomic and Metabolomic Plasma Biomarkers Associated with Pulmonary Hypertension Syndrome in Broilers. Duaa. Almansaf [1, 2], Rohana. Liyanage[3], Douglas. Rhoads[1, 2] (*Will be Submitted soon to Poultry Science*).

CHAPTER 1

Literature Review

The History of the Domestic Chickens

Chickens are rich in several ideal nutrients because they mainly contain proteins in both products (meat and eggs), a good source of energy, and the best dietary source of several vitamins and minerals (Marangoni et al., 2015). According to several studies, a chicken diet helps consumers reduce body weight and obesity, decrease the incidence of cardiovascular disease, and lower the risk of developing Type 2 diabetes (Hu et al., 2005). Archaeological, morphological, and historical evidence proved that chickens were domesticated in Southeast Asia more than 5,400 years ago (Darwin et al.,1875; Fumihito et al., 1996; Storey et al., 2012). Then, domestic chickens reached China, Europe, and Western Asia (Granevitze et al., 2009; Tixier-Boichard et al., 2011). Genetic studies based on mitochondrial DNAs (mtDNA) sequences from several gallinaceous birds, the red junglefowl (RJF, Gallus gallus gallus) is the prominent ancestor of the domestic chickens (Fumihito et al., 1994; Hillel et al., 2003). However, other mitochondrial and genomic studies show that other ancestors participate in the history of domestic chickens (Nishibori et al., 2005; Oka et al., 2007; Miao et al., 2013). In addition, mtDNAs have a low ability to identify detailed demographics from the past (Larson, & Burger, 2013). On the other hand, other modern techniques such as Whole Genome Resequencing are promising for revealing a complete story of the domestication process (Wang et al., 2016). There is a controversial issue about which, where, and when, were the domestic chickens originally descended. Theories on domestic chickens originating from RJF, (Gallus gallus) in different sites in Southeast Asia are still questionable (Wang et al., 2020). In addition to the RJF, Ceylon jungle fowl, Java or green jungle fowl, and grey jungle fowl are other major Gallus species mentioned in the literature. Each species has different characteristics, and early domestication has influenced genotyping (Stevens & Lewis 1991). Religious and cultural reasons, food, and decoration were the main aims behind the initial

domestication of chickens in the Middle East, Africa, and Southeast Asia. Moreover, in the United States and Europe, chickens became popular because they have been used to study human diseases.

Domestic Chickens in the Industry

In the USA, broiler production increased from 413 million to 1.1 billion bounds between 1940 to 1945. Alabama, Georgia, and Arkansas are the highest states in broilers production, producing broilers for many decades (Figure 1.1). According to the US Department of Agriculture, the USA is the first chicken producer globally and the biggest exporter after Brazil. In 2013, the chicken production in the USA poultry industry reached 8.22 billion broilers, and their weight was 50.6 billion pounds. In Europe, especially in Netherlands and Germany, the demand for American broilers has continued to grow. Therefore, 173 million pounds were produced (Bishop et al., 2015). In the 1940s, chickens required a long time (approximately 16 weeks) to achieve the desired weight (2 to 2.5 kg). After selective breeding was incorporated, broilers reached the standard weight within less than seven weeks (Bugos, 1992; Griffin et al.,1994; Bishop et al., 2015).

Chickens, in general, were selected in the 1990s for particular traits such as eggs and meat production in both Europe and the USA. The selection occurred at that time due to the fact that RJF has a lower-body weight (about two pounds) and lays 10 to 12 eggs/ year. Because of intensive genetic selections, which have been done through several generations within broiler lines (meat-type chickens) and layer lines (egg production type), the current commercial broilers have a higher body weight (~ 4 times) and grater breast muscle (~8 times) than un-selected ones. Furthermore, egg-type chickens can lay more than 300 eggs/ year. The genetic selection also increases the production of both main pectoralis muscles by 79% in males and 85% in males and minor pectoralis muscles by 30% in males and 37% in females. Selective breeding helps study the mechanism of tissue growth, lowering the time and reducing the costs of raising each flock,

including food, housing, heating, and lighting. In addition, breeding in the poultry industry has other advantages such as high selection intensity, which can be done in a short time and at a large scale (Griffin et al.,1994; Deeb et al., 2002; Zuidhof et al., 2005; Jacob et al., 2011; Buzala et al., 2016). As shown in Figure1.2, from 1925 to 2012, broilers almost doubled in weight, the number of weeks required to reach market weight has decreased by 56%, the feed needed per pound of chickens have been dropped by 61%, and the mortality rate has been reduced by 78% (Bishop et al., 2015). Chicken consumption among Americans has increased since 2012, while there was a decline in the consumption of both pork and beef (Figure 1.3) (Larsen, 2012).

Since the 1990s, several breeding companies have been established, and a large number of geneticists have been employed. In the early stage of genetic selections, geneticists improved the random breeding by using white broilers such as Athens Canadian Random Bred Control (ACRBC) as the experimental population (Havenstein et al., 2003). That gives a better result, such as changing the bird's phenotype, reducing mortality rate, and controlling diseases (Flock et al., 2005). However, unexpected results of practicing genetic selection can alter the bird's traits because the gene of interest can be linked with an undesirable gene (Ellegren, 2005). Moreover, various metabolic diseases associated with genetic selection and rapid growth have existed in the poultry industry for more than 30 years. That has negatively impacted the poultry industry. Ascites or Pulmonary Arterial Hypertension (PAH), Fatty Liver and Kidney Syndrome (FLKS), skeleton diseases (Griffin, 1993), and immune disorders are examples of diseases that affect the poultry industry (Leshchinsky, & Klasing, 2001).

The poultry breeding and production system developed by modern poultry breeding companies can be visualized in a pyramidal breeding structure (Figure 1.4). The pure pedigreed line is at the top of the pyramid scheme, and each level indicates one generation. Cobb-Vantress

company is an example of a modern poultry breeding company. It generates over 50 birds/pedigree. These birds are precisely evaluated for their phenotypes, health, and fitness (Flock et al., 2005; Laughlin et al., 2007; Paxton et al., 2010; Katanbaf et al., 2010). The pure pedigree line, which consists of a small population, gives the parental generation of the great grandparents' stock. Then, the great grandparents provide the generation of the grandparents' stock. The first crossing, in the pyramid scheme, occurs between the grandparents' levels. Then, the parents are mated to provide the commercial broilers, which will be available in the poultry industries for meat and egg production. As shown in Figure 1.5, the pure pedigree line mainly consists of two major lines of both sexes. The four populations are maintained under specific genetic selections to improve some economic traits such as meat production and growth rate in male populations while egg production and growth rate in female populations. The four populations used the great grandparents to produce grandparents' stock (first hybrid). Then, the grandparents produce the parents (second hybrid). Finally, the parent's generation is mated to produce the commercial broilers (double hybrid). Based on some statistical studies, there was an estimation of 400,000 pedigree birds, which represent a range of 35 to 40 pure lines, are the major ancestor of approximately 400,000 million broilers (Pollock, 1999).

If a conventional breeding scheme or natural selection is followed today, the broilers should be fed with high-efficiency feed. Broilers are allowed to consume a large quantity of food for the first two weeks to maximize their skeleton before more muscle is obtained. The general public has the misconception that hormones, which are illegal not only in the USA but also in the majority of other countries, are a major part of broilers' diets and are in charge of their rapid growth. Broilers grow quickly due to their breeding improvement, good management, and nutrition (Jacob et al., 2011).

The Chicken Genome

We have expanded our knowledge about avian genomics in the last few years since several articles related to avian genome sequencing have been published (Bravo et al., 2021). The first bird and domesticated animal whose genome has been sequenced is the chicken. To begin with, a proposal was approved in 2002 by the National Human Genome Research Institute to sequence the chicken genome. As a result, the Genome Sequencing Center at Washington University's medical school sequenced around 1.2 billion bp in 2003 utilizing a Whole Genome Sequencing method that specialized in Sanger sequencing. Then, after one year, the preliminary results of the genome sequences were published and available to public databases. A DNA from an inbred strain of female RJF Gallus gallus was chosen to create the first genome sequencing draft (Fumihito et al., 1994; Ellegren, 2005; Groenen et al., 2009). Even though this draft represents approximately 86% coverage of the chicken genome, the major histocompatibility complex (MHC) on chromosome 16 and the single copy of the sex chromosomes (Z and W chromosomes) were poorly represented as well as 5 % to 10% of genes were not included in the assembly (Burt, 2005). The avian genome reference assembly has been improved over time due to having more effective genome assembly software, which helps remove artifacts. The new chicken genome assembly (Gallus gallus 5.0) was published in 2015 (Ye et al., 2011; Schmid et al., 2015). According to early studies, bird genomes are substantially smaller than mammalian genomes. The size of a chicken genome is 1230.26 MB which is approximately one-third the size of the mammalian genome with an estimated range of 20,000 to 23,000 genes, while the size of the chicken mitochondrial genome is 16.775 MB (Desjardins et al., 1990; Waltari et al., 2002; Ellegren, 2005). Though the evolutionary significance of this variation is unknown, one theory suggests that birds' limited genome size evolved to meet their physiological demands on flight (Butler, 2016). The karyotype

of chicken is made up of 39 chromosome pairs, as shown in Figure 1.6: there are ten pairs of large autosomes (chromosomes 1-10), 28 pairs of microchromosomes (chromosomes 11-38), and one pair of sex chromosomes (chromosome W and Z). Males carry two copies of chromosome Z (ZZ), whereas females carry only one copy of chromosome Z and one copy of chromosome W (ZW) (Masabanda et al., 2004).

There are some motivating factors in sequencing the chicken genome. First, the chicken, which descended from dinosaurs 310 million years ago, is the first non-mammalian vertebrate sequenced. The evolutionary tree of life includes chickens as well. Secondly, understanding chickens' genomes helps provide a general picture of how genes in vertebrates have developed and been modified. By classifying the chicken as an outgroup, it is easy to distinguish the ancient traits of mammalian biology. The chicken is an excellent model for biomedical research. They also have been used in several biological studies, especially in developmental biology, including the embryonic development of vertebrates, and studying distinct branches of biology, including immunology, virology, microbiology, and oncogenesis (Brown et al., 2003). Moreover, the chicken is significant for agriculture because it is one of the primary sources of proteins, and having its genome sequenced would help identify Quantitative Traits Locus (QTL) related to either meat or egg production and animal health (International Chicken Genome Sequencing Consortium, 2004; Burt, 2005).

Using the single nucleotide polymorphisms (SNPs) in the genomic project enables a large portion of the whole genomic variation to be identified and evaluated. It can be accomplished by examining several unlinked regions in synonymous and non-synonymous SNPs (Eltanany & Distl, 2010). Besides sequencing the chicken genome, a genetic variation map was generated 2.8 million SNPs based on comparing three different types of domestic chickens (broiler, layer, Silkie) to their

common ancestor RJF (International Chicken Polymorphism Map Consortium, 2004). Further studies proved that almost 90% of the SNPs are valid, while 70% are common SNPs that differentiate between various domestic breeds. In other words, the nucleotide variation between these domestic chickens and RJF is 5 SNP/kb, which means that three domestic chickens are extremely inbred relative to their ancestor (Groenen et al., 2000; Muir et al., 2003). The chicken genome contains more SNPs than mammals. Research suggests the chicken genome acquires six times the number of SNPs (~7,000,000 SNPs) than the mammalian genome and contains a significant number of microsatellites (~375,000) (Eltanany & Distl, 2010). SNPs are responsible for genetic diversity. Therefore, SNP arrays have been widely used to study the population's genetic structure using many uniformly distributed markers on all chromosomes. This method allows the identification and mapping of a large number of copy number variations (CNVs) on the genome (Muir et al., 2008; Gärke et al., 2012). Various genetic resources for chickens with excellent coverage are available and have been improved by bacterial artificial chromosome (BAC) based library production (Wallis et al., 2004). The development of QTL is significant because it contributes to identifying molecular markers associated with phenotypic variations and diseases (Hocking, 2005). QTL is a genetic locus on a chromosome where allelic variation is statistically linked to particular phenotypes. Scientists identify QTL by comparing the genetic make-up and certain features of the targeted group to the control group. Then, they use a statistical test to assess if a DNA region or certain gene is involved in the trait of interest (Grisel,2000). Macrochromosomal traits, chromosome distribution, and genomic size has been defined using a combination of genetic and physical mapping (Ladjali-Mohammedi et al., 1999). Currently, whole-genome sequencing (WGS) and genome-wide association studies (GWAS) are two practical approaches for determining a chromosomal region that is linked to the trait of interest

through building a genetic map with a high density (Gheyas et al., 2013; Liz et al., 2016). The genetic map can be used for QTL mapping associated with several commercial features mentioned earlier (Wong et al., 2004; Hillier et al., 2004).

Genetic Selection in the Poultry Industry

Current genotyping technologies with the development of molecular genetics has made it possible to integrate molecular data into animal genetic improvement. Moreover, these strategies allow switching to the use of markers that are in linkage disequilibrium through the population (Dekkers, 2005). Over the last 60 years, the program of genetic selection has resulted in a faster development rate and improved meat production in broilers (meat-type chickens), lowering the slaughter age, decreasing the energy and the amount of feed necessary to produce birds with the desired weight (Bradshaw et al., 2002; Tallentire et al., 2016). From 1975 to 2005, the rate of growth grew by more than 400% (Zuidhof et al., 2014), and almost 85-90% of this growth was due to genetic selection (Havenstein et al., 2003). This remarkable achievement was accomplished by phenotypic selection and breeding based on an estimation of breeding values (EBV) without knowing the number of genes (Dekkers, 2005). Utilizing this would increase the frequency of the beneficial alleles or decrease the frequency of the undesirable ones. (Meuwissen et al., 2001). Due to the publishing of the chicken genome sequence in 2004 and having advanced technology with an excellent computing system, several poultry breeding industries were highly interested in using the genomic data to help them in selecting and breeding. Since Mendel proposed the possibility of what we call “internal variables” that can be passed down from generation to generation, the link between genotypic and phenotypic makeup has sparked a biological interest. In the past few years, a large number of technologies have been enhanced to detect DNA variations and have a better

understanding of how DNA variation influences traits. As a result, various strategies and methodologies have been improved and participated in enhancing breeding programs.

Restriction Fragment Length Polymorphisms (RFLPs)

Restriction Fragment Length Polymorphisms (RFLPs) were the first molecular techniques that allow genetic mapping and identifying individuals based on genetic variations through using restriction enzyme-digested DNA and probes for certain genes or genomic dispersed repetitive elements (Powell et al., 1996; Edwards et al., 2007). This approach was successful in detecting significant deletions or insertions in DNA, as well as single base alterations within the restriction enzyme digesting site. Furthermore, RFLPs were used to detect DNA variations that impact commercially important traits. This technique was used to identify gene location, diagnose the genetic disorder, and determine parentage. Nevertheless, this method was too expensive and time-consuming, which limited individuals' numbers that can be genotyped. Thus, RFLPs were not recently used in the poultry breeding industries (Lamont, 1990; Fulton, 2012).

Microsatellites (MS) or Simple Sequence Tandem Repeats (SSTRs)

Since the development of the polymerase chain reaction (PCR), it can be easy to amplify a minimal amount of the DNA. Microsatellites (MS) or simple sequence tandem repeats (SSTRs), which were used as a molecular marker, are short repetitive DNA sequences of short length. These repetitive sequences can be detected by PCR (Powell et al., 1996; Milan et al., 1998; Hillel et al., 2003; Dalvit et al., 2008; Li et al., 2004). Then, the amplified region or PCR product can be further examined using polyacrylamide gel electrophoresis (PAGE) with a high throughput property (Suwabe et al., 2002; Wang et al., 2003). MS is multiallelic, codominant, and extremely polymorphic. MS is widely distributed across the genome (Fulton, 2012). It has been proven that MS plays an important role not only in identifying genetic variations but also in determining the

phylogenetic tree of some organisms within the same species (Buchanan et al., 1994; Vanhala et al., 1998). All the previous characteristics suggested that microsatellites can be an excellent selection marker. However, detecting MS at first was prohibitively costly. later, the cost had been dramatically dropped once they have been identified. An MS-based genomic map was highly improved to uncover genetic regions associated with different commercial traits, but these regions were too large, including hundreds of genes. Even though the given number of MS markers and the experimental population sizes were inadequate to help to determine the gene of interest, this initial study revealed those genetic variations that are responsible for commercial traits could be identified (Fulton, 2012). MS can be affected by some major evolutionary mechanisms involving gene flow, mutations, and genetic drift, but natural selection does not have any impact on MS (Kikuchi et al., 2002; Estoup et al., 2002).

Quantitative Trait Loci (QTL)

Quantitative trait locus (QTL), which is a statistical tool, shows the relationship between two various types of data, including genotypic information or molecular markers and phenotypic information or trait measurements. The main purpose of QTL is to show why there is a variation in some complex phenotypes and what are the genetic basis behind that. Furthermore, this approach can provide more specific details about not only gene positions but also genetic interactions and gene numbers (Kearsey; Lynch et al., 1998; Miles et al., 2008). The easiest way is to do a single marker analysis where each marker locus has a range of trait values that are different from other markers. Although the analysis of variance (ANOVA) has been widely used to find QTL, it does not predict both sizes and positions of the QTL effect (Jones et al., 2006). QTL mapping is beneficial for different fields of study involving evolution, agriculture, and medicine (Miles et al., 2008). Moreover, QTL analyses are a powerful tool for identifying genomic

regions that co-segregate within a particular trait in F2 generations or the family of Recombinant Inbred Line (RIL) (Alonso-Blanco et al., 1998; Kowalski et al., 1994). To demonstrate, scientists need two factors to start a QTL analysis. First, they require at least two different types of organisms with genetic differences in the trait of interest. Second, they must have genetic markers for genotyping purposes. Various types of markers have been used for QTL mapping involving MS, RFLPs, SNPs, and transposable element positions (Casa et al., 2000; Vignal et al., 2002; Henry, 2006; Miles et al., 2008). To start the QTL mapping, the heterozygous F1 individuals are crossed based on a specific breeding scheme. Then, the new generations (F2) are scored for genotypes and phenotypes. Genetic markers associated with QTL can influence the trait of interest only if they show a high allelic frequency. To determine the QTL for a trait influenced by many genes (it could be tens or even hundreds of genes), the parental lines should have the same traits with different alleles. Then, the F1 generation can be reassorted based on recombinant alleles with distinct phenotypic values (Miles et al., 2008). Scientists were able to identify QTL associated with evolution or ecology for unmanipulated natural populations through using sibships, pedigree, and hybrids information (Mott et al., 2000; Slate, 2005; Shaw et al., 2007; Baack et al., 2008). Ten years ago, the animal genome QTL database (http://www.animalgenome.org) published 125 articles discovering over 2,400 QTL for 248 phenotypes. The phenotypes that underwent studies were related to metabolic disorders, egg quality and production, growth, specific disease resistance, and chickens' behavior. The majority of all these QTL analyses were determined using 200 to 350 of MS as well as creating new lines by doing multiple crosses between different types of breeds, including broilers and egg-laying chickens. The control regions, which have been observed between new lines, are responsible for the large phenotypic variations. Only a few breed QTL studies were published, and it is possible that different poultry breeding companies conducted

further studies which have not been reported in the literature (Fulton, 2012). There are two main issues in QTL mapping. First, the ability to identify a QTL depends on the sample size, experimental design, and the type of statistical approach used for data analysis (Majumde et al., 2005). The second issue is what are the nature and marker distributions of chromosomes (Gibbs et al., 2003; Wall et al., 2003). There are other issues in QTL mapping that can affect the determination of phenotypic values, including genotype-environment interactions and gene-gene interactions or epistasis (Majumde et al., 2005). Since 2003, QTL mapping has been expanded to include more informative results such as measuring the gene expression or eQTL and knowing the protein quantity produced by specific genes or pQTL (Schadt et al., 2003; Foss et al., 2007; Cheverud et al., 2008).

Marker-Assisted Selection (MAS) in Poultry

Marker-Assisted Selection (MAS) is a branch of molecular breeding (MB) that has been defined as the application of genetic manipulation at a molecular level to enhance the traits of interest not only in animals but also in plants (Ribaut et al., 2010; Jiang et al., 2013). To apply the MAS in poultry, several studies have been done in chickens using markers with a random distribution in the genome or MS. This helps in identifying a large number of QTL related to meat production (Tatsuda et al., 2001; Nones et al., 2006; Ambo et al., 2008; Campos et al., 2009). Other researchers have focused on identifying SNPs in functional genes and assessed their relationship with targeted QTL (Boschiero et al., 2013; Felício et al., 2013; Pértille et al., 2015). In the past few years, next-generation sequencing (NGS) has become popular since it has been used to conduct GWAS to identify a novel QTL as well as update regions mentioned in the literature (Sun et al.; Morris et al., 2013; Park et al., 2013; Moreira et al.; Godoy et al., 2015; Pértille et al., 2017). The aim was to use the previous information for MAS since several significant

genomic regions related to the traits of interest have been identified. Then, individuals with superior performance were chosen at an early age based on specific DNA markers. Although these DNA markers used for selection might not be the main cause for phenotypic variations, they were at least close to the targeted regions that could be used for selection (Fulton, 2012). MAS has been widely used in the breeding programs of chicken industries. They used MAS to reduce the frequency of undesirable alleles or enhance the frequency of the beneficial alleles (Meuwissen et al., 2001; Siegel et al., 2006). For example, MAS has some practical restrictions. To begin with, recombination might appear in the next generations only if the DNA markers were not close to the region of interest, such as a lack of positive correlation between a marker allele and a significant trait. Moreover, the correlation between a marker allele and a significant trait was family or population specific. This has created several MAS limitations within pure line populations. The negative association between two or more traits other than the trait of interest was the other problem with MAS. For instance, the major goal of the commercial breeding program through MAS is to have a bird of good quality. However, MAS for large eggs might have other unfavorable traits such as markers responsible for feed efficiency, shell strength, or body weight (Fulton, 2012).

Genome-Wide Association Studies (GWAS)

Genome-Wide Association Studies (GWAS) are a complementary and powerful approach to QTL mapping (Zhang et al., 2014). GWAS looks for genetic variations associated with a disease of interest in two different groups (disease cases and unaffected control). It has been possible to do GWAS due to identifying large numbers of SNPs across the genome as well as realizing that the majority of these SNPs can determine prevalent genetic variations (Gibbs et al., 2003; Witte, 2010). Gu et al. conducted GWAS to identify SNPs associated with body weight in chickens. They used an F2 resource population that has been developed from White Plymouth Rock X Silky Fowl;

they found that several important SNPs can cause late growth at 7-12 weeks of age. These SNPs, which are in chromosome 4, are associated with LIM domain-binding factor 2 (LDB2) gene (2011). Another study has shown that there are seven SNPs and four genes located in the same region (chromosome 4) and related to body weight. These genes are Leucine Aminopeptidase 3 (LAP3), Transmembrane Anterior-Posterior Transformation 1(TAPT1), LIM Domain Binding 2 (LDB2), and Ligand Dependent Nuclear Receptor Corepressor Like (LCORL) (Liu et al., 2013). A group of scientists has conducted GWAS on F2 crosses between a fast-growing commercial broiler (Cobb-Vantress) and a local Chinese breed (Beijing-You chickens). They found that there are 14 genes responsible for meat quality traits. Some of these genes are microsomal glutathione S-transferase 1 (MGST1), natriuretic peptide B (NPPB), sterol regulatory element-binding transcription factor 1 (SREBF1), and protein tyrosine kinase (TYRO3) (Sun et al., 2013). GWAS has become popular recently in genetic research, and before that, linkage and candidate gene studies were used to find the genetic basis of diseases (Botstein et al., 1980). Linkage studies have a lower power because they assess markers widely distributed across the genome to see if these markers are passed to the next generations or not. Moreover, linkage studies are not able to uncover disease-related genetic risk factors. Candidate gene studies focus on the relationship between genetic variations and disease of interest, but they skip most of the genome. Consequently, candidate gene studies can miss a lot of causative regions or genes and detect a lot of false-positive associations (Hirschhorn et al., 2002; Lohmueller et al., 2003). GWAS has more power than the two previous approaches because GWAS has successfully identified hundreds of variations linked to great numbers of diseases. Most of these variations are novel; chromosomal regions or related SNPs in genes have never been linked to the disease before (Hindorff et al., 2009). Although GWAS represents an improvement over the limitations of QTL studies, they introduce other issues.

However, when they are carried out together, they can correct each other's limitations (Korte et al., 2013). QTLs have hundreds of related genes, while GWAS generates a significant number of unrelated genes or nucleotides. Even though GWAS are restricted to organisms with genetic resources, a combination of these two approaches has a beneficial advantage through enhancing the statistical power with great mapping of linked genes or even nucleotides (Miles et al., 2008). Currently, next-generation sequencing (NGS) and DNA hybridization help in identifying significant structural variations known as number variation (CNV). The CNV is a genomic polymorphism that results in changing the number of copies of DNA through duplications or insertions and deletions (INDELs). The CNV usually ranges from 1kbp to 5Mbp and is one of the most important contributors to phenotypic variations in plants and animals (Clop et al., 2012; Yan et al., 2014). A large number of studies have been conducted to prove that CNV is responsible for several human diseases such as autism spectrum disorders, cardiovascular diseases, and peripheral arterial diseases (Pollex et al., 2007; Girirajan et al., 2013; Koller et al., 2020).

Single Nucleotide Polymorphisms (SNPs)

Approximately 2.8 million single nucleotide polymorphisms (SNPs) were identified and published by the Beijing Genome Institute once the 2004 chicken genome had been released. SNPs are "single nucleotide variants within the DNA sequence," and INDELs account for almost 10% of these variants (Brandstrom et al., 2007; Fulton, 2011; Yan et al., 2014). SNPs can be either heterozygous or homozygous. The simplest way of SNP genotyping after targeting a particular region containing a candidate gene is to sequence PCR products from the gene. Sometimes, it might be difficult to determine the true polymorphisms when two peaks exist because of sequence artifacts. When there is a single peak at one position, the SNP is homozygous. However, the SNP is heterozygous if duple peaks are observed (Figure1.7) (Vignal et al., 2002). SNPs may be found

within the coding, non-coding sequences of genes or between genes (known as intergenic regions) (Miles et al., 2016). To identify SNPs, scientists compared the RJF genome sequence to partial genome data of 4 chickens from 3 various types. These chickens are 1 White Leghorn: 2 commercial broilers: and 1 Silkie (Fleming, 2016). The identified SNPs help to create all the basis for an SNP genotyping platform (Fulton, 2011). In protein-coding regions, a point mutation is a heritable alteration in the genetic region in which a single nucleotide is replaced by another. Synonymous, non-synonymous, and nonsense are three distinct types of mutations. To begin with, even though synonymous mutations change the DNA sequences, they do not change the encoded amino acids. Therefore, synonymous mutations are functionally silent and evolutionary neutral. On the other hand, non-synonymous mutations change both DNA and protein sequences, and they undergo natural selection. Similarly, nonsense mutations change the protein sequences because they introduce a stop codon and produce incomplete protein products (Chu et al., 2019). The majority of detected SNPs have little biological effect when they are located in the non-coding region or exist in synonymous mutations. According to the DNA analysis of human beings, any two individuals are 99.99% identical, whereas only 0.1% hold all the genetic variations (Crow, 2002). A transition: pyrimidine-pyrimidine (C ⇔ T) or purine-purine (A ⇔ G) exchanges, or a transversion: pyrimidine-purine or purine-pyrimidine (G ⇔ C, G ⇔ T, A ⇔ C, A ⇔T) exchanges are two significant mechanisms involved in the mutations. The mutations are considered to be random if the ratio for transitions or transversions is 0.5 (Vignale et al., 2002).

SNP Chips

Genomic-based selections can be achieved if high-density SNP chips have been developed. It enables genotyping to be done quickly, for a large number of animals, and at a minimal cost (Wolc et al., 2016). The first chicken SNP chips contained only 3K SNPs, and this has been

insufficient (Muir et al., 2008). In 2008, a grant proposal of approximately $5 million was approved by the USDA for SNP genotyping of organisms with a genome sequence. This was limited to only two animal species (cattle and chickens). The higher support was for the cattle SNP chips. Consequently, the USDA grant for the chicken SNP chips was insufficient. Therefore, several other commercial breeding companies, including Hendrix Genetics and Cobb-Vantress, accepted and supported the initial proposal for this project. As a result of these monetary contributions, the 60K SNP chip, which has been developed by Illumina, Inc., became private and limited. The same company, Illumina, Inc., designed another SNP chip (42K SNPs). The second chip was completely funded by other companies such as Lohmann Tierzucht, Hy-Line International, and EW Group. Thus, the 42K SNP chip was not available to the public (Groenen et al., 2011; Fulton, 2012; Kranis et al., 2013). These two medium-sized chips played a significant role in genomic selection studies as well as they involved some genetic variations from layers and broilers. In 2013, a high-density chip (600K SNPs) was developed with sponsorship from the Roslin Institute and the BBSRC. This high-density chip was publicly available, and it contains genetic variations from not only white and brown layer breeders but also broiler breeders (Kranis et al., 2013). Surprisingly, despite the significant increase in high-density chips, the medium-density chips, which contain thousands of SNPs, are the most recent ones used for genomic selections in poultry breeding. The SNPs used in the medium chips are carefully selected from the high-density chip (600K). There are three main reasons for shifting back to medium-density chips. First, due to technological advances, the price of medium-density chips has highly decreased, and they are now equivalent to low-density chips. Second, we only need tens of thousands of SNPs because this number can provide us with an accurate estimation of genomic breeding values (Ilska et al., 2014). Third, the genomic prediction process is simplified using a medium-density chip

rather than imputation from high to lower densities. As a result, medium-density chips will be an excellent choice, soon, for a massive number of genomic selections in poultry (Wolc et al., 2004; Habier et al., 2009).

Next-Generation Sequencing (NGS)

Next-generation sequencing (NGS) is a deep or highly parallel DNA sequencing method that has revolutionized genomic research (Davey et al., 2011). Furthermore, NGS detects the nucleotide order of the whole genome and targeted RNA. NGS is powerful since it can sequence the entire human genome in a short period of time (it could be one day). In comparison, the previous technology (Sanger sequencing) took over a decade to complete the final draft of the human genome. NGS can be widely applied in clinical studies to improve patient care, and that involves identifying mosaic mutations, studying rare somatic variations, and detecting novel human microbiomes (Behjati et l., 2013; Swierniak et al., 2016; Malla et al., 2019). NGS has been used to uncover new RNA variants and splicing regions or measure gene expression (Twine et al., 2011). This technology examines the effect of epigenetic factors including DNA-protein interactions and genome-DNA methylation (Movassagh et al., 2011). NGS is a cost-efficient substitute for SNP genotyping methods, and it has the opportunity to improve both GWAS as well as whole-genome selection (WGS) in chicken egg and meat production (Campbell et al., 2015). This approach helps in detecting genetic variations across the whole chicken genome that are linked to functional changes such as chromosome rearrangements, short INDELs, CNVs, and SNPs (Baxter et al., 2011). Different methods such as microarrays, qRT-PCR, ChIP-seq, protein analysis, siRNA, and in situ hybridization are used to eliminate or verify genetic variations once NGS data has been stored and potential causative regions or genes have been identified (Schmid et al., 2015). The high-density chip was successfully paired with WGS to detect the responsible

gene for talpid2 mutation, and RNA-seq was performed to confirm the association between the C2CD3 gene and this disorder (Chang et al., 2014). Likewise, WGS has been conducted at the University of California for different developmental mutations involving limbless, eudiplopodia, diplopodia-3, diplopodia-4, and stumpy (Schmid et al., 2015).

Copy Number Variations (CNVs)

A segment of genomic sequences, which range from 1000bp to million base pairs, can be present in different quantities relative to the normal genome, and that is called copy number variations (CNVs). Large-scale variations can be seen under a microscope. Therefore, they are commonly detected and examined using microscopy during karyotyping (Feuk et al., 2006; Conrad et al., 2010; Wang& Byers, 2014). Scientists, for a short period, ignored doing any research due to the lack of appropriate studying tools. Nevertheless, during the last decade, advanced DNA technology, including sequencing and microarray, has made it possible to identify CNVs in different genomes. That results in increasing the CNV studies in different organisms, especially humans (Carter, 2007). CNVs can be detected by comparing the DNA sequence of various cells within the same organism or species. CNVs have resulted from sequence translocation, insertion, inversion, deletion, substitution, or a combination of all these processes. An insertion results in the addition of a genetic segment, whereas a deletion results in the loss of a genetic segment (Mahmoud et al., 2019). Over the last few years, chicken CNVs have become a popular subject in scientific research. Chicken CNVs research is motivated by a variety of interests from both a basic biological point of view and a poultry science perspective (Kanginakudru et al., 2008; Griffin et al., 2008). Array comparative genomic hybridization (aCGH), which was the first effective method for scanning the genome for CNVs, was used to identify CNVs in chickens, humans, and other species (Pinkel & Albertson, 2005; Bertone et al., 2006; Fiegler et al., 2006). Moreover, CNVs

were detected using SNP arrays. Even though the main purpose of designing SNP arrays was SNP genotyping, it is possible to detect CNVs using this technique due to the formation of abnormal hybridization. This occurs when both a CNV region and an SNP probe are in the same position (Colella et al., 2007; Wang et al., 2007). Using SNP arrays in identifying CNVs makes a difference in the combination of GWAS with SNP arrays. This method is particularly popular in human studies (Jakobsson et al., 2008; Wheeler et al., 2013). Several studies were conducted to detect CNVs in animals involving chickens (Jia et al., 2013). In this review, we will discuss several examples related to the identification of CNVs across the chicken genome using GWAS analysis. Griffin et al. reported that there are 12 CNVs found in RJF/domestic chickens. The size of CNVs was estimated to be 90kb and 127kb, which were distributed on micro and macro- chromosomes (2008). Other studies have focused on copy number variation regions (CNVRs) which have been defined as the sum of overlapping CNVs found in two various specimens (Upadhyay et al., 2017). The CNVRs in chickens account for 8.3% of the genome (Wang& Byers, 2014). Crooijmans et al. identified the largest CNVs in commercial line/ experimental line. The total number of CNVs was 3,154, 1556 of which were CNVRs. The largest CNVs were observed in chromosome Z, with an average size of 46.3kb (2013). A study found a relationship between CNVs and Marek's disease (MD) (Xu et al., 2017). MD, which is caused by alphaherpesvirus, is a major issue in the poultry industry (Engel et al., 2012). In four chicken lines, a total of 45 CNVs have been identified, 28 of them play a significant role in cell proliferation and immune response, and two CNVs are responsible for MD resistance. This new finding may result in more effective techniques for genetic manipulation of disease resistance in chickens (Luo et al., 2013). The CNVs can impact phenotypes because they have the potential to alter the function of the proteins if they are implicated in protein-coding. In addition, the CNVs affect gene expression if they are linked to the

regulatory regions of the gene. The CNVs might influence up to 500Mb away (Henrichsen et al., 2009). There are several traits associated with CNVs in chickens, such as dark brown plumage color, late feathering, dermal hyperpigmentation, and pea-comb (Elferink et al., 2008; Wright et al., 2009; Dorshorst et al., 2010; Gunnarsson et al., 2011).

Epigenetics in Poultry

several economically significant features in animal production show a phenotypic variation because of environmental and polygenic factors. Although several QTLs for agronomic traits have been detected, the fundamental genes for most of these traits are still ambiguous. GWAS revealed that genetic variation is the major cause of diversity in complex traits (Manolio et al., 2009; Frésard et al., 2013). According to genetic and epidemiological analysis in both animals and humans, epigenetic marks can be passed down across generations and impact the phenotypes of offspring (Jablonka et al., 2009). Epigenetics has been defined as the phenotypic changes without alterations in DNA sequence, and that are passed down from generation to generation (Ho et al., 2010). According to Russo et al., epigenetics is "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in the DNA sequence" (1996). Bird stated that; "epigenetics is the structural adaptation of chromosomal regions to register, signal or perpetuate altered activity states" (2007). The epigenetic mechanisms are essential gene regulators which play a significant role in establishing the heritable modifications in gene expression without changing the nucleotide sequences (Rivera et al., 2013). These mechanisms involve DNA methylation, histone modification, chromatin remodeling, noncoding regulatory RNA (lncRNA, miRNA, snoRNA), and DNA packaging. Moreover, epigenetic marks are involved in determining active transcription or gene silencing patterns, as well as contribute to tissue-specific gene expression (Youngson et al., 2008; Jin et al., 2011; Mazzio et al.; Feil, 2012; Jeltsch et al., 2016).

Epigenetics marks can be passed from cell to cell during pedigree development, and they can influence individual phenotypes if they receive them at an early age. Epigenetic marks can influence the phenotypes of future generations through intergenerational effects caused by epigenetic modifications obtained during embryogenesis or via epigenetic marks passed down over generations through gametes (Jablonka et al., 2009; Daxinger et al., 2012). The expansion rate in publications and research on epigenetics has considerably grown up in different organisms because of the rapid advancement of NGS technology and the sharp decline in costs. Unfortunately, there is little research on the relationship between epigenetic mechanisms and commercially significant traits in chickens. It is still a puzzle how epigenetics impacts gene expression (how much, when, and where) in chickens. Nevertheless, there is an expectation of having more epigenomic data published in the near future, which will dramatically increase our insight into the genome-phenome interactions (Frésard et al., 2013).

