

**TRANSCRIPTOME AND HISTOLOGIC ANALYSIS  
OF CARDIAC DIFFERENCES BETWEEN  
MODERN BROILER AND HERITAGE CHICKENS**

by

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Animal Science

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## **ABSTRACT**

This project focuses on characterizing differentially expressed genes in the left ventricle of the heart, comparing the Heritage chicken line, selectively bred for meat production until the 1950's, and Ross 708, a modern fast-growing broiler line, during normal post-hatch growth at 6, 21, and 42 days of age. Since Ross 708 broilers have a reduced normalized heart size and increased susceptibility to heart failure compared to Heritage birds, this research may offer insight into the pathological consequences of selection for rapid growth on the heart, and may further explain the development of left ventricular enlargement that is seen in the modern broiler. Most differences in gene expression are observed at 21 days post hatch, in genes related to collagen fiber remodeling in the extracellular matrix, inflammatory cytokines, and expression of developmental proteins in Ross 708 compared to Heritage. This may indicate a mechanism for coping with pressure or volume overload due to the increased body size and slower rate of growth in the heart in Ross 708. Histologic analysis of cardiac tissue between groups shows that there is no significant increase in myofiber diameter between 21 and 42 days post hatch within the Ross 708 line, but average myofiber diameter is larger in Heritage than Ross 708 at 28 and 42 days post hatch. Some lesions are observed in the myocardium when transverse sections of heart stained with hematoxylin and eosin are analyzed, but there was no evidence of ascites or heart failure in birds from either line. Slightly thickened interstitial collagen is observed in the

Ross 708 line at 21, 28 and 42 DPH compared to the Heritage line when transverse sections of the myocardium are compared with a trichrome stain.

## **Chapter 1**

### **INTRODUCTION**

Following a peaks in mortality of commercial broiler flocks at 3-5 days post hatch (DPH) due to stress from maladjustment and starvation, there is a gradual increase in mortality from 30 DPH until harvest due to myocardial infarction, ascites, and lameness from multifactorial leg weakness [1], [2]. The cause of heart failure around 40 DPH is thought to be related to the increased growth rate and body mass of modern broilers [3]. As modern broilers age, their heart size relative to body weight tends to decrease when compared to Heritage chickens of the same age [4]. Although Ross 708 birds gain body mass faster overall, they only gain 5mg of heart tissue per gram of body weight, whereas Heritage chickens gain 7mg of heart tissue per gram of body weight. The larger body size and smaller heart of the Ross 708 bird puts additional biomechanical stress on the heart, and may lead to myocardial hypertrophy, extracellular matrix remodeling, and heart failure from increased workload on the heart [5–7].

Blood enters the right atrium from systemic circulation through the anterior and posterior vena cava, and is then ejected into the right ventricle, which contracts to pump blood to the lungs [8]. Simultaneously, blood enters the left atrium from the lungs and is ejected into the left ventricle, which contracts to pump blood into systemic circulation via the aorta. In chickens, end stage heart failure usually manifests as a result of ascites, a metabolic disorder in which pulmonary hypertension and right ventricular heart failure causes a buildup of fluid in the lungs with

subsequent leakage into the coelomic cavity [9]. This increase in pressure also prevents the tricuspid valve, located between the right atrium and ventricle, from closing completely, which can in turn increase pressure in the veins, and liver, thereby further increasing leakage of fluid into the coelomic cavity [10], [11]. Endocardiosis can also distort atrio-ventricular valves in broiler chickens afflicted with ascites and cause insufficient closure, regurgitant blood flow, and result in pulmonary hypertension [12], [13]. Gross pathology and histopathology shows that both ascites-afflicted and unaffected broiler chickens exhibit enlargement of the left ventricular wall at 7-10 DPH [14].

The pathology of chronic heart failure (CHF) has been studied in mammals, including mice and humans [6], [15–18]. The first phase of the disease usually involves a trigger, such as myocardial infarction, biomechanical stress, or abnormalities due to genetic defects (congenital heart disease) [19]. Broiler chickens are specifically known to have a high incidence of arrhythmias [20], abnormalities in the extracellular matrix proteins from genetic mutations [21], hypoxemia due to reduced blood flow efficiency and low pressure in capillaries [22], and ascites [5].

Because multiple factors play a role in the maladaptive response of the heart to long-term stress, studies have been carried out to uncover the mechanism of myocardial remodeling and fibrosis in animal models [23–25]. Connective tissue growth factor (CTGF) and other accompanying pathways and proteins have a function in the response to cardiac overload in mice, rats, and rabbits [26]. Basal expression of CTGF is maintained for developmental processes, while its upregulation tends to cause cardiac hypertrophy [27] and induction of Akt signaling for physiologic growth [25], [28]. Cardiac myocytes are terminally differentiated at hatch, and do not increase



in number with age through mitosis, but rather increase in size due to hypertrophy in response to mechanical stimulus such as pressure or volume overload or exercise [29], [30] through mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase/ protein kinase B (PI3K/ Akt) signaling [31]. Cardiomyocyte hypertrophy is achieved through the addition of contractile units in one of two ways: in parallel (thickening), which is classified as concentric, or in series (elongating), which is eccentric [32], [33]. Concentric hypertrophy decreases the volume of the ventricular chambers in response to high blood pressure. Eccentric hypertrophy increases the volume of the ventricular chamber to increase cardiac output in response to high blood volume [32], [29].

Cardiac hypertrophy also involves changes in molecular characteristics of cardiac cells. Cytoskeletal proteins in the contractile units interact with cell adhesion molecules, both to anchor sarcomeres to the ends of cardiomyofibers and to transmit signals to stimulate hypertrophy under stress [34]. The hypertrophic process also involves the release of cytokines, increased growth factor signaling, apoptotic signals, and expression of developmental genes that are normally repressed in the mature heart [35]. Chronic stress induced by pressure or volume overload can cause an increase in activity from fibroblasts, resulting in interstitial fibrosis [6], [22]. In chronic heart failure, fibroblastic activity can either be the result of myocardial degeneration (reparative fibrosis) or a potential cause for degeneration (interstitial fibrosis) [41]. Prolonged upregulation of CTGF gives rise to a fibrotic response, such as when it is coupled with the upregulation of transforming growth factor beta (TGF $\beta$ ) [24]. This profibrotic response causes extracellular matrix remodeling, proliferation of

fibroblasts, and increased deposition of extracellular matrix, which can stiffen the walls of the heart leading to poor contractility and eventual failure [42].

By using transcriptome sequencing (RNAseq), differences in gene expression levels can be easily compared between the Ross 708 and Heritage lines. A transcriptome encompasses all RNA transcribed from a particular genome, and is used jointly with next-generation sequencing (NGS) to further develop the field of transcriptomics [43]. Once a library of short sequences has been synthesized, they can be sequenced and mapped to a reference genome where any subsequent reads are counted [44]. The result is raw data that is converted to RPKM (reads per kilobase per million mapped reads) values in order to measure gene expression levels. RPKM is used to quantify gene expression, and is derived by normalizing raw sequencing data using gene length and total number of reads [45]. Data can be analyzed using a number of software packages to obtain results.

Histology is the study of microscopic characteristics of tissues. In routine histologic analysis, tissues are first fixed in formalin to preserve its structure and then dehydrated and embedded in paraffin wax to create tissue blocks [46]. Following sectioning using a microtome, tissue sections are affixed to microscope slides and stained to allow differentiation between cells based upon staining characteristics such as color and staining intensity. The most common stains utilized are hematoxylin and eosin (H&E), which allows the researcher to distinguish tissue lesions, such as inflammation, characterized by hypercellular loci, and tissue degeneration, characterized by hypereosinophilia [47]. Special stains, such as Masson's trichrome (MT) can be used to distinguish collagen in connective tissues from muscle fibers or epithelial tissues [46].

## **Chapter 2**

### **HYPOTHESIS**

Given disproportionately reduced heart growth to rapid body growth observed in Ross 708 broilers, we predict that resultant cardiac stress will manifest as increased expression of genes involved in hypertrophy, ECM organization, collagen deposition, and sarcomere activity in transcriptome comparison with the Heritage line.