The Effect of Genetic Selection on Broiler Welfare

Intensive genetic selection for commercially significant traits such as growth rate, body weight, and feed efficiency have increased production and efficiency in poultry breeding companies (Havenstein et al., 1994; Hunton, 2006; Elfick, 2012; Tavárez et al., 2016;). In today's world, breeding programs with extremely precise estimated breeding values are achievable due to the availability of accurate technologies and a better understanding of how gene networks are connected to production traits. Therefore, broiler production grew by more than 400% between 1957 to 2005 despite a 50% decrease in feed conversion (Barton, 1994; Zuidhof et al., 2014). The production rate of livestock has dramatically increased because of genetic selection. Nevertheless, in addition to a beneficial increase in production, species that have been chosen for high productive efficiency appear to be more susceptible to immunological, behavioral, and physiological

abnormalities. There are more than 100 articles that have been published associated with unfavorable (cor)related consequences of intensive genetic selection for high production efficiency in pigs, broilers, and dairy cattle in terms of reproductive, health, and metabolic features (Rauw et al., 1998). The genetic selection might cause animals to lose their homeostasis, which leads to several diseases and, as a result, poor animal welfare. In this review, we will be focusing on the genetic selection in poultry and discuss several examples of unfavorable side effects of extensive genetic selection for high production efficiency. To begin with, genetic selections to increase body weight in turkey result in having a considerably heavy male, which prevents natural breeding (Rauw et al., 1998). If natural breeding occurs, it causes damage to the hens' backs (Hunton, 1984). In addition, increased body weight reduces fertility in broiler breeders (Dunnington, 1990; Liu et al., 1994). Furthermore, genetic selection to increase body weight has changed the growth style in broilers (Anthony et al., 1991). Chickens with a high body weight developed faster, the rate of lipolysis decreased, and that resulted in increased fat deposition (Chambers et al., 1981; Barbato et al., 1984; Calabotta et al., 1985). Sinsigalli et al. (1987) suggested that increased fat deposition in broilers is linked to elevated insulin and glucagon level in the blood, as well as insulin resistance. In broiler breeders, excessive genetic selection for a high body weight produces a large number of eggs. However, most of the eggs are defective (e.g., soft-shelled, shell-less, extra calcified, double yolk) (Van Middelkoop et al., 1976). Dunnington and Siegel (1996) indicated that the major cause of defective eggs is a lack of synchronization between ovulation and egg packaging. Moreover, genetic selection for body weight may evolve a poor immunological performance. For instance, when the broilers with higher body weight are challenged by lamb erythrocytes, they demonstrate fewer antibody responses (Miller et al., 1992). By comparing the commercial1991 broiler lines to random-bred 1957 baselines, a higher mortality rate was observed in commercial broilers at 42

days of age due to leg problems and ascites. Furthermore, the commercial broiler lines can be affected more by tibial dyschondroplasia (~47.5%) than random-bred baseline (2.2%) (Havenstein et al., 1994). It was reported that the incidence of dyschondroplasia was 28% in roosters selected for a high body weight compared to 3% in those selected for a low conversion rate of feed. In addition, broilers with high body weight have shown a greater risk of mortality (~7.4%) than those with a low conversion rate of feed (2%), and the major cause of mortality was heart and circulation disorders and infectious diseases (Leenstra, 1993). Broilers selected for a high body weight showed several skeletal problems including long bone distortions, tibial dyschondroplasia, rickets, chondrodystrophy, and other infectious diseases that induce skeletal problems such as retrovirus, *Mycoplasma synovia*, and *Staphylococcus aureus*. According to estimates, 3.2 percent of broiler losses were attributable to skeletal anomalies in terms of condemnation, culling, and death. That negatively impacts the economy because the poultry industry lost $0.16/broiler or $120 million/year (Cook, 2000). Moreover, skeletal maturity and strength are regulated by several factors involving gender, growth aging, toxins antinutrient, nutrition, disease, physical loading, endocrine, and genetic factors (Rath et al., 2000). Intensive genetic selection for rapid growth produces broilers that are susceptible to heart-related problems such as chronic heart failure (ascites, hypoxia) and acute heart failure (SDS) (Olkowski et al., 1998; Korte et al., 1999; Olkowski, 2007). My research has focused on one of the most significant metabolic conditions, that being ascites. Ascites or pulmonary hypertension (PH) has been defined as the accumulation of fluid in the abdominal cavity; a condition referred to as “water belly” (Figure1.8). The goal of this dissertation is to study the genetics of PH in broilers as well as to identify biomarkers relevant to the disease.

The Function of Heart and Lung

The avian heart is divided into four chambers: left and right ventricles, as well as left and right atria. The blood is passed from the right ventricle to the pulmonary artery, which then transports it to the lungs for oxygenation. Then, the oxygen-rich blood is pumped by the left ventricle for systemic circulation. There are two major differences between left and right ventricles. First, the right ventricle functions at a lower pressure, whereas the left ventricle functions at a higher pressure. Second, the muscle mass in the left ventricle is higher than in the right ventricle (Olkowski et al., 1998; Tekeli et al., 2014). The left and right ventricles maintain distinct systolic pressures, which cause the size differences. Therefore, more systolic pressures can be produced by the left ventricle under normal circumstances (Tarrant, 2016). When a broiler chick hatches, it weighs 40g and can grow to 4000g in two months. If we grow at the same rate, the weight of infants would be 300kg in 8 weeks. This massive growth cannot be sustained if there is no equal increase in the function of both the heart and lungs. This includes increasing the cardiac output after chick hatches. The range of cardiac output should be from 480 mL/hour for a 40-g chick to 48L/hour for a 4-kg broiler ((Wideman et al., 2013). The circulating blood return to the heart for oxygenation. The cardiac pulmonary system initiates once the deoxygenated blood re-enters the heart via the right atrium. The right ventricle takes the deoxygenated blood and pumps it to the lung via the pulmonary artery. In a healthy bird, the pulmonary artery pressure is approximately 20mmHg (Chapman & Wideman, 2001; Tarrant, 2016).

Ascites or Pulmonary Hypertension Syndrome (PHS) in Chickens

Ascites syndrome, pulmonary hypertension syndrome (PHS), or pulmonary arterial hypertension (PAH) in modern poultry is the negative result of intensive genetic selection for high body weight. PHS was identified in the 1950s as a serious issue that affected broilers under

abnormal conditions such as cold weather, high ambient altitude, and hypoxia (Smith et al., 1954). In the 1980s, the PHS rate was highly increased in broilers due to the fast-growing rate and feed conversion. PHS is mainly caused by a number of processes, but all of them lead to pulmonary hypertension and right ventricular failure. As a result, the level of oxygen in other tissues is low due to the high demand for oxygen during fast growth. PHS has become a popular subject and has been thoroughly tested (Julian, 1993; Owen et al., 1994; Scheele, 1996). Pavlidis et al. (2007) reported that the mortality of ascites in broilers had reached 30%. Ascites is responsible for 8% of the 361million broilers' death annually. Ascites have negatively impacted the economy in the United States because the cost of ascites was estimated to be $100 million each year. Recently, PHS is no longer a problem due to intensive genetic selections against this disease that have been conducted by commercial breeding companies. Modern broilers can acquire the desired weight in 60% less than the old broilers. However, the capacity of cardiac and pulmonary in both old and modern broilers are the same. As a result, the cardiopulmonary system in both broilers is forced to work at or near its physiological limitation (Baghbanzadeh, & Decuypere, 2008). The pulmonary capacity does not always provide enough oxygen to support rapid growth. That makes it more difficult to manage the energy balance in severe circumstances, such as cold weather or high altitude. (Luger et al., 2003). Schmidt et al. (2003) conducted a study in modern and 1950s unselected broilers to compare the differential growth of breast and heart. At five weeks of age, the breast muscle in the old broilers accounted for 9% of total body weight, while the breast muscle in the modern broilers accounted for 18% of total body weight. Conversely, the heart size in modern broilers reduced at 14 days of age, indicating that genetic selection for rapid growth in breast muscle resulted in a heart muscle reduction. That might cause cardiovascular complications since the small heart in the modern broilers has to work to maintain the rapid growth of the broilers

(Konarzewski et al., 2000). Various factors might cause PHS. To begin with, primary factors such as chemicals, heat, cold, and certain nutrients might decrease vasodilation due to a higher metabolic rate, or these factors might cause pulmonary hypertension that triggers ascites in broilers due to the higher resistance to pulmonary blood flow by: (i) a reduction in the pulmonary vascular capacity; or (ii) the rigidity of red blood cells or higher blood viscosity. Secondary factors, including excessive sodium ratio in water or feed, might cause not only increased blood flow but also an increase in the resistance to pulmonary blood flow (Julian, 2000). Other studies proved that germ-negative bacteria such as *Salmonella typhimurium* and *Escherichia coli* could induce PHS in meat-type chickens. That occurs because the germ-negative bacteria contain lipopolysaccharide (LP) in their outer membrane, and respiratory exposure to this type of bacteria increases the incidence of PHS. LP induces pulmonary hypertension by inducing pulmonary vasoconstriction (Tottori et al., 1997; Chapman et al., 2005).

The Physiology and Etiology of PHS

PHS is a multifactorial disorder that is influenced by genetic, environmental, physiological, and nutritional factors. That triggers several subsequent complications, which end with ascites (Baghbanzadeh, & Decuypere, 2008). PHS in broilers is linked to right ventricular hypertrophy (RVH) and pulmonary hypertension that stimulates by various factors such as increased muscle mass, air quality, cold or heat, and elevated sodium rate. There are significant characteristics of broilers or meat-type chickens which make them susceptible to PHS. The broilers' lungs are small, firm, and anchored in the thoracic cavity. Both respiratory capillaries and blood capillaries form a rigid system. The physiology and morphology of the respiratory system in broilers are crucial in increasing the incidence of PHS. The small body size, heavy and large breast muscle, and high pressure on alveoli play a significant role in triggering PHS (Julian, 1993). Broilers with PHS can

be characterized by the presence of ascitic fluid in the abdominal cavity, the congestion of central venous, liver cirrhosis, and right-sided cardiac failure. PHS is developed in broilers during their fast growth because of the high oxygen demand. This might stimulate cardiac output, which increases the pressure in both lungs and the right ventricle (RV). The RVH gets thicker to face the sudden higher pressure, and that leads to high pressure in pulmonary capillaries and arteries (Figure1.9). The right atrioventricular (AV) valve gets thicker, too. When the right AV fails, both cardiac output and hypertension decrease. However, the pressure in the vena cava, right atrium, portal vein, and sinus venous increase (Chapman and Wideman, 2001). Others suggested that increasing the blood flow through the pulmonary vasculatures inhibits red blood cells from staying for a long time on the respiratory surface to get enough oxygen, resulting in hypoxemia (Olkowski et al., 1998). Elevated blood pressure in the veins causes portal hypertension in the liver, which induces plasma leakage into the peritoneal space. PHS is recorded only if there is plasma accumulation in the abdominal cavity (Wideman and French, 2000).

Methods for inducing PHS

The genetic foundations of disorders like PHS have long been studied using broilers. Broilers are not the only domestic animals that develop PHS, but they are more prone to develop this disease. This makes broilers a good model for PHS research. To study the PHS in broilers, the targeted population must first be challenged so it develops PHS. There are different methods used in literature to induce PHS. Using a pulmonary artery clamp, Wideman and Kirby (1995a) set off a number of pathophysiological progressions to induce PHS in broilers. The incidence of PHS among broilers was raised by the surgical clamp, and the ranges were between 68% to 90%. On the other hand, the control group (broilers without a surgical clamp) showed a lower PHS incident (0 % to 12%). Another study from the same research group proved that injecting the intravenous

broilers with microparticles induced PHS. This occurred due to microparticles occluding a small part of the pulmonary arteriole, increasing the cardiac output, followed by hypoxia development (Wideman and Erf, 2002). Environmental methods found to be involved in the development of PHS such as cold stress (Lubritz & McPherson, 1994; Balog et al., 2003; Ipek& Sahan, 2006), dietary supplementation (Villar-Patiño et al., 2002; Wang et al., 2013; Fathi, 2016), lighting (Al-Masri et al., 2012), and high altitude (Özkan et al., 2010). As mentioned previously, PHS was identified in the 1950s among broilers grown at high ambient altitudes (Smith et al., 1954). At the University of Arkansas, Dr. Anthony and his group developed a hypobaric chamber that works under a partial vacuum to reduce oxygen partial pressure and stimulates the high altitude (Figure1.10). The hypobaric chamber is an advanced and beneficial method since it develops different populations with PHS for several generations (Owen et al., 1990; Tellez et al., 2005; Dey et al., 2018; Balog et al., 2000). In this dissertation, the hypobaric chamber is the only method used to induce PHS in broilers.

Divergent Selection for Developing Ascites Research Lines

Divergent selection is a powerful and excellent method used to generate different ascites experimental lines (Balog et al., 2003). In 1994, Dr.Anthony and his colleagues (at the University of Arkansas, Department of Poultry Science) initiated the selection program for ascites development. Essentially, chickens from a commercial elite line were raised in a hypobaric chamber to mimic the high altitude (9000ft above sea level). The hypobaric chamber was provided with a partial vacuum to decrease the partial oxygen pressure and induce the PHS. Susceptible to PHS (SUS line) and resistant to PHS (RES line) are the main genetic lines that were developed for further studies (Balog and Anthony, 2000; Tellez et al., 2005; Pavlidis et al., 2007). In 1995, these groups, which are the main population, were moved to the poultry research building at the

University of Arkansas. These birds were raised for six weeks in a hypobaric chamber. Meanwhile, several significant data were collected, such as mortality rate, body weight, the accumulation of plasma in the abdominal cavity or the pericardium, heart and liver shape, and the major cause of death. They recorded information about dead birds, such as gender, the date of death, and body weight. At the end of the experiment (usually six weeks), all the surviving birds were terminated, then they were necropsied to evaluate birds for PHS and remove the important organs (liver, heart, spleen) for further examination. Birds were classified into SUS or RES based on the collected information, including the ratio of the right ventricle (RV) to the total ventricle (TV) and the presence of ascetic fluid in the peritoneal cavity. Series breeding was conducted using 24 Sire families (three females and one male per Sire) to reproduce the targeted line with several generations as well as generate siblings to collect mortality rates for further selection. Nevertheless, both SUS and RES lines were developed after a few generations of divergent selection (Figure 1.11). In addition to the previous two lines, the relaxed unselected (REL) line was randomly mated to serve as (the original population or the control group) and showed 75.3% of PHS incidence. However, it was obvious that the incidence of PHS in the SUS line was the highest (95.1%), while the incidence of PHs in the RES was the lowest (7.1%) (Figure1.12) (Balog, 2003; Pavlidis et al., 2007; Wideman et al., 2013).

Genetic Markers for PHS

The heritability rate, mortality, and incidence of PHS are three major differences between the three lines (SUS, RES, REL) (Pavlidis et al., 2007; Krishnamoorthy et al., 2014). Nowadays, the chicken genome has been extensively studied to identify the molecular basis of a specific phenotype (Theron et al., 2001). Therefore, it is very significant to determine the molecular modifications that occurred in these lines as a result of selection. Scientists started by performing

the candidate gene approach. A mutation in the bone morphogenetic protein receptor-II (BMPR 2) gene is one of the major causes of PHS in humans (Deng et al., 2000; Rudarakanchana et al., 2002; Morisaki et al., 2004). The PHS in humans is physiologically and genetically similar to this in broilers (Druyan and Moschandreou, 2012). Therefore, a study has been done by Cisar et al. to investigate whether mutations in the BMPR 2 gene play a significant role in initiating PHS in broilers or not. By comparing the RNA sequences of both ascitic and non-ascitic commercial broiler lines to those of White Leghorn chicken, there were 14 SNPs detected in the BMPR2 gene of the commercial line, and there were no mutations associated with PHS. In addition, in the coding region of the gene, there were 12 synonymous substitution SNPs. However, these SNPs did not change the protein sequence. Moreover, the BMPR2 gene expression was measured by RT-qPCR, and the results revealed that the expression level of the gene is the same in the two groups (2003). A second generation (F2) was generated through double reciprocal crosses of RES and SUS lines. Then, A genome-wide SNP was performed to find chromosomal regions associated with PHS susceptibility. The statistical analysis illustrated a relationship between a region on chromes 9 (12 to 13Mbp) and PHS phenotype in both experimental lines (RES and SUS) and the commercial line. The susceptibility to PHS was gender-specific because females were more affected than males. Several candidate genes were involved in this region, such as urotensin 2 domain (UTS2D), an angiotensin II type 1 receptor (AGTR1), and Serotonin Receptor/Transporter type 2B (5HT2B) (Burks et al., 2011Wideman et al., 2013; Krishnamoorthy et al., 2014). Moreover, other studies identified several regions associated with the PHS phenotype that are distributed across the genome. These regions are seven regions on chromosome 4, three regions on chromosome 9 (13.5-14.8Mbp, 15.5-16.3Mbp), and one region on chromosome 27 (2.0-2.3Mbp) (Wideman et al., 2013). In addition to the previous candidate genes identified in chromosome 9,

angiotensinogen cleaving enzyme (ACE) is a candidate gene found to be related to ascites susceptibility (Wideman et al., 2013). The candidate genes UTS2D, AGTR1, and ACE were found to be correlated to some aspects of hypoxia or high blood pressure in humans or mice (Watanabe et al., 2006; Djordjevic and Goerlach, 2007; MacLean, 2007; Chung et al., 2009; Simonneau et al., 2014). Another study showed an association between regions on chromosomes 2, 4, 5,6, 8, 10, 27, and 28 and PHS (Rabie et al., 2005). In our lab, Dey et al. performed a GWAS on the REL line and commercial elite line to identify regions associated with PHS susceptibility or resistance. The statistical analysis illustrated a significant relationship between chromosome 9 (11.8 to 13.6Mbp) and PHS. Within this region, two candidate genes (UTS2D and AGTR1) were identified earlier and confirmed by Dey et al. to be associated with PHS (2017). Another subsequent GWAS was conducted in our lab on REL at 16 and 18 generations using a 60K SNP chip. Two significant regions on chromosome Z (60Mbp) and chromosome 2 (70Mbp) were found to be relevant to PHS incidence (Tarrant). Myocyte enhancer factor 2C (MEF2c) gene is a candidate gene located inside the Z chromosomal region and is responsible for PHS resistance in females. On the other hand, the melanocortin-4-receptor (MC4R) gene and Cadherin 6 (CDH6) are two candidate genes identified within the 2 chromosomal regions and associated with PHS resistance in males (Tarrant et al., 2017). However, these two regions were not effective in MAS breeding. Therefore, our research group conducted WGR in the REL line and identified 31 regions associated with the PHS trait (Figure1.14). Two of these regions were thoroughly studied by Dey et al. (2018) and Parveen et al. (2020). As a result, the carboxypeptidase Q (CPQ) gene on chromosome 2 (127.62 to 127.75 Mbp) and leucine-rich repeat transmembrane neuronal 4 (LRRTM4) gene on chromosome 22 (3.80 to 3.90Mbp) related to PHS resistance in male broilers. These two genes were the first

identified through GWAS and WGR that was demonstrated to be associated with ascites through additional genotyping.

In humans GWAS, CPQ gene, or plasma glutamate carboxypeptidase (PGCP) gene was found to be associated with heart rate, hypertension, and blood pressure. Both CPQ and LRRTM4 have not been identified before as markers for PHS resistance in male broilers. Male broilers are prone to PHS due to their larger body weight and increase oxygen requirement for rapid growth. However, according to GWAS results, male and female broilers showed a higher SNP frequency for CPQ and LRRTM4. There was one attempt of using these two genes in MAS, and they were validated by Lee et al. (manuscript in review).

SYNOPSIS

The major focus of this dissertation is on the genetic, proteomic, and metabolomic studies of pulmonary hypertension in fast-growing broilers. The first study (chapter 2) emphasizes measuring the CPQ gene expression level among eight tissues (heart, liver, kidney, thigh, breast, spleen, lung, thymus) from unchallenged, six-week-old REL line birds from both genders with specific genotypes (homozygous reference, heterozygous, homozygous non-reference). Moreover, further analysis was performed on previous next-generation sequencing (NGS) data, which show a large chromosomal deletion (~125Kbp) affecting the CPQ gene. The deletion is spanning from intron 4 to ~ 40 kbp downstream of exon 8. The aim of the second chapter (chapter 3) was to identify proteins, fatty acids, lipids, and small molecules in REL line birds “non-challenge with no ascites (NC-NA), challenge with no ascites (C-NA), challenge with ascites (CA)”. Various mass spectrometry analysis (tandem mass spectrometry “LC-MS)/MS”, gas chromatography-mass spectrometry “GC-MS”, liquid chromatography-tandem mass spectrometry “LC-MS”) was done

to determine the proteomic and metabolomic changes concerning hypobaric challenge and ascites. Finally, chapter 4 summarizes the results and highlights the major conclusion of this dissertation.

TABLES AND FIGURES

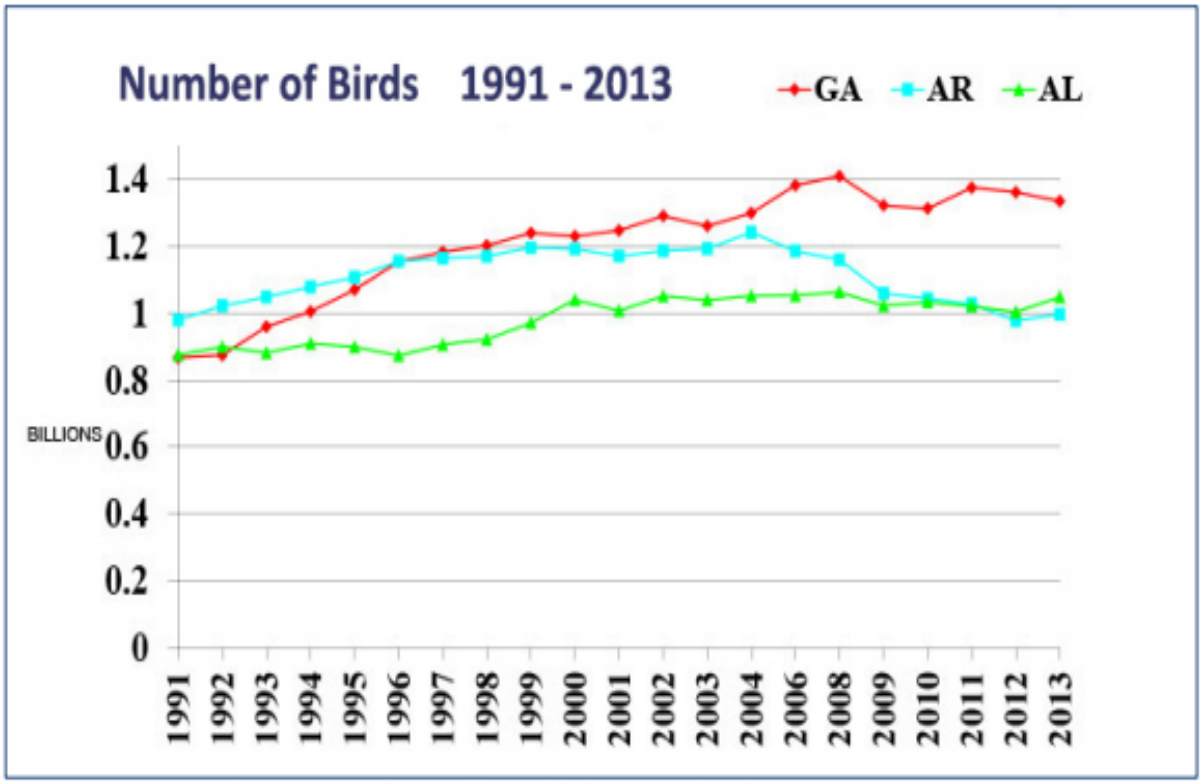

Figure 1. 1. Broilers Production in the Top States (GA, AR, AL) from 1991to 2013 (Bishop et al., 2015).

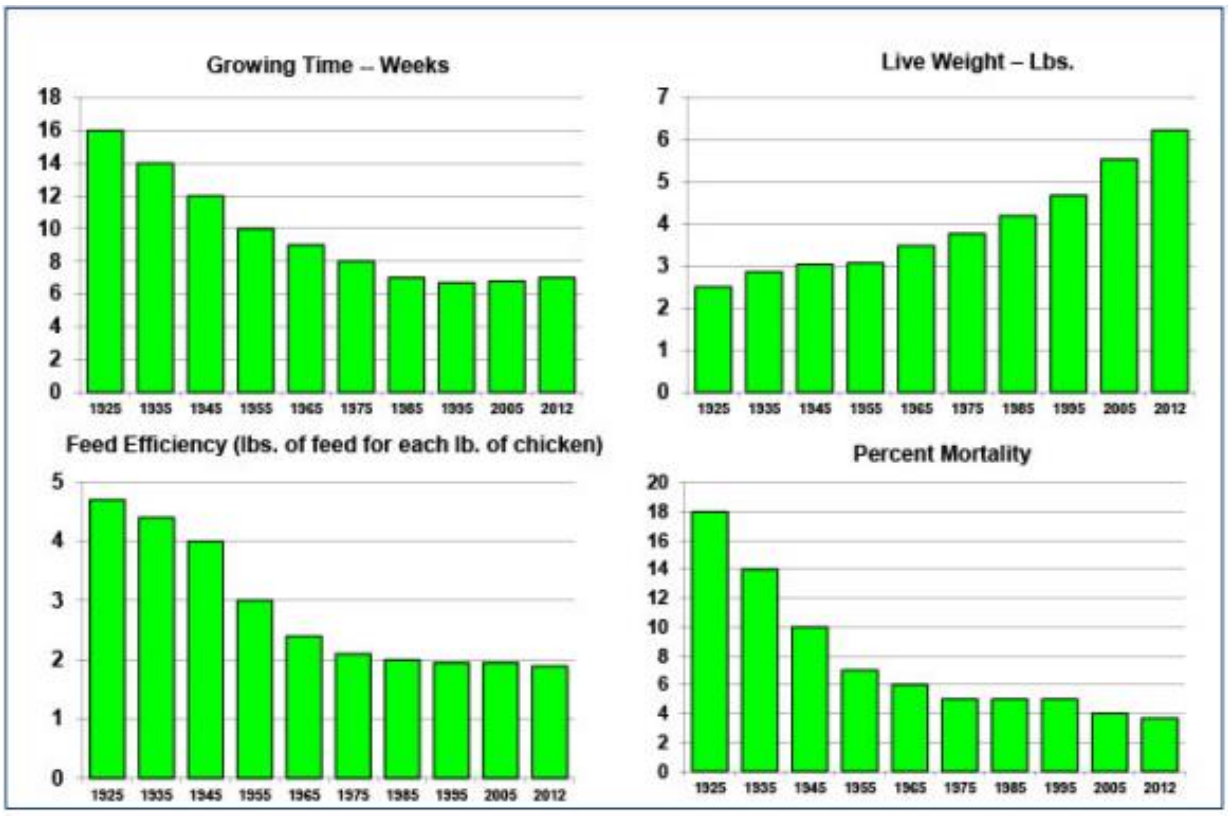

Figure 1. 2.Broilers Performance Trends. Broiler performance has improved dramatically as a result of research and innovation (Bishop et al., 2015).

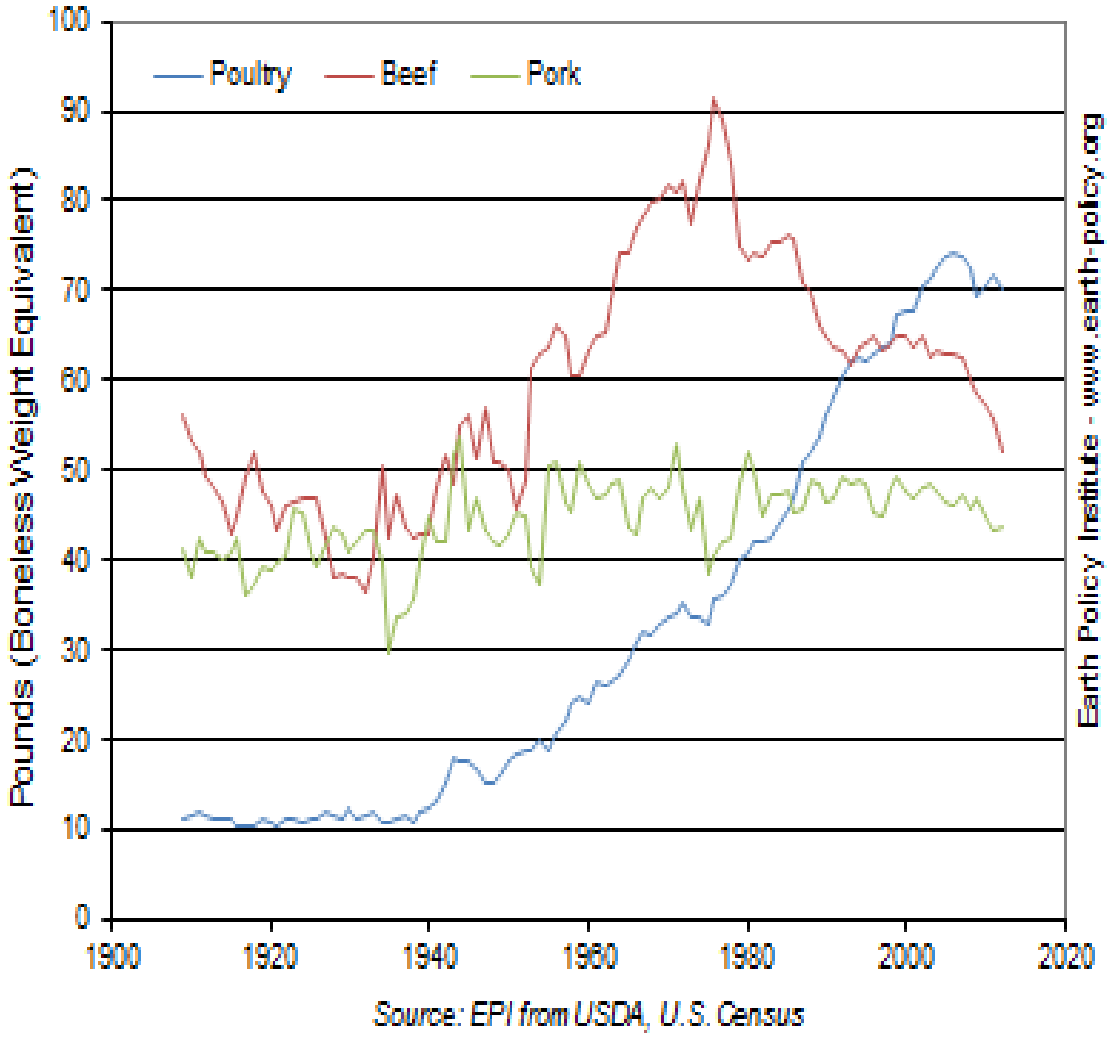

Figure 1. 3. The Meat Consumption (Chickens, Beef, and Pork) in the USA (Larsen, 2012).

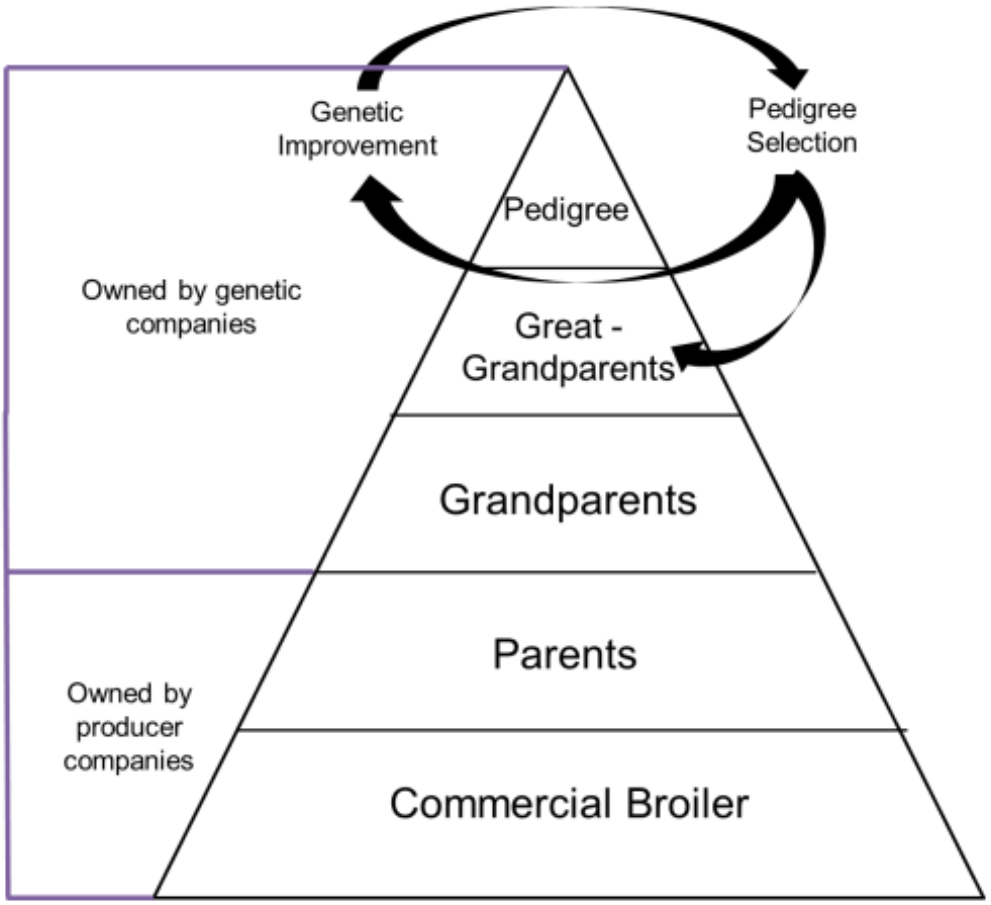

Figure 1. 4.Breeding Schemes Used in Modern Broiler Industries (Tarrant, 2016).

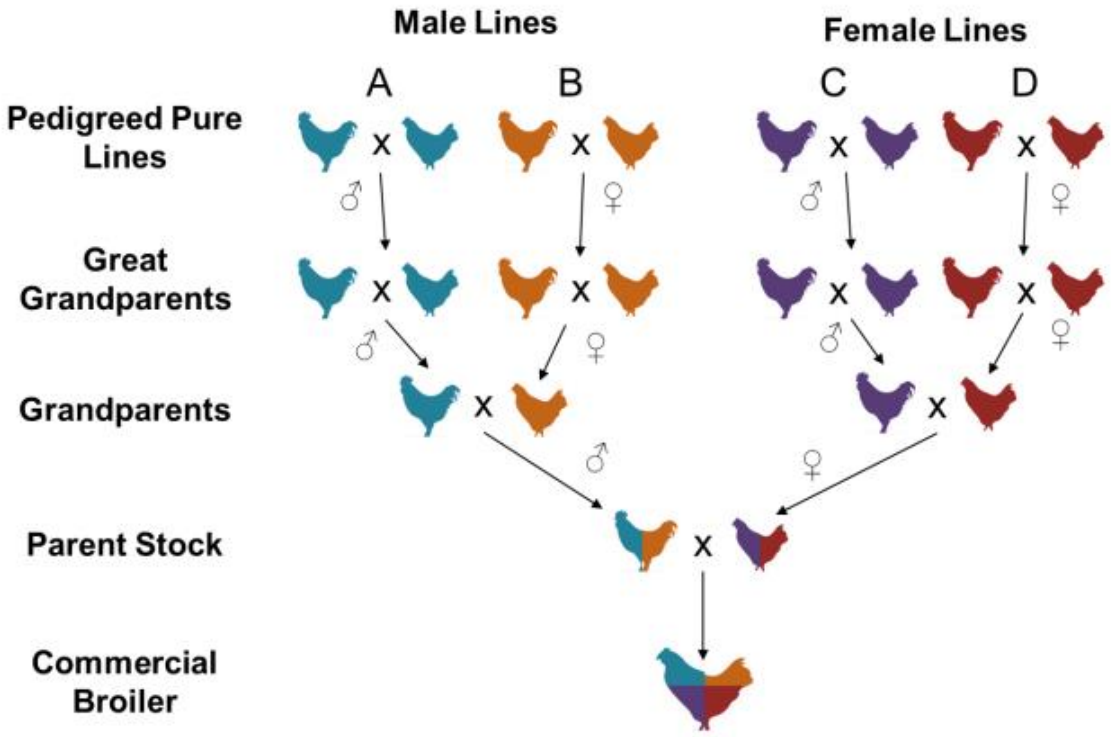

Figure 1. 5.Line Crossing Observed at Each Level of in Poultry Breeding Pyramid (Tarrant, 2016).

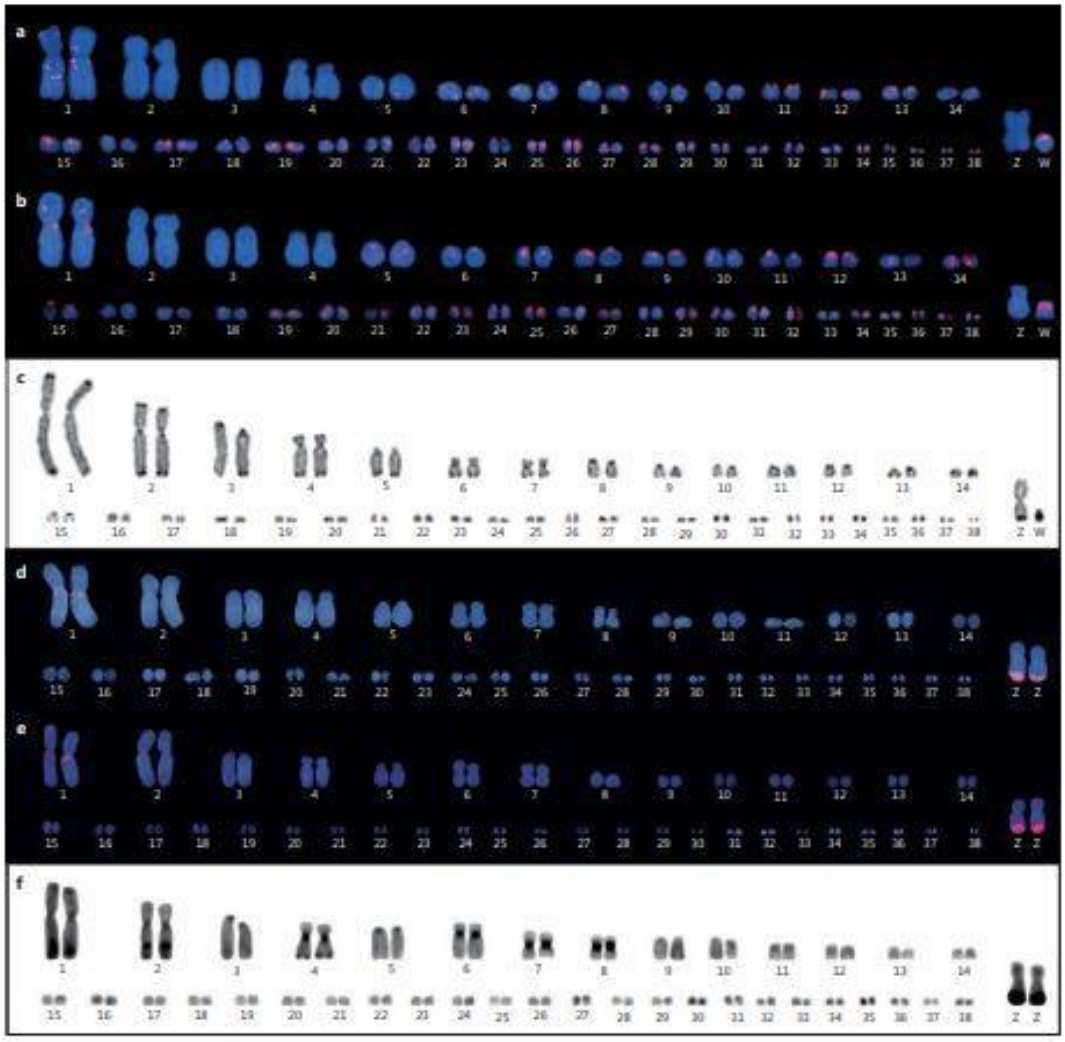

Figure 1. 6.The Karyotype of Chicken. The chicken karyotype is made up of 39 chromosome pairs (Castillo et al., 2010).

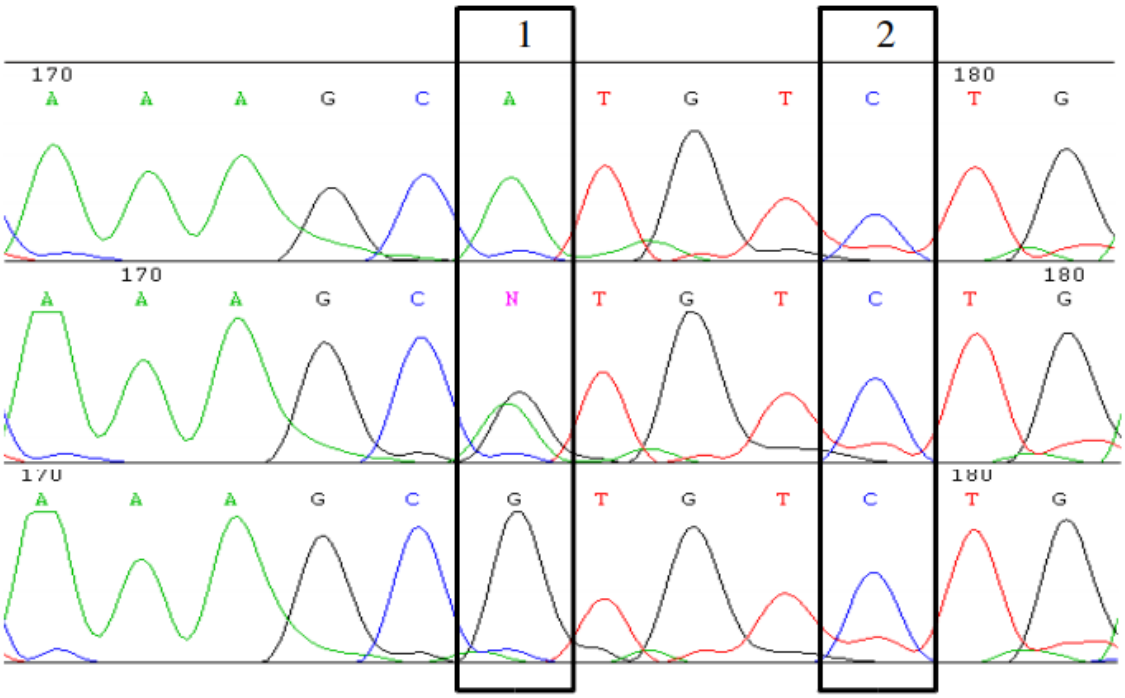

Figure 1. 7.Sanger Sequencing of PCR Product. SNPs can be identified by sequence alignment. Box 1 represented the first allele for three different genotypes (homozygous AA, heterozygous AG, and homozygous GG). Box 2 represented the second allele (homozygous CC, heterozygous CT, and homozygous CC) (Vignal et al., 2002).

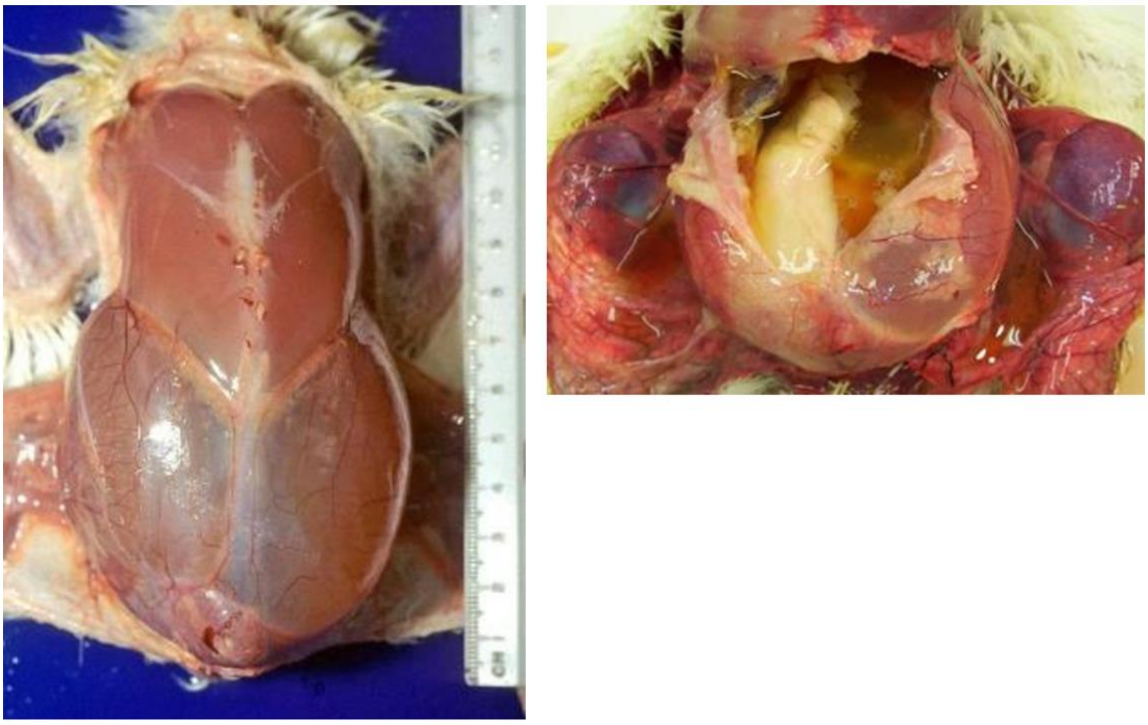

Figure 1. 8.Ascites Symptoms in Broilers. One of the most significant symptoms is the accumulation of fluid in the abdominal cavity (Dey, 2017).

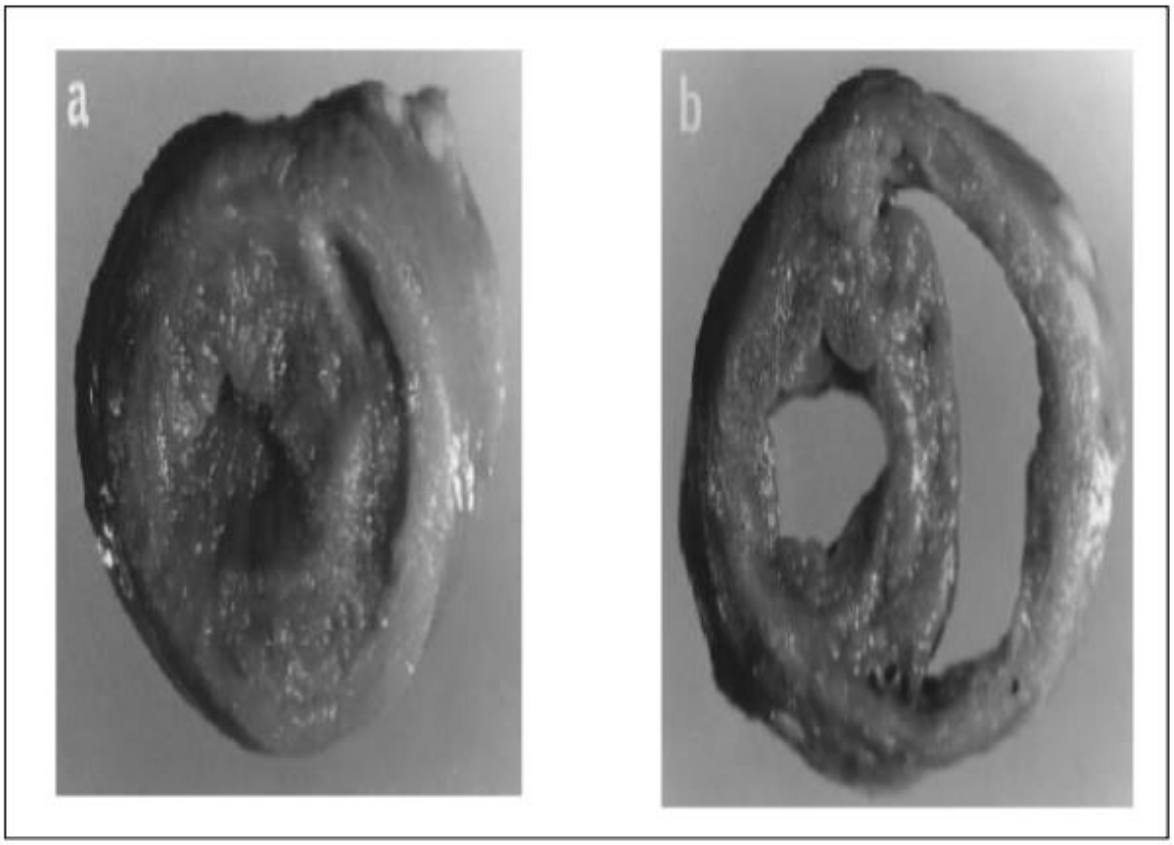

Figure 1. 9.A Cross-Sectional of Two Ventricles. (A) a normal heart; (B) an ascitic heart with ventricular dilation (Olkowski et al.,2001)

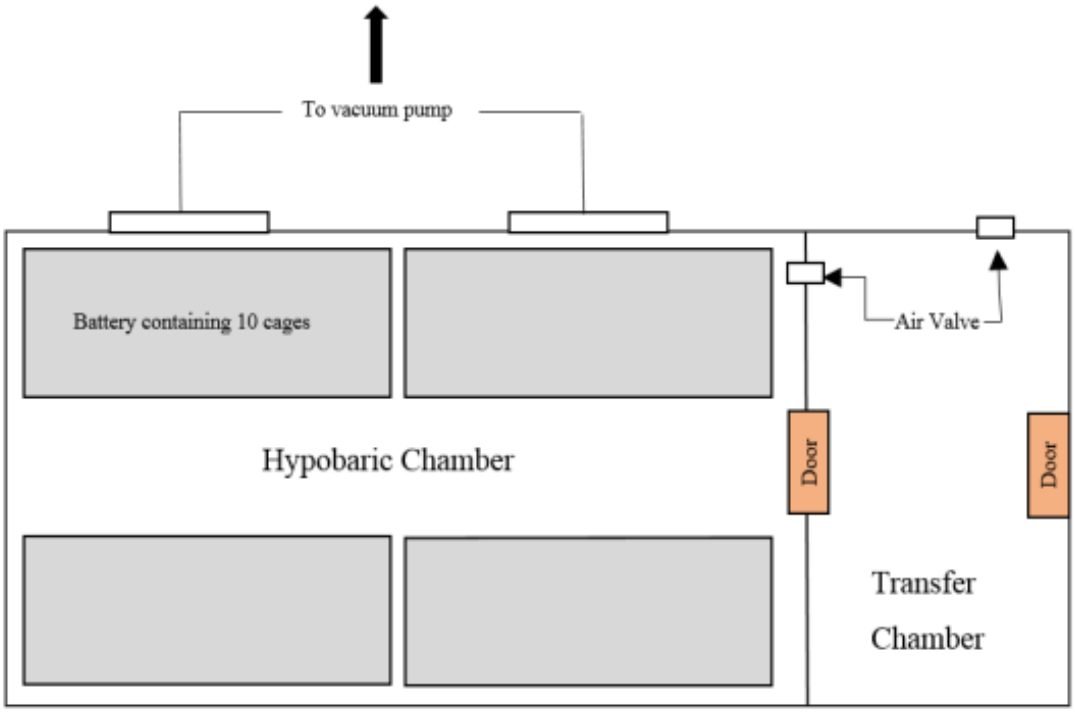

Figure 1. 10.A Paradigm of a Hypobaric Chamber used at the University of Arkansas to induce Ascites in Broilers.

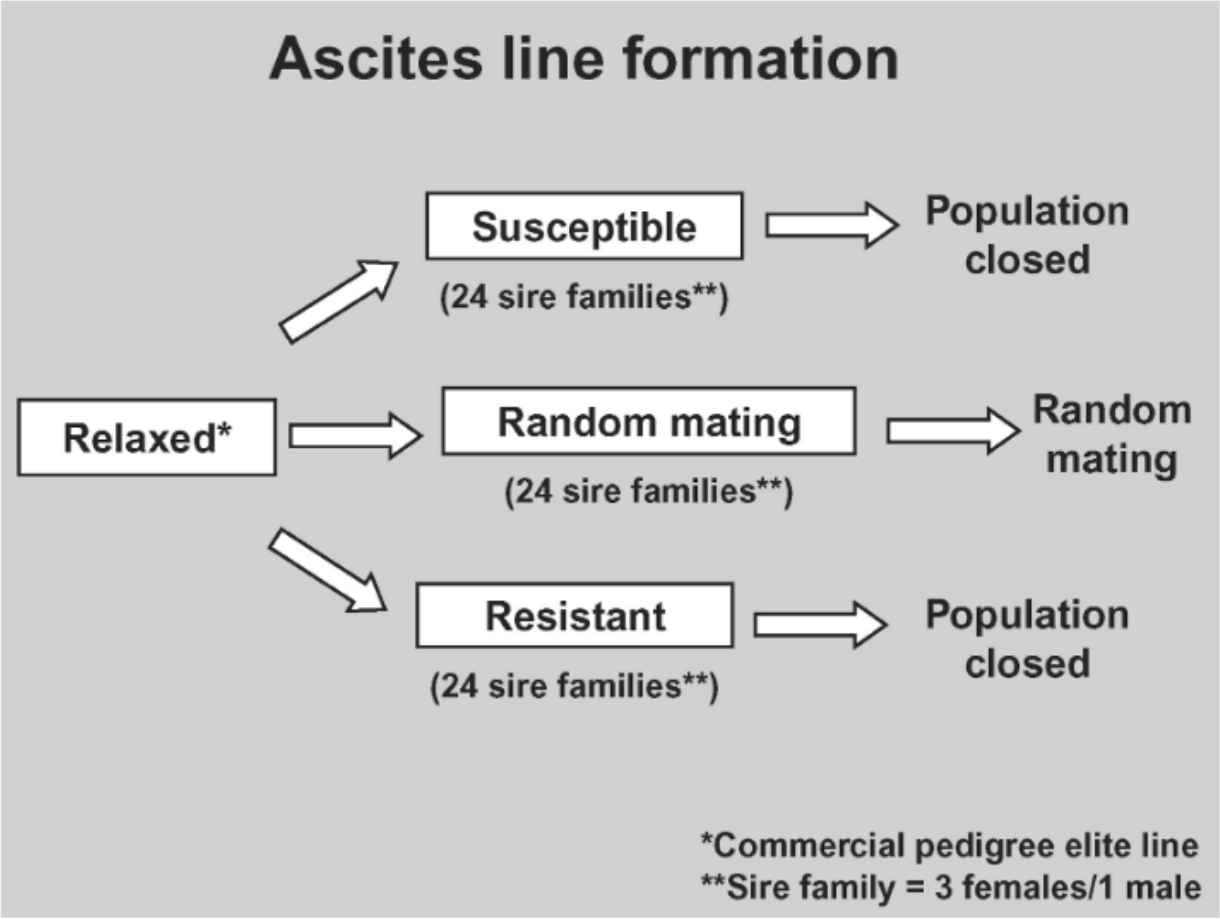

Figure 1. 11.The Development of Three Major Lines (SUS, RES, and REL) from the Commercial Pedigree Line (Pavlidis et al., 2007).

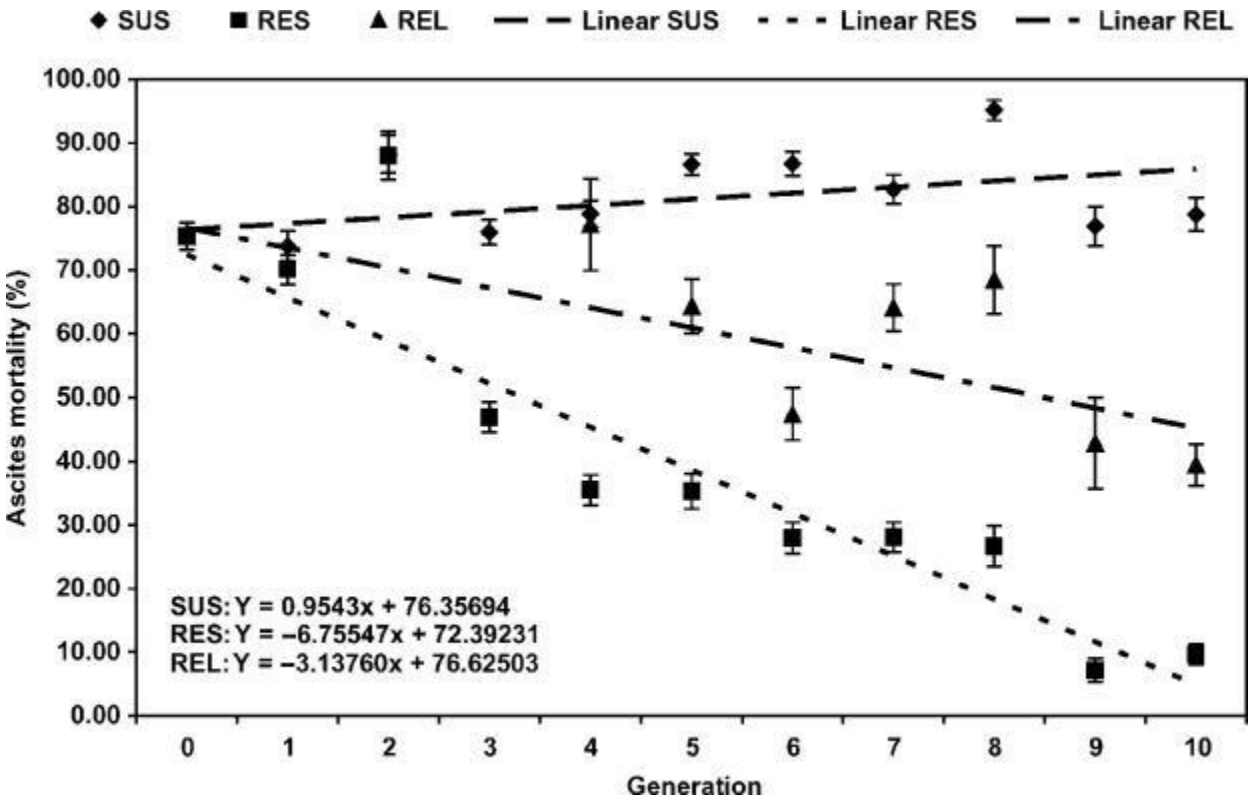

Figure 1. 12.The Three Major Lines (SUS, RES, and REL) with Multiple Generations are Differently Affected by Ascites (Pavlidis et al., 2007).

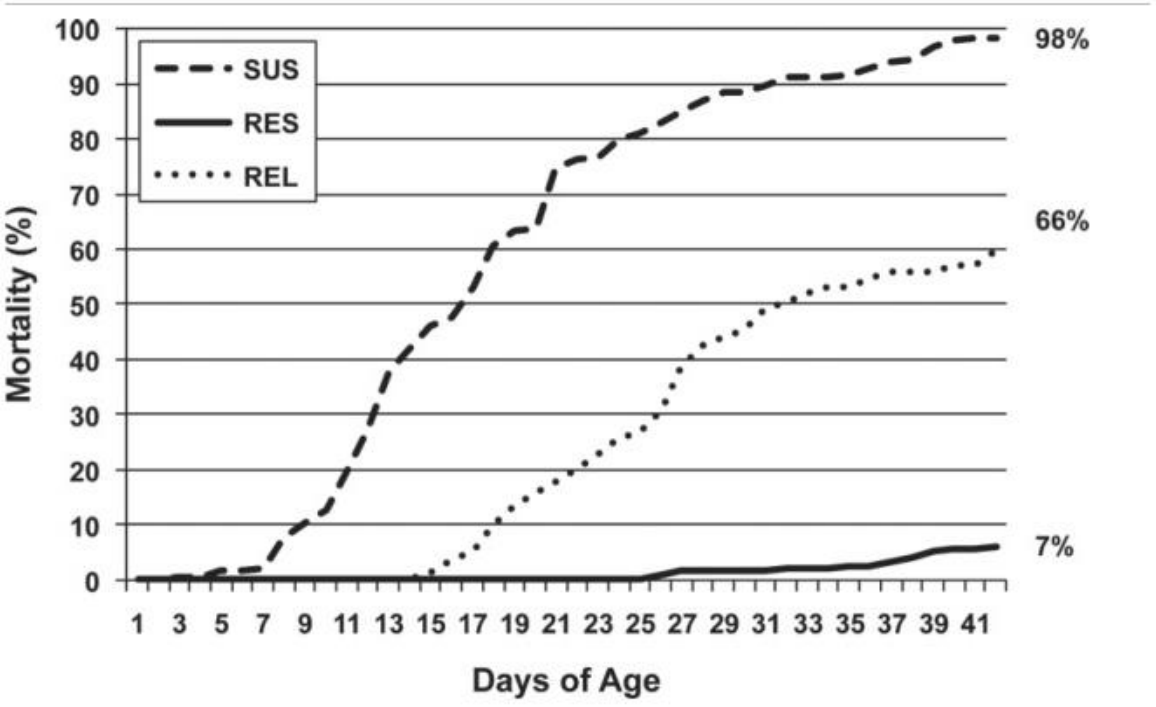

Figure 1. 13.The Cumulative Ascites Mortality Obtained from Three Broiler Lines (SUS, RES, and REL) at Generation 14 (Wideman et al., 2013).

	Mbp			Res-Sus SNP frequency		
Chr	Start	Stop	Size	Male	Female	Genes within Region
1	48.18	48.41	0.23	30%	30%	APLD,GPRC5A,HEBP1,FAM234B,GSG1, EMP1,MIR6581
1	170.483	170.53	0.05	40%	0%	CAB39L
1	175.68	175.87	0.19	-25%	30%	PDS5B
1	182.31	182.46	0.15	40%	20%	AASDHPPT,KBTBD3,MSANTD4
1	183.65	183.95	0.30	35%	35%	DCUN1D5,MMP13,MMP10,MMP3,MMP7,BIRC2
2	22.86	23.03	0.17	50%	0%	SAMD9L,HEPACAM2,VP50
2	34.47	34.61	0.14	40%	-20%	PLCL2
2	91.85	91.92	0.07	50%	0%	CNDP2,FAM69C
2	95.14	95.22	0.08	-30%	0%	CDH19
2	122.75	122.83	0.08	45%	-25%	CA2
2	126.97	127.09	0.12	40%	20%	CPQ**
3	37.27	37.36	0.09	-30%	40%	RYR2
3	48.98	49.00	0.02	-20%	30%	RMND1
3	50.41	50.44	0.03	40%	0%	SCAF8
3	100.70	101.10	0.40	0%	-30%	OSR1
4	36.72	36.90	0.18	40%	0%	GRID2
5	13.26	13.29	0.03	0%	50%	DDTNFR23, CARS
5	25.44	25.47	0.04	-50%	25%	SPTBN5
6	28.82	28.87	0.05	0%	40%	ABLIM1
10	6.49	6.54	0.05	40%	30%	TJP1
14	1.48	1.66	0.18	0%	30%	LMTK2,BHLHA15,TECPR1,BRI3,BAIAP2L1, NPTX2
20	9.06	9.10	0.04	0%	-40%	PXDNL,PCMTD2
22	4.40	4.48	0.08	60%	20%	LRRTM4
27	7.85	7.98	0.13	-30%	0%	RAMP2,WNK4,COA3,BECN1,PSME3,AOC3, G6PC,PTGES3L,RPL27,IFI35,VAT1,RND2
28	0.59	0.63	0.05	-25%	0%	TIMM44,HNRNPM
Z	18.60	18.73	0.13	-25%	-50%	PDE4D
Z	19.10	19.50	0.40	25%	50%	ZSWIM6,KIF2A
Z	33.87	33.90	0.03	25%	25%	SLC24A2

Figure 1. 14.Data from Whole Genome Resequencing in REL Shows 31 Regions Associated with Ascites (Parveen et al., 2020).

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CHAPTER 2

A 125 Kilobase Deletion Affecting the Carboxy Peptidase Q Gene in Broilers

ABSTRACT

Ascites syndrome is a pathophysiological condition resulting from unmet body demand for oxygen in fast-growing broilers. The high demand for oxygen increases the pressure within the pulmonary circulation, leading to the accumulation of fluid in the abdominal cavity and increasing the mortality rate. Previously we used whole-genome resequencing to identify 28 gene regions related to ascites. One of these regions is a 127 kbp region on chromosome 2 encompassing the 3' end of the Carboxypeptidase Q (CPQ) gene. The CPQ gene; also known as Plasma Glutamate Carboxypeptidase, encodes a metallopeptidase that plays a significant role in the cleavage of dipeptides into amino acids. Gene expression assays measured the CPQ gene expression among different tissues (heart, liver, kidney, thigh, breast, spleen, lung, and thymus) using the housekeeping gene TATA-box binding protein (TBP) as reference. These results indicated that the CPQ gene is highly expressed in the liver (*p*-value=0.008) in chickens. Based on reading depth analysis of our next-generation sequence data, some broilers carry a CPQ gene deletion spanning from intron 4 to ~40 kbp downstream of exon 8. We developed PCR primers that span the deleted region allowing us to fine map the exact limits of the deletion, which appears to have resulted from the recombination of two CR1 repeats. We used exonuclease PCR assays to genotype for relative levels of exon 2 and 8 to quantify the prevalence of the deletion in our REL ascites research line; an unselected closed population derived from a commercial broiler elite line from the 1990s. Whether this deletion exists in other populations and the relationship of the deletion to ascites phenotype will need to be determined.

INTRODUCTION

Pulmonary hypertension syndrome (PHS), also known as ascites syndrome, is a metabolic disease affecting fast-growing broilers due to increased meat yield and rapid growth (Julian, 1993; Khajali & Wideman, 2016). The PHS cause increasing blood pressure which elicits several pathophysiological progressions involving elevated oxygen requirement, central venous and right ventricle congestion, heart and liver failure, and the accumulation of ascitic fluid in the abdomen to form a water belly, and results in birds' death (Wideman, 2000). The PHS is attributed to a reduction in cardiac output (Wideman & Kirby, 1995). However, the major cause of PHS is still ambiguous till now, which makes it idiopathic. Therefore, broilers are an excellent model for idiopathic pulmonary arterial hypertension in humans (Wideman & Hamal, 2011). A higher body rate requires more oxygen, which has been pronounced in modern male broilers. Thus, males are prone to develop ascites relative to females (Baghbanzadeh & Decuypere, 2008; Closter, 2014). To reduce the incidence of ascites in the United States, several environmental and nutritional factors have been controlled involving temperature adjustment, lighting reduction, nutritional control, and ventilation (Aftab & Khan, 2005; Fairchild & Lacy, 2006; Baghbanzadeh & Decuypere, 2008). Nevertheless, the worldwide economic loss has been estimated to be $100 million annually (Tarrant et al., 2017). Genetic factors are associated with PHS due to the high heritability of ascites-related traits, including ventricular hypertrophy (Pakdel, 2004). Thus, identifying ascites-related quantitative trait loci (QTL) could assist reduce the economic loss due to the genetic selection against susceptibility (Parveen et al., 2020).

Currently, several genetic markers have been developed for marker-assisted selection (MAS) (De Greef et al., 2001; Pakdel et al., 2005; Li et al., 2015). In our lab, several single nucleotide polymorphisms (SNPs) based genome-wide association studies (GWAS) were

performed to identify a possible genetic marker associated with ascites and used for MAS (Krishnamoorthy et al., 2014; Dey et al., 2017; Tarrant et al., 2017). Several potential regions were identified on chromosomes 1, 2, 4, 9, and Z. However, these regions were not used for MAS because they showed a minor association with ascites. A whole-genome-resequencing (WGR) was performed by Alia Parveen, a previous Ph.D. student in our lab, to determine the associated regions for ascites resistance or susceptibility on both chromosomes 2 and 9 (Parveen et al., 2020). A new region (127.65-127.75Mbp) was identified on chromosome 2, which spans a large part of the gene known as the Carboxypeptidase Q (CPQ) or plasma glutamate carboxypeptidase (PGCP) gene. This gene was found to be responsible for high blood pressure or hypertension in humans and associated with ascites resistance in broilers. Shatovisha Dey determined that the CPQ gene is highly expressed in three major ascitic organs (heart, lung, liver) in the different genotypes (Dey et al., 2018). In this study, we expanded the gene expression study by looking at the CPQ gene expression in five additional tissues. Additionally, we used Next-generation-sequencing (NGS) data and PCR to investigate a possible large deletion affecting the CPQ gene, which had been initially suggested by Dr. Giri Athrey of Texas A&M (personal communication). The deletion could affect ascites resistance or susceptibility.

MATERIALS AND METHODS

Animals Resources

All the animal processes (breeding, hatching, and growing out) were done at the University of Arkansas Poultry Research farm. An approval from the University of Arkansas Institutional Care and Use Committee was obtained to do this work (the protocol numbers were 15039, 15040,

21087, and 21088). All the birds and DNAs that have been used in this study are from the relaxed unselected (REL) line at the 18th generation (Pavlidis et al., 2007; Dey et al., 2018).

Genomic Data

All the genome positions correspond to those in the March 2018 assembly of the *Gallus_gallus*-6 genome (The accession ID: GCF_000002315.5). The required genomic sequences for particular genes or chromosomal regions were obtained from either the NCBI (National Center for Biotechnology Information (nih.gov)) or the UCSC genome browser (UCSC Genome Browser Home).

DNA Extraction from Blood

A total volume (10ul) of the blood samples was collected from the birds at four days of age through towing venipuncture. DNA extraction was done using a rapid protocol as described previously and stored at -20℃ (Bailes et al., 2007).

DNA Extraction from Tissues

The frozen tissues, which were stored in RNA later (SIGMA; St.Louis, MO) at -20℃, were cut into small pieces (50-100mg) and rinsed with 1X cold phosphate buffer saline (PBS). Then, the tissues were homogenized in 1ml TE buffer (10 mM Tris-HCl, 1 mM Na_2 EDTA pH 7.5) using a Bullet Blender homogenizer (Next Advance, Inc., Averill Park, NY). Autoclaved beads were used to homogenize the tissues, and bead size was adjusted depending on the particular tissue. Sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5%, and the samples were incubated for 10 minutes at 50℃. RNAse was added to a final concentration of 200ug/ml, and the samples were incubated for 30 minutes at 37℃. A solution of 250mM of EDTA was added to the samples to bring to 10mM and then incubated for 10 minutes at 50℃. The solution was made 100ug/ml for Pronase E and incubated overnight at 37℃. The DNA was extracted three times with

50:50 phenol: chloroform and two times with chloroform. Sodium acetate (3 M, pH 5.2) was added to 0.3 M, followed by DNA precipitation using 95% alcohol. The samples were centrifuged at 4℃ for 20 minutes at 12000 x g to produce a DNA pellet. The DNA pellet was rinsed with 70% alcohol, dried, and dissolved in Te (10 mM Tris-HCl 0.1 mM EDTA pH 7.5). The DNA was then evaluated for quantity using fluorimetry using Hoechst 33258 (GLOMAX Multi Jr, Promega 91 Corp., Madison, WI) and optical purity, A260/280 (NanoVue, GE Healthcare Biosciences, MA, USA).

Designing Primers, Probes, and PCR Sequencing

The sequence of the DNA for the Jungle Fowl CPQ gene on *Gallus_gallus*-6 genome Chr 2 at 126,884,501-127,048,754 was downloaded from the UCSC genome browser. Manipulation and annotation of the CPQ gene sequences were done using the SeqBuilder software (DNASTAR Lasergene Suite 16). Design of PCR primers and probes utilized Primer3 (version 0.4.0; http://bioinfo.ut.ee/primer3-0.4.0/primer3/) with adjusting general primers parameters to primer size (18-24bp), G/C content (40-60%), and annealing temperature (55-65℃). Primer and probe uniqueness was checked by BLAT searches in the UCSC genome browser. Primers and probes were synthesized by Integrated DNA Technologies (IDT; Coralville, IA, USA). Primers and probes are listed in Table 2.1. PCR was performed in a T-100 thermal cycler (BioRad Laboratories, Hercules, CA). The total PCR volume was 20ul, which contained 1X Taq Buffer (1mM MgCl2, 30ug/ml BSA, 50mMTris-Cl pH8.3), 0.5uM of both forward and reverse primers, 0.2mM dNTPs, 4U of Taq polymerase, and 2ul with an approximate concentration of 100ng/ul chicken DNAs. The PCR protocol was; an initial denaturation of 90℃ for 30 seconds, 40 cycles of 90℃ for 15 seconds, the primer specific- annealing for 30 seconds (Table 2.1), 72℃ for 1 minute, and a final extension of 72 ℃ for 3 minutes, followed by infinity at 4℃. The PCR protocol was optimized based on each primer pair to amplify a specific region on the mRNA or CPQ gene. The positive

control consisted of the master mix plus 2ul of white leghorn (WL) DNA, while the negative control did not contain DNA. To evaluate the quality, 5 ul of the PCR product was run in 1.5% of agarose gel. The remaining PCR products for selected samples were purified for sequencing using the RapidTip pipette tips following the manufacturer's protocol (Diffinity Genomics, West Chester, Pennsylvania), quantified using Hoechst 33258, mixed with 5uM of either forward or reverse primer, then submitted for capillary sequencing (Eurofins Genomics, Louisville, KY). AB1 files were viewed, aligned to the CPQ gene, and further analyzed using SeqManPro 17 (DNASTAR, Madison, WI).

Exonuclease Allelic Discrimination Assay (SNP genotyping)

TaqMan 5'-exonuclease assays were used for genotyping the birds based on specific SNPs in the CPQ gene. Quantitative PCR (qPCR) was in a CFX96 real-time thermocycler (Bio-Rad Laboratories, Inc., Hercules, California). The total volume for each reaction was 20 ul containing the previously mentioned PCR reagents plus 0.25 uM of the two probes (Table 2.1). The PCR cycling was 90 ℃ for 30 seconds, 10 cycles of 90℃ for 15 seconds, and a specific annealing temperature as shown in Table 2.1 for 60 seconds, followed by 30 cycles of the same temperatures and times including plate reads after each cycle. Each sample was run in triplicates, and the amplification profile was examined to score the birds for specific genotypes. Selective qPCRs were confirmed for genotype by capillary sequencing (see above).