Histologically, it is expected that Ross 708 will show evidence of hypertrophy (thicker cardiomyofibers), a higher incidence of myocardial ECM remodeling, and more interstitial collagen as compared to Heritage birds.

## **Chapter 3**

### **MATERIALS AND METHODS**

#### **Bird Growout**

Heritage line eggs were obtained from Chet Utterback of the University of Illinois at Urbana- Champaign, and Ross 708 eggs were retrieved from Mountaire Farms in Millsboro, Delaware. The eggs were incubated at 37°C until hatch, where females were culled in order to simplify the experimental design and reduce sex-specific effects. The chicks were placed into large colony houses warmed to 33°C, and the temperature was reduced by 3°C each week until the house temperature reached 24°C at 21 DPH. The birds were given food and water ad libitum. The Agricultural Animal Care and Use Committee Approval form for the experiment that contains this procedure can be found in Appendix A.

#### **Tissue Collection**

At 6, 21, and 42 DPH, Ross 708 and Heritage were necropsied and left ventricular heart tissue was collected for subsequent RNA extraction. Tissue was immediately flash frozen in liquid nitrogen and stored at -80°C to maintain RNA integrity. Heart tissue used for histologic analysis was collected during a second necropsy at 21, 28, and 42 DPH and preserved in 10% neutral-buffered formalin. Tissue collection was part of a larger study, so age of the birds at the time of necropsy differs due to availability. A layout of when each bird was grown and what sample was collected can be found in Appendix B.

### **RNA Extraction and QC**

Total RNA from left ventricular tissue was extracted using a Qiagen RNeasy fibrous Tissue Kit. RNA quality was assessed using the RNA Integrity Number (RIN) provided by the RNA Nano Lab Chip run on the Agilent 2100 Bioanalyzer. Only RNA with a RIN above 9 was used to prepare indexed libraries for high throughput transcriptome sequencing.

### **Transcriptome Library Synthesis and QC**

RNA seq libraries were prepared according the manufacturer's instructions with the Illumina TruSeq RNA Prep Kit. Molecular concentrations of each library were obtained using the Qubit broad range double stranded DNA assay and diluted to 10uM. The indexed libraries were pooled sent to the DBI Core Sequencing Facility (Newark, DE) where they were sequenced on the Illumina G2 and RPKM values were calculated using the ERANGE software. Results were uploaded to the Big Bird database for retrieval.

A total of 28 transcriptome libraries were used in this study. Fourteen transcriptome libraries were synthesized for both the Ross 708 and Heritage lines. For 6DPH, three libraries were used for each bird line, at 21 DPH, six were used for each bird line, and at 42DPH five were used for each bird line.

### **Transcriptome Library Analysis**

RPKM data were downloaded from the database and all statistical analyses were done using the JMP 10 software. Differential expression was calculated using the following formula:

$$\log_2 \left( \frac{\text{Ross RPKM Avg}}{\text{Heritage RPKM Avg}} \right)$$

RPKM values for genes with a 1.5-fold difference ( $\log_2$  above 0.585 for Ross 708 and below -0.585 for Heritage) were clustered separately for each treatment group and bird type. In BioLayout, the minimum correlation coefficient was set to 0.95 (Pearson's Coefficient, R, 0.7 min). The correlation coefficient minimum was lowered to 0.85 in order to compensate for a lack of enriched clusters at 0.95 and 0.9 minimum coefficients when necessary.

Clustered genes were evaluated using the Database for Annotation, Visualization and Integrated Discovery (DAVID) to determine ontological terms, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapper to find enriched pathways. Groups with redundant and least descriptive gene ontology (GO) terms were removed using a Perl script and the resulting list was paired with bird type for comparative study. This analysis focuses on differences between bird types and differences in development between time points.

### **Histology Slide Preparation and Analysis**

Heart specimens were trimmed, processed, sectioned and stained with H&E and MT by Joanne M. Kramer of the College of Agriculture and Natural Resources Comparative Pathology Lab at the University of Delaware (Newark, DE). Slides were analyzed for inflammation and myofiber degeneration by the author with subsequent review by Dr. Erin Brannick, a certified veterinary anatomic pathologist of the College of Agriculture and Natural Resources at the University of Delaware.

Photomicrographs were captured with a Nikon DS-Fi2 camera and myofiber diameter was measured manually using NIS Elements software. Five measurements

were taken from one field of view for each slide, in fields devoid of visible myofiber degeneration or inflammation. The T-test in JMP10 was used to determine if there was a difference in average myofiber diameter across the Ross 708 and Heritage lines at 21, 28, and 42 days post hatch, and within the Ross 708 and Heritage lines between 21 and 42 DPH.

A total of 66 slides were analyzed, 33 with H&E, and 33 with MT. Twelve slides were analyzed for samples collected at 21DPH, which included six Ross 708 and six Heritage line birds. Twelve slides were analyzed for samples collected at 28DPH, seven from the Ross 708 line and five from the Heritage line. Nine slides were analyzed from samples collected at 42DPH, six from the Ross 708 line and three from the Heritage line.

## Chapter 4

### TRANSCRIPTOME ANALYSIS: 6 DAYS POST HATCH

Table 1 Differentially expressed genes and their respective functional categories between Ross 708 and Heritage at 6 days post hatch.

Heritage		Ross 708	
Functional Category	Genes	Functional Category	Genes
Chaperone	HSPA8, HSP90AA1	Angiogenesis	RSF, ELK, POFUT1
		Apoptosis	BID, ASNS, BIRC5, GCLM, SOX9, TRAF3, SGK1, RHOB
		Immunoglobulin	IL18R1, CD86, BTN1A1, LRRN1, CD4, PTPRU, TAPBPL, NEGR1

### Heritage

Table 1 contains functional categories and genes within each key category from differentially expressed genes in Ross 708 and Heritage at 6DPH. When comparing differentially expressed gene lists from D6 Heritage to D6 Ross, BioLayout clustering results were inconclusive because of low enrichment scores from DAVID at 0.95, 0.90 and 0.85 minimum clustering coefficients. When a non-manipulated list of genes with 1.5-fold expression difference was analyzed with DAVID, functional annotation clusters were muscle and chaperone activity, due to HSP70 and HSP90. These HSPs are present at intermediate levels in heart tissue, and there is no significant fluctuation in expression in the instance of heart failure or thermal stress [48]. HSP90 forms complexes with actin to promote polymerization [49], and HSP70



regulates cardiac hypertrophy by increasing histone deacetylase 2 (HDAC2) activity [50], [51].

### **Ross 708**

The comparison of differentially expressed gene lists between D6 Ross to D6 Heritage, yields developmental activity such as hematopoiesis and angiogenesis, both of which could be a response to increase in oxygen demand that arises from normal heart growth and development [52]. The presence of a response to DNA damage is most likely due to double-stranded breaks during mitotic division of cells.

The functional category apoptosis includes genes ASNS, BIRC5, GCLM, SGK1, TRAF3, BID, RHOB. Asparagine synthetase (ASNS) has an inhibitory effect on apoptosis, and when highly upregulated, causes the suppression of c-Jun N Terminal Kinase (JNK or MAPK8) signaling, which further aides in the suppression of the apoptotic response [53]. Along with ASNS, baculoviral IAP repeat containing 5 (BIRC5) [54], glutamate-cysteine ligase (GCLM) [55] and serum and glucocorticoid-responsive kinase (SGK1) [56], all have roles in inhibiting apoptosis. SGK1 also activates potassium, sodium, and chloride channels [57], and interacts with PDK-1, a protein which indirectly regulates the phosphatidylinositol 3 kinase/ protein kinase B (PI3K/ Akt) pathway, which promotes hypertrophic growth of cardiac myocytes [58].

In contrast, Tumor necrosis factor receptor-associated factor 3 (TRAF3) [59] and BH3 interacting domain death agonist (BID3), are cleaved by caspase to induce cardiomyocyte apoptosis [60–62]. This protein is a member of the B-cell lymphoma 2 (Bcl-2) family, which induces apoptosis when triggered by intrinsic signals, such as growth factors, hypoxia, DNA damage, or oxidative stress [61]. It is suggested that mechanical factors regulate apoptosis in the neonatal heart, which can be due to

normal structural remodeling during growth [63]. Ras homolog gene family, member B (RhoB) also induces apoptosis as shown by its role in modulating cell death in neoplastic cells carrying unrepaired double-stranded DNA breaks [64].