Measuring the Expression of the CPQ gene

Tissue Collection and Preparation

At six weeks of age, seven males and five female birds with particular genotypes were euthanized either by cervical dislocation or CO_2 inhalation. The tissue samples (breast, heart, thigh, spleen, liver, thymus, lung, and kidney) were collected immediately at necropsy. Samples were

placed in RNA Later (Thermo Fisher Scientific, Waltham, MA) in 2 ml microfuge tubes. The ratio of the RNA Later to tissue was at least 10:0.5 (v:v). The tube was mixed well, equilibrated at 4℃ overnight, and then stored at -20℃ until RNA extraction.

RNA Extraction, cDNA Synthesis, and Gene Expression Assay

Samples were homogenized using a Bullet Blender, as above. The homogenate was extracted using the Trizol Reagent (Thermo Fisher Scientific, Waltham, MA) based on Trizol RNA Isolation Protocol. The extracted RNA was assessed for purity (A 260/280) and quantity using NanoVue spectrophotometry (GE Healthcare Biosciences, MA, USA). The minimum A 260/280 ratio was 1.6. RNA integrity was assessed by running 5 ul of the isolated RNA by electrophoresis in 1.5% agarose gels in 0.5X TBE (50 mM Tris, 25 mM Borate, 1 mM Na_2EDTA, pH 8.3). Ethidium bromide (0.5ug/ml) was used for staining. The gels were scanned for fluorescence (ex 523 nm: em 600 nm) using a Typhon 9600 scanner (GE Health Care, Piscataway, NY). All the RNA samples showed three strong bands of different sizes (5S, 18S, and 28 S rRNA). RNA samples were tested for genomic DNA contamination using primers specific to genomic DNA to confirm no amplification of a specific PCR product. Five micrograms of total RNA were used for first-strand cDNA synthesis using SuperScript III Reverse Transcriptase (Thermo Scientific, USA) based on the manufacturer's instructions. Single-step RT-qPCR was in a CFX96 real-time thermocycler (Bio-Rad Laboratories, Inc., Hercules, California) to measure CPQ gene expression level. The total reaction volume was 20ul, and each reaction had the previous PCR reagents with the target-specific primers (Table 2.1) plus 1x EvaGreen dye (Biotium Inc., Fremont, CA). Each assay was in triplicates with negative (no DNA) control. The PCR protocol was the initial denaturation at 90℃ for 30 seconds, 10 cycles of 90℃ for 15 seconds, 55℃ for 30 seconds, 72℃ for 60 seconds, followed by 35 cycles of the same temperature and time with each cycle followed

by a plate read. To confirm the product specificity, a high-resolution melt (HRM) curve was performed post PCR. TATA-box binding protein (TBP) gene was used as a reference to normalize gene expression data (Radonić et al., 2004). Ct data were analyzed in Microsoft Excel to compute ΔCt and $\Delta\Delta Ct_{(Avg.)}$.

Statistical Analysis

All statistical calculations were done in Excel. The student T-test was used to determine whether there is a significant difference between different genders with different genotypes within the same line. A significant difference was assumed with a p-value < 0.05.

RESULTS

In a previous study, CPQ gene expression was evaluated for an association between different genotypes with gene expression levels (Dey et al., 2018). The gene expression assay was performed on healthy six-week-old REL line birds with different genotypes (homozygous reference, heterozygous, and homozygous non-reference for intron 6 SNPs). Expression focused on the lung, heart, and liver, as these may be the organs most impacted by ascites. The expression results revealed that homozygous birds homozygous for the non-reference SNPs showed the highest gene expression in all three tissues (lung, heart, and liver) (Dey et al., 2018). Therefore, we aimed to determine the potential effect of genotype on CPQ gene expression in additional tissues. Eight tissues (breast, heart, thigh, spleen, liver, thymus, lung, and kidney) were chosen based on the expression of CPQ in *Homo sapiens* (NCBI RNAseq summaries). The selected tissues were collected from unchallenged, six-week-old REL line birds from both genders with specific genotypes. The relative gene expression level was determined using RT-qPCR, using TBP as reference (Table 2.2). We found that CPQ gene expression level was significantly increased in the

liver of homozygous non-ref males (*p*-value = 0.0080). However, there are no significant differences in the CPQ expression level in other tissues from homozygous non-ref males and all tissues from homozygous non-ref females. We could not measure the gene expression for some heterozygous male birds even though we designed multiple primers to amplify various exonic regions of the gene including exons 5, 6, 7, and 8. High-Resolution Melt (HRM) analyses post PCR implied no specific amplification in these particular samples (Figure 2.1). The PCR products were evaluated by agarose electrophoresis to further confirm the lack of specific PCR products (Figure 2.2).

Further analysis was performed on previous next-generation sequencing (NGS) data obtained from REL line birds phenotyped for ascites in the hypobaric chamber. The NGS data was for 8 libraries prepared from eight pools of REL line DNAs with each pool containing DNAs from 10 birds of each gender and ascites phenotype (resistant or susceptible) with two replicates (eight pools representing 2 phenotypes x 2 genders x 2 replicates). The Illumina HiSeq 2500 was used to sequence each pool and generate data with approximately 67Gb from each pool. Then, DNAStar NGen was used to align the sequence reads to the *Gallus_gallus*-6 genome. The alignments were quantified for SNPs and CNVs using ArrayStar. The NGS analyses included read depth for NGS data from each pool. Our NGS results showed that there are significant differences in SNP frequencies in the CPQ gene region. There was an obvious decline in the NGS read depth starting from around 126,970k to 127,095k, suggesting a chromosomal deletion of approximately 125kbp. Thus, some birds appear to carry a deletion of this region beginning in intron 4 (Figure 2.3) to ~40kbp downstream of exon8 (Figures 2.4). Further analyses were performed on resistant and susceptible REL males and females to determine if this chromosomal deletion affected ascites resistance and susceptibility. In Figure 2.5, the read depth for the phenotype pools is plotted with

orange dots for resistant (R), and gray dots for susceptible (S). There are more reads in the deletion region of resistant females than susceptible females, whereas susceptible males have more reads in the region of the deletion than the resistant males, suggesting that the chromosomal deletion is more frequent in resistant females and susceptible males. Therefore, this chromosomal deletion may be associated with both resistance and susceptibility.

Two probes (exon 2 vs. exon 8) were designed for the exonuclease qPCR test to genotype different target DNA samples for the deletion. The TaqMan assay was applied first to archived DNA samples from the REL line phenotyped for ascites (resistant and susceptible) in the hypobaric chamber and genotyped for CPQ-intron 6 SNPs as either homozygous ref [T/A], heterozygous [Y/M], homozygous non-ref [C/C] (Dey et al., 2018). Exon 2 and exon 8 primers and probes (Table 2.1) were used as a way to screen the birds for deletion. We genotyped over 175 DNA samples from REL line birds by visually inspecting the amplification profile and scored them for "without the deletion" or "with the deletion". Specifically, if the amplification profile shows two signals, "HEX and FAM", the sample lacks the deletion. While, if the amplification profile shows one signal "FAM", it means that the sample contains the deletion. Then, a statistical analysis was performed to obtain *p*-values for the Chi-test of observed vs expected. Our results revealed that there is a significant association (*p*-value = 0.048) between this chromosomal deletion and intron 6 heterozygous birds with the deletion. However, this association does not apply to the homozygous ref birds with the deletion (*p*-value = 0.41) and the homozygous non-ref birds with the deletion (*p*-value = 0.081) (Table 2.3). To clarify, the 125kbp chromosomal deletion might increase the ascites resistance and susceptibility in heterozygous birds only, whereas neither homozygous ref nor non-ref birds are affected by the deletion.

An additional 27 DNA samples were genotyped for the deletion. These were REL line male birds for which we had produced RNAseq data for the right heart ventricle. The samples were from healthy birds raised at low altitudes and birds with and without ascites raised at simulated high altitudes in the hypobaric chamber (Licknack, unpublished). The main goal of the RNAseq experiment was to quantify the transcriptomic alterations in the right ventricle upon hypobaric challenge and then with the development of ascites. The birds were phenotyped for ascites resistance (No Ascites "NA") and susceptibility (Ascites "A") and genotyped for the specific CPQ SNPs in intron 6 and exon8 (Table 2.4). For the intron 6 CPQ SNPs, 37% of the homozygous non-ref birds (C/C) were resistant, 30% of the homozygous non-ref were susceptible, and 15% of the homozygous ref (T/A) were resistant, and 4% of the homozygous ref were susceptible. The heterozygous birds (Y/M) showed an equal percentage of resistant and susceptible (~7.4%). For the exon 8 CPQ SNP, 30% of the homozygous non-ref birds (C/C) were resistant, 30% of the homozygous non-ref were susceptible, and 15% of the homozygous ref (G/G) were resistant, and 7.4% of the homozygous ref were susceptible. Almost 15% of the heterozygous birds (C/G) were resistant, while only 4% were susceptible. For the chromosomal deletion analysis, we ran all the 27 DNA samples by qPCR with different primers flanking the deletion (CNVFxR, CNVF1xR1, CNVF1xR2; Table 2.1). The PCR product was sent for sequencing for further confirmation and to specifically map the recombination breakpoint. The results suggested that 100% of the birds with genotypes for intron 6 and 8 of CC-S or YM-S contained chromosomal deletion. Meanwhile, 50% of the birds with the YM-C genotype contained the deletion. The most plausible explanation for such findings is that the birds must be either triploid or higher for this region for birds to be heterozygous for SNPs in intron 6 and 8 but yet contain a deletion spanning both intron 6 and exon 8. To clarify, the CC-S birds are heterozygous for exon 8, and yet there is at least one copy that is

deleted. Moreover, the YM-C birds are heterozygous for intron 6 and contain a copy that is deleted (Table 2.4).

Sanger sequence analysis of PCR products from birds carrying the deletion localized the recombination site to a 3-base region (Figure 2.7). To illustrate, both left and right boundaries are connected at the recombination point TCC. Within the approximately 125 kbp deletion, there are 44 long interspersed nuclear elements (LINEs) (Figure 2.6), The SeqManUltra alignment was used to align the two LINEs that appear to have recombined as the left and right boundaries. The left and right deletion LINEs were downloaded from the UCSC genome browser and aligned with sequences from three deletion PCR products (Figure 2.7). Our SeqManUltra analysis proved that two LINEs connect the left to the right flank at the recombination point TCC. These two LINEs belong to the CR1 family (Figure 2.6). These data indicated that the REL line birds have a large heterozygous deletion (~125kbp), including exons 5-8 of the CPQ gene.

DISCUSSION

The Carboxypeptidase Q (CPQ) gene or human plasma glutamate carboxypeptidase (PGCP) gene, which encodes a metallopeptidase, is a member of the peptidase M28 family. The molecular weight of its functional protein is 56-kDa and plays a significant role in the cleavage of hydrophobic amino acids in the circulating dipeptides (Gingras et al., 1999). The CPQ has been found to facilitate the hydrolysis of thyroglobulin (Tg) precursor into thyroxine (Ahmetov et al., 2016). In humans, this gene showed an association with blood pressure, hypertension, electrocardiography, and heart rate. Its functional protein is conserved in all vertebrates. The CPQ

protein in chickens shows an identity of 70% to other vertebrates such as rats, mice, monkeys, and humans (Dey et al., 2018).

In a previous study, the genotyping data of different broiler lines revealed that homozygous non-ref birds (for both intron 6 SNPs and exon 8 SNP) are more resistant to ascites. This relationship is more pronounced in males rather than females. The heterozygous genotype does not show a high ascites resistance, suggesting that the non-ref allele is recessive. The exon 8 SNP changes the encoded proteins with lysine at 444 residue substituted by asparagine (K444N). This substitution occurs in the exposed region of the enzyme, not in the catalytic site. Thus, K444N would impact protein-protein interactions and possibly affect the activity of CPQ on circulating dipeptide hormones responsible for the homeostasis of blood pressure. Previous gene expression data showed that homozygous non-ref birds had a higher gene expression in three major tissues (heart, lung, liver) compared to the homozygous ref. This indicates that the non-ref SNPs in the 3' region of the CPQ gene may influence regulatory DNA sequences which then alter gene expression. However, in our study, the gene expression results disagree that heart and lung do not show a high expression level of CPQ mRNA in homozygous non-ref in both genders. In addition, other tissues including, the breast, thigh, spleen, thymus, and kidney, show normal gene expression. We only observed a significant gene expression in the liver of homozygous non-ref males relative to homozygous ref males (Table 2.2). This suggested that the CPQ gene is regulated in a gender-specific manner where it is highly expressed in the major ascitic organ, and the mature protein or its mRNA level increases the ascites resistance.

Analysis of previous NGS data demonstrates a chromosomal deletion of 125kbp in the CPQ gene region to 40kbp downstream of exon 8. The role of this chromosomal deletion on ascites is still under investigation since both genders from the same line showed different associations.

Ascites or pulmonary hypotension is a metabolic disease in broilers with higher body weight. Generally, male broilers have a higher body weight than females, so they are more likely to develop ascites due to their high oxygen demand (Closter et al., 2012). The manifestation and effect of ascites are more prominent in males. Therefore, this chromosomal deletion could be associated with ascites susceptibility.

Our sequence analyses revealed that 100% of the birds with intron 6+exon 8 SNP genotypes of CC-S or YM-S showed chromosomal deletion. The genotype CC-S consists of homozygous non-ref for intron 6 SNPs and heterozygous for missense exon 8 SNP. Dey et al. (2018) demonstrated that this genotype shows a strong association with ascites resistance and high CPQ gene expression. Nevertheless, our gene expression data argue that this genotype showed no expression in eight tissues This might occur due to the chromosomal deletion starting from intron 4 to 40kbp downstream of exon 8, suggesting that this deletion could increase the ascites susceptibility.

Even though the CPQ gene shows a strong relationship with high blood pressure and hypertension in humans, the main role of its mature protein is unknown. Dey *et al.* (2018) showed a positive correlation between the CPQ gene and avian pulmonary hypertension. Another study showed that broilers with pulmonary hypertension developed plexiform lesions similar to those in humans (Wideman et al., 2013). These new data agree with Dey *et al.* (2018) that the CPQ gene is highly expressed in the liver for some alleles. This may contribute to having a better understanding of the role of this gene in human hypertension as well as developing new treatments for some forms of human hypertension. Moreover, the thyroxin hormone is released through the hydrolysis of thyroglobulin by CPQ protein (Luger et al., 2001). This hormone is tri-iodinated in the kidney and liver, and it is secreted in broilers under normal environmental and physiological

conditions such as low temperature, a higher metabolic rate, and increased oxygen demand. The thyroxin hormone is significantly upregulated in the plasma of unaffected birds, while the affected birds are not able to generate a sufficient amount of this hormone. Thus, a higher expression level of the CPQ gene in homozygous non-ref males would provide the birds with ascites resistance because they could increase the production of thyroxin during a high metabolic rate and increase oxygen demand (Luger et al., 2001). However, the lack of gene expression in heterozygous recombinant (CC-S) birds with the deletion are prone to ascites development. Therefore, the plasma thyroxin level is not sufficient, which inhibits the birds from adapting to hypoxia.

In summary, our gene expression results showed that the CPQ gene is normally expressed in all tissues except the major ascitic organ (liver). The overexpression in the liver is considered gender-specific since it has been observed in broiler males but not in females. Further NGS analysis proved that some heterozygous birds showed a deletion affecting the 3' end of the CPQ gene. However, our results did not strongly support the association between the 125K chromosomal deletion and ascites phenotypes. Therefore, this chromosomal deletion is still a candidate region for future study of relevance to ascites or pulmonary hypertension in broilers. Our new findings give significant hints to recognizing the mechanisms that underlie this deletion and affect the ascites phenotypes.

TABLES AND FIGURES

Table 2. 1.primers, probes, and conditions for qPCR, PCR, and RT-qPCR. For each CPQ SNP locus: position is the base pair position on chromosome 2 according to the 2015 genome assembly; Primers are 5'-3' for forward (F-) and Reverse (R-); Probes are 5'-3' associated with SNP127.70, and the first allele (P1TA/CC) is labeled with FAM while the second allele (P2TA/CC) is labeled with HEX. The melting temperature (˚C) is the temperature used for both qPCR and RT-qPCR. The highlighted red SNPs are the SNPs being used for genotyping.

Assay	**Locus**	**Position (bp)**	**Primers/Probes (5'>3')**	**˚C**
CPQ intron 6 SNPs	CPQ 127.705F	Chr2:127670338	Cctaattgcactgcctttgc	**60**
	CPQ 127.705R	Chr2: 127670568	Actgattctgtggccttcct	
	CPQp1TA/CC	Chr2: 127670526	HEX-ctacaataaaagaagagttgattc	
	CPQp2TA/CC	Chr2: 127670526	FAM-ccacaatacaaagaagagttga	
CPQ exon 8 SNPs	CPQex8F	Chr2:127708143	CGTGATGACCTCAGTAAATACTTCTGG	**56**
	CPQex8R	Chr2:127708591	CCTGCTGCAAAGGATAAGTTTGCATAC	
	CPQex8P1	Chr2:127708196	HEX-TGCTTTGGATCCTGAACTGTC	
	CPQex8P2		FAM-TGGTTTGGATCCTGAACTGTC	
CPQ RT-qPCR	CPQ_cDNAex7F	Chr2:127677758	CAATTTCTGGATGAGGGACGGAG	**55**
	CPQ_cDNAex8R	Chr2:127708283	CAGCATCTCCTCCATGTCAGC	
	CPQex5F	Chr2:127649442	CGGAGGAGCATTTATATCATGGG	
	CPQex6R	Chr2:127664424	TGTTCTTCTCCTGTCCATAGCA	
	CPQex6F	Chr2: 127664446	AGGAGGAATAGGTGCTGAGC	
	CPQex7R	Chr2:127677761	CTCCGTCCCTCATCCAGAAA	

Table 2.1. (continue).

Assay	Locus	Position (bp)	Primers/Probes (5'>3')	˚C
TBP RT-qPCR	TBPF	Chr3:40848487	GAACCACGTACTACTGCGCT	**55**
	TBPR	Chr3:40846952	CTGCTGAACTGCTGGTGTGT	
CPQ deletion SNPs	CPQx2F	Chr2:127564601	Gccacgatatgctggcaggt	**61**
	CPQx2R	Chr2:127564355	Gcacattctccagcccgtct	
	CPQx2P	Chr2:127564398	HEX-ccaaaagcatttgaaggcatcag	
	CPQx8F	Chr2:127708135	Ccagcttgcgtgatgacctc	
	CPQx8R	Chr2: 127708376	Cgtgcaaaatcagggtgaaaaaagg	
	CPQx8P	Chr2:127708226	FAM-ctgtgctgctgtttggactg	
CPQ left and right boundaries SNPs (CNV)	CPQCNVF	Chr2:127627767	ACCGCACATCTTGGCATCAT	**69**
	CPQCNVR	Chr2: 127756139	GTGGCAGGGGAACTAAGGTT	
	CPQCNVF1	Chr2:127627825	AGTGCAGGTCCAGAGATTCCA	
	CPQCNVR1	Chr2:127756140	TTGTGGCAGGGGAACTAAGGT	
	CPQCNVR2	Chr2:127756161	Gcaacgaggagcctacatcat	

Table 2. 2.The CPQ mRNA expression values for homozygous non ref REL line compared to the homozygous ref using TBP as a reference. Higher expression of the gene is considered at P<0.05.

Tissue Type	Male		Female	
	***p*-value**	**FC**	***p*-value**	**FC**
Breast	0.322	0.096	0.39	0.42
Heart	0.44	0.002	0.34	0.46
Thigh	0.069	0.079	0.27	0.007
Spleen	0.15	0.039	0.48	0.011
Liver	0.008	0.0022	0.40	0.22
Thymus	0.136	0.017	0.20	0.015
Lung	0.36	0.060	0.26	0.27
Kidney	0.23	0.016	0.41	0.51

Table 2. 3.Genotype data for CPQ-intron 6, exon2, and exon 2 and 8 for REL line. The genotype frequencies were determined for the ascites susceptible (S) and resistant (R). The REL line samples were genotyped for intron 6 and the deletion based on their gender. The p-values for a simple Bonferroni (Adj Pval) of chi square test of observed vs. expected are present.

	With the deletion												
Line	Geno-type		All				Male				Female		
		Count	All Freq	R Freq	S Freq	Adj Pval	Count	R Freq	S Freq	Adj Pval	Count	R Freq	S Freq	Adj Pval
REL	TA	3	0.036	0.065	0	0.41	2	0.071	0	1.005	1	0.055	0	1.24
	YM	35	0.42	0.28	0.64	0.048	18	0.32	0.69	0.28	12	0.22	0.66	0.178
	CC	45	0.54	0.65	0.35	0.081	21	0.60	0.30	0.63	17	0.72	0.33	0.49
	Without the deletion												
Line	Geno-type		All				Male				Female		
		Count	All Freq	R Freq	S Freq	Adj Pval	Count	R Freq	S Freq	Adj Pval	Count	R Freq	S Freq	Adj Pval
REL	TA	23	0.25	0.19	0.30	0.96	12	0.24	0.315	1.900	7	0.15	0.25	1.47
	YM	6	0.065	0.043	0.11	0.66	2	0	0.10	0.31	4	0.1	0.125	2.44
	CC	63	0.68	0.76	0.58	1.004	30	0.76	0.57	1.41	26	0.75	0.625	1.96

Table 2. 4.Genotype data for CPQ-intron 6, CPQ-exon 8, and chromosomal deletion for 27 REL line samples. This table includes all the significant information for those samples involving the samples number, the wing band number, and the genotyping based on PCRseq. Only six birds showed a heterozygous deletion.

Sample #	Wing band #	Genotyping of intron 6 based on PCRseq	Genotyping of X8 based on PCRseq	Phenotype (NoAscites "NA" or Ascites "A")	Sample#	Wing band #	Genotyping of intron 6 based on PCRseq	Genotyping of X8 based on PCRseq	Phenotype (NoAscites "NA" or Ascites "A")
1	12014	TA	C	NA	15	12056	YM	G	A
2	11818	CC	G	NA	16	11890	YM	C	NA
3	11954	TA	C	NA	17	12043	CC	S (C/G)	NA
4	11906	CC	C	A	18	12105	CC	C	A
5	11979	CC	G	NA	19	12168	CC	C	A
6	12011	TA	C	NA	20	12044	YM	S (C/G)	NA
7	12198	YM	C	A	21	12164	CC	C	A
8	11926	TA	C	A	22	12017	CC	C	NA
9	12190	CC	G	A	23	12167	CC	C	NA
10	12022	TA	C	NA	24	12009	CC	S (C/G)	NA
11	11973	CC	G	NA	25	12117	CC	C	A
12	11901	CC	G	NA	26	12050	CC	C	A
13	12185	CC	C	NA	27	12159	CC	S (C/G)	A
14	12086	CC	S (C/G)	NA					

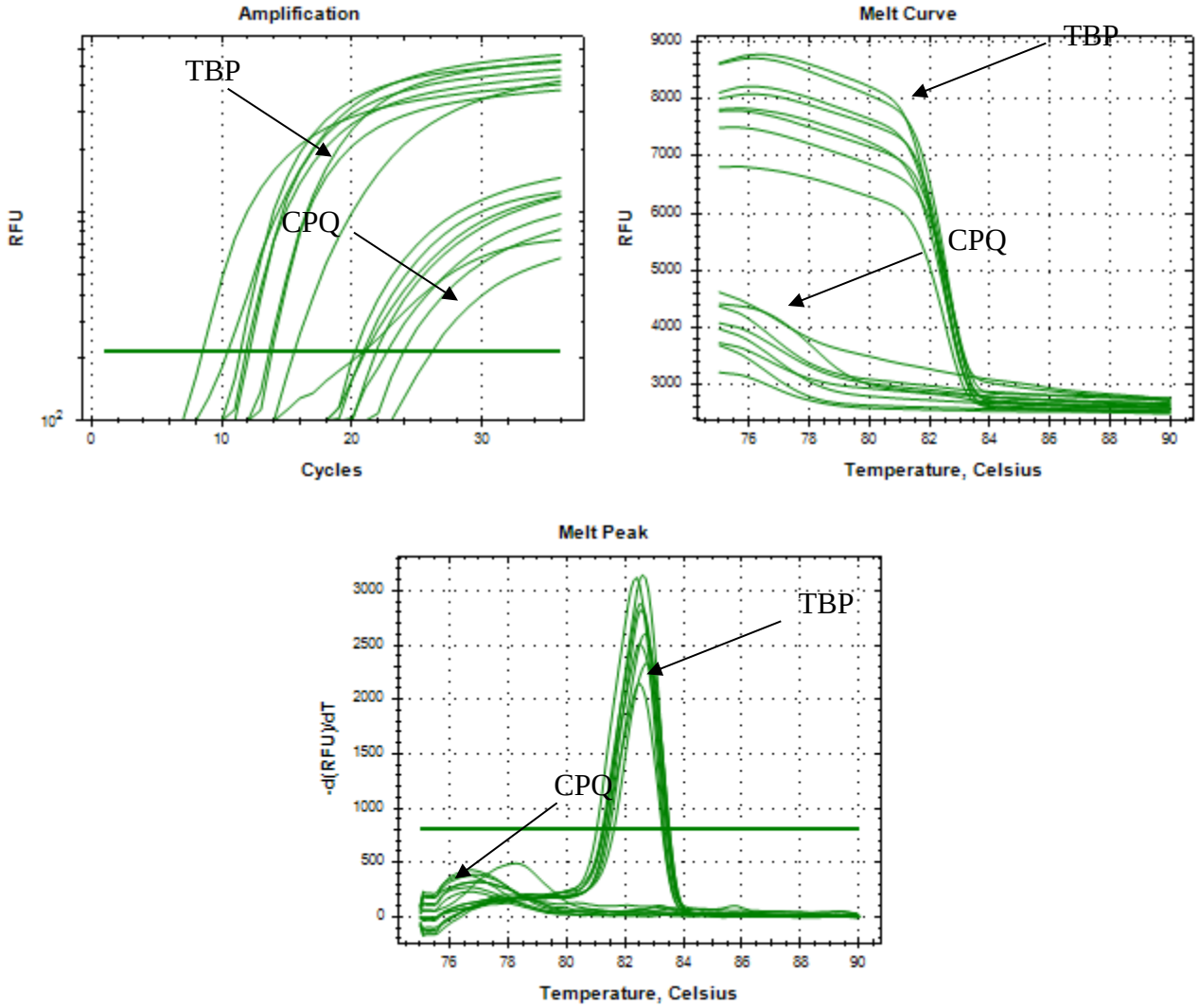


Figure 2. SEQ Figure_2. * ARABIC 1.CPQ mRNA expression levels assayed by RT-qPCR for CPQ gene in eight tissues breast, heart, thigh, spleen, liver, thymus, lung, and kidney) in heterozygous using PCR primers flanking exon 7 and 8. The amplification profile shows that heterozygous samples amplify with both TBP and CPQ primers. However, the High-Resolution Melt (HRM) analyses post PCR implied no specific amplification in these particular samples.

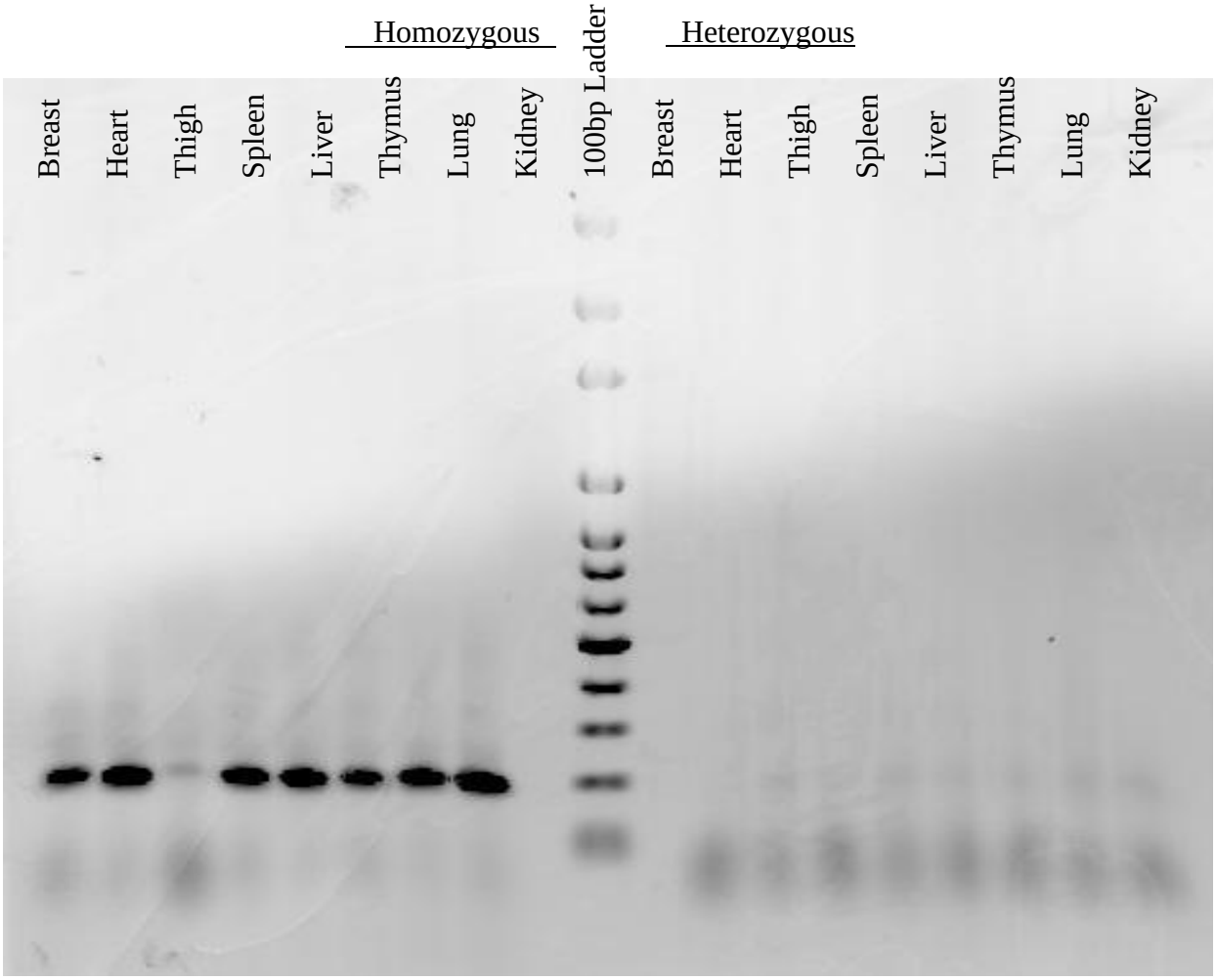


Figure 2. 2.Agarose gel (1.5%) of CPQ mRNA expression levels assayed by RT-qPCR for CPQ gene in eight tissues (breast, heart, thigh, spleen, liver, thymus, lung, and kidney) in homozygous and heterozygous using PCR primers flanking exon 7 and 8.

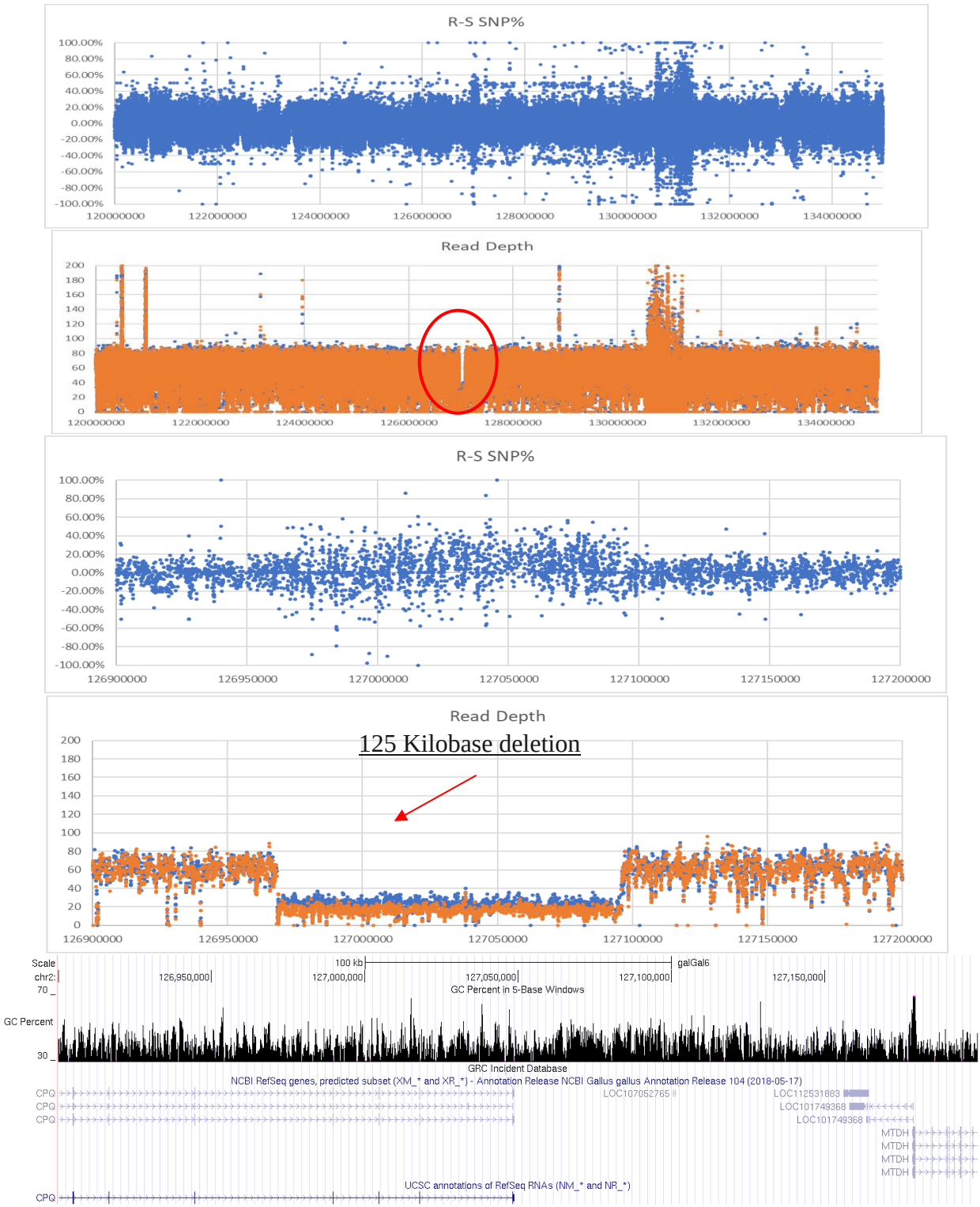


Figure 2. 3.NGS: A 125-kb deletion is seen by a significant decline in NGS read depth. Deletions from 126,970k to 127,095k = 125kbp.

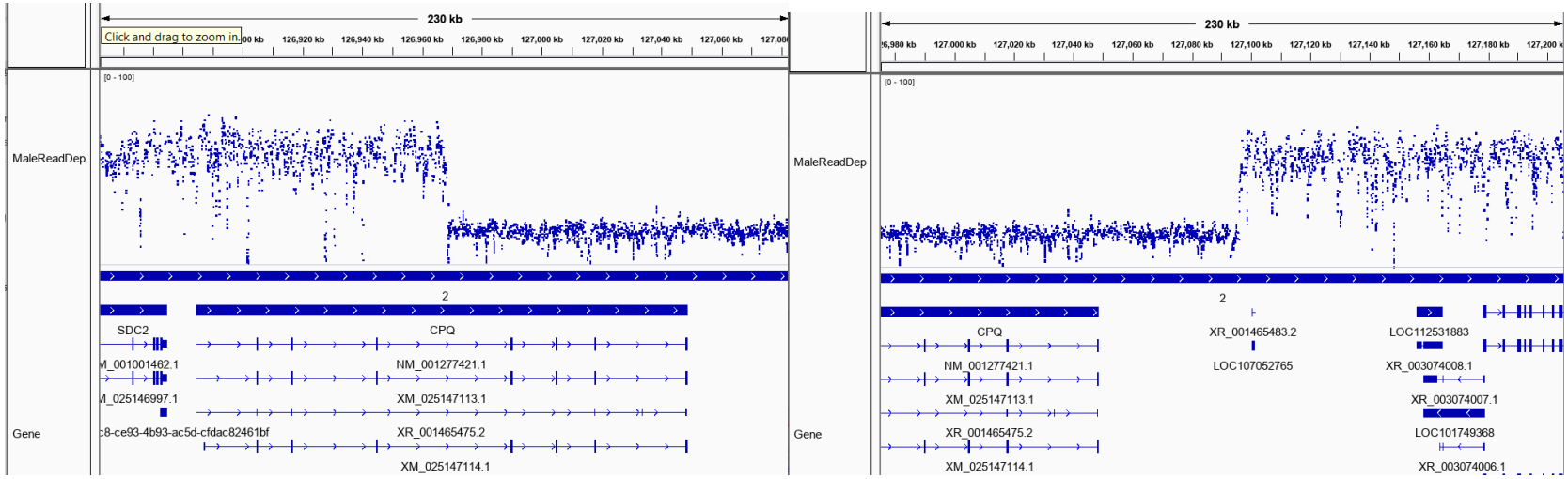
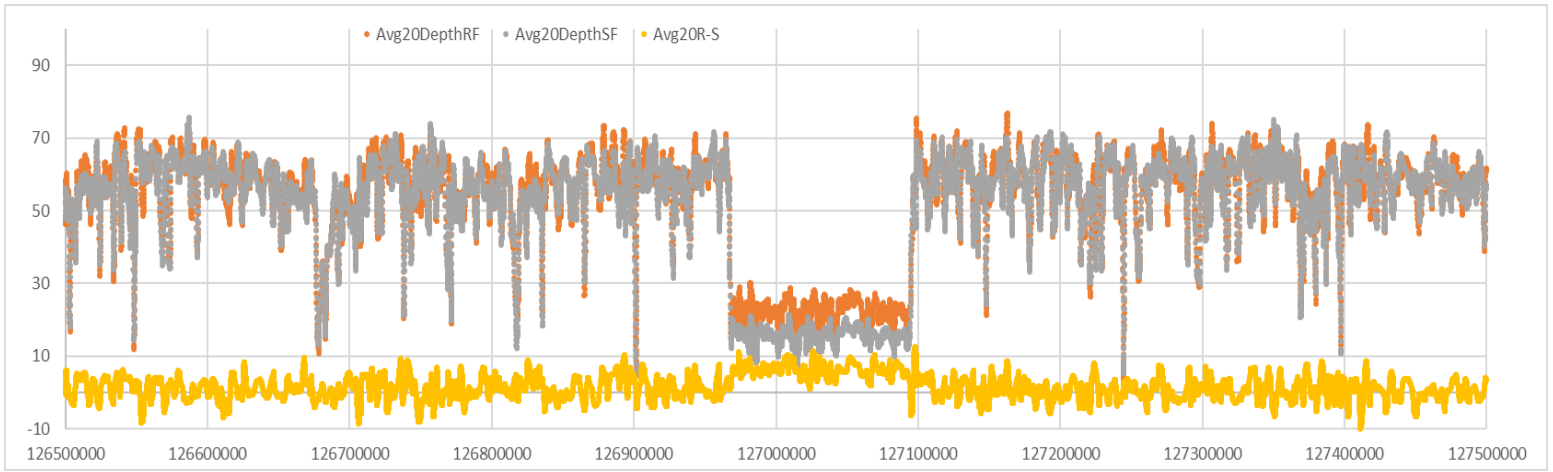


Figure 2. 4.NGS: A 125-kb deletion is evidenced by a significant decline in NGS read coverage. The deletion appears to span from 126,970k to 127,095k = 125kbp.

A.



B.

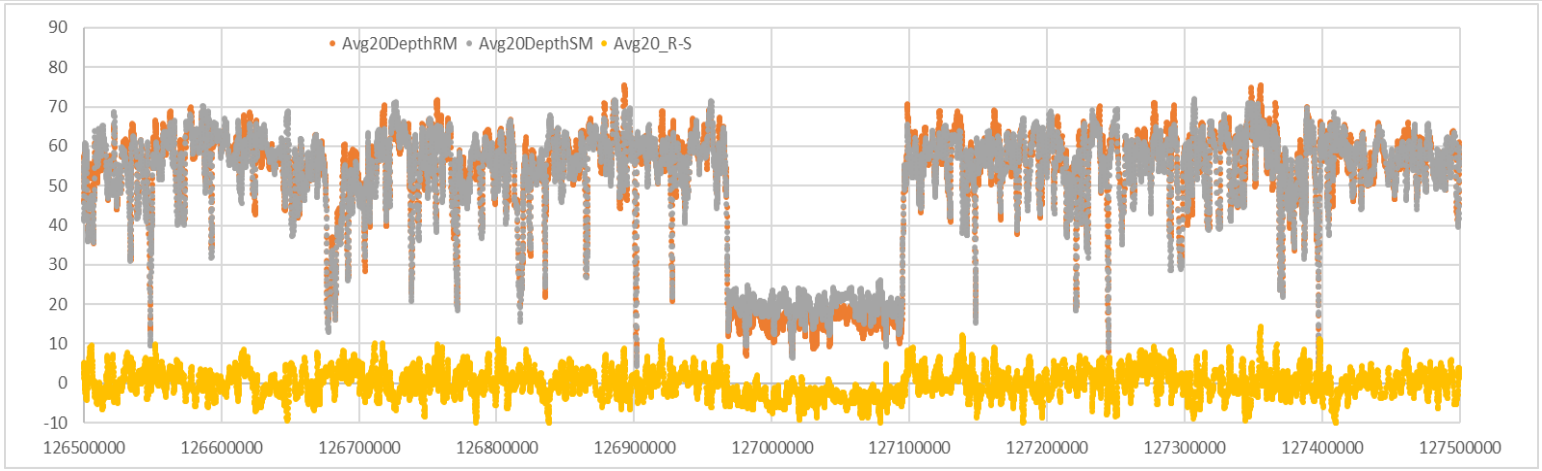


Figure 2. 5.Depiction of significant chromosomal deletion in both R=resistant and S=susceptible for M=males and F=females. A is the read depth in females while B is for males.

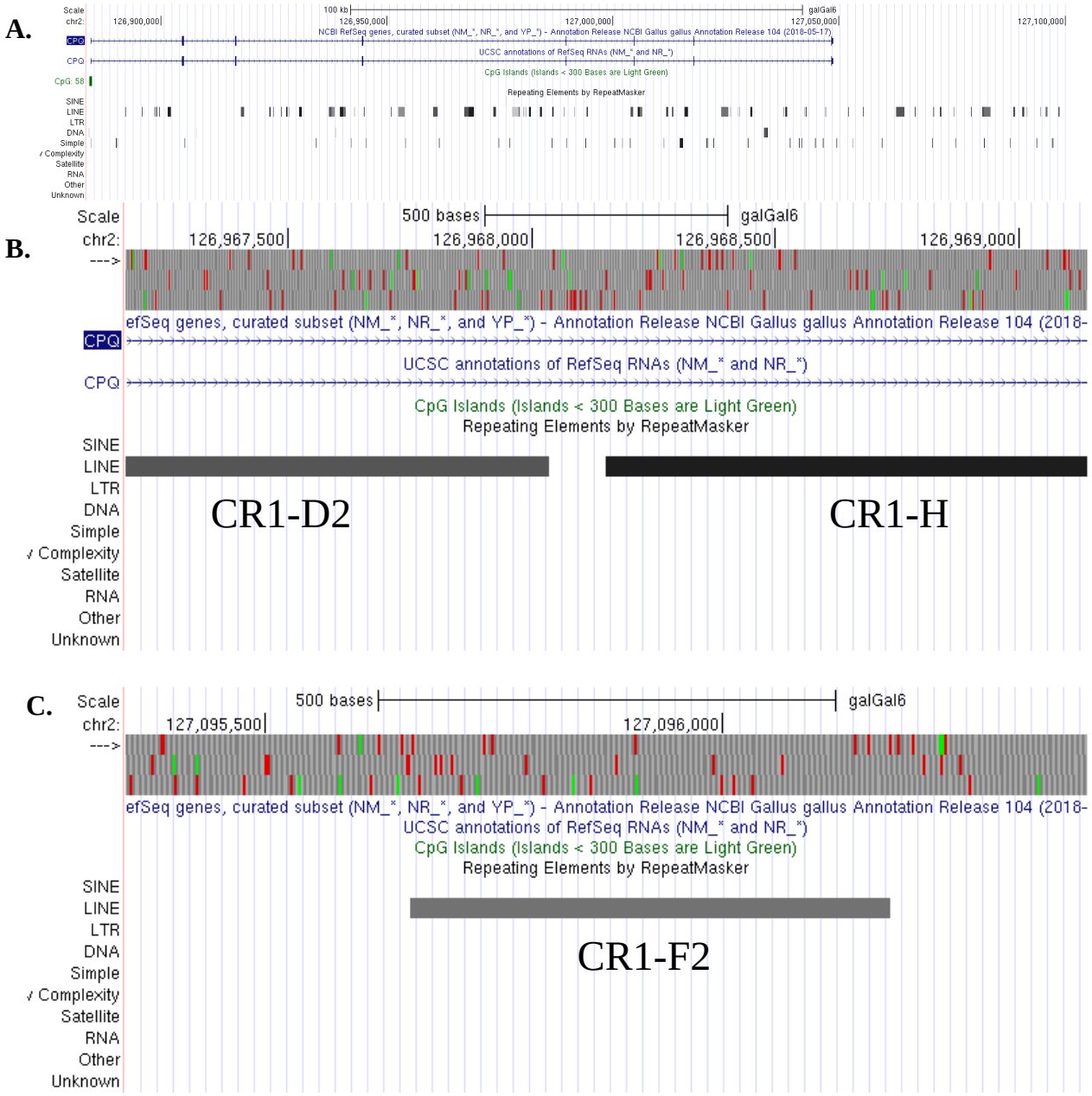


Figure 2. 6.A general picture of the long interspersed nuclear elements (LINEs) identified in the CPQ gene and CPQ gene deletion region using the UCSC genome browser. Panel A shows the number of LINEs in the CPQ gene region (red bracket delimits the deletion). Panel B is the LINEs present at the left boundary. Panel C is the LINE present at the right boundary.

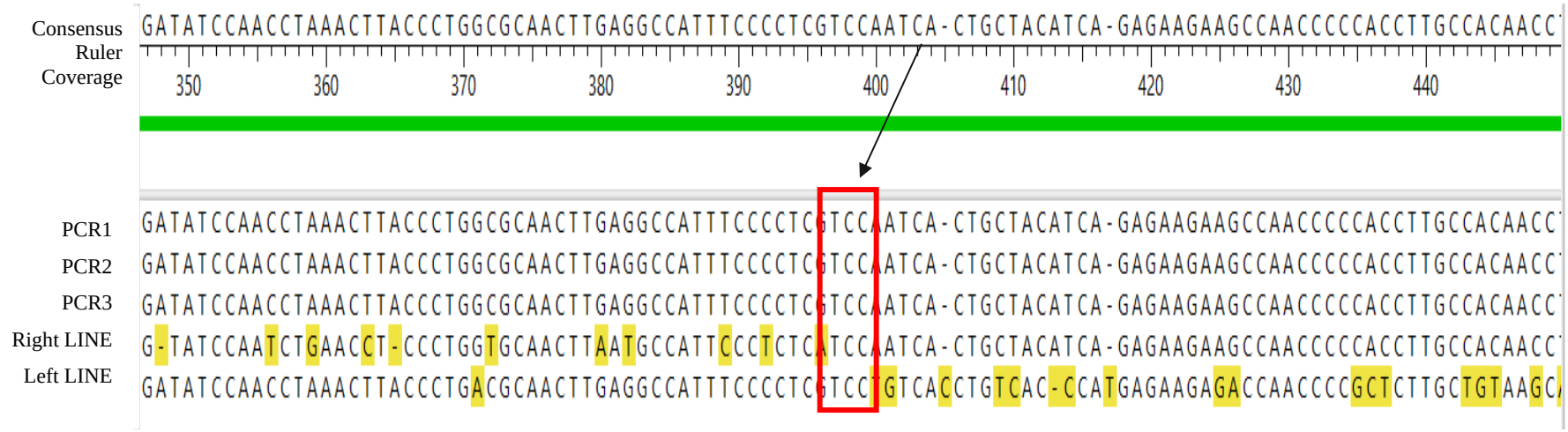


Figure 2. 7.Identification and analysis of 125kbp in CPQ gene. A different set of primers for CNVs (CNVFR, CNVF1R1, CNVF1R2) were used to amplify the recombination junction (red box), and the sequencing results were comparted with the available chicken genome sequence (Galgal6). The left and right LINEs sequences were downloaded from the UCSC genome browser and aligned with the three PCR products using the SeqManUltra to recognize the recombinant point.

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CHPTER3: Proteomic and Metabolomic Studies to Identify Plasma Biomarkers Associated with Pulmonary Hypertension Syndrome (PHS) in Fast-Growing Broilers

Almansaf, Duaa[1, 2], Liyanage, Rohana[3], Rhoads, Douglas D [1, 2, *]

*** Correspondence: drhoads@uark.edu**

1 – Cell and Molecular Biology Program, University of Arkansas, Fayetteville, AR, USA

2 – Department of Biological Sciences, University of Arkansas, Fayetteville, AR, USA

3 – Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR, USA

ABSTRACT

Pulmonary hypertension syndrome (PHS) is a multifunctional metabolic disease, affecting broiler chickens. These broilers show a distended belly formed from the accumulation of the fluid in the abdominal cavity. This disease is a major issue in the poultry industry due to considerable economic losses annually. We analyzed three different phenotypic groups in this study: non-challenge with no ascites "NC-NA", challenge with no ascites "C-NA", and challenge with ascites "CA". The challenge was exposure to hypoxic conditions in a hypobaric chamber. Proteomic and metabolomic changes in the plasma of the healthy birds (NC-NA, C-NA) and affected birds (CA) were explored using tandem mass spectrometry (LC-MS)/MS), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-tandem mass spectrometry (LC-MS) to identify molecular biomarkers in proteins, total fatty acids, lipids, and small molecules. The LC-MS)/MS spectra analysis illustrated elevated levels of apolipoprotein A-I (APOA1), ovotransferrin (TF), hemoglobin subunit beta (HBBA), beta-2-glycoprotein 1 precursor (a protein induced by APOH gene), angiotensinogen (AGT), peroxiredoxin-1 (PRDX1), calcium-binding protein (P22), prothrombin (F2), and coagulation factor IX (F9) and reduced level of fibrinogen alpha chain (FGA), fibrinogen gamma chain precursor (FGG), fibrinogen beta chain (FGB), fibronectin (FN1), IgG Fc-binding protein (Fcγ BP), complement factor H isoform X1 (produced by CFH gene), glutathione peroxidase (GPX3) and von Willebrand factor precursor (VWF) with the onset of ascites. The GC-MS spectra analysis showed a reduced level of stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2) concerning ascites. The LC-MS spectra analysis illustrated a high level of phosphatidylcholine (PC) (34:2), PC (38:3), PC (38:1), PC (36:5), PC (36:3), PC (36:2), PC (40:1), PC (36:1), PC (40:0), and cholesterol ester (CE) 16:1 and a low level of PC (36:4; O) in the plasma for the affected broilers. Further small molecule analysis showed a higher

abundance of PC (18:2(9Z,12Z) \/0:0), PC (0:0/18:1(6Z), LysoPI (20:4(5Z,8Z,11Z,14Z)/0:0), PC (0:0/16:0), phosphatidylethanolamine (PE) (18:2(9Z,12Z)/0:0), and PC (14:0/O-1:0) and a lower abundance of ubiquitin, L-aspartyl-L-glutamate and dehydroepiandrosterone sulfate in response to ascites. These results suggest that these proteins, fatty acids, lipids, and small molecules might be beneficial as noninvasive biomarkers for pulmonary hypertension or ascites in broilers.

KEYWORDS: ascites, proteomics, metabolomics, upregulated, downregulated, proteins, fatty acids, lipids, small molecules, LC-MS/MS, GC-MS, LC-MS.

INTRODUCTION

Pulmonary hypertension syndrome (PHS) or ascites syndrome (AS) is a multifactorial metabolic disease influenced by physiological, environmental, genetic, and nutritional factors (Decuypere et al., 2000; Baghbanzadeh, & Decuypere, 2008). PHS is characterized by several structural alterations involving thrombosis, vasoconstriction, and adverse pulmonary vascular remodeling, resulting in elevated arterial pressure, right ventricular hypertrophy (RVH), and ends up with heart failure (Peacock, 1990; Cottrill & Chan, 2013; Lannan et al., 2014). PHS is caused by elevated blood flow or pulmonary vascular resistance (Julian, 1986). Prolonged PHS, which is driven by increasing demand for oxygen, leads to liver damage and fluid accumulation in the abdominal cavity (Buys, 1999; Luger et al., 2003; Singh et al., 2011). PHS is a worldwide issue and seems to be a common disease in poultry because modern boilers are more susceptible to it (Olkowski, 2007; Wideman et al., 2013; Khajali & Wideman, 2016; Biswas, 2019). This is problematic for the poultry industry since %5 of broilers dies after ascites development (Wang et al., 2012). There was an estimation of economic loss of $ 1 billion/per year due to PHS (Maxwell, 1997; Jacobson & Brownell, 2000; Wideman et al., 2001). Identification of biomarkers for the early stages of PHS would be valuable in monitoring the disease (Rafikova et al., 2016). Idiopathic pulmonary hypertension (IPAH), in humans, remains a serious and progressive disease with an unknown cause that often leads to a failure in the right ventricle (RV), considerable morbidity, and early mortality (Firth et al., 2010; Mathai & Hassoun, 2012). There is no specific remedy for IPAH even though significant advancements have been recently achieved in its pathology (Barst, 1999; Humbert et al., 2014). Animal models have been used in IPAH studies to simulate the clinical pathophysiology of this disease (Zaiman et al., 2005; Firth et al., 2010).

At the University of Arkansas, fast-growing broilers have been used as models for PHS studies due to their breeding feasibility and short generation interval. Relax unselected (REL) line, susceptible (SUS) line, and resistant (RES) line are three research lines developed through divergent selection by Dr. Anthony and his associates. PHS was induced by raising the broilers in a hypobaric chamber to mimic the high altitude (9000ft above sea level) (Pavlidis et al., 2007). At the end of the experiment, three groups were developed: non-challenge with no ascites (NC-NA), challenge with no ascites (C-NA) and challenge with ascites (CA).

Proteomic and metabolomic studies help researchers in exploring specific proteomes and metabolomes directly involved in a targeted disease state (Hu et al., 2020). Previous studies have used proteomic and metabolomic profiles of PHS to detect associated molecular biomarkers in broilers (Wang et al., 2012; Shen et al., 2014; Shi et al., 2017; Ge et al., 2020). In the current study, we have conducted proteomic and metabolomic studies on male broilers from three different groups (NC-NA, C-NA, CA) to identify proteins, total fatty acids, lipids, and small molecules that contribute to the disease. The major aim of this study is to find molecular biomarkers prior and post PHS development. Then, we linked the identified molecular biomarkers to several biological processes and pathways to know which pathways/processes responded to the challenge and disease.

In this study, all the experiments were performed on plasma samples with six replicates for each group (NC-NA, C-NA, CA) obtained from REL line male birds at 2 weeks of age. Proteins and metabolites (fatty acids, lipids, and small molecules) were extracted and submitted for mass spectrometry analysis. The generated data were further analyzed using various bioinformatics programs to compare significantly proteomic and metabolomic changes (p-value <0.05) in NC-NA relative to C-NA and C-NA relative to CA (Figure 3.1).

MATERIALS AND METHODS

Birds Stock and Sample Collection

All animal procedures took place at the University of Arkansas Poultry Research farm. This work followed procedures approved by the University of Arkansas Institutional Care and Use Committee; protocol number (Approval Numbers 15039, 15040, 21087, 21088). Three groups were taken from an unselected relaxed (REL) line at the 18th generation, which was randomly mated. Then, the chicks were moved to cages in a hypobaric chamber for six weeks to produce the Challenged group, or to identical cages at ambient pressure (2700ft) to produce the Unchallenged group. Both chambers and cages were provided with a mesh floor, trough feeders, and nipple water. The hypobaric chamber's simulated altitude was set to 9500ft, and the airflow was adjusted to 17 m^3/min. The initial temperature in the hypobaric chamber was 90 ^oF and was gradually dropped to 80 ^oF over time until the sampling date (Balog and Anthony, 2000; Tellez et al., 2005; Pavlidis et al., 2007). A daily mortality rate was recorded, and necropsy was done on the dead birds to identify the major cause of the death and determine the bird's gender. Birds with ascites were identified based on the presence of ascitic fluid in the abdominal cavity (Druyan et al., 2007; Tarrant et al., 2017). In addition, there are other symptoms associated with ascites including right ventricle to total ventricle ratio, a flaccid heart, and liver lesion (Dey et al., 2018). Blood samples were collected from 2 weeks old male broilers for plasma extraction as described previously (Chamorro et al., 2013) for further proteomic and metabolomic studies. At six weeks of age, surviving birds were terminated by cervical dislocation and assessed for ascites phenotype (Pavlidis et al., 2007; Tarrant et al., 2017). Three groups were identified at the end of the experiment: non-challenge with no ascites (NC-NA), challenges with ascites (CA), and challenges with no ascites (C-NA).

Plasma Sample Preparation for Proteomic, Lipidomic, Metabolomic, and Total Fatty Acids Analysis

A total volume of 80ul of plasma sample was mixed with 1.5ml of the extraction solvent Chloroform/Methanol/Water in a1:3:1 ratio in 2ml Eppendorf tube. The mix was vortexed for 20 seconds, stored at 4^{o}C for 5 minutes, and then centrifuged at 13000g for 3 minutes at 4^{o}C. The supernatant from each tube was transferred to a 2ml glass screw-top vial. Both the supernatant and pellet were stored at -80^{o}C. The supernatant was submitted for total fatty acids, intact lipids, and small molecules analyses using Gas Chromatography-mass spectrometry (GC-MS), Liquid Chromatography-Mass Spectrometry (LC-MS), and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS), respectively (Fang et al., 2015; Warren, 2018; Serafim et al., 2019). Pellets were used for protein analysis as described below. To prepare a quality control (QC) sample, an equal volume from each sample (both cases "C-NA and CA" and the controls "NC-NA" and C-NA) was mixed.

Proteomic Analyses

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The plasma extract pellet was resuspended in a 2x Tris-glycine SDS loading buffer (100mM Tris HCL: 4% SDS: 0.2% bromophenol blue:200mM dithiothreitol: 20% glycerol) at PH 6.8. A reducing agent (10x of 2-mercaptoethanol) was added to the samples, which were then incubated for 2 minutes at 85^{o}C. The protein samples, three biological replicates from each condition (described), and a total of ten samples including the QC control, were loaded in a 4-20% gradient tris-glycine gel (Navex Wedge Well, Invitrogen, Thermo Fisher Scientific). The gel was run in 1x Sodium Dodecyl Sulfate (SDS) running buffer in 200V, 100mA, and stained with Coomassie Brilliant Blue dye.

Trypsin Digestion for Protein Identification

Each gel lane corresponding to biological samples was divided into five sections (Figure 3.2). Each subsection of the gel was cut into small pieces and washed with 1ml of 25mM ammonium bicarbonate for 30 minutes. Then, the gel segments were destained with 50:50 H_2O: acetonitrile (ACN, Bio-Rad Laboratories, Hercules, CA, USA) in 25mM ammonium bicarbonate for 60 minutes, followed by gel dehydration with 100% acetonitrile for 15 minutes. A protein disulfide bond reduction was achieved by incubating the dried gel fragments for 60 minutes at 55^{o}C in 10mM dithiothreitol (DTT, Bio-Rad) in 25mM ammonium bicarbonate. The excess DTT was completely removed, then 55mM of iodoacetamide (IAA, Bio-Rad) in 25mM ammonium bicarbonate was added and incubated for 60 minutes in the dark at room temperature to carbamidomethylate the cysteines after the reduction. The excess IAA was discarded, and the gel segments were further washed with 25mM ammonium bicarbonate before being dehydrated with ACN for 15 minutes. After that, the dehydrated gel pieces were dried by a vacuum and treated with 10 ng/ul of MS Grade Trypsin in 25mM ammonium bicarbonate enough to cover the dried gel pieces and stored at 4^{o} C for 30 minutes to increase the penetration of trypsin into the dried gel. This was followed by an extra 200ul of 25mM ammonium bicarbonate and incubation overnight at 37^{o}C. Gel supernatant, which contains all the tryptic peptides on the gel, was transferred into a new Eppendorf tube. To extract the remaining tryptic peptides stuck in the gel, the gel pieces were resuspended in 5% of formic acid with 60% of ACN. The gel segments were briefly vortexed and then transferred and combine with the same Eppendorf tube. This solution, which had all the tryptic peptides, was fully evaporated, and reconstituted in 50ul of 0.1% formic acid before being used for proteomic analysis using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS)/MS).

Mass Spectrometry Analysis for Proteomics

LC-MS/MS was performed using an Agilent 1200 series micro-flow high-performance liquid chromatography (HPLC) coupled to a Bruker Amazon SL quadrupole ion trap mass spectrometer (Bruker Daltoniks Inc., Billerica, MA, United States) with a captive spray ionization source as described earlier. Tryptic peptides were separated by using a C18 capillary column (150 mm × 0.1 mm, 3.5 μm particle size, 300 Å pore size; ZORBAX SB) with 5–40% gradients of 0.1% formic acid (solvent A) and ACN in 0.1% formic acid (solvent B). Solvent flowed at a rate of 4 μL/min over 300 min each.

Bruker Data Analysis 4.0 software was used to generate peak lists from LC-MS/MS chromatogram for Mascot database search using MASCOT v 2.2 search engine (Matrix Science). In the Mascot search, parent ion and fragment ion mass tolerances were set at 0.6 Da with cysteine carbamidomethyl fixed modification and methionine oxidation as variable modifications. For the identification of proteins, a Mascot search was performed against the Gallus UniProt database. Proteins identified with a 95% confidence limit were further filtered by considering the false discovery rate (FDR). Only the proteins that fall into less than 5% FDR were used. FDR was calculated in during the Mascot search by simultaneously searching the reverse sequence database. Uncharacterized Gallus proteins were identified based on gene sequence similarities tentatively. For evaluation of differentially expressed proteins, Mascot.dat files were exported to Scaffold Proteome Software version 5.0.1 (http://www.proteomesoftware.com), and quantitative differences were determined based on a 95% confidence limit.

The protein functional annotation was determined using David Functional Annotation Bioinformatics Microarray Analysis (DAVID Functional Annotation Bioinformatics Microarray Analysis (ncifcrf.gov) and PANTHERdb (www.pantherdb.org). To obtain associated KEGG and

Reactome pathways and protein-protein interactions, the differential expressed proteins were submitted to STRING (STRING: functional protein association networks (string-db.org).

Fatty Acids Methyl Ester Preparation for Total Fatty Acids Analysis

Nitrogen gas was used to dry 100ul of plasma extract supernatant, and 1ml of 2% of sodium methoxide (Sigma Aldrich, St.Louis, MO, USA) was directly added to the dried samples. The samples were incubated for one hour at room temperature. The solution was transferred into a 5ml glass vial. The fatty acid methyl esters (FAMEs) were extracted by addition of 1ml of water plus 500ul of hexane, the solution was vortexed, then the top hexane layer was transferred to HPLC vials. The FAMEs were resolved by Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

Gas Chromatography/Mass Spectrometry

Samples were analyzed using Agilent 6890N gas chromatograph with 5973 single quadrupole mass selective detector (GC/MS) equipped with Agilent 7683B autosampler. Agilent 30m G3903-63011 DB-FastFAME column with an internal diameter of 0.25 mm and 0.25 µm film liquid layer was used for the analysis. One microliter of the sample was injected in spitless mode into the injector port where the temperature was maintained at 280^{o} C. Temperature of the oven was changed linearly from 40^{o} C to 220^{o} C at the rate of 12^{o} C/min followed by five minutes hold at 220^{o} C. The carrier gas was helium, and the flow rate is variable throughout the run as the method used constant pressure. Electron impact ionization filament was turned on after 3.0 minutes delay time to avoid the solvent front. The mass spectrometer was fully optimized using the software so that all the lenses involved in ion extraction and the detector voltage were set to the optimum values before running the assay. Quadrupole was also tuned and calibrated the same way before the assay. The mass spectrometer was scanned in the m/z range of 30 - 500 in fast scanning mode.

Identification and Quantification of GC-MS data for FAMEs

Each of the fatty acid methyl esters were identified using their individual standard retention times for the same GC-MS method used to analyze all the biological samples and using their NIST (National Institute of Standards and Technology) 70 eV electron impact ionization fragment ion spectra for the pure compound. FAMEs are quantified using total intensity normalization and these normalized intensities were used for statistical analysis.

Lipid Analysis and Metabolomic Analysis (details)

Lipids and metabolites were analyzed using Shimadzu-IT-TOF high-resolution mass spectrometer coupled to Shimadzu LC-20AD. Lipids were separated using two SunFire™ C18 columns (4.6 x 250 mm;5 µm) connected in series. An isocratic gradient consisting of isopropanol/methanol/chloroform (4:2:1, v/v/v) with 35 mM ammonium acetate was used as the mobile phase, with a flow rate of 0.4 mL/min. Small molecule metabolites were separated using a Bio-wide Pore C8 reverse phase column (4.6 mm × 15 cm, 5 µm) (Supelco, St. Louis, MO) was used with a solvent flow rate of 0.7 mL/min using a 0.1% formic acid/acetonitrile gradient of 0–100% over 70 min. Mass spectrometry analysis was conducted in positive ion mode.

Lipids and Metabolomics Data Analysis

Compounds lists containing mono isotopic m/z values and their corresponding intensities for both lipids and metabolites were created using Shimadzu LabSolutions software. Lipids were identified using exact mass and the fatty acid composition from FAMES studies. Metabolite compounds were identified using exact mass as well as their fragmentation pattern. The mc ID (http://www.mycompoundid.org/mycompoundid_beta2/doc/contact.jsp) and metFrag (https://msbi.ipb-halle.de/MetFrag/) were used to identify small molecule metabolites. Each compound expression differences between different groups were calculated using the ratio (fold

change) of the normalized intensities of the compounds in each condition. Volcano plots, $\log_{10}$(*p-value*) as a function of $\log_2$(fold change) were created to visually recognize the differences between groups for total fatty acids, intact lipids, and small molecules using the excel program. The *p*-values less than 0.05 from the student T-test were used to determine the significantly changed compounds/metabolites. MetaboAnalyst 5.0 (MetaboAnalyst) was used to identify the biological pathways affected by PHS or AS. LIPIDS MAP (LIPID MAPS), Human Metabolome Database (HMD) (Human Metabolome Database (hmdb.ca)), and METLIN (https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage) were used to identify significantly changed metabolites.

RESULTS

Proteomics Identification and Quantification

In this study, the unselected REL line was raised at ambient pressure (2700t) to produce non-challenge with no ascites (NC-NA) and in a hypobaric chamber (9500ft) to generate challenge with no ascites (C-NA) and challenge with ascites (CA). Plasma samples were collected from three different groups (NC-NA, C-NA, CA) for proteomic study. The proteomic data were undergone two different comparisons using two different controls and two cases. The purpose of using two controls and two cases in the current study is to look at the proteomic changes before and after ascites development since both C-NA and CA were raised at high altitudes. In the first comparison, NC-NA was the control, while the C-NA was the case. A comparison between NC-NA vs. C-NA was applied to determine the differentially expressed proteins with respect to a hypobaric challenge. In the second comparison, C-NA was the control, whereas CA was the case. C-NA was compared to CA to identify the differentially expressed proteins with respect to ascites.

Based on the Scaffold Q+S viewer (version:5.0.1), a total of 148 individual proteins were identified in broiler plasma. In terms of presence/absence (Figure 3.3A), 141 proteins were present in all three treatments (NC-NA, C-NA, CA). There were 4 proteins (ovotransferrin "TF", tubulin alpha chain "TUBA1A1", glutathione "GSTA4L", coagulation factor IX "F9") only identified in the two challenged groups C-NA and CA, not in NC-NA. There were 2 proteins (histone H4 "H4-I", histone H2B "HIST1H2B5L") present in NC-NA and CA, not in C-NA. There was a single protein (uncharacterized protein) present only in NC-NA and C-NA, not in CA. To identify the uncharacterized protein, a BLASTp was performed on two exclusive unique peptides which consist of 48 amino acids. The non-redundant(nr) protein database at NCBI was queried. The very top hit identified the estimated protein to be chicken immunoglobin heavy chain "IgH" protein. The query coverage was 72%, and the identity was 98.02%. Four proteins (TF, TUBA1A1, GSTA4L, F9) were induced in NC-NA vs. C-NA in response to the hypobaric challenge. In addition, the level of immunoglobin heavy chain "IgH" declined below detectable in C-NA vs. CA with the occurrence of ascites.

The quantitative proteomic profile and statistical analysis of the student T-test revealed that 131 proteins remained unchanged in NC-NA, relative to C-NA and in C-NA compared to CA ($p< 0.05$) (Table 3.1, Figure 3.3B). However, 9 proteins were significantly upregulated, and 8 proteins were significantly downregulated in C-NA compared to CA in response to ascites. The following proteins were found to be more abundant in the C-NA group when compared to the CA group ($p< 0.05$): apolipoprotein A-I (APOA1), ovotransferrin (TF), hemoglobin subunit beta (HBBA), beta-2-glycoprotein 1 precursor (a protein induced by APOH gene), angiotensinogen (AGT), peroxiredoxin-1 (PRDX1), calcium-binding protein (P22), prothrombin (F2), and coagulation factor IX (F9). However, the abundance of other proteins including fibrinogen alpha chain (FGA),

fibrinogen gamma chain precursor (FGG), fibrinogen beta chain (FGB), fibronectin (FN1), IgG Fc-binding protein (FcγBP), complement factor H isoform X1 (produced by CFH gene), glutathione peroxidase (GPX3) and von Willebrand factor precursor (VWF) was lower in C-NA group compared to CA group ($p<0.05$) (Table 3.2).

Several biological processes and pathways were identified by uploading the gene ID numbers of differentially expressed (upregulated/ downregulated) proteins to David Functional Annotation Bioinformatics Microarray Analysis, PANTHERdb, and STRING. There are 27 various biological processes that are implicated in ascites development, one of which is shared by upregulated and downregulated proteins (Figure 3.4). Among the listed biological process in Figure 3.4, several biological processes were upregulated and downregulated to induce ascites, such as acute phase response, blood coagulation, fibrinolysis, platelet activation, and platelet aggregation. The KEGG and Reactome pathways which initiate ascites are listed in Table 3.3. There are three major pathways whose components are upregulated and four other pathways whose components are downregulated. The most significant pathway is blood hemostasis, where a large number of both upregulated and downregulated proteins are involved in the processes. Three upregulated proteins; apolipoprotein A-I (APOA1), beta-2-glycoprotein 1 precursor (produced by APOH gene), and ovotransferrin (TF), are responsible for platelet degranulation. Two upregulated proteins belong to the intrinsic pathway, including prothrombin (F2) and coagulation factor IX (F9). Moreover, these two proteins are involved in some metabolic processes, such as protein metabolism. Five downregulated proteins; fibrinogen alpha chain (FGA), fibrinogen beta chain (FGB), fibrinogen gamma chain precursor (FGG), fibronectin (FN1), and Von Willebrand factor precursor (VWF), play an important role in platelet degranulation, initiating MAPK signaling pathway, forming a fibrin clot, and serve as integrin signaling (Table 3.3). Figure 3.4 illustrates

the number of upregulated and downregulated proteins that undergo various biological processes. Protein-protein interactions were evaluated to have a better insight into how proteins are involved in the protein network and initiate ascites. STRING database is an appropriate resource for representing protein-protein interactions. Figure 3.5 shows the potential interactions between upregulated and downregulated proteins using STRING.

Metabolomic Identification

The plasma samples from the same treatments (NC-NA, C-NA, CA) were used for metabolomic studies to identify common and changed metabolites (total fatty acids, intact lipids, small molecules). NC-NA was compared to C-NA to identify the significantly changed metabolites in response to a hypobaric challenge, followed by another comparison between C-NA and CA to determine the significantly changed metabolites in response to ascites.

Identification and Quantification of Total Fatty Acids

In our experiment, one step rapid method was performed using sodium methoxide (CH_3 ONa) to hydrolyze any lipids to their fatty acid components and methylate for GC-MS studies. This detects total fatty acid compositions in biological samples (Bielawska et al., 2010). Serum total fatty acids profiles of NC-NA vs. C-NA and C-NA vs. CA are summarized in Table 3.4. Saturated fatty acids (palmitic acid and stearic acid), monounsaturated fatty acids (palmitoleic acid and oleic acid), and polyunsaturated fatty acids (linoleic acid) were identified in the broiler plasma. There were no significant changes in fatty acids comparing NC-NA to C-NA. However, a comparison of C-NA and CA showed that stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2) were significantly decreased with the onset of ascites. Palmitic acid and palmitoleic acid were unchanged with the onset of ascites (Table 3.4). Both significant and non-significant total fatty acids were eluted in the first 10 minutes (Figure 3.6).

Identification and Quantification of the Intact Lipids in Broiler Plasma by LC-MS

An LC-MS method was used in this study to quantify the intact lipids in broiler plasma. The major aim is to see the significantly changed lipids pre and post ascites development. To study several lipid classes in broiler plasma, total lipids were extracted from six plasma samples for each group (NC-NA, C-NA, CA) as described in the method section. Then, the samples were analyzed by LC-MS in positive ion mode electrospray ionization (ESI). In the positive ion mode, protonated lipid molecules$[M+H]^+$ were detected. Base peak chromatograms of the plasma extract showed several visible peaks between the 0- and 40-minutes retention time (RT). However, most of the significantly changed lipids eluted from 22 to 34 minutes RT. Mass spectra with relative mass to charge ratio (m/z) from 723 to 846 correspond to different major putative lipid classes (Table 3.5).