Immunoglobulin activity related to differentiation of myeloid cells can be attributed to resident immune cells in the heart and extramedullary hematopoiesis (EMH), which can be present in virtually any tissue in birds, but especially in the heart[9]. Antigen-presenting lymphocytes, B and T cells, are mediated by clusters of differentiation (CD86, CD4) [39]. Cluster of differentiation 86 (CD86) is a membrane protein that has two isoforms, the first of which can act as a T-cell activator, and the second acting as a negative regulator of activation [65]. CD4 is expressed in T cells, B cells, granulocytes, and macrophages and may also regulate T cell activation [66].

## Chapter 5

### TRANSCRIPTOME ANALYSIS: 21 DAYS POST HATCH

Table 2      Differentially expressed genes and their respective functional categories between Ross 708 and Heritage.at 21 days post hatch.

Heritage		Ross 708	
Functional Category	Genes	Functional Category	Genes
Chaperone	HSPA8, HSP90AA1	pro-inflammatory/ pro-apoptotic	TNAIP6, TNFAIP8L3, TNFRSF8, TNFRSF6B, TNFRSF13B, C1QTNF8, C1QTNF9, IL1R1
		Regulation of apoptosis	SOX9, TBX3, BCL6, TGFB, GLI3
		Proteinaceous extracellular matrix	OGN, LAMB4, WNT5B, LUM, ACAN, WNT11, POSTN, EPYC, COL11A1, COL4A6, COL4A5, ANXA2
		Cell-cell adhesion	PCDH1, LOC768740, ACAN, SOX9, COL11A1, CDH5, TGFB2
		MAPK signaling	EVI1, MAPK4K4, IL1R1, PDGFB, MAPK12, RPS6KA2, MAPKAPK3, PP3CA, MAPK10, AKT3, TGFB2, RASA2
		Embryonic morphogenesis	TBX3, PRRX1, GLI3
		VEGF signaling	MAPK12, MAPKAPK3, PP3CA, PIK3R3, AKT3
		ErbB signaling	MAPK10, PIK3R3, AKT3

## **Heritage**

Table 2 contains functional categories and genes within key categories from differentially expressed genes in Ross 708 and Heritage at 21DPH. Enriched terms in Heritage at this stage are far fewer in number than the Ross 708 counterpart. Similar to 6dph, Heritage at 21dph show activity related to HSP70 and HSP90 chaperones.

## **Ross 708**

Proinflammatory and pro-apoptotic cytokines (TNFAIP6, TNFRSF8, TNFAIP8L3, TNFRSF6B, C1QTNF9, TNFRSF13B, C1QTNF8) and interleukin receptor 1 (IL1R1) can be differentially regulated as a response to chronic heart failure [67], [68]. TNFAIP6 is known to interact with inter- $\alpha$ -inhibitor (I- $\alpha$ -I) and enhance serine protease activity of I- $\alpha$ -I, which is involved in the protease network associated with inflammation [69]. When prolonged, inflammatory response in the heart leads to oxidative stress, reduced vasodilation and increased apoptosis [70].

IL1R1 recognizes IL1A and IL1B, and has a role in increasing matrix metalloproteinase expression [71]. IL1B confers a maladaptive response in reaction to cardiac stress when cardiac inflammation is prolonged, along with regulating genes in immune-related pathways such as apoptosis, interleukin, TGF $\beta$ , and PI3K signaling [72]. Apoptosis includes activity from TBX3, BCL6, SOX9, TGF $\beta$ , and GLI3. TBX3 negatively regulates apoptosis [73], and inhibition of apoptosis is achieved by BCL6 through transcription repression [74], [75], and SOX9 through activation of PIK3/Akt signaling [76].

Extracellular matrix organization is controlled by TGF $\beta$ 2 and POSTN. A target gene of TGF $\beta$ 2, periostin (POSTN) is an extracellular matrix adhesion protein [77], that also has a role in heart valve development, cardiomyopathy [78], and hypertrophy

[37]. It can be significantly upregulated in response to mechanical stretch and pro-fibrotic TGF $\beta$  signaling in cases of pressure overload [37], while a decrease can cause changes in extra-cellular matrix that cause structural defects in heart valves [79]. POSTN is known to interact with TGF $\beta$  and is unique to cardiac fibroblasts [80]. Increased activity of TGF $\beta$  typically triggers SMAD signaling, which has a role in promoting fibrosis and left ventricular hypertrophy [81].

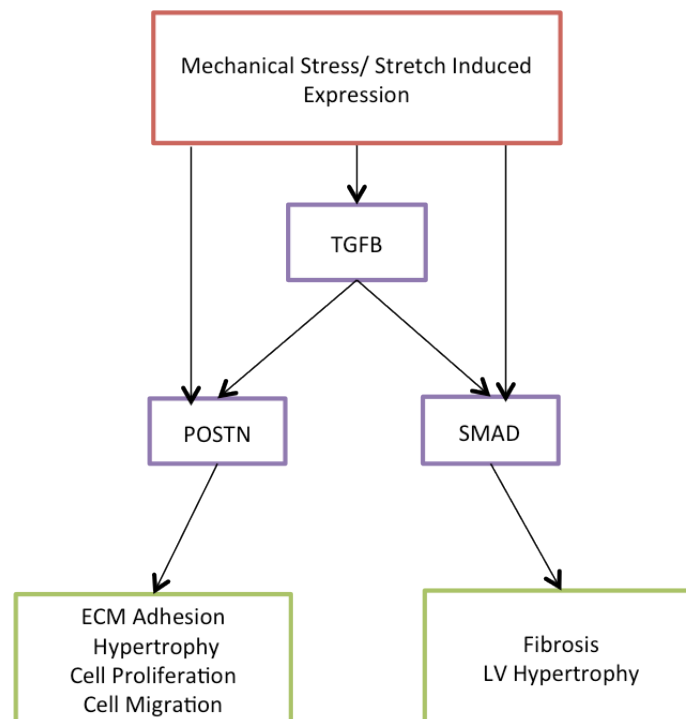


Figure 1 The interactions, causes and effects of TGF $\beta$  upregulation in Ross 708 at 21 days post hatch.

There is an increase in collagen fibril gene expression (COL11A1, COL4A5, COL4A6, ACAN, EPYC, LAMB4, PDGF $\beta$ ), cell adhesion (PCDH1, LOC768740,

ACAN, COL11A1, CDH5, TGF $\beta$ 2) and the proteinaceous extracellular matrix (OGN, LAMB4, WNT5B, LUM, ACAN, WNT11, POSTN, EPYC, COL11A1, COL4A6, COL4A5, ANXA2). Laminin (LAMB4) and type IV collagens (COL4A5, COL4A6) are basement membrane components [82], [83]. Cell proliferation, adhesion, and migration are all results of increased collagen deposition and play a key role in developmental processes in the extracellular matrix via fibroblastic activity [84]. Extracellular matrix protein turnover may be contributing to the enrichment of terms pertaining to zinc and calcium metalloproteinases, which includes collagenase [85], [58]. Collagen fiber formation and turnover in the heart is normal under developmental circumstances and usually occurs at a slow rate [85], but upregulation can be the product of cardiac stress or injury [86–88]. Vascular- endothelial cadherin (CDH5), expressed by vascular endothelial cells, is integral to proper vascular development by organizing adherens junctions and resisting apoptotic signals through vascular endothelial growth factor (VEGF) signaling [89], [90].

Cardiac fibroblasts, which deposit and arrange collagen into matrices, are usually not actively dividing or depositing collagen fibrils into the extracellular matrix in the mature heart [32], [84], and the rate of collagen synthesis is usually similar to degradation [88]. The proliferation of these fibroblasts involves MAPK activity (MAPK12, MAPK10), and interaction of growth factors with cell surface receptors [40], [91], [92].