Using the LIPID MAPS, a total of 46 lipids were identified in all groups (NC-NA, C-NA, CA) (Figure 3.7). Based on the quantitative comparison, 33 lipids remained unchanged in all groups, whereas a comparison of NC-NA to C-NA showed significant downregulation of two lipids (PC (36:1), and PC (40:0) in response to a hypobaric challenge. On the other hand, 11 upregulated and 2 downregulated lipids were identified in C-NA compared to CA with the onset of ascites. The following lipids were found to be significantly higher in C-NA when compared to CA [phosphatidylcholine (PC) (34:2), PC (38:3), PC (38:1), PC (36:5), PC (36:3), PC (36:2), PC (40:1), PC (36:1), PC (40:0), and cholesterol ester (CE) 16:1] (p<0.05). Only PC (36:4; O) was significantly lower in C-NA compared to CA (Table 3.5, Figure 3.9).

The KEGG ID and HMD ID of total fatty acids and lipids were uploaded into the MetaboAnalyst 5.0 system for visual and pathway analysis. Pathway enrichment analysis shows that several biological pathways are associated with the upregulation and downregulation of fatty

acids and lipids. These pathways include bile acid biosynthesis, alpha-linolenic acid, and linoleic metabolism, plasmalogen synthesis pathway, biosynthesis of unsaturated fatty acids, arachidonic acid metabolism, glycerophospholipids metabolism, steroid biosynthesis, and mitochondria beta-oxidation of long-chain saturated fatty acids. Table 3.6 and Figure 3.10 summarize the major biological pathways induced with ascites in broilers, including the pathway name, total fatty acids and lipids involved in each pathway, total fatty acids and lipids significantly differentiated in this study, *p*-value, false discovery rate (FDR), and KEGG and HMD ID for each pathway.

Identification and Quantification of Small Molecules

Further analytical studies were performed using an LC-MS/MS to see the significantly changed small metabolites among the three groups (NC-NA, C-NA, CA). The obtained data "small molecules data" was applied to fold change analysis for univariate evaluation. In volcano plots, upregulated and downregulated small molecules were indicated by positive and negative values, respectively (Figures 3.13 & 3.14). Fold changes were evaluated for statistically significant changes based on the standard deviation of the fold change between groups. The volcano plot verified highly differential small molecules ($p<0.05$) (Figures 3.13&3.14). Statistical analysis showed that a total of 62 small molecules demonstrated a differential response. Out of these, 9 small molecules were classified as glycerophospholipids, a steroid hormone, dipeptide, and small proteins (Figure 3.12). The Venn diagram shows the number of common and significantly changed small molecules in NC-NA, C-NA, and CA. The overlapping section in the Venn diagram shows a total of 104 small molecules that were common in all three groups (Figure 3.11). We did a mass spec fragmentation to identify some of the most significant small molecules. Based on the identified metabolites listed in Table 3.7, the following small molecules PE (18:2(9Z,12Z)/0:0), PC (0:0/16:0), and PC (14:0/O-1:0) were significantly downregulated in NC-NA vs. C-NA in

response to a hypobaric challenge. In contrast, several small molecules including PC (18:2(9Z,12Z) \/0:0), PC (0:0/18:1(6Z), LysoPI(20:4(5Z,8Z,11Z,14Z)/0:0), PC (0:0/16:0), PE (18:2(9Z,12Z)/0:0), and PC (14:0/O-1:0) were highly upregulated in C-NA compared to CA. Other small molecules such as Ubiquitin, L-aspartyl-L-glutamate, and dehydroepiandrosterone sulfate were highly downregulated in C-NA compared to CA. We only focused on identifying the significantly changed (upregulated/ downregulated) small molecules related to C-NA vs. CA to find a biomarker directly response to ascites in broiler chickens using several metabolic databases, involving LIPIDS MAP, Human Metabolome Database (HMD), METLIN (Table 3.7).

The pathway analysis was done on significantly changed small molecules with low *p*-values (Table 3.7) using *Gallus gallus* as the pathway database to connect their biological roles to various signaling pathways. According to MetaboAnlyst 5.0 and KEGG, glycerophospholipids metabolism, steroid hormone biosynthesis, and ribosome are three major pathways significantly changed to induce the disease (Tables 3.8&3.9).

DISCUSSION

Fast-growing broilers (C-NA and CA) were raised in a hypobaric chamber to simulate the high altitude (9000ft above sea level). Only the CA group developed ascites since they underwent hypobaric hypoxia, where broilers increased their oxygen demand. As a result, the biological system was stressed due to the lack of oxygen required for mitochondrial metabolism. Moreover, proteins started to be modified by post-translation modifications or phosphorylation (Chandel et al., 1996). Prolonged hypoxia caused alterations in gene expression, and it eventually led to cell death once the adaptive mechanisms were depleted (Schumacker et al., 1993). The major aim of this study was to find proteins and metabolites (total fatty acids, intact lipids, small molecules) in

broiler plasma associated with ascites. This helped to identify several molecular biomarkers that can differentiate the healthy birds from the affected ones.

Our results indicated that ascites developed because of upregulated and downregulated proteins involved in various biological processes. The identified proteins were found to be associated with different biological pathways, involving blood hemostasis, protein metabolism, extracellular matrix interaction, focal adhesion, and signal transduction. Blood homeostasis is the most significant pathway where both upregulated and downregulated proteins participate in the interaction network.

The upregulation of hemostatic proteins such as apolipoprotein A-I (APOA1), beta-2-glycoprotein 1 precursor, or apolipoprotein H (produced by APOH gene), ovotransferrin (TF), prothrombin (F2), and coagulation factor IX (F9) disrupted the platelet degranulation and intrinsic pathway. Apolipoprotein A-I (APOA1) is an interesting protein identified in C-NA compared to CA. APOA1, a member of the APOA1/A4/E family, is produced mainly in the liver (Ahmad et al., 2013). APOA1 is the primary functional and structural component of cholesterol or high-density lipoprotein (HDL), being found in the extracellular space, and plays a significant role in cholesterol hemostasis (Zhang et al., 2020). Several studies showed that APOA1 had multifactional roles in apoptosis, immunity, and bacterial and viral infections (Mangaraj et al., 2016). This protein is known as an inflammatory protein since it contributes to chronic and acute inflammatory disorders (Zhang et al., 2020). APOA1 is also involved in decreasing the incidence of cardiovascular diseases by removing fat particles (Di et al., 2016). It participates in protecting the cardiovascular system because of its anti-aggregatory and anti-clotting effect on platelets (Nkosi, 2019; Mangaraj et al., 2016). APOA1 protects the airway function and the pulmonary artery along with preventing respiratory inflammation and collagen deposition (Wang et al., 2010).

Plasma expression of APOA1 was significantly abundant (*p*-value <0.05) in C-NA compared to CA, indicating the ant-inflammatory and anticoagulant roles of APOA1. This new finding was confirmed by the high-altitude pulmonary edema (APE) in humans, which caused an upregulation of APOA1 due to the inflammatory response (Ahmad et al., 2011). Therefore, APOA1 is classified as an acute-phase protein (APP). Beta-2-glycoprotein 1 precursor or apolipoprotein H (APOH) is another interesting APP protein whose expression was significantly abundant in the plasma from C-NA compared to CA. APOH belongs to the family of complement control proteins (Erkan et al., 2014). APOH is one of the most significant apolipoproteins which contributes to heart development in fast-growing broilers. APOH binds and transfers lipids in blood circulation and prevents the accumulation of triglycerides in the heart (Nielsen et al., 2002). Under severe circumstances or diseases, APOH binds to the pathogen or its protein during the acute phase to elicit an innate immunity response. (Stefas et al., 2011). In this study, APOH protein level was highly upregulated ($p<0.05$) in C-NA vs. CA, supporting the anti-inflammatory response of APOH. Another APP detected in C-NA vs. CA is ovotransferrin (TF). TF, which is a transmembrane glycoprotein found in plasma and egg white, plays a critical role in ferric-ion delivery and is responsible for iron metabolism (Giansanti et al., 2012). Several studies revealed that TF serves as APP in broilers, and it increased under viral, chemical, and bacterial-induced inflammation (Xie et al., 2002a; Xie et al., 2002b). Another study showed that the TF concentration was altered under microbial and inflammatory stress. Thus, TF is a biomarker of inflammatory and infectious diseases in broilers (Rath et al., 2009). In this study, TF was highly upregulated (*p*-value <0.05) in C-NA relative to CA, supporting the potential anti-inflammatory role of TF. A further interesting protein identified in C-NA compared to CA is prothrombin (F2). F2 is a plasma protein synthesized in the liver, involved in blood clotting and maintaining normal hemostasis

(Hoffman & Monroe, 2001). In previous studies, chickens with bacterial and fungal infections that initiated inflammation would increase the expression of F2 (Roy et al., 2014). This happened due to the defect in the common coagulation and intrinsic pathways. The higher expression of F2 is associated with defective hepatic metabolism since this protein is a coagulation factor produced by the liver (Fernandez et al., 1995). In the current study, the concentration of F2 was significantly upregulated (p-value <0.05) in the plasma of C-NA compared to CA, suggesting the homeostatic alterations as a part of an anti-inflammatory and pathophysiologic response to ascites.

Other upregulated proteins were identified in C-NA plasma and might be a biomarker for ascites in fast-growing broilers. These proteins are hemoglobin subunit beta (HBBA), angiotensinogen (AGT), peroxiredoxin-1 (PRDX1), and calcium-binding protein (P22). Hemoglobin, which is an oxygen carrier and regulator component, is an iron-rich protein distributed in all living organisms. This protein helps the organisms to adjust to different environmental conditions (Melo et al., 2003). Previous studies proved that high expression of hemoglobin might be linked to a hypoxic response since this protein is beneficial in providing the tissues with oxygenated blood (Storz, 2007; Gordeuk et al., 2016). Hypoxic stress changes the oxygen affinity and influences the oxygen transport properties of hemoglobin (Mairbäurl,1994). The hemoglobin oxygen affinity allows for rapid alterations in oxygen binding and release. Therefore, changes in the primary structure of the globin chain, which influence oxygen intake and transport to tissues, are thought to be responsible for this adaption (Ahmad et al., 2013). In the present study, the upregulation of hemoglobin subunit beta (HBBA) in C-NA compared to CA, supports the potential role of HBBA in adapting to hypoxia. A further significant protein found in C-NA vs. CA is angiotensinogen (AGT). The AGT, which is synthesized in the liver, is a component of a hormonal system called the renin-angiotensin system (RAS). The RAS plays a

significant role in regulating blood pressure and fluid balance (Wong, 2016). Several studies showed a strong positive correlation between the plasma level of angiotensinogen and high blood pressure in humans and mice (Walker et al., 1979; Kimura et al., 1992; Jeunemaitre et al., 1992). Until now, AGT has not been reported as a plasma biomarker for PHS in broilers. Only one study showed that brain AGT was overexpressed in broilers with PHS (Hassanpour et al., 2019). In the current study, AGT was highly upregulated in C-NA, relative to CA, providing the primary information for the possible effect of AGT in PHS in broilers. Plasma AGT could be a potential molecular biomarker for the affected broilers, and this might be a new finding. Another interesting protein identified in C-NA vs. CA is peroxiredoxin-1(PRDX1). PRDX1, which is a member of the ubiquitous family (peroxiredoxin), is involved in regulating cellular redox homeostasis (Ding et al., 2017). Based on physiological and pathophysiological studies, PRDX1 serves as a modulator for cardiovascular events (Schroder et al., 2008; Jing et al., 2011; Guo et al., 2018). The high expression level of this protein refers to cardiac hypertrophy, which causes pulmonary arterial hypertension in mice (Tang et al., 2020). In this study, PRDX1 was significantly upregulated in CA, supporting that PRDX1 induces right ventricular hypertrophy, which subsequently results in a right-ventricular failure in broilers. PRDX1 might be a potential biomarker for PHS in chickens. Another important protein detected in C-NA vs. CA is a calcium-binding protein (P22). The P22, which is a member of the EF-hand superfamily, is a structural and functional protein of sarcoplasmic and endoplasmic reticulum membranes (Milner et al., 1992; Barroso et al., 1996; Andrade et al., 2004). Prolonged endoplasmic reticulum (ER) stress causes smooth muscle and endothelial cell dysfunction. This results in inadequate vascular remodeling and intense pulmonary vasoconstriction, which subsequently leads to pulmonary arterial hypertension in mice (Dromparis et al., 2013; Hu et al., 2020; Pan et al., 2020; Pu et al., 2020). In this study, the P22 was highly

upregulated in C-NA compared to CA. The high level of P22 in broiler plasma could result from excessive ER stress and results in ascites development. The elevated level of this protein might be a possible biomarker for ascites in broilers.

There are five downregulated acute phase proteins (APPs): fibrinogen alpha chain (FGA), fibrinogen beta chain (FGB), fibrinogen gamma chain precursor (FGG), fibronectin (FN1), and von Willebrand factor precursor (VWF) resulted in dysfunctioning of the hemostatic system. All the first three proteins (FGA, FGB, FGG) were classified under the fibrinogen family, which is a type of glycoprotein synthesized in the liver. The FGA binds to FGB and FGG to form fibrinogen which plays a central role in coagulation or blood clot formation. Chickens infected with different types of bacteria such as *Streptococcus zooepidemicus, E. coli*, and *E. tenella* stimulated hyperfibrinogenemia as an acute phase response (infection-induced inflammatory) (Springer et al., 1974; Georgieva et al., 2010; Roy et al., 2014). Similarly, humans and chicken fibrinogen increased when exposed to stress (Lazzarino et al., 2015; Ellins et al., 2017; Nwaigwe et al., 2020). Henderson et al. proved that the level of fibrinogen highly decreased in patients with ascites since fibrinogen was degraded within the ascitic fluid (fibrinogenolysis) (1980). In the present study, the three major types of fibrinogens (FGA, FGB, FGG) were significantly downregulated (p-value<0.05) in the plasma of C-NA compared to CA, supporting the occurrence of fibrinogenolysis in affected broilers. Therefore, fibrinogen might be a molecular biomarker for ascites in broilers. Further work should be done on ascitic fluid to prove the previous statement. Fibronectin (FN1) is a further hepatic glycoprotein, classified as extracellular matrix protein, and was identified in C-NA compared to CA. The FN1 was highly expressed in patients with PHS since this protein is involved in the proliferation of smooth muscle cells (Clausell & Rabinovitch, 1993; Jones et al., 1997). Patients suffering from PHS exhibited overexpression of FN1 since FN1 is a major

component in developing plexiform lesions (Tuder et al., 2007). Hamal et al. found that broilers with idiopathic pulmonary arterial hypertension (IPAH) had a strong expression of FN1 in their plasma due to the thickening of their pulmonary arterioles, and the proliferation of their smooth muscle cells, and the formation of their plexiform lesions (2012). Changes in FN1 expression were associated with the physiology and etiology of the disease. However, in our study, the level of FN1 was significantly downregulated (p-value<0.05) in C-NA compared to CA, suggesting that broilers with PHS developed hepatic damage (Höfeler & Klingemann, 1984). Another downregulated protein identified in C-NA vs. CA was the von Willebrand factor precursor (VWF). The VWF, which is an adhesive glycoprotein, plays a significant role in hemostasis and thrombosis (Luo et al., 2012). The VWF was reported to be a molecular biomarker of endothelial dysfunction since a high level of this factor is present in atherosclerotic and inflammatory vascular disorders (Blann & Lip, 1998). Patients with pulmonary hypertension are characterized by several pathophysiological features, including a severe bleeding event (anticoagulation) (Opitz et al., 2009). A low level of VWF has been linked to bleeding, especially in cardiovascular events (Sadler, 2009; Roldán et al., 2011). In the current study, the VWF was highly downregulated (p value<0.05) in C-NA relative to CA, supporting the idea of bleeding in the cardiovascular system at the late stage of PHS.

There are other downregulated proteins identified in the broiler plasma and could be a possible biomarker for ascites or pulmonary hypertension in broilers. These proteins are IgG Fc-binding protein (FcγBP), complement factor H isoform X1 (produced by CFH gene), and glutathione peroxidase (GPX3). The IgG Fc-binding protein (FcγBP) is an interesting protein detected in C-NA compared to CA. The FcγBP is produced by mucin-producing cells in different mucosae, involving respiratory mucosa (Kobayashi et al., 2021). The FcγBP could be involved in

the anti-inflammatory function and cell protection (Gazi et al., 2008). Airway obstruction can be developed as a result of inflammation and mucus plugging (Fernandez-Bonetti et al., 1983). The inflammatory response is a characteristic of pulmonary hypertension in broilers (Dorfmüller et al., 2003; Low et al., 2015). However, the role of FcγBP in PHS is unknown. There is no specific interpretation of why FcγBP was downregulated in C-NA vs. CA. This is a new finding and potential biomarker which needs further work to be done for further confirmation. Another important protein identified in C-NA vs. CA is complement factor H isoform X1(CFH). The CFH, which is a glycoprotein, is essential in enhancing the defensive immune system by regulating complement activation. Nevertheless, the dysregulation of the protective complement activation might damage tissues, as proven in different ischemic, autoimmune, and inflammatory disorders (Ricklin et al., 2016; Frid et al., 2020a). Previous studies unveiled the relative participation of classic, alternative, and lectin activation pathways in the complement activation induced by tissue remodeling and damage. The most important pathway is the activated alternative pathway which regulates the pro-proliferative and proinflammatory alterations in the lung. A low level or absence of CFH activated the complement alternative pathway involved in the PHS and its pathogenesis, especially in affected individuals with high mortality risk (Rhodes et al., 2017; Li et al., 2019; Frid et al., 2020b). In this study, the CFH was significantly downregulated in C-NA vs. CA, indicating the activation of complement alternative pathways following the PHS induction. The last significant protein detected in C-NA vs. CA is glutathione peroxidase (GPX3). The GPX3, which is a cellular antioxidant enzyme, defense against oxidative stress by controlling the reactive oxygen species (ROS) (Forgione et al., 2002; Blankenberg et al., 2003; Van, 2015). ROS leads to PHS since it is an inflammatory mediator during hypoxia and ischemia. Furthermore, the ROS is produced by activated macrophages as a result of cellular metabolism in the mitochondria and

causes severe damage to the vascular endothelium of pulmonary blood vessels (Irodova et al., 2002). A low level of GPX3 in the plasma was pronounced in individuals with PHS (Miyamoto et al., 2003; Masri et al., 2008; Mikhael et al., 2019). In the current study, the GPX3 was highly downregulated in C-NA compared to CA, supporting the central role of GPX3 in defending against oxidative stress, which developed ascites in fast-growing broilers.

In the current study, we used targeted GC-MC metabolomics to examine the possibility of plasma total fatty acids as a disease biomarker in broilers with ascites by comparing the results of NC-NA to C-NA to CA. Our hypothesis was metabolites were highly differentiated in broilers with PHS. A total of 6 total fatty acids were measured from different groups of broilers (NC-NA, C-NA, CA). Then, the data were analyzed using different statistical analyses mentioned earlier. We found that broilers from the C-NA group had a higher level of stearic acid (C18:0) and oleic acid (C18:1) and a lower level of linoleic acid (C18:2) in a statistically significant way (Table 3.5) when compared to the CA group. It has been reported that mitochondria functions are influenced by different types of fatty acids. For instance, mitochondrial respiration and fatty acid compositions are changed by different hormones (Clejan et al., 1980). Mitochondrial beta-oxidation is a significant metabolic process that breaks down long-chain fatty acids and releases energy in the form of ATP (Talley & Mohiuddin, 2021). The fatty acids can either be stored as triglycerides or undergo beta-oxidation (Brittain et al., 2016). In PHS, either hypoxia, which activates hypoxia-inducible factor 1-alpha (HIF-1 α), or oxidative stress, which results from increasing the ROS and decreasing antioxidant enzymes, inhibits beta-oxidation of long-chain fatty acids (Chen et al., 2020). That leads to massive accumulations of saturated and unsaturated fatty acids (Huang et al., 2014; Chen et al., 2020). Furthermore, increasing the ROS was seen in chickens with PHS (Cawthon et al., 2001). Therefore, chickens with PHS have different

mitochondrial functions because of different fatty acid content or different ratio of saturated and unsaturated fatty acids (Iqbal et al., 2002). Patients with hypertension showed a low level of unsaturated fatty acids linoleic acid (C18:2) (Tsukamoto & Sugawara, 2018). Several studies demonstrated an inverse relationship between linoleic acid and blood pressure (Wang et al., 2008; Wang et al., 2011; Zeng et al., 2014). A high level of linoleic acid may induce relaxation in the coronary artery in pigs and acts as a substrate for vasoactive prostaglandins in mice (Iacono & Dougherty, 1993; Pomposiello et al., 1998). In our study, the level of saturated and unsaturated fatty acids s such as stearic acid (C18:0) and oleic acid (C18:1) was significantly increased in C-NA relative to CA, indicating that broilers with PHS had different mitochondrial functions. They underwent beta-oxidation, which triggered the accumulation of these two fatty acids. On the other hand, linoleic acid was highly downregulated in C-NA vs. CA, which initiates hypertension.

The LC-MS and LC-MS/MS metabolomics were used to identify potential plasma intact lipids and small molecules as a molecular biomarker for PHS in fast-growing broilers. Novelty, the present study demonstrated different glycerophospholipids [phosphatidylcholine (PC) and phosphatidylethanolamine (PE)], lysophosphatidylinositols (LysoPIs), and a sterol lipid [cholesterol esters (CE)] profile changes in C-NA, relative to CA. Emerging literature indicated that higher levels of plasma total fatty acids and lower levels of high-density lipoprotein cholesterol could elicit an abnormal metabolization of the lipids in patients with PHS. That leads to elevate lipid deposition in the cardiomyocytes of the right ventricle (RV), RV lipotoxicity, and RV hypertrophy (Heresi et al., 2010; Hemnes et al., 2014; Talati & Hemnes, 2015; Brittain et al., 2016; Hemnes et al., 2019). Our results agree with the previous study since saturated fatty acids (stearic acid) and unsaturated fatty acids (oleic acid) were significantly increased in C-NA vs. CA ($p<0.05$). Therefore, the following lipids: PC (34:2), PC (38:3), PC (38:1), PC (36:5), PC (36:3),

PC (36:2), PC (40:1), PC (36:1), PC (40:0), CE 16:1, PC (18:2(9Z,12Z) \/0:0), PC (0:0/18:1(6Z), LysoPI(20:4(5Z,8Z,11Z,14Z)/0:0), PC (0:0/16:0), PE (18:2(9Z,12Z)/0:0), and PC (14:0/O-1:0) were statistically significant abundant in C-NA relative to CA (p<0.05). In addition, our metabolomic analysis showed a possible new finding. Among the most significantly changed metabolites, one phosphatidylcholine PC (36:4; O) was significantly lower in C-NA compared to CA. This could be a possible association with PHS since the PC is the main phospholipid that provides the endothelial cells with arachidonic acid (Thomas et al., 1984). Prostacyclin, which is produced by arachidonic acid metabolism from the vascular wall, is a potent vasodilator. In patients with PHS, an imbalance of prostacyclin production reflects the activation of platelets and induces an abnormal reaction of the pulmonary endothelium (Christman et al., 1992).

There are other small metabolites identified in the plasma of C-NA vs. CA using LC-MS/MS. One of the most important small metabolites is ubiquitin. Ubiquitin, which is a small protein (76 amino acids), plays a central role in targeting protein for proteasomal degradation by a process known as the ubiquitin-proteasome system (UPS) (Rathinasabapathy & West, 2018; Wade et al., 2018). The pro-inflammatory response in the late stage of PHS activates several transcription factors, one of which is nuclear factor kappa B (NF-κB). The NF-κB is mainly activated in pulmonary vascular cells, lymphocytes, and macrophages ((Price et al., 2013). The activation of NF-κB is associated with vascular remodeling and cardiovascular hypertrophy (Zhang et al., 2014). Similarly, the UPS plays a significant role in PHS development because it is involved in pulmonary vascular remodeling (Wade et al., 2018). Previous studies demonstrated a strong positive relationship between UPS and NF-κB pathway in PHS (Zhang et al., 2014). Another study proved that the level of total ubiquitin increased in patients with hypertrophic cardiopathy, indicating that the UPS is dysfunctional, and the total protein degradations are

decreased in heart failure (Zolk et al., 2006). On the other hand, Wade et al. demonstrated that patients with PHS showed a reduction in the net ubiquitin involved in protein degradation due to the development of hypoxia (2018). In this study, the ubiquitin was significantly downregulated in the C-NA, suggesting the adaptive response of ubiquitin in broilers with PHS against hypoxia. Another important metabolite identified in the plasma is dehydroepiandrosterone sulfate (DHEA-S). DHEA-S is a male sex hormone expressed in both genders. It is responsible for the production of male and female sex hormones (testosterone and estrogen) (Webb et al., 2006). DHEA-S assists in controlling the blood pressure and heart rate in humans (Schunkert et al., 1999). This hormone plays a significant role in stress response, especially in domestic animals and humans (Gabai et al., 2020). Several studies proved that a reduced level of DHEA-S is associated with PHS in humans and mice (Hampl et al., 2003; Ventetuolo et al., 2016; Baird et al., 2018). In addition, DHEA-S was used as a pharmacological drug to minimize chronic hypoxia that induces pulmonary hypertension in mice (Hampl et al., 2003). In our study, DHEA-S was significantly downregulated in C-NA compared to CA. DHEA-S could be a potential biomarker for PHS in broilers.

In conclusion**,** our data revealed that there were significant changes in proteomes and metabolomes (fatty acids, lipids, small molecules) in the C-NA group relative to the CA group due to anti-inflammatory, pathophysiologic, hypoxia, and oxidative stress- responses which develop ascites. We were able to identify some molecular biomarkers similar to what has been published so far. Furthermore, many of the identified biomarkers are new findings that need further work to be proved. The candidate biomarkers might give some explanations of how the ascites developed in chickens. These new biomarkers could be a beneficial tool for early ascites detection.

TABLES AND FIGURES

Table 3. 1. The common unchanged proteins in NC-NA relative to C-NA and C-NA relative to CA.

Protein #	Accession Number	Gene Name	Identified Proteins
1	A0A1D5NW68	ALB	Serum albumin OS=Gallus gallus OX=9031 GN=ALB PE=4 SV=1
2	F1NK40	A2ML4	Alpha-2-macroglobulin-like protein 1 OS=Gallus gallus OX=9031 GN=A2ML4 PE=4 SV=4
3	A0A1D5P9F9 (+1)	C3	Complement C3 precursor OS=Gallus gallus OX=9031 GN=C3 PE=4 SV=1
4	Q197X2	APOB	Apolipoprotein B OS=Gallus gallus OX=9031 GN=APOB PE=2 SV=1
5	F1NV02	APOB	Apolipoprotein B OS=Gallus gallus OX=9031 GN=APOB PE=4 SV=2
6	Q90633		Complement C3 OS=Gallus gallus OX=9031 PE=2 SV=1
7	P02112	HBB	Hemoglobin subunit beta OS=Gallus gallus OX=9031 GN=HBB PE=1 SV=2
8	P02789		Ovotransferrin OS=Gallus gallus OX=9031 PE=1 SV=2
9	Q4ADJ6	TFEW	Ovotransferrin OS=Gallus gallus OX=9031 GN=TFEW PE=2 SV=1
10	Q4ADG4	TFEW	Ovotransferrin OS=Gallus gallus OX=9031 GN=TFEW PE=2 SV=1
11	A0A1D5NVI0	HBBA	Hemoglobin subunit beta OS=Gallus gallus OX=9031 GN=HBBA PE=3 SV=1
12	A0A1D5PCD2		Alpha-2-macroglobulin isoform X2 OS=Gallus gallus OX=9031 PE=4 SV=2
13	P01994	HBAA	Hemoglobin subunit alpha-A OS=Gallus gallus OX=9031 GN=HBAA PE=1 SV=2
14	A0A1D5P6F4		IgGFc-binding protein OS=Gallus gallus OX=9031 PE=4 SV=1
15	A0A1D5PU94	C4A	Complement C4 OS=Gallus gallus OX=9031 GN=C4A PE=4 SV=1
16	P02001	HBAD	Hemoglobin subunit alpha-D OS=Gallus gallus OX=9031 GN=HBAD PE=1 SV=1
17	F1NMZ3 (+1)	HBBR	Hemoglobin subunit epsilon OS=Gallus gallus OX=9031 GN=HBBR PE=3 SV=1
18	A0A1L1S0P1	PIT54	PIT54 protein precursor OS=Gallus gallus OX=9031 GN=PIT54 PE=4 SV=1
19	E1C7A7 (+1)	VTN	vitronectin precursor OS=Gallus gallus OX=9031 GN=VTN PE=4 SV=2
20	O93601	APOAIV	Apolipoprotein AIV OS=Gallus gallus OX=9031 GN=apoAIV PE=2 SV=1

Table 3.1. Continued.

Protein #	Accession Number	Gene Name	Identified Proteins
21	F1NEQ4	A2ML2	Alpha-2-macroglobulin isoform X2 OS=Gallus gallus OX=9031 GN=A2ML2 PE=4 SV=4
22	R9PXM5	IGLL1	Ig lambda chain V-1 region precursor OS=Gallus gallus OX=9031 GN=IGLL1 PE=4 SV=2
23	F1NWX6	PLG	Plasminogen OS=Gallus gallus OX=9031 GN=PLG PE=3 SV=2
24	A0A1D5PD98	C5	Complement C5 OS=Gallus gallus OX=9031 GN=C5 PE=4 SV=1
25	A0A1D5NXA6 (+1)	ITIH3	Inter-alpha-trypsin inhibitor heavy chain 3 OS=Gallus gallus OX=9031 GN=ITIH3 PE=4 SV=1
26	E1BZE1	AHSG	Alpha 2-HS glycoprotein OS=Gallus gallus OX=9031 GN=AHSG PE=4 SV=1
27	A0A1I7Q422 (+1)	TTR	Transthyretin OS=Gallus gallus OX=9031 GN=TTR PE=3 SV=1
28	A0A1D5P1E3 (+1)		Alpha-2-macroglobulin isoform X2 OS=Gallus gallus OX=9031 PE=4 SV=1
29	F1NTM6	TFR2	Transferrin receptor protein 1 OS=Gallus gallus OX=9031 GN=TFR2 PE=4 SV=3
30	O93510	GSN	Gelsolin OS=Gallus gallus OX=9031 GN=GSN PE=2 SV=1
31	P01875		Ig mu chain C region OS=Gallus gallus OX=9031 PE=2 SV=2
32	F1NVF3	GC	Vitamin D-binding protein precursor OS=Gallus gallus OX=9031 GN=GC PE=4 SV=1
33	B3VE14	ITIH2	Inter-alpha inhibitor heavy chain 2 OS=Gallus gallus OX=9031 GN=ITIH2 PE=2 SV=1
34	A0A1D5P5V5	C4	complement 4 precursor OS=Gallus gallus OX=9031 GN=C4 PE=4 SV=1
35	A0A1D5PV66	LOC100857892	Alpha-tectorin protein OS=Gallus gallus OX=9031 GN=LOC100857892 PE=4 SV=1
36	A0A1D5PLZ2 (+1)	SERPIND1	Serpin family D member 1 OS=Gallus gallus OX=9031 GN=SERPIND1 PE=3 SV=1
37	Q5ZHM4	RCJMB04_35g11	Hypothetical protein RCJMB04_35g11 OS=Gallus gallus OX=9031 GN=RCJMB04_35g11 PE=2 SV=1
38	F1NA58		Serpin family G member 1 OS=Gallus gallus OX=9031 PE=3 SV=3
39	A0A146F050	KRT6A	Type II alpha-keratin IIC OS=Gallus gallus OX=9031 GN=KRT6A PE=2 SV=1

Table 3.1. Continued

Protein #	Accession Number	Gene Name	Identified Proteins
40	E1C7T1	SPIA1	Alpha-1-antitrypsin isoform X1 OS=Gallus gallus OX=9031 GN=SPIA1 PE=3 SV=1
41	P10184	OIH	Ovoinhibitor OS=Gallus gallus OX=9031 GN=OIH PE=1 SV=2
42	F1P2M6	LOC419851	Complement component 4 binding protein, GPI-anchored precursor OS=Gallus gallus OX=9031 GN=LOC419851 PE=4 SV=1
43	Q5ZMQ2	ACTG1	Actin, cytoplasmic 2 OS=Gallus gallus OX=9031 GN=ACTG1 PE=1 SV=1
44	A0A1D5PEY8		Complement C8 gamma chain OS=Gallus gallus OX=9031 PE=3 SV=1
45	E1C6U2 (+1)	C7	Complement component 7 OS=Gallus gallus OX=9031 GN=C7 PE=4 SV=2
46	A0A1L1RKA9 (+1)	HRG	Histidine rich glycoprotein OS=Gallus gallus OX=9031 GN=HRG PE=4 SV=1
47	A2N883	VH1	VH1 protein (Fragment) OS=Gallus gallus OX=9031 GN=VH1 PE=2 SV=1
48	A0A160F7C1 (+1)		Corticosteroid binding globulin OS=Gallus gallus OX=9031 PE=2 SV=1
49	Q7LZS1		12K serum protein, beta-2-m cross-reactive (Fragment) OS=Gallus gallus OX=9031 PE=1 SV=1
50	H9L385	HPX	Hemopexin OS=Gallus gallus OX=9031 GN=HPX PE=4 SV=3
51	A0A1D5PV72		Immunoglobulin heavy chain OS=Gallus gallus OX=9031 PE=4 SV=1
52	A0A1L1RNR4	KNG1	Kininogen 1 OS=Gallus gallus OX=9031 GN=KNG1 PE=4 SV=1
53	A0A1D5P3R2 (+1)	CA2	Carbonic anhydrase 2 OS=Gallus gallus OX=9031 GN=CA2 PE=4 SV=1
54	E1C7C1	C8B	Complement component C8 beta chain precursor OS=Gallus gallus OX=9031 GN=C8B PE=4 SV=2
55	F1NJU5	C8A	Complement C8 alpha chain OS=Gallus gallus OX=9031 GN=C8A PE=4 SV=2
56	E1BY93	JCHAIN	Immunoglobulin J chain precursor OS=Gallus gallus OX=9031 GN=JCHAIN PE=4 SV=1
57	P41263	RBP4	Retinol-binding protein 4 OS=Gallus gallus OX=9031 GN=RBP4 PE=1 SV=1
58	A0A1D5NTU8		Uncharacterized protein OS=Gallus gallus OX=9031 PE=3 SV=1
59	P01038 (+1)		Cystatin OS=Gallus gallus OX=9031 PE=1 SV=2

Table 3.1. Continued

Protein #	Accession Number	Gene Name	Identified Proteins
60	SERPINC1	F1NLP7	Antithrombin-III OS=Gallus gallus OX=9031 GN=SERPINC1 PE=3 SV=2
61	F1NSC8	IGLL1	IgL OS=Gallus gallus OX=9031 GN=IGLL1 PE=4 SV=4
62	A0A1D5PMQ5	KRTC42L	Uncharacterized protein OS=Gallus gallus OX=9031 GN=KRTC42L PE=3 SV=2
63	F1NDN6		Keratin 12 OS=Gallus gallus OX=9031 PE=3 SV=3
64	Q90W37	COL2A1	Alpha 1 type IIA collagen OS=Gallus gallus OX=9031 GN=COL2A1 PE=2 SV=1
65	A0A1D5NW11 (+2)	KRT19	Keratin, type I cytoskeletal 19 OS=Gallus gallus OX=9031 GN=KRT19 PE=3 SV=1
66	P81475		Complement factor B-like protease (Fragment) OS=Gallus gallus OX=9031 PE=1 SV=1
67	A0A1D5NTE9	CFD	Complement factor D OS=Gallus gallus OX=9031 GN=CFD PE=3 SV=1
68	B8ZX71	C6	Complement C6 OS=Gallus gallus OX=9031 GN=C6 PE=2 SV=1
69	A0A1D5PU00	AMBP	Protein AMBP precursor OS=Gallus gallus OX=9031 GN=AMBP PE=4 SV=1
70	A0A1D5PAQ0		Immunoglobulin heavy chain OS=Gallus gallus OX=9031 PE=4 SV=1
71	A0A1D5PBP6	CP	Ceruloplasmin OS=Gallus gallus OX=9031 GN=CP PE=3 SV=1
72	Q9W6U9	gp130	Glycoprotein 130 OS=Gallus gallus OX=9031 GN=gp130 PE=2 SV=1
73	E1BZN8	HGFAC	HGF activator OS=Gallus gallus OX=9031 GN=HGFAC PE=3 SV=2
74	A0A1L1RK76	TUBA1A1	Tubulin alpha chain OS=Gallus gallus OX=9031 GN=TUBA1A1 PE=3 SV=1
75	A0A1D5PW77	LOC776376	C-reactive protein OS=Gallus gallus OX=9031 GN=LOC776376 PE=4 SV=1
76	A7UEB0 (+1)	OGCHI	Alpha-1-acid glycoprotein OS=Gallus gallus OX=9031 GN=OGCHI PE=2 SV=1
77	Q90933		Neuron-glia cell adhesion molecule (Ng-CAM) OS=Gallus gallus OX=9031 PE=2 SV=1
78	F1NSC7		Immunoglobulin lambda light chain OS=Gallus gallus OX=9031 PE=4 SV=3
79	F1NHT5	FETUB	Fetuin-b precursor OS=Gallus gallus OX=9031 GN=FETUB PE=4 SV=2

Table 3.1. Continued

Protein #	Accession Number	Gene Name	Identified Proteins
80	F1P4N9	POSTN	Periostin precursor OS=Gallus gallus OX=9031 GN=POSTN PE=4 SV=1
81	P62801 (+1)	H4-I	Histone H4 OS=Gallus gallus OX=9031 GN=H4-I PE=1 SV=2
82	E1C7H6	SERPINF1	Serpin family F member 1 OS=Gallus gallus OX=9031 GN=SERPINF1 PE=3 SV=2
83	A0A1D5PRS4 (+1)	GPI	Glucose-6-phosphate isomerase OS=Gallus gallus OX=9031 GN=GPI PE=3 SV=1
84	P02457	COL1A1	Collagen alpha-1(I) chain OS=Gallus gallus OX=9031 GN=COL1A1 PE=1 SV=3
85	A0A0K0PUH6	RARRES2	Chemerin OS=Gallus gallus OX=9031 GN=RARRES2 PE=2 SV=1
86	F1P4U3	SPP2	Secreted phosphoprotein 24 OS=Gallus gallus OX=9031 GN=SPP2 PE=4 SV=3
87	P00337	LDHB	L-lactate dehydrogenase B chain OS=Gallus gallus OX=9031 GN=LDHB PE=1 SV=3
88	Q9YGP0		Clusterin OS=Gallus gallus OX=9031 PE=2 SV=1
89	E1BSP0	C1QA	Complement C1q A chain OS=Gallus gallus OX=9031 GN=C1QA PE=4 SV=2
90	E1BWI0	DSP	Desmoplakin OS=Gallus gallus OX=9031 GN=DSP PE=4 SV=4
91	A0A1L1RPW4 (+1)	COL12A1	Collagen alpha-1(XII) chain OS=Gallus gallus OX=9031 GN=COL12A1 PE=4 SV=1
92	F1NQ20 (+1)	COL9A1	Collagen alpha-1(IX) chain OS=Gallus gallus OX=9031 GN=COL9A1 PE=4 SV=2
93	E1BWG1	F13A1	Coagulation factor XIII A chain OS=Gallus gallus OX=9031 GN=F13A1 PE=4 SV=2
94	A0A1D5NW85 (+1)	EXFABP	Extracellular fatty acid-binding protein OS=Gallus gallus OX=9031 GN=EXFABP PE=3 SV=1
95	A0A1D5PGB2	COL11A2	Complement C1q C chain OS=Gallus gallus OX=9031 GN=COL11A2 PE=4 SV=1
96	F1N9H4	EEF1A2	Elongation factor 1-alpha OS=Gallus gallus OX=9031 GN=EEF1A2 PE=3 SV=3
97	F1NAR5		Serpin family F member 2 OS=Gallus gallus OX=9031 PE=3 SV=2

Table 3.1. Continued

Protein #	Accession Number	Gene Name	Identified Proteins
98	Q9W6J2	GSTA4L	Glutathione S-transferase class-alpha OS=Gallus gallus OX=9031 GN=GSTA4L PE=2 SV=1
99	A0A1D5P140	SPEN	Spen family transcriptional repressor OS=Gallus gallus OX=9031 GN=SPEN PE=4 SV=1
100	A0A1D5P5L2	F5	Coagulation factor V OS=Gallus gallus OX=9031 GN=F5 PE=3 SV=2
101	A0A346RQZ3 (+2)	HSP70	Heat shock protein 70 OS=Gallus gallus OX=9031 GN=HSP70 PE=3 SV=1
102	F1N9S7 (+1)	ANXA1	Annexin OS=Gallus gallus OX=9031 GN=ANXA1 PE=3 SV=1
103	E1C206	SPIA5	Alpha-1-antitrypsin isoform X1OS=Gallus gallus OX=9031 GN=SPIA5 PE=3 SV=3
104	A0A1L1RNY8	H2AFX	Histone H2A OS=Gallus gallus OX=9031 GN=H2AFX PE=3 SV=1
105	A0A1D5PC92	HIST1H2B5L	Histone H2B OS=Gallus gallus OX=9031 GN=HIST1H2B5L PE=3 SV=1
106	A0A1D5P0Y6 (+1)	DST	Dystonin OS=Gallus gallus OX=9031 GN=DST PE=4 SV=1
107	A0A1D5P9K1		Obscurin, cytoskeletal calmodulin and titin interacting RhoGEF OS=Gallus gallus OX=9031 PE=4 SV=2
108	P11533	DMD	Dystrophin OS=Gallus gallus OX=9031 GN=DMD PE=2 SV=1
109	A0A1D5NW25 (+1)	VPS13D	Vacuolar protein sorting 13 homolog D OS=Gallus gallus OX=9031 GN=VPS13D PE=4 SV=1
110	A0A1D5P1M0	MYH11	Myosin-11 OS=Gallus gallus OX=9031 GN=MYH11 PE=3 SV=1
111	Q90824		Cytotactin 200kD OS=Gallus gallus OX=9031 PE=2 SV=1
112	G1K338	TUBB2B	Tubulin beta chain OS=Gallus gallus OX=9031 GN=TUBB2B PE=3 SV=2
113	A0A1D5NY79	RAB12	RAB12, member RAS oncogene family OS=Gallus gallus OX=9031 GN=RAB12 PE=4 SV=1
114	A0A1D5PC18 (+2)	H3F3C	Histone H3 OS=Gallus gallus OX=9031 GN=H3F3C PE=3 SV=1
115	E1BZI5 (+1)	SYNE2	Spectrin repeat containing nuclear envelope protein 2 OS=Gallus gallus OX=9031 GN=SYNE2 PE=4 SV=3

Table 3.1. Continued

Protein #	Accession Number	Gene Name	Identified Proteins
116	F1NLS8	TTBK1	Tau-tubulin kinase 1 OS=Gallus gallus OX=9031 GN=TTBK1 PE=4 SV=2
117	R4GJN9	ETAA1	ETAA1, ATR kinase activator OS=Gallus gallus OX=9031 GN=ETAA1 PE=4 SV=2
118	P13648	LMNA	Lamin-A OS=Gallus gallus OX=9031 GN=LMNA PE=2 SV=1
119	Q04619	HSP90AB1	Heat shock cognate protein HSP 90-beta OS=Gallus gallus OX=9031 GN=HSP90AB1 PE=2 SV=1
120	A0A1D5PY99 (+2)	NME2	Nucleoside diphosphate kinase OS=Gallus gallus OX=9031 GN=NME2 PE=3 SV=1
121	F1P1U0		Dynein axonemal heavy chain 8 OS=Gallus gallus OX=9031 PE=4 SV=3
122	A0A1D5PEM3 (+1)	SYNE1	Nesprin-1OS=Gallus gallus OX=9031 GN=SYNE1 PE=4 SV=1
123	F1NLZ9		Ryanodine receptor 2 OS=Gallus gallus OX=9031 PE=4 SV=3
124	A0A1D5PDR9 (+1)	DYNC2H1	Dynein cytoplasmic 2 heavy chain 1 OS=Gallus gallus OX=9031 GN=DYNC2H1 PE=4 SV=1
125	A0A1D5PAH0	MYCBP2	MYC binding protein 2, E3 ubiquitin protein ligase OS=Gallus gallus OX=9031 GN=MYCBP2 PE=4 SV=1
126	A0A1D5PX29	UTRN	Utrophin OS=Gallus gallus OX=9031 GN=UTRN PE=4 SV=1
127	F1NKL4	DYNC1H1	Dynein cytoplasmic 1 heavy chain 1 OS=Gallus gallus OX=9031 GN=DYNC1H1 PE=4 SV=3
128	A0A1D5P1G1 (+2)	SASH1	SAM and SH3 domain containing 1 OS=Gallus gallus OX=9031 GN=SASH1 PE=4 SV=1
129	F1NGU3	LRPPRC	Leucine rich pentatricopeptide repeat containing OS=Gallus gallus OX=9031 GN=LRPPRC PE=4 SV=2
130	F1NHM9 (+1)	PGAM1	Phosphoglycerate mutase OS=Gallus gallus OX=9031 GN=PGAM1 PE=3 SV=3
131	P54939 (+2)	TLN1	Talin-1 OS=Gallus gallus OX=9031 GN=TLN1 PE=1 SV=2

Accession Number	Gene Name	T-Test (*p*-value): (*p* < 0.05)	↑↓	Log Fold Change	Identified Proteins	Molecular Weight
A0A1L1RJF5 (+1)	APOA1	0.021	↑	0.7	Apolipoprotein A-I OS=Gallus gallus OX=9031 GN=APOA1 PE=3 SV=1	32kDa
A0A1D5P4L7	TF	0.013	↑	2.2	Ovotransferrin OS=Gallus gallus OX=9031 GN=TF PE=3 SV=1	78kDa
F1P4V1	FGA	0.0011	↓	0.09	Fibrinogen alpha chain OS=Gallus gallus OX=9031 GN=FGA PE=4 SV=2	87kDa
Q90864	HBBA	0.048	↑	0.2	Beta-H globin OS=Gallus gallus OX=9031 GN=HBBA PE=3 SV=1	16kDa
E1BV78	FGG	0.00010	↓	0.06	Fibrinogen gamma chain precursor OS=Gallus gallus OX=9031 GN=FGG PE=4 SV=2	50kDa
F1NUL9 (+1)	FGB	0.00014	↓	0.05	Fibrinogen beta chain OS=Gallus gallus OX=9031 GN=FGB PE=4 SV=2	55kDa
F1NJT4	FN1	0.036	↓	0.8	Fibronectin OS=Gallus gallus OX=9031 GN=FN1 PE=4 SV=3	272kDa
E1C7P4	CFH	0.015	↓	0.6	Complement factor H isoform X1 OS=Gallus gallus OX=9031 GN=CFH PE=4 SV=2	148kDa
F1NPJ8	GPX3	0.02	↓	0.4	Glutathione peroxidase OS=Gallus gallus OX=9031 GN=GPX3 PE=3 SV=2	24kDa
A0A1D5PS90		0.003	↓	0.2	IgGFc-binding protein OS=Gallus gallus OX=9031 PE=4 SV=1	23kDa
A0A1D5PNU2	APOH	0.0078	↑	2.0	Beta-2-glycoprotein 1 precursor OS=Gallus gallus OX=9031 GN=APOH PE=4 SV=1	41kDa
F1NDH2	AGT	0.01	↑	4.4	Angiotensinogen OS=Gallus gallus OX=9031 GN=AGT PE=3 SV=2	51kDa
A0A1D5PLV4 (+3)	VWF	0.00015	↓	0.07	Von Willebrand factor precursor OS=Gallus gallus OX=9031 GN=VWF PE=4 SV=1	306kDa
P0CB50	PRDX1	0.022	↑	16	Peroxiredoxin-1 OS=Gallus gallus OX=9031 GN=PRDX1 PE=1 SV=1	22kDa
Q90940	P22	0.034	↑	6	Calcium-binding protein OS=Gallus gallus OX=9031 GN=P22 PE=2 SV=1	22kDa
A0A1L1RV31 (+2)	F2	0.013	↑	7	Prothrombin OS=Gallus gallus OX=9031 GN=F2 PE=3 SV=1	69kDa
F1NWP1	F9	0.016	↑	0	Coagulation factor IX OS=Gallus gallus OX=9031 GN=F9 PE=3 SV=1	52kDa

Table 3. 2. Differentially expressed proteins found in C-NA relative to CA. The table includes the accession number, gene name, T-test, or p-value (p-value <0.05), the up (↑) and down (↓) arrows (the up arrow (↑) is the upregulated proteins in the C-NA relative to the CA, while the down arrow (↓) is the downregulated proteins in the C-NA relative to the CA), the fold change, the protein name, and the molecular weight.

Table 3. 3.KEGG and Reactome pathways enrichment analysis. Each biological pathway has a KEGG or Reactome ID number, does different functions, and is affected by a number of upregulated and downregulated proteins (count in gene set)

NO.	PATHWAY ID	PATHWAY NAME	BIOLOGICAL PROCESS OR MAJOR FUNCTIONS	COUNT IN GENE SET
Upregulated pathways				
1	R-GGA-114608	Hemostasis	Platelet degranulation	3
2	R-GGA-159763	Metabolism of proteins	Transport of gamma-carboxylated protein precursors from the endoplasmic to the Gologi apparatus	2
3	R-GGA-159782	Metabolism of proteins	Removal of amino terminal peptides from gamma-carboxylated proteins	2
4	R-GGA-140837	Hemostasis	Intrinsic pathway of fibrin clot formation	2
Downregulated pathways				
1	Gga04512	ECM-receptor interaction		2
2	Gga04510	Focal adhesion		2
3	R-GGA-354194	Hemostasis	GRB2: SOS provides linkage to MAPK signaling for integrins	5
4	R-GGA-354194	Singal transduction	GRB2: SOS provides linkage to MAPK signaling for integrins	5
5	R-GGA-372708	Hemostasis	P 130Cas linkage to MAPK signaling for integrins	5
6	R-GGA-372708	Singal transduction	P 130Cas linkage to MAPK signaling for integrins	5
7	R-GGA-354192	Hemostasis	Integrin signaling	5
8	R-GGA-354192	Singal transduction	Integrin signaling	5
9	R-GGA-216083	Extracellular matrix interactions	Integrin cell surface interactions	5
10	R-GGA-114608	Hemostasis	Platelet degranulation	5
11	R-GGA-140875	Hemostasis	Common pathway of fibrin clot formation	3

Table 3. 4.Free fatty acids of broiler plasma from NC-NA, C-NA, and CA. Values with the red color show significantly changed free fatty acids in C-NA, relative to CA ($p<0.05$). The up arrow (↑) is the upregulated fatty acids in the C-NA relative to the CA, while the down arrow (↓) is the downregulated fatty acids in the C-NA relative to the CA. NC means no significant changes

Retention time (RT) (min)	***p*-value (NC-NA vs C-NA)**	***p*-value (C-NA vs CA)**	**Free fatty acids (FFA)**	**Change trend (↑↓)**
				C-NA vs CA
6.5	0.32	0.21	Palmitic acid (C16:0)	NC
6.7	0.16	0.18	Palmitoleic acid (C16:1)	NC
7.9	0.53	0.02	Stearic acid (C18:0)	↑
8.2	0.82	0.03	Oleic acid (C18:1)	↑
8.7	0.39	0.02	Linoleic acid (C18:2)	↓
11.3	0.77	0.85		NC

Abbreviation: NC-NA= non challenge / no ascites, C-NA= challenge without ascites, CA= challenge with ascites, FC= no significant change.

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Table 3. 5.Possible peak lists with their mass to charge ratio (m/z) and corresponding $[M+H]^+$ peak for 13 lipids. Values with the red color show significant differentiation of the listed lipids ($p<0.05$). The up arrow (↑) is the upregulated lipids in the C-NA relative to the CA, while the down arrow (↓) is the downregulated lipids in the NC-NA vs. C-NA and C-NA vs. CA

Retention time (RT) (min)	m/z	*p*-values (NC-NA vs C-NA)	FC (NC-NA vs C-NA)	*p*-values (C-NA vs CA)	FC (C-NA vs CA	Lipids	Change trend (↑↓)	
							NC-NA vs C-NA	C-NA vs CA
22.483	758.573	0.26	3.65	0.006	0.41	PC (34:2) $[M+H]^+$	NC	↑
23.9	812.614	0.13	3.17	0.004	0.39	PC (38:3) $[M+H]^+$	NC	↑
22.525	816.622	0.045	3.32	0.005	0.31	PC (38:1) $[M+H]^+$	NC	↑
22.4	780.557	0.48	3.29	0.010	0.30	PC (36:5) $[M+H]^+$	NC	↑
22.519	784.589	0.22	3.29	0.005	0.35	PC (36:3) $[M+H]^+$	NC	↑
23.312	786.606	0.40	3.72	0.04	0.51	PC (36:2) $[M+H]^+$	NC	↑
23.323	798.545	0.40	2.6	0.015	1.06	PC (36:4; O) $[M+H]^+$	NC	↓
23.489	844.654	0.17	3.60	0.02	0.48	PC (40:1) $[M+H]^+$	NC	↑
23.708	824.599	0.36	2.63	0.03	0.98	??	NC	↓
24.522	788.618	0.017	3.05	0.023	0.48	PC (36:1) $[M+H]^+$	↓	↑
24.616	846.674	0.011	2.86	0.016	0.39	PC (40:0) $[M+H]^+$	↓	↑
26.8	623.621	1.53	0.17	0.006	0.23	CE 16:1 $[M+H]^+$	NC	↑
33.947	723.64	0.28	2.67	0.001	0.34	??	NC	↑

Table 3. 6.List of integrating enrichment analysis pathways of fatty acids and lipids using MetaboAnlyst 5.0.

Pathway name	Match status		*P*-value	-log (P)	FDR	Impact	Pathway KEGG ID
	In set	In background					
Linoleic acid metabolism	2	4	1.98E-4	3.92	0.017	1.0	gga00591
Biosynthesis of unsaturated fatty acids	3	36	4.62E-4	3.33	0.019	0.0	gga01040
Primary bile acid biosynthesis	2	46	0.018	1.72	0.52	0.082	gga00120
Alpha-linolenic acid metabolism	1	13	0.061	1.21	1.0	0.0	gga00592
Arachidonic acid metabolism	1	32	0.145	0.83	1.0	0.0	gga00590
Glycerophospholipids metabolism	1	35	0.15	0.80	1.0	0.085	gga00564
Steroid biosynthesis	1	42	0.18	0.72	1.0	0.0	gga00100

Abbreviation: FDR= false discovery rate.

Table 3. 7.List of significantly changed metabolites (C-NA vs. CA) with their retention time (RT), molecular mass to charge ratio (m/z), p-value or t-test, the fold change (FC), the compound name and formula, the identification number (HMDB ID, KEGG ID, BioCyc ID, catalog number, LM ID). Values with the red color show significant differentiation of the listed small molecules ($p<0.05$). The up arrow (↑) is the upregulated small molecules in C-NA relative to CA, while the down arrow (↓) is the downregulated small molecules in C-NA vs. CA.

Retention time (RT) (min)	m/z	*p*-values (NC-NA vs C-NA)	FC (NC-NA vs C-NA)	*p*-values (C-NA vs CA)	FC (C-NA vs CA)	Small molecules (name+ formula)	Compound	Change trend (↑↓) (C-NA vs CA)
14.981	857.367	0.061	0.240	0.016	4.010	Ubiquitin	Gene ID: 395796	↓
26.744	263.088	0.102	0.514	0.017	2.477	L-aspartyl-L-glutamate (C9H12N2O7)	CPD-20353	↓
26.751	369.171	0.112	0.572	0.025	2.101	Dehydroepiandrosterone sulfate (C19H28O5S)	C04555	↓
35.927	478.303	0.002	1.601	2.68E-06	0.268	PE (18:2(9Z,12Z)/0:0) (C23H44NO7P)	LMGP02050011	↑
36.205	520.339	0.075	1.496	0.015	0.513	PC (18:2(9Z,12Z) \/0:0) (C26H50NO7P)	LMGP01050035	↑
38.832	496.339	0.001	1.215	1.549E-05	0.708	PC (0:0/16:0) (C24H50NO7P)	LMGP01050074	↑
39.075	522.361	0.102	1.617	0.033	0.494	PC (0:0/18:1(6Z)) (C26H52NO7P)	LMGP01050079	↑
45.319	482.323	0.007	1.465	7.45E-06	0.330	PC (14:0/O-1:0) (C23H48NO7P)	LMGP01080020	↑
58.239	621.311	0.789	1.079	0.046	0.520	LysoPI(20:4(5Z,8Z,11Z,14Z)/0:0) (C27H51O12)	HMDB0061690	↑

Table 3. 8.List of integrating enrichment analysis pathways of small molecules using MetaboAnlyst 5.0.

Pathway name	Match status		*p*-value	-log (P)	FDR	Impact	Pathway KEGG ID
	In set	In background					
Glycerophospholipids metabolism	1	35	0.047	1.32	1.0	0.031	gga00564
Steroid hormone biosynthesis	1	71	0.09	01.01	1.0	0.0	gga00140

Table 3. 9.Significant information related to the small molecule (ubiquitin) using KEGG, UniProt, and David Functional Annotation Bioinformatics Microarray Analysis.

Pathway ID	Pathway name	Count in network	FDR	Functional annotation linked to biological process
gga03010	Ribosome	10	1.15e-18	DNA repair
				Modification-dependent protein catabolic process
				Protein ubiquitination
				Translation

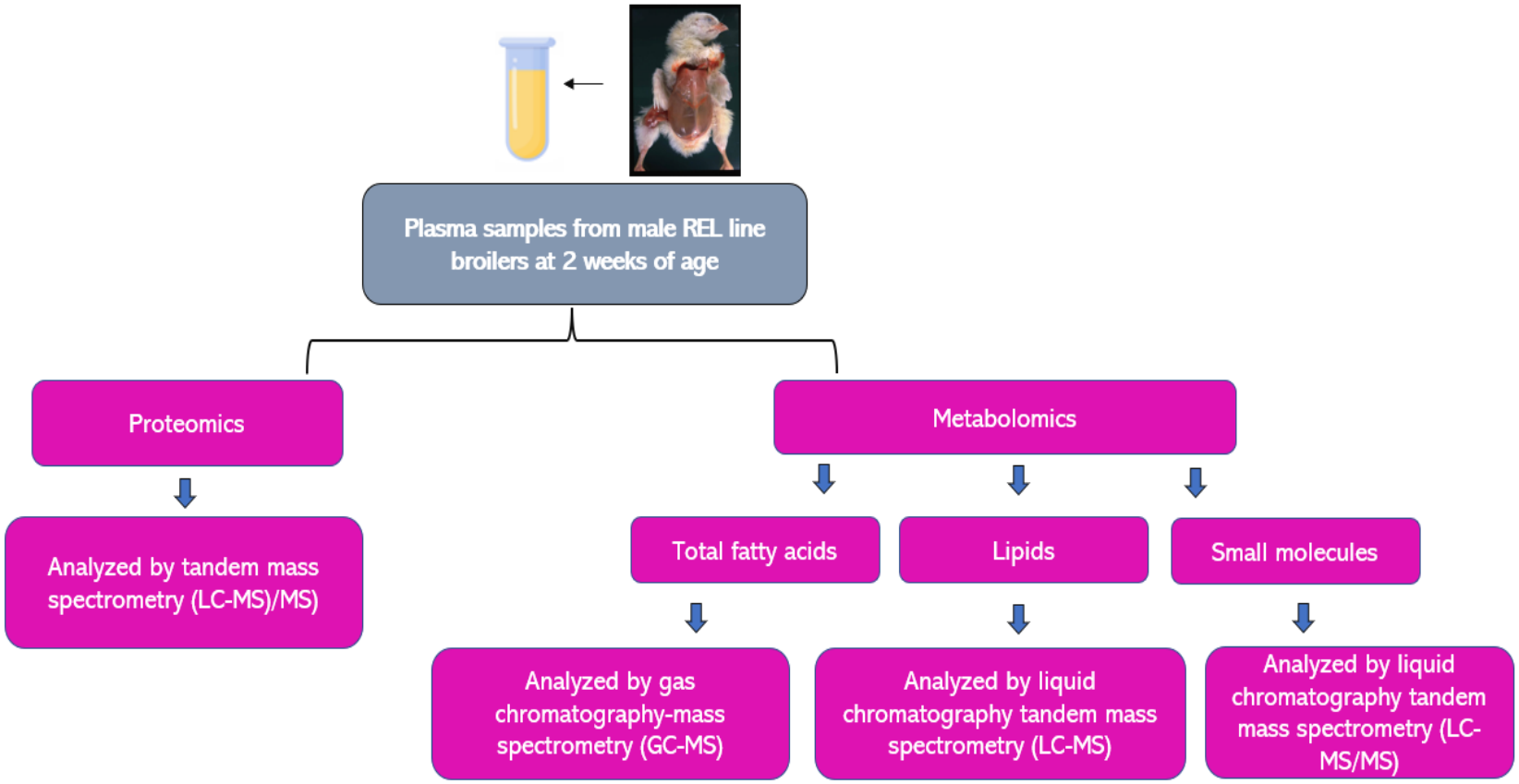


Figure 3. 1.Flow chart of the experimental design. A total of 27 birds at 2 weeks of age were used in this study. The plasma samples were collected from three different groups (NC-NA, C-NA, CA) for proteomic and metabolomic studies. Proteins, total fatty acids, lipids, and small molecules were extracted and submitted for mass spectrometry analyses.

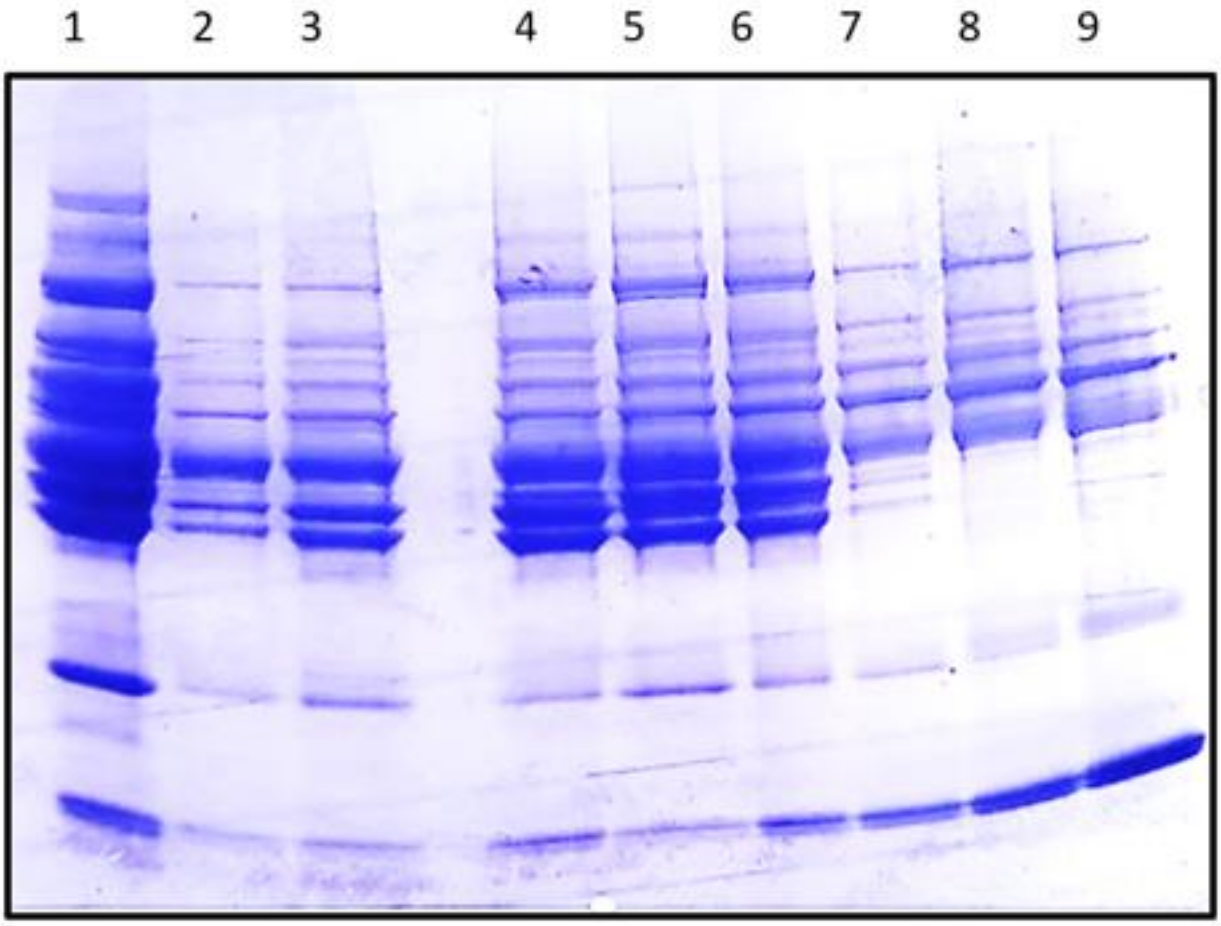
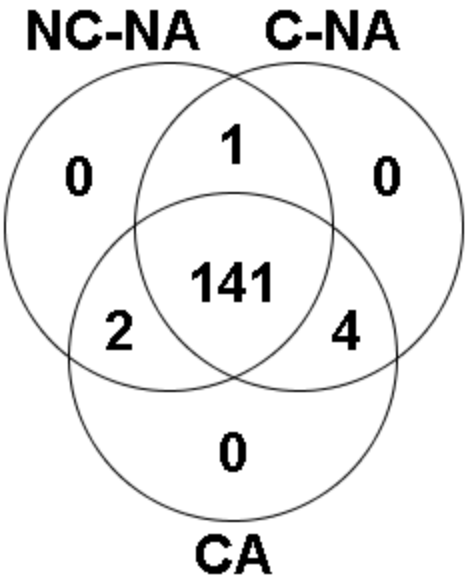


Figure 3. 2.SDS-PAGE analysis for fast-growing broiler proteins. Lanes 1,2, and 3 are protein samples from the NC-NA group, lanes 4,5, and 6 are protein samples from the C-NA group, and lanes 7, 8, and 9 are protein samples from the CA group.

A.



B.

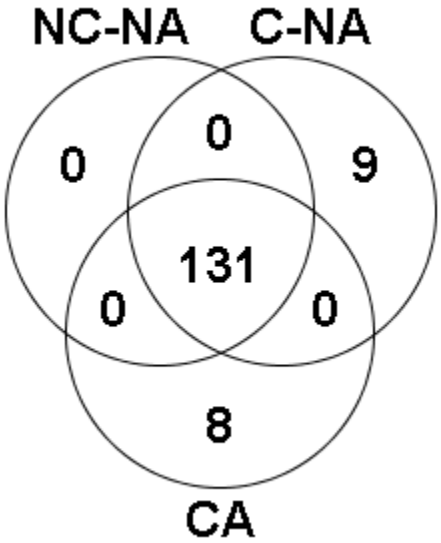


Figure 3. 3.**Venn diagrams illustrate the common and differentially expressed proteins in the three major groups (NC-NA, C-NA, CA);** (A) is the individual proteins based on the presence and absence; (B) is the individual proteins based on the quantitative profile.

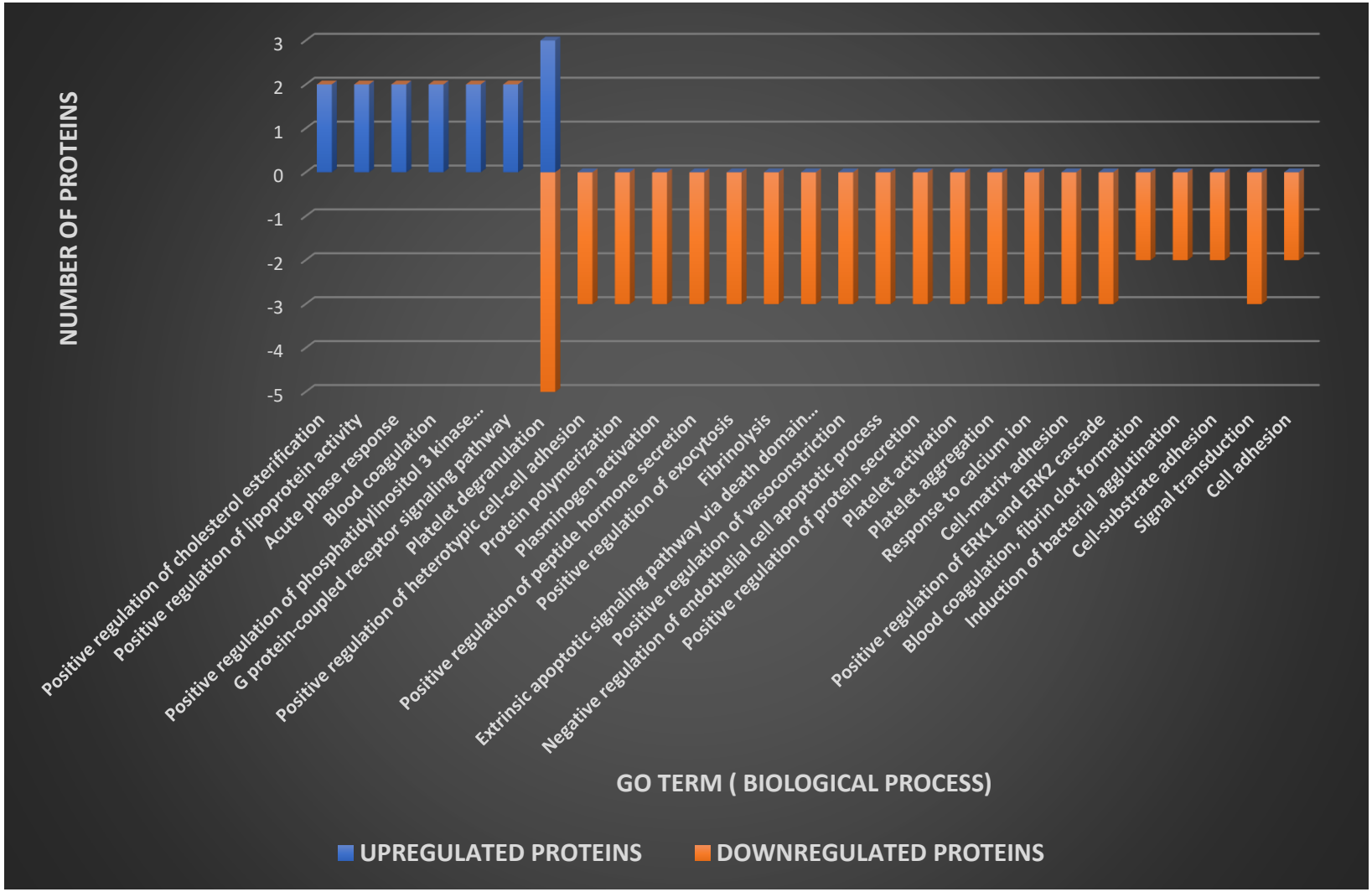


Figure 3. 4.Upregulated and downregulated proteins in different biological processes contribute to ascites induction in fast-growing broilers.

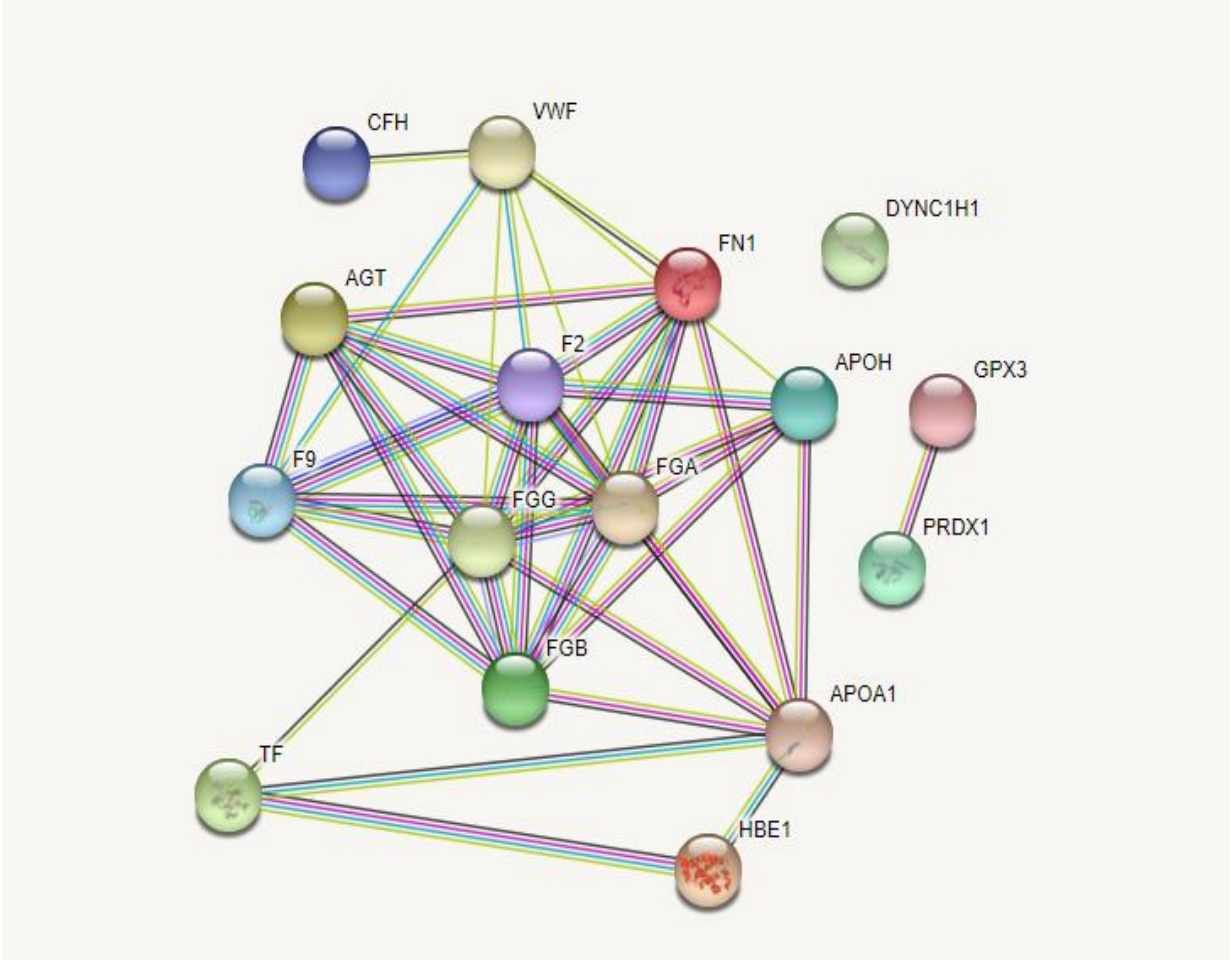


Figure 3.5.The protein-protein interactions (the interaction between upregulated and downregulated proteins) using STRING.