MAPK12 is a stress- activated protein kinase. This protein is a member of the p38 MAPK group, which responds to cellular stressors such as proinflammatory cytokines or physical stress [93]. The activity of p38 MAPKs is positively correlated with concentric cardiac hypertrophy in response to pressure overload [94]. MAPK10

is a paralog of MAPK12, and is also known as JNK3. The JNK family of MAPK proteins has a role in regulating apoptosis [95].

Both the ErbB (PIK3R3, AKT3, MAPK10) and the VEGF (MAPK12, MAPKAPK3, PPP3CA, PIK3R3, AKT3), signaling pathways are enriched, which interact with the PI3k/AKT signaling pathway [96], [97]. The ErbB signaling pathway mediates cell-to-cell interactions in the heart through phosphorylation [98]. PIK3R3 encodes for p55 $\gamma$ , a regulatory subunit in class I $\alpha$  of the PI3K family, which is known to have a role in cell growth, proliferation, differentiation, motility, survival and intercellular trafficking [99], [100]. Upregulation of the p55 $\gamma$  subunit indicates that the pathway is being activated for physiologic or adaptive reasons [101].

PI3K/Akt signaling can also be activated in the failing heart, such as in the case of ischemia or pressure overload-induced hypertrophy. Activation of Akt signaling after ischemic injury prevents apoptosis of cardiomyocytes [102] and improves function in surviving cardiomyocytes [103]. Ribosomal s6 kinase 2 (RPS6KA2) promotes cell survival by inducing PI3K/Akt signaling [104], and can also activate members of the MAPK family [105].

AKT3 is part of the serine/ threonine kinase family, which regulates the cellular response to insulin levels, glucose availability, and growth factors [106]. High feed intake or low insulin levels can cause hyperglycemia, which increases s6 kinase 1 activity, which controls PI3K activity through inhibitory phosphorylation [107], [108]. By extension, PI3K signaling can be activated by a decrease in glucose availability by preventing inhibitory phosphorylation by s6 kinase 1.

Hypertrophy induced by PI3k/Akt signaling usually results in non-altered dimensions (mixed hypertrophy) rather than concentric or eccentric hypertrophy alone

[109]. Myosin heavy chain, (MYH1-3 and 6-8) myosin binding protein, myosin light chain 1, myosin light chain kinase 2, and myosin 1B, 3B, 5A, M3, Z1 and Z3 are differentially expressed in Ross 708 compared to Heritage, which is also indicative of hypertrophy [110], [111]. G-Protein Coupled Receptors (GPCR) (GNAZ, GPR39, GPR123, GNG10, PPYR1, FZD3, CALCRL, GPR17, HTR1D, and GPR153) respond to extracellular stimuli such as pro-inflammatory signals and hemostatic processes, and also activate PI3K signaling [112].

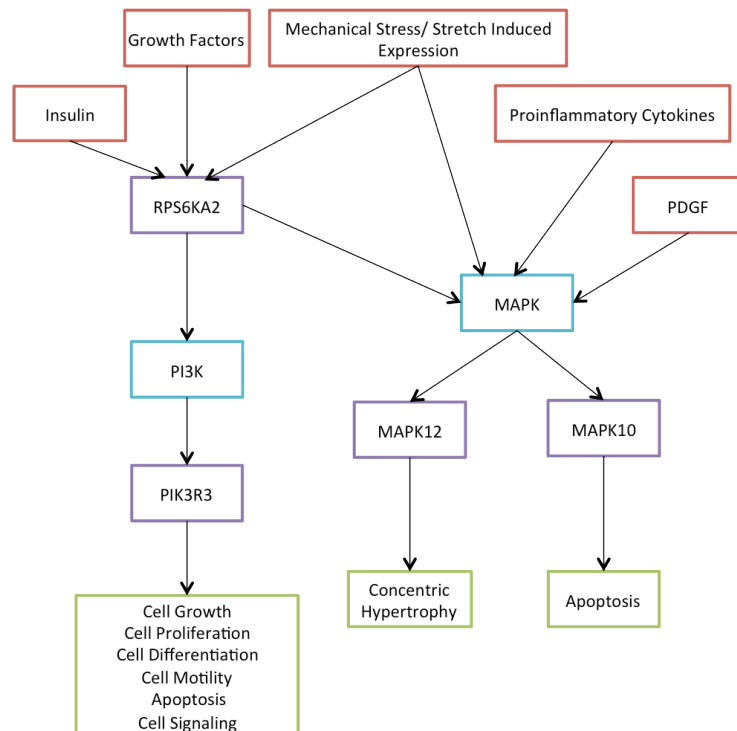


Figure 2 Interactions, causes and effects of the upregulation of p38 and JNK MAP kinases and the members of the PI3K signaling pathway in Ross 708 at 21 days post hatch.



Kinase activity is also enriched for platelet-derived growth factor (PDGFB), which has a mitogenic role [113] and activates PI3K, MAPK and PKC $\gamma$  signaling, and MAP4K4, which in turn activates the JNK pathway by sensing death receptors, inflammatory cytokine receptors, and G-protein coupled receptors [114–116].

There is an abundance of GO terms pertaining to embryologic development due to 3 proteins in particular: GLI3, TBX3, and PRRX1. When RPKM averages are compared between Ross and Heritage at 21dph, they are slightly higher in Ross 708 for all four genes. (Figure 3) These three gene products are involved in several developmental processes, by acting as transcriptional regulators that have a role in heart development and angiogenesis [117].

Developmental transcription factors can also be activated by heart disease [118]. The GLI family zinc finger 3 (GLI3) promoter region contains a binding site for myocyte enhancer factor 2A (MEF2A), a transcription factor that has a role in inducing left ventricular chamber dilation and hypertrophic growth in response to pressure overload [119], [38]. The T-box3 (TBX3) promoter contains a binding site for NK2 homeobox 5 (Nkx2-5), which increases survivability of myocytes [120], and has an indirect role in the induction of hypertrophy [38], [121]. Paired related homeobox 1 (PRRX1) promoter contains a binding site for nuclear factor  $\kappa$ B (NF $\kappa$ B), which positively regulates hypertrophy [122], [38]. Formin1 (FMN1) is responsible for fascia adherens formation and actin polymerization in the heart is activated when there is enlargement of myocardial cells [123]. When the expression of GLI3, PRRX1, TBX3, and MEF2A at 21dph is compared to 6dph, there is a slight difference in expression. In Ross 708, expression of GLI3 and MEF2A is slightly higher at 6dph, and expression of PRRX1 and TBX3 is slightly higher at 21dph.