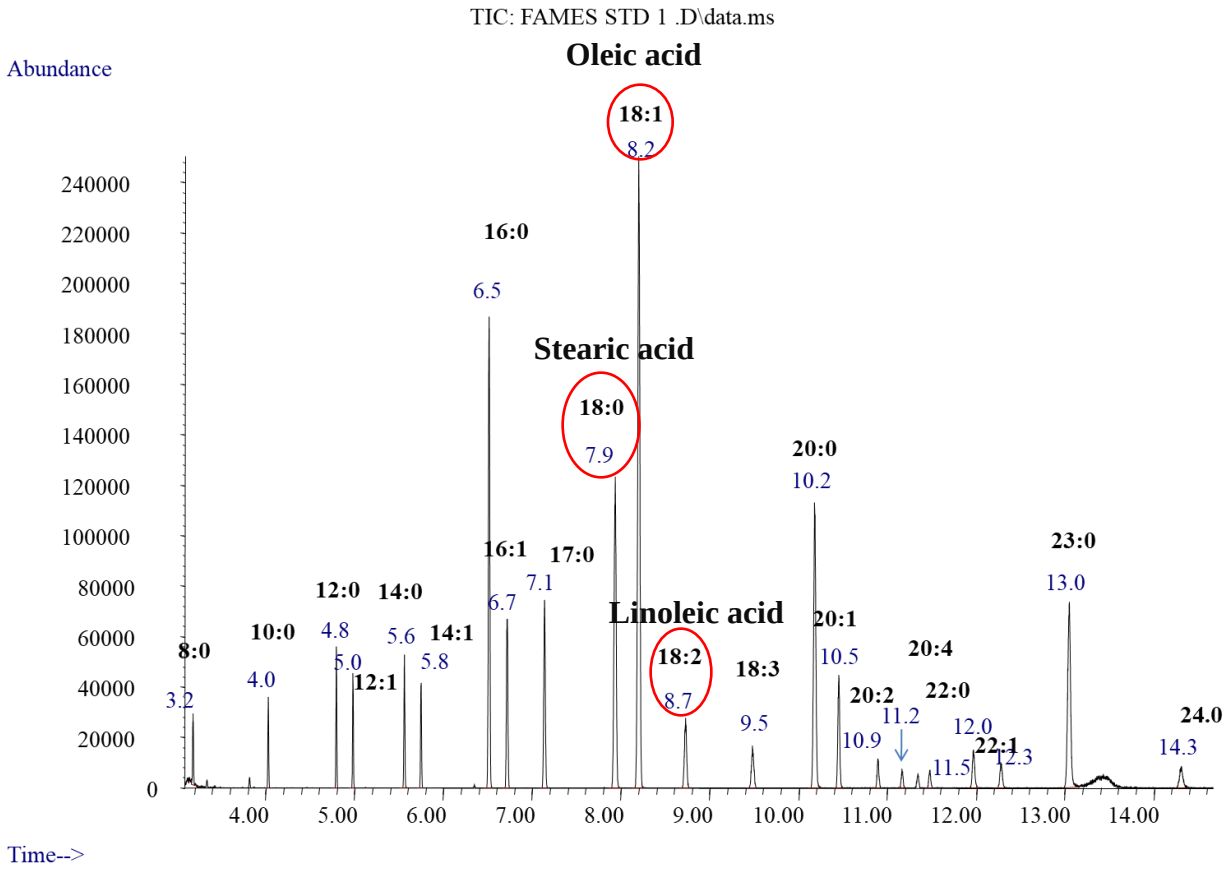


Figure 3. 6.GC-MS total ion analysis of plasma free fatty acids (FFAs) from NC-NA, C-NA, and CA groups. Each peak corresponds to specific fatty acids. Three FFAs show significant differentiation (p-value <0.05) in C-NA vs. CA but not in NC-NA vs. C-NA (red circle).

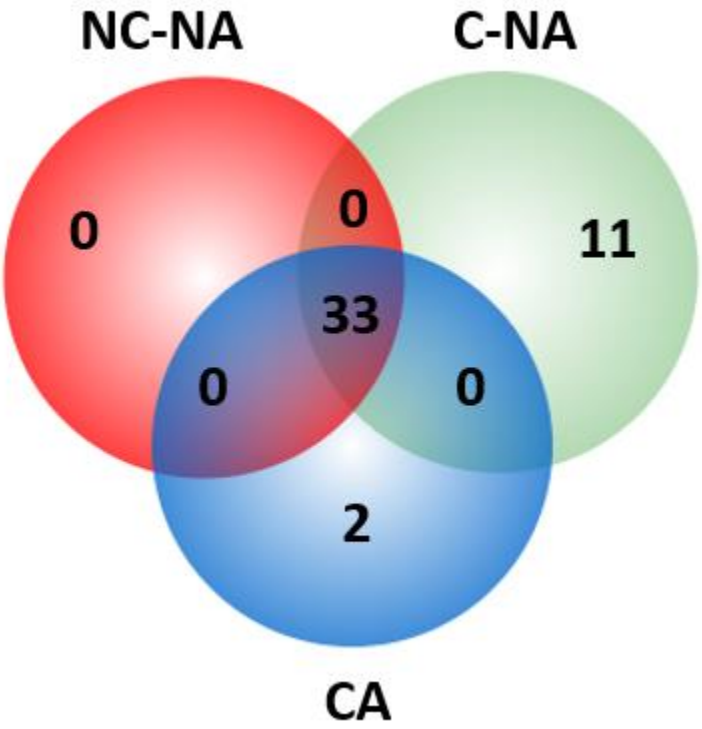


Figure 3. 7.Venn diagram shows the common and significantly changed lipids (based on the quantitative profile).

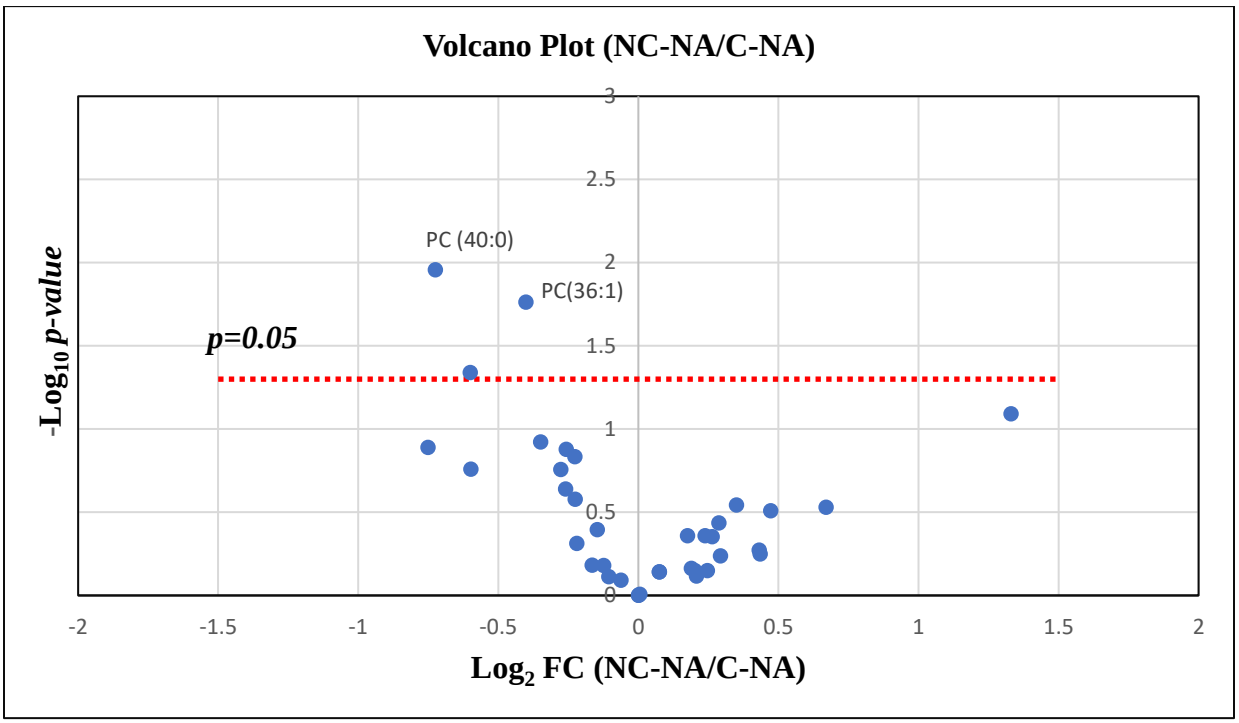


Figure 3. 8.The volcano plot shows the lipid compositions of plasma samples from NC-NA vs. C-NA. The ratio of intensity for peaks from NC-NA and C-NA samples were plotted in the x-axis [log2(NC-NA/C-NA)]. The y axis represents the negative logarithm "base 10" of the p-value or t-test. The data points with highly significant differences (low p-values) represent above the red horizontal line ($p<0.05$). Upregulated lipids are on the positive (right) side of the red horizontal line, while downregulated lipids are on the negative (left) side of the red horizontal line.

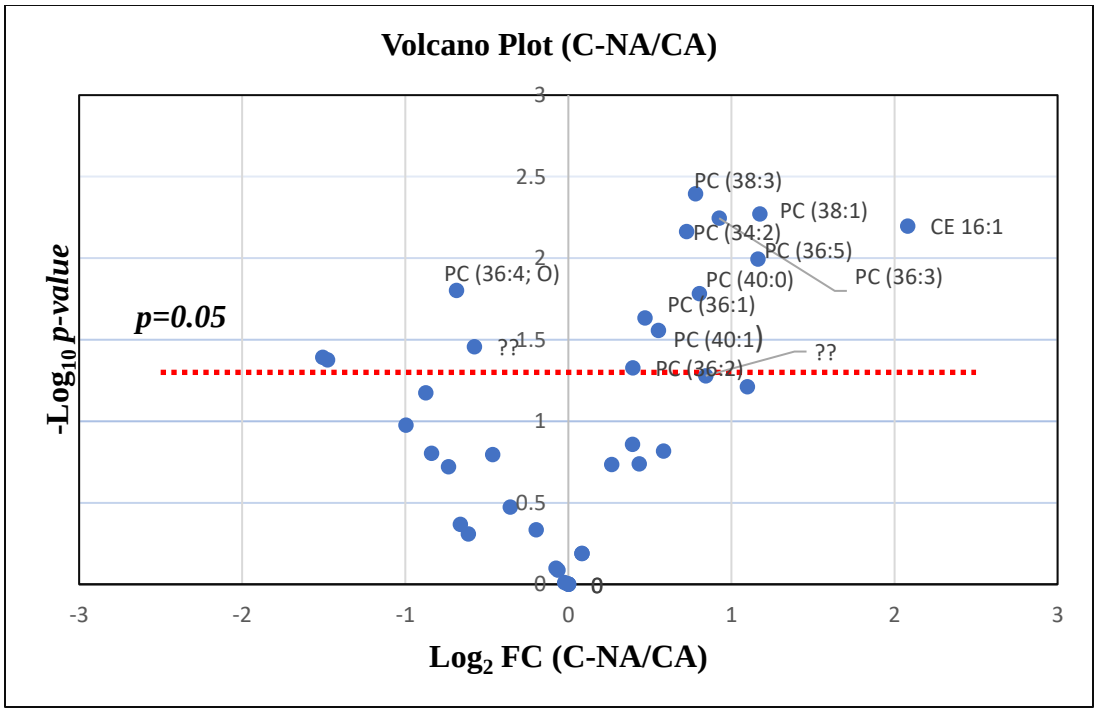


Figure 3. 9.The volcano plot shows the lipid compositions of plasma samples from C-NA vs. CA. The ratio of intensity for peaks from C-NA and CA samples were plotted in the x-axis [log2(C-NA/CA)]. The y axis represents the negative logarithm "base 10" of the p-value or t-test. The data points with highly significant differences (low p- values) represent above the red horizontal line ($p<0.05$). Upregulated lipids are on the positive (right) side of the red horizontal line, while downregulated lipids are on the negative (left) side of the red horizontal line.

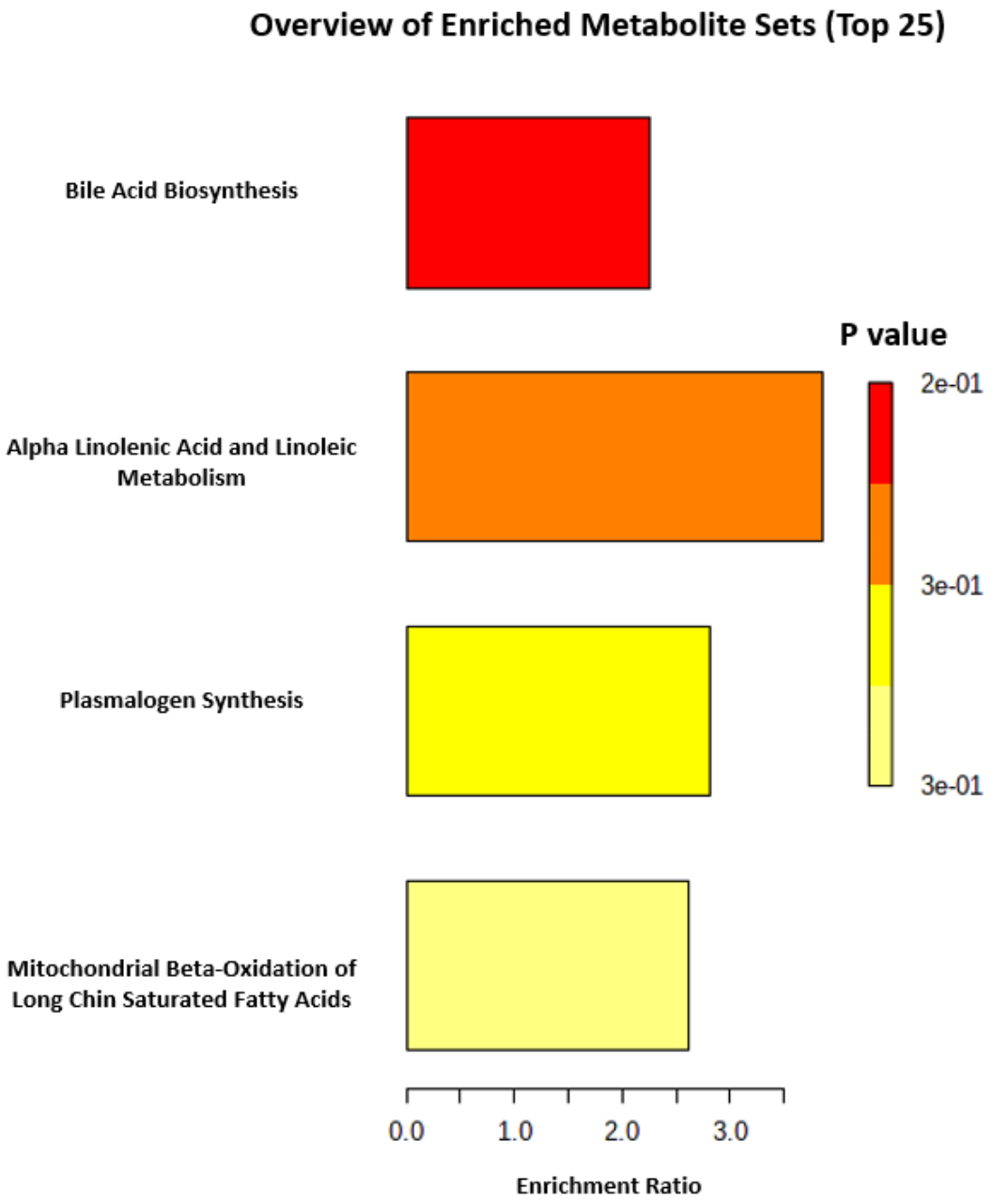


Figure 3. 10.The overview of metabolic pathways which induced ascites or pulmonary hypertension based on SMPDB. The metabolic analysis was performed using downregulated and upregulated fatty acids and lipids.

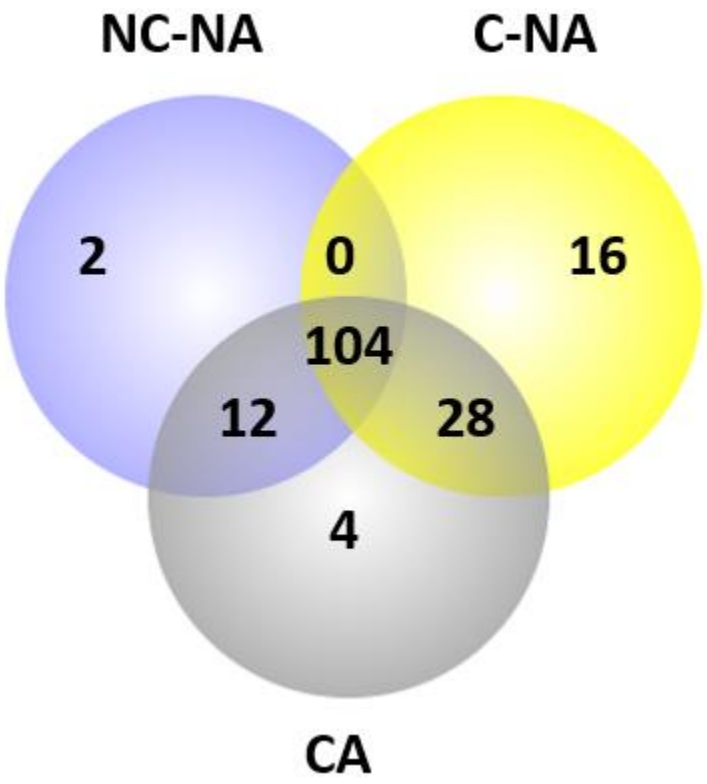


Figure 3.11. The Venn diagram shows the common and significantly different small molecules based on the quantitative profile.

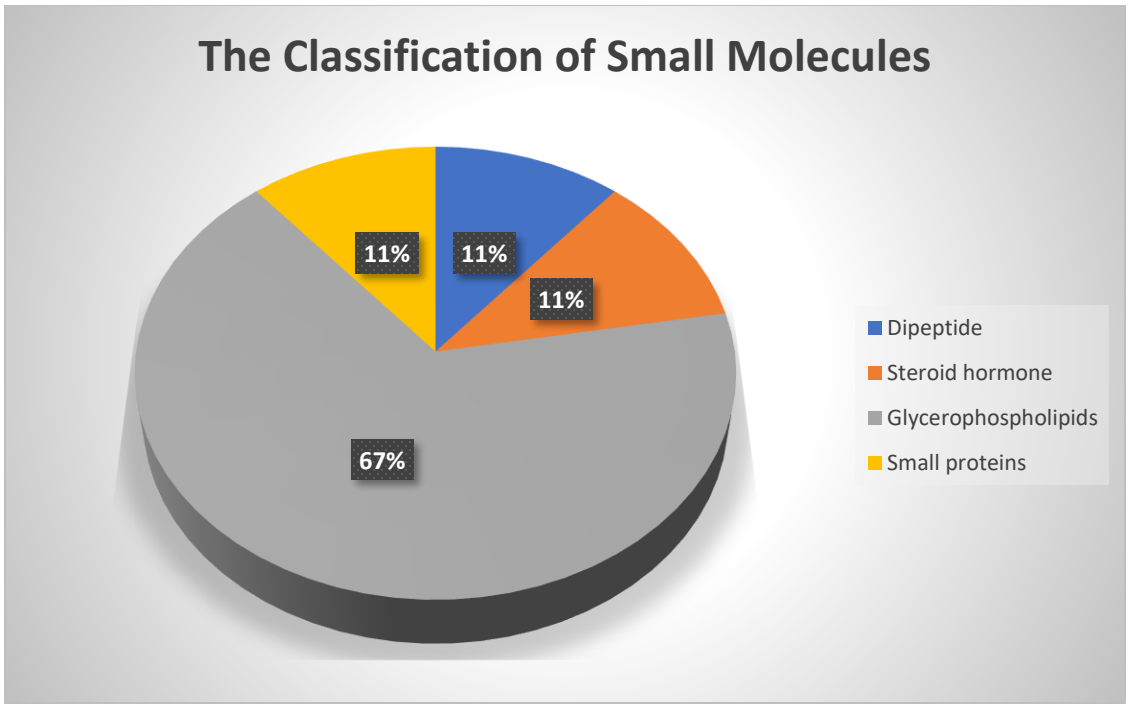


Figure 3. 12.A pie chart shows different classifications of small molecules.

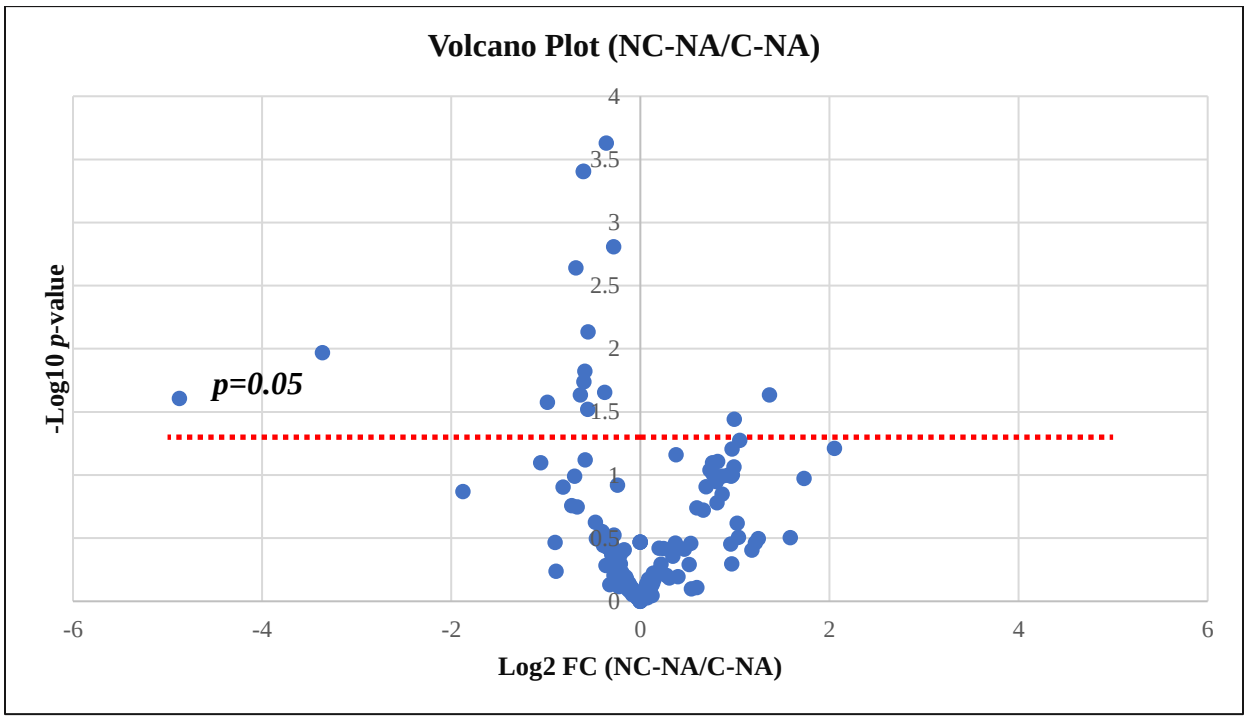


Figure 3. 13.The volcano plot shows the small molecules in plasma samples from NC-NA vs. C-NA. The ratio of intensity for peaks from NC-NA and C-NA samples were plotted in the x-axis [log2(NC-NA/C-NA)]. The y axis represents the negative logarithm "base 10" of the p-value or t-test. The data points with highly significant differences (low p-values) represent above the red horizontal line ($p<0.05$). Upregulated small molecules are on the positive (right) side of the red horizontal line, while downregulated small molecules are on the negative (left) side of the red horizontal line.

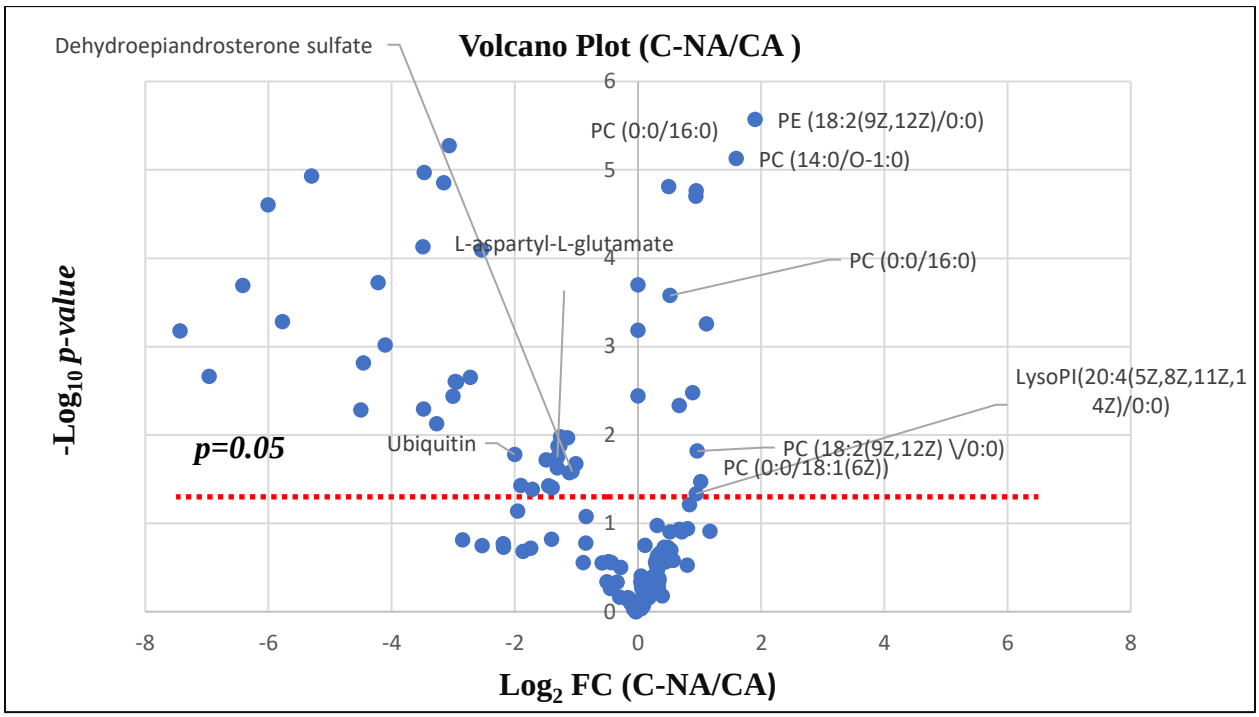


Figure 3. 14.The volcano plot shows small molecules in plasma samples from C-NA vs. CA. The ratio of intensity for peaks from C-NA and CA samples were plotted in the x-axis [log2(C-NA/CA)]. The y axis represents the negative logarithm "base 10" of the p-value or t-test. The data points with highly significant differences (low p-values) represent above the red horizontal line ($p<0.05$). Upregulated small molecules are on the positive (right) side of the red horizontal line, while downregulated small molecules are on the negative (left) side of the red horizontal line.

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CHAPTER 4

Summary & Conclusion

Poultry meat is a valuable food because it is rich in high nutritional value. It is a good source of digestible proteins with a minimal level of collagen (Kralik et al., 2018). The poultry meat is a good source of moderate energy content and contains unsaturated lipids, minerals such as," zinc, copper, and iron," and essential vitamins such as "pantothenic acid, vitamin B 6, and thiamin". Several epidemiological studies showed a strong association between good health and consumption of poultry meat since it has reduced the development of obesity and overweight, type 2 diabetes, and cardiovascular diseases (Marangoni et al., 2015; Richi et al., 2015). In the 1990s, chickens were genetically selected in both Europe and the USA for particular traits such as eggs and meat production (Elfick, 2012). Selective breeding helps study the mechanism of tissue growth, lowering the time and reducing the cost of raising each flock (Tallentire et al., 2016). In the early stage of the genetic selection, geneticists were able to change the birds' phenotypes, reduce the mortality rate, and control diseases that affect the poultry (Flock et al., 2005).

There are different metabolic diseases associated with rapid growth and genetic selections which have existed in the poultry industry for more than 30 years. Examples of these are fatty liver and kidney syndrome, skeleton diseases, and ascites or pulmonary hypertension (Leshchinsky & Klasing, 2001). Ascites syndrome or pulmonary hypertension syndrome is a consequence of genetic selection for higher body mass (Smith et al., 1954). Broilers with ascites syndrome are characterized by having a big belly due to the accumulation of fluid in the abdominal cavity. It is a serious issue in the poultry industry because the mortality rate of broilers was 30%, and the economic loss was estimated to be $100 annually (Pavlidis et al., 2007).

In 1994, Dr.Anthony and his colleagues (at the University of Arkansas, Department of Poultry Science) initiated the selection program for ascites development. Essentially, chickens

from an elite commercial line were raised in a hypobaric chamber to mimic the high altitude (9000ft above sea level). The hypobaric chamber was provided with a partial vacuum to decrease the partial oxygen pressure and induce ascites. Susceptible to ascites (SUS line) and resistant to ascites (RES line) is the main genetic lines that were developed for further studies (Balog et al., 2000; Tellez et al., 2005; Pavlidis et al., 2007).

In our lab, several studies were performed to examine the genetic basis of the ascites phenotype. To begin, a second-generation (F2) was generated through double reciprocal crosses of RES and SUS lines. Then, a genome-wide SNP was performed to find chromosomal regions associated with PHS susceptibility. The statistical analysis illustrated a relationship between a region on chromes 9 (12 to 13Mbp) and ascites phenotype in both experimental lines (RES and SUS) and the commercial line. The susceptibility to ascites was gender-specific because females were more affected than males. Several candidate genes were involved in this region, such as urotensin 2 domain (UTS2D), an angiotensin II type 1 receptor (AGTR1), and Serotonin Receptor/Transporter type 2B (5HT2B) (Burks et al., 2011; Wideman et al., 2013; Krishnamoorthy et al., 2014). Another genome-wide association study was conducted by Dey et al. (2017) and illustrated a significant relationship between chromosome 9 (11.8 to 13.6Mbp) and ascites. Within this region, two candidate genes (UTS2D and AGTR1) were identified earlier and confirmed by Dey et al. (2017) to be associated with ascites. Another subsequent genome-wide association study was done on unselected relax lines at 16 and 18 generations. Two significant regions on chromosome Z and chromosome 2 were found to be relevant to ascites (Tarrant et al., 2017). A whole-genome resequencing study was conducted by our research group, which identified 31 regions associated with ascites. One of which is the carboxypeptidase Q (CPQ) gene on

chromosome 2 (127.62 to 127.75 Mbp) (Dey et al., 2018). This gene has been used in marker-assisted selection studies (manuscript under review).

Chapter 2 summarizes the CPQ gene expression data of relaxed unselected line, where we measured the gene expression in eight tissues (heart, liver, kidney, thigh, breast, spleen, lung, thymus) using RT-qPCR. Then, the gene expression data were normalized to the reference gene TATA-box binding protein (TBP) gene. We found that CPQ gene expression level was significantly increased in the liver of homozygous non-ref males. However, there are no significant differences in the CPQ expression level in other tissues from homozygous non-ref males and all tissues from homozygous non-ref females. We could not measure the gene expression of heterozygous samples. Therefore, further analysis was done on previous next-generation sequencing data to determine if there is a copy number variation or a chromosomal deletion affecting the gene expression. Based on reading depth analysis of our next-generation sequence data, some broilers carry a CPQ gene deletion spanning from intron 4 to ~40 kbp downstream of exon 8. The size of the deletion was estimated to be ~125Kbp. A TaqMan assay was designed to genotype the birds for the deletion and investigate the association of this deletion with the ascites phenotype. PCR primers were developed to span the deleted region allowing us to fine map the exact limits of the deletion, which appear to have resulted from the recombination of two CR1 repeats. This chromosomal deletion is still a candidate region for future study of relevance to ascites or pulmonary hypertension in broilers. Our new findings give significant hints to recognizing the mechanisms that underlie this deletion and affect the ascites phenotypes.

Chapter 3 summarizes the mass spectrometry data, where we analyzed the proteomic and metabolomic changes for 18 male birds (6 no challenged with no ascites “NC-NA” vs. 6 challenged with no ascites “C-NA” vs. 6 challenged with ascites “CA”). The major aim of this chapter is to

identify noninvasive biomarkers for early ascites detection. The *p*-values less than 0.05 from the student T-test were used to determine the significantly changed proteins/metabolites. We were able to identify eight upregulated proteins (apolipoprotein A-I “APOA1”, ovotransferrin “TF”, hemoglobin subunit beta “HBBA”, beta-2-glycoprotein 1 precursor “a protein induced by APOH gene”, angiotensinogen “AGT”, peroxiredoxin-1 “PRDX1”, calcium-binding protein “P22”, prothrombin “F2”, coagulation factor IX “F9”) and nine downregulated proteins (fibrinogen alpha chain “FGA”, fibrinogen gamma chain precursor “FGG”, fibrinogen beta chain “FGB”, fibronectin “FN1”, IgG Fc-binding protein “FcγBP”, complement factor H isoform X1 “produced by CFH gene”, glutathione peroxidase “GPX3”, von Willebrand factor precursor “VWF”) in C-NA vs. CA with the onset of ascites. The level of some fatty acids (stearic acid “C18:0”, oleic acid “C18:1”, linoleic acid “C18:2”) was upregulated in C-NA vs. CA with respect to ascites. We could identify a large number of lipids and small molecules which were upregulated in C-NA vs. CA in response to ascites. Examples of the upregulated lipids are phosphatidylcholine “PC” (34:2), PC (38:3), PC (38:1), PC (36:5), PC (36:3), PC (36:2), PC (40:1), PC (36:1), PC (40:0), and cholesterol ester “CE” (16:1). Examples of small molecules are PC (18:2(9Z,12Z) \/0:0), PC (0:0/18:1(6Z), LysoPI (20:4(5Z,8Z,11Z,14Z)/0:0), PC (0:0/16:0), phosphatidylethanolamine “PE” (18:2(9Z,12Z)/0:0), and PC (14:0/O-1:0). Furthermore, PC (36:4; O) was the only lipid that was downregulated with the onset of ascites. Three small molecules (ubiquitin, L-aspartyl-L-glutamate, dehydroepiandrosterone) were downregulated in C-NA vs. CA. Most of the identified proteins/metabolites are candidate biomarkers that explain how ascites evolve in broiler chickens.

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CHAPTER 5

Appendix

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Nicholas Anthony

FROM: Craig N. Coon, Chairman

DATE: Apr 3, 2015

SUBJECT: IACUC Approval

Expiration Date: Apr 5, 2018

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

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

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

CNC/aem

cc: Animal Welfare Veterinarian

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199
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UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Nicholas Anthony

FROM: Craig N. Coon, Chairman

DATE: Apr 8, 2015

SUBJECT: IACUC Approval

Expiration Date: Apr 8, 2018

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

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

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

CNC/aem

cc: Animal Welfare Veterinarian

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
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DIVISION OF AGRICULTURE
RESEARCH & EXTENSION
University of Arkansas System

To: Sara Orlowski
Fr: Billy Hargis - Ag-IACUC Chair
Date: January 15th, 2021
Subject: IACUC Approval
Expiration Date: January 14th, 2024

The Division of Agriculture Institutional Animal Care and Use Committee (Ag-IACUC) has APPROVED your protocol # 21087 *General Rearing of Selected Chicken and Quail Populations*.

In granting its approval, the Ag-IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the Ag-IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond January 14th, 2024 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy, the Ag-IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Sara Orlowski, Joseph Haltz, Travis Tabler, Maricela Maqueda, Craig Maynard, Clay Maynard, Garrett Mullenix, Sami Dridi, Liz Greene, Alison Ferver, Nima Emami, Duaa Almansaf, Layla Almitib, Alyssa Papineau, Ehsan Sheiksamani, Joshua Lyte, and Kirsten Shafer. Please submit personnel additions to this protocol via the modification form prior to their start of work.

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

BMH/tmp

21087

DIVISION OF AGRICULTURE
RESEARCH & EXTENSION
University of Arkansas System

To: Sara Orlowski
Fr: Billy Hargis - Ag-IACUC Chair
Date: January 15th, 2021
Subject: IACUC Approval
Expiration Date: January 14th, 2024

The Division of Agriculture Institutional Animal Care and Use Committee (Ag-IACUC) has APPROVED your protocol # 21088 *Utilization of Hypobaric Hypoxia to Induce Ascites in Broiler Chickens*.

In granting its approval, the Ag-IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the Ag-IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond January 14th, 2024 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy, the Ag-IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Sara Orlowski, Joseph Hiltz, Travis Tabler, Maricela Maqueda, Kirsten Shafer, Sami Dridi, Liz Greene, Duaa Almansaf, Layla Almitib, Alyssa Papineau, and Ehsan Sheiksamani. Please submit personnel additions to this protocol via the modification form prior to their start of work.

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

BMH/tmp

21088