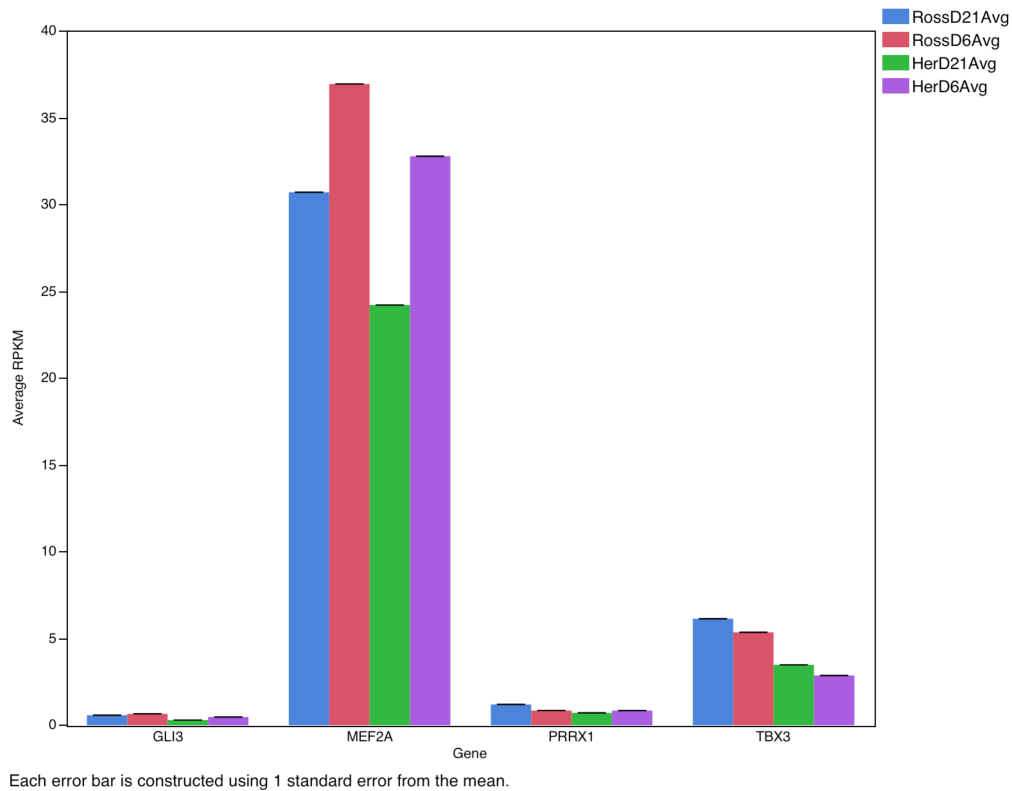


Figure 3 Differences in developmental gene expression between Ross 708 at 6 and 21 days post hatch, and Heritage at 6 and 21 days post hatch.

Complement control proteins (CR2, C1R, MASP2) contain a domain that functions in distinguishing self from non-self in the immune response [124]. However, in ischemic heart disease, chemotactic activity of the complement system is directly related to the duration of the hypoxic state [125]. The presence of C1R (R component of complement component 1) suggests that C1s may also be present, which bind together to form complement component 1 (C1). C1 has been found to bind to the mitochondrial membrane of cardiac cells [126] and has an anti-apoptotic role in cells damaged by hypoxia [127].

## Chapter 6

### TRANSCRIPTOME ANALYSIS: 42 DAYS POST HATCH

At 42 dph, there were no enriched functional annotation terms from DAVID in Ross 708 or Heritage when BioLayout minimum correlation coefficient was set to .9. KEGG Pathway Mapper also yielded no results. This may be due to the nature of the cardiac pressure overload response. Typically, both synthesis and degradation of the collagen network in the heart is upregulated soon after the stressor is introduced. Synthesis of these fibers returns to normal four weeks after the initial stress [88], which, based on previous studies, might occur at 14dph [4].

Table 3 Differentially expressed genes in Ross 708 and Heritage at 42 days post hatch.

Ross 708	Heritage
MOCOLN3	LOC771360
LOC422071	CYP27C1
LOC772115	GFRA3
LUZP2	LCOR
C14orf143	UTS2R
LOC768337	LOC428238
LOC770168	FAM123A
AGTRL1	NDST3
LOC42832	KY
MEF2B	LOC772146
	C6orf157
	LOC415661
	LOC770313

Table 3, cont.

<b>Ross 708</b>	<b>Heritage</b>
	NETO1
	GCNT7
	C17orf48
	GRIN3A
	LOC771179
	TRIM7
	CCDC60
	LOC769357
	GIP
	HEBP2

## Chapter 7

### TRANSCRIPTOME ANALYSIS: HERITAGE AGE COMPARISON

Table 4 Differentially expressed genes and their respective functional categories in the Heritage line between 6 and 21 days post hatch.

6 Days Post Hatch		42 Days Post Hatch	
Functional Category	Genes	Functional Category	Genes
Kinesin	KIF23, KIFC1, KIF4A, KIF11, KIF18B	WNT signaling	WNT5B, WNT11, WNT8A, FZD7, FZ-8, WNT2B FZD9, DKK3
Extracellular matrix	SPOCK3, LOC395532, ADAMTS3, ADAMTS2, ENTPD2, COL11A1, ADAMTS19, DHRS11, THBS4	Response to oxygen levels	EDNRA, KCNMA1, EPAS1, PLAUI, ADA, CITED2
		SCY	CCL1, CCL20, CX3CL1, LOC395914
		Lymphocyte mediated immunity	PTPRC, CRTAM, TAP2, CXORF9, CD80, CD3E, CD4, CD74, STAT5B, IL2RG, CARD11, B2M
		Immunoglobulin	IL1R1, CD8A, MDGA1, NFASC, MERTK, NEGR1, NTM
		Myeloid cell differentiation	TNFSF11, LMO2, MAFB, STAT5B, ADIPOQ

## **6 Days Post Hatch**

Table 4 contains functional categories and genes within each key category from differentially expressed genes in Heritage at 6DPH and 42DPH. Differentially regulated terms from 6DPH as compared to 42DPH are mostly related to developmental functions. Kinesin (KIF23, KIFC1, KIF4A, KIF11, KIF18B) is an intracellular motor protein that moves along microtubules, to move organelles, large molecules, and aids in cell division [128]. Chromosome assembly, mitosis, helicase activity, double stranded break repair, and acetylation, which are most likely due to developmental fibroblastic and endothelial activity via mitosis rather than maturation of cardiac myocytes. Organelle, chromosome, and protein localization, cell activation and cell motility are all also related to the activation of mitosis and movement of cardiac fibroblasts and cytoskeletal components due to development and deposition and arrangement of extracellular matrix proteins [80], [129].

Extracellular matrix activity includes ECM-receptor interaction, proteinaceous extracellular matrix activity, and metalloproteinases. As stated previously, metalloproteinases can be involved in either fiber degradation or in formation and deposition into the extracellular matrix. G protein signaling aids in transcription, cell motility, contractility and secretory functions. Hemopoietic activity in the heart at this stage is further substantiated by the tendency of birds to exhibit EMH [9].

## **42 Days Post Hatch**

Wnt receptor-signaling is involved in cell-to-cell communication, cell proliferation and cell differentiation in both the fetal and adult heart, for maintenance of cardiac muscle [130]. Differences in fibroblast activity and proliferation between the two stages of development could be contributing to differential expression. Cell-

cell adhesion involves the Pleckstrin homology domain in regards to intracellular signaling or cytoskeletal components for myocardial remodeling in response to external stimuli [131]. Cytoskeletal activity is related to RAS/ GTPase activating proteins [132] and Src kinase [133].

Enrichment of genes EPAS1, EDNRA, KCNMA1, CITED2, XDH, PLAUG, NUDT19, and SCP2 suggests a potential response to hypoxia. Expression of EPAS1 is enhanced response to hypoxic conditions, and in turn controls expression of other proteins that are regulated in response to low oxygen levels [134], [135]. Endothelin receptor A (EDNRA) has a high affinity for EDN1, the expression of which is increased during hypoxia [136]. Calcium signaling (KCNMA1) stimulates hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) expression [137], and CITED2 is known to lower levels of HIF1 $\alpha$  in response to hypoxia [138]. Xanthine dehydrogenase (XDH) is a precursor of xanthine oxidase and its expression is increased during hypoxia [139–141]. Apoptotic activity is repressed by BCL-2 that could be hypoxia-induced [142]. Imbalances between oxidants and antioxidants can cause a hypoxic state which can be characterized by increased peroxisome (PEX5L, EHHADH, HMGCL, HSD17B4) activity [141]. Increased demand for oxygen, such as in high growth phases and fast growing broilers, can also exacerbate hypoxia [143].

Heparin- binding EGF has a role in cardiac development, function, and hypertrophy. It is a chemotactic glycoprotein which is essential for normal heart function [144]. It can influence several members of the ErbB signaling pathway, including ERBB4, which in turn acts on PI3K to initiate the PI3K/AKT pathway [145], [146].

Members of the SCY family (CCL1, CCL20, CX3CL1, LO395914) of proteins are involved in mitogenic (MAPK), chemotactic, and inflammatory processes. Birds of this stage are exhibiting inflammatory characteristics, made evident by enriched terms such as lymphocyte mediated immunity, (PTPRC, CRTAM, TAP2, CXORF9, B2M) T cell activation (PTPRC, CD80, CD3E, CXORF9, IL2RG, ADA), immunoglobulin (IL1R1, CD8A, MDGA1, NFASC, MERTK, NEGR1, NTM), and negative regulation of myeloid cell differentiation (TNFSF11, LMO2, MAFB, STAT5B, ADIPOQ).



## Chapter 8

### TRANSCRIPTOME ANALYSIS: ROSS 708 AGE COMPARISON

Table 5 Differentially expressed genes and their respective functional categories in the Ross 708 line between 6 and 21 days post hatch.

6 Days Post Hatch		42 Days Post Hatch	
Functional Category	Genes	Functional Category	Genes
Fibronectin	PTPRU, LRRN1, NEO1	Immunoglobulin	BLB2, BLB1, HAVCR1, ICOS, CD79B, TGM4, EMB, LOC425256
Muscle cell differentiation	BARX2, LOC768344, NEO1, NTN1	Response to oxidative stress	PRDX6, GPX4, GPX3
Epithelium development	ALDH1A2, ALDH1A3, GATAD2A, CA2	Cytoskeletal organization	CAV3, SBDS, GSN
Angiogenesis	TBX20, ELK3, MYH9		
Regulation of apoptosis	IFIH1, ALDH1A3, MITF, DYRK2, MEF2A, CASP7, CIDEA, EIF2AK2		

#### 6 Days Post Hatch

Table 5 contains functional categories and genes within each key category from differentially expressed genes in Heritage at 6DPH and 42DPH. Fibronectin is an extracellular matrix protein that binds to intermembrane proteins for cell migration and growth via laminin, extracellular matrix organization, and fibrillar collagen. Enrichment of terms related to hydroxyproline, such as procollagen and proline

hydroxylase can all be attributed to an increase in the deposition of noncollagenous extracellular matrix, which aid in adding strength to collagen and forming the triple helix conformation of collagen fibers [88].

At this stage, muscle cell differentiation is enriched, along with epithelium development, vascular development, tissue morphogenesis, and angiogenesis. There is immune activity via immunoglobulin, lymphocyte activation, and leukocyte differentiation, which, as stated previously, is most likely due to resident immune cells and EMH in the chicken heart. There is also apoptotic activity which is most likely due to Serine/Threonine Kinase receptors, which play a role in the regulation of cell proliferation, programmed cell death, apoptosis, cell differentiation, and embryonic development.

#### **42 Days Post Hatch**

Ross 708 birds at 42DPH compared to 6DPH have enriched terms pertaining to immunoglobulin activity (BLB2, BLB1, ICOS, TGM4, B-MA2). There are three genes that are described as response to oxidative stress, peroxidase activity, and antioxidant activity. (PRDX6, GPX4, GPX3) The presence of peroxiredoxin 6 (PRDX6) and glutathione peroxidases (GPX3, 4) decreases oxidative injury to ischemic areas of the heart during reperfusion [147], [148].

Cytoskeletal activity pertaining to actin and cell adhesion molecules (CAMs), also may be related to GTP binding for GTPase signal transduction in regulatory functions. Cytoskeletal activity is also been related to hypertrophy of cardiac myocytes and heart failure due to chronic pressure or volume overload [149], [150].

## **Chapter 9**

### **HISTOLOGIC ANALYSIS**

#### **Hematoxylin and Eosin**

At 21DPH, one of six (16.7%) Heritage showed multifocal myofiber degeneration. (Figures 7 & 8) Five (83.3%) had no significant lesions, though one showed EMH. (Figure 5) Three of six (50%) Ross 708 at 21DPH showed mild or minimal myofiber degeneration including one showing EMH. One (16.7%) showed mild multifocal myocardial degeneration. Two (33.3%) had no significant lesions.

At 28DPH in Heritage, no significant lesions were observed in the five samples. Out of seven, two (28.6%) Ross 708 had hyper cellular foci. Five (71.4%) had no significant lesions other than EMH.

At 42DPH, one of three Heritage exhibited vascular associated hypercellular foci and lymphoplasmacytic and histocytic epicarditis. (Figure 6) One exhibited hypercellular foci and one had no significant lesions. One out of six (16.7%) Ross 708 exhibited a lymphohistiocytic nodule and moderate multifocal degeneration. One (16.7%) showed vascular- associated hypercellular foci. (Figure 9) Three (50%) showed no significant lesions, and one showed no significant visible lesions other than EMH.

The higher incidence of myocardial degeneration in Ross 708 at 21DPH supports the idea that Ross 708 may be undergoing cardiac remodeling at that stage. Cardiac remodeling is typically characterized as pathological in nature, and although it can provide immediate benefit in the case of volume or pressure overload, a prolonged

remodeling status can have detrimental effects. The presence of these microscopic lesions with no gross evidence of cardiac disease could suggest that there are no congenital defects causing the higher incidence of ascites in broiler chickens, and offers further evidence that the disproportionate growth of the breast muscle and other organs trigger development of subclinical heart lesions which, in turn, could predispose birds to develop ascites upon concurrent exposure to detrimental environmental or health conditions.

Based upon a difference of means test performed in JMP10 the average cardiomyofibers diameter was significantly different between Ross 708 and Heritage at 21, 28 and 42 DPH. (Table 2) At 21 DPH, Ross 708 myofiber diameter was larger than Heritage, but the converse was true at 28 and 42 DPH. When average myofiber diameter was compared between Ross 708 at 21DPH and 42DPH, the P-value shows that there is not a significant difference at an  $\alpha$  level of either 0.99 or 0.95 (p-value-.0855). This means that over time, the average diameter of Ross myofibers did not increase. The same comparison in Heritage yields a significant difference at an  $\alpha$  level of .99 (p-value- <0.0001). This means that average myofiber diameter at 42DPH in the Heritage line is significantly larger than myofiber diameter at 21DPH. (Figure 4)

Masson's Trichrome staining shows no striking differences in collagen content between the Ross 708 and Heritage lines at 21, 28, and 42 days of age. However, subjectively, there is a subtle increase in the amount of collagen staining in the Ross 708 line either indicating an increase in interstitial collagen or a higher number of small capillaries surrounded by collagen in the myocardium. True foci of fibrosis were only present in one Heritage bird at 21DPH, and one at 42DPH, neither of which were

accompanied by inflammation, and in a single Ross 708 bird at 42DPH that was also not accompanied by inflammation.

Table 6 Comparison of average myofiber diameter in Ross 708 vs. Heritage at 21, 28, and 42 days post hatch.

Age	Line	N	Mean (μm)	Std. Dev.	Min (μm)	Max (μm)	P-value
21 DPH	Heritage	60	6.558	1.547	3.7	11.35	.0046
	Ross	60	7.365	1.512	4.83	11.4	
28 DPH	Heritage	115	7.597	1.145	5.2	10.04	.0001
	Ross	110	6.879	1.089	4.79	10.48	
42 DPH	Heritage	50	7.9902	1.133	5.84	10.77	.0001
	Ross	110	6.979	1.111	4.78	10.53	

A p-value less than 0.01 indicates significance at a 99% level of confidence.

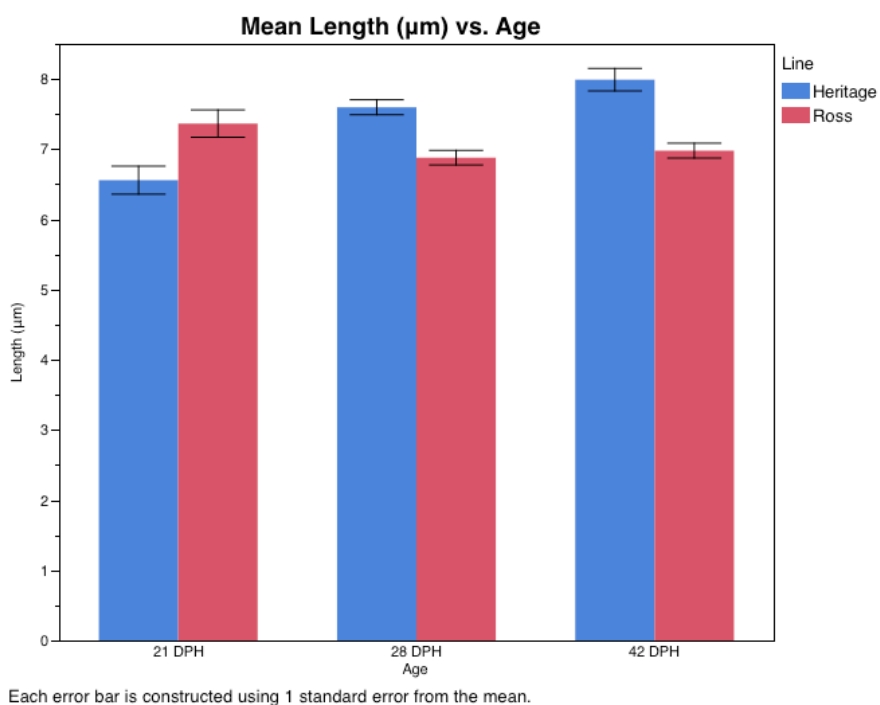


Figure 4 Differences in myofiber diameter at 21, 28, and 42 days post hatch between Ross 708 and Heritage.

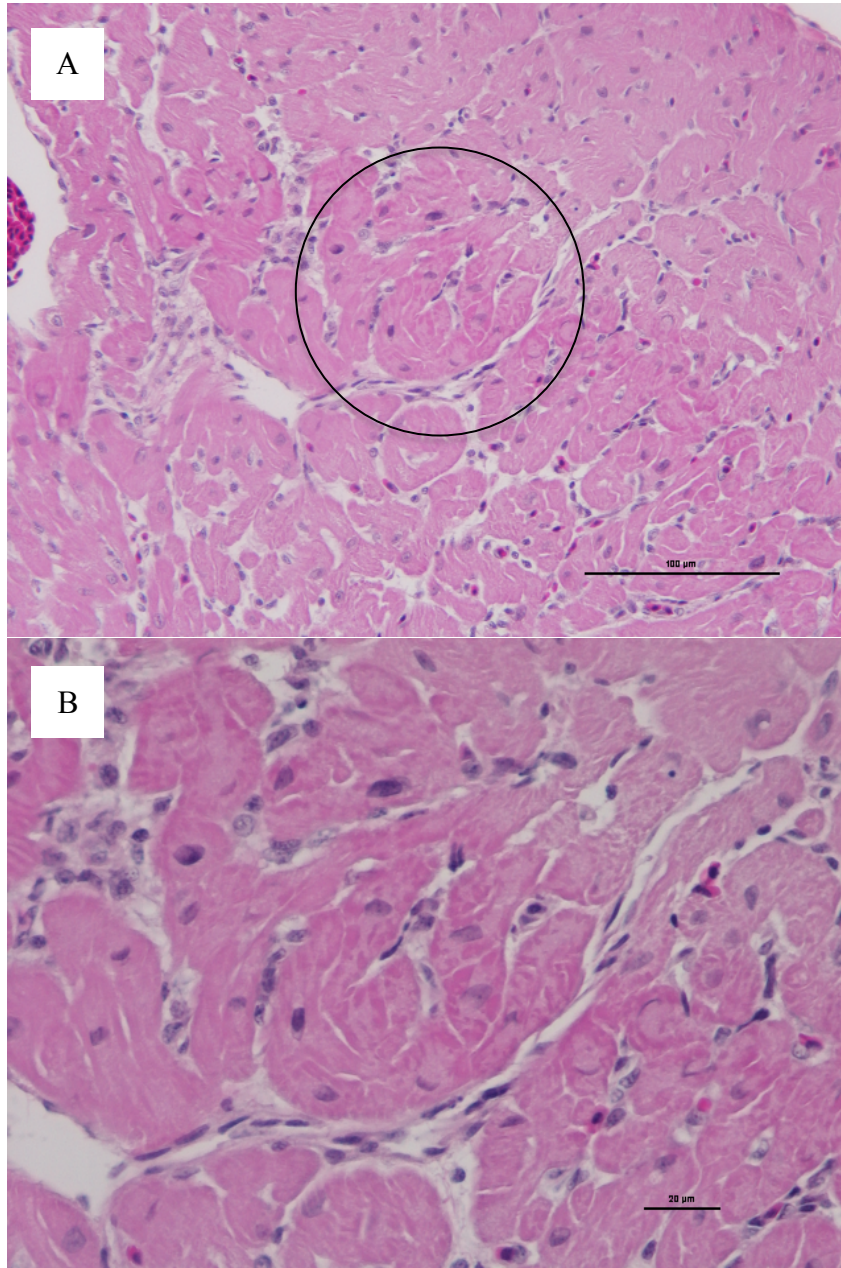


Figure 5 Heart, H&E, Heritage, 42DPH; Representative image of myofiber degeneration at 100x (A) and 400x (B) magnification (transverse). Disrupted contractile units are peripheralized and condensed (hypereosinophilic).

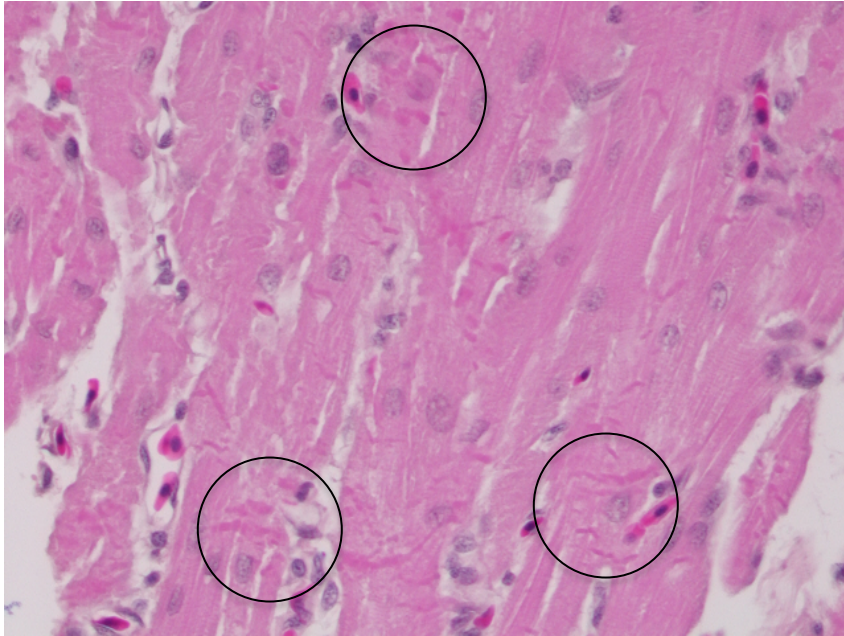


Figure 6 Heart, H&E, Ross 708, 21 days post hatch, 400x magnification; Representative image of longitudinal myofiber degeneration. Areas of contraction band condensation are circled.



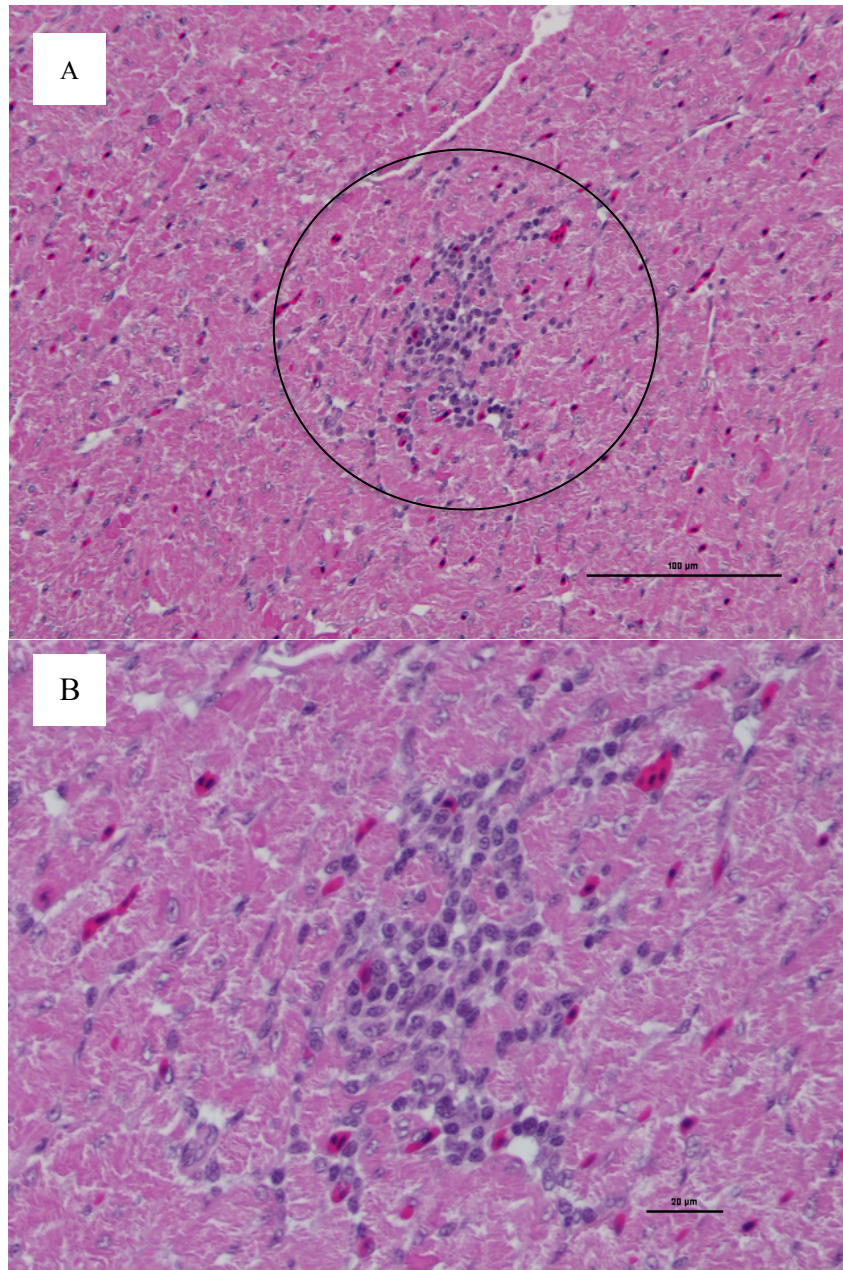


Figure 7 Heart, H&E, Heritage 21 days post hatch; Representative image of extramedullary hematopoiesis in both lines at 200x (A) and 400x (B) magnification, comprised of a mixture immature and mature lymphoid and myeloid cells.



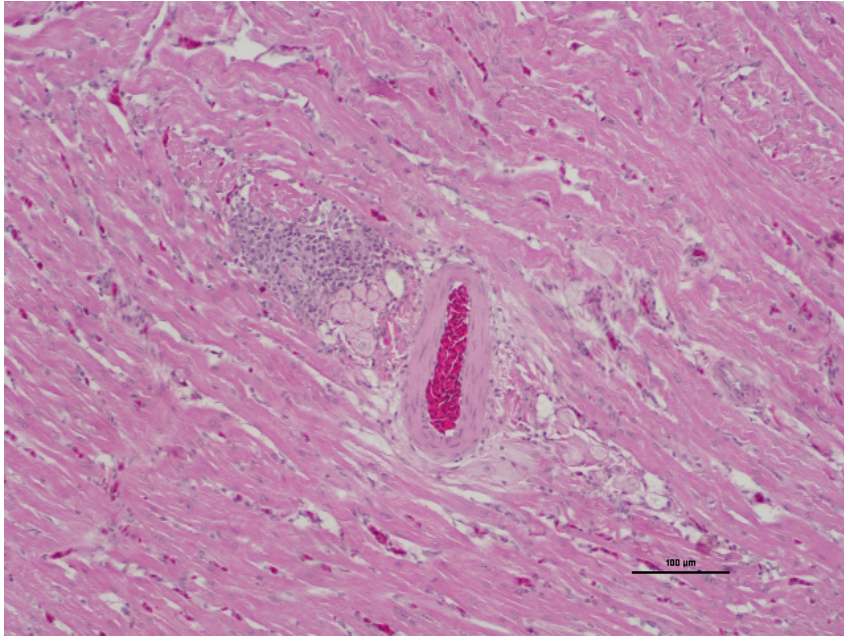


Figure 8 Heart, H&E, Ross 708, 42 days post hatch, 100x magnification; Representative image of vascular-associated hypercellular focus. The lesion, comprised of dense aggregates of spindle- shaped cells adjacent to vessels may potentially indicate vascular or extracellular matrix remodeling and is not observed in association with inflammation or extramedullary hematopoiesis.

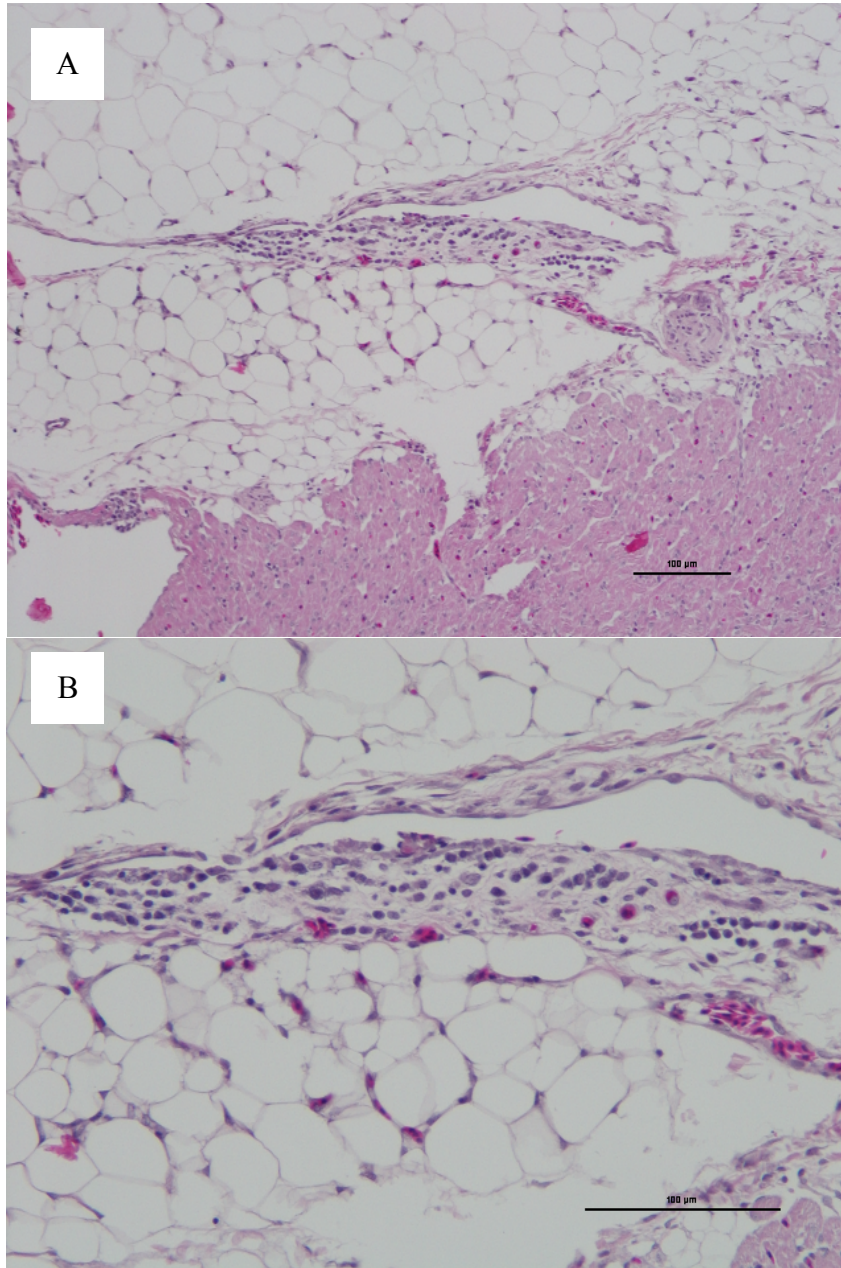


Figure 9 Heart, H&E, Heritage 42days post hatch; Representative image of epicardial inflammation at 100x (A) and 200x (B) magnification.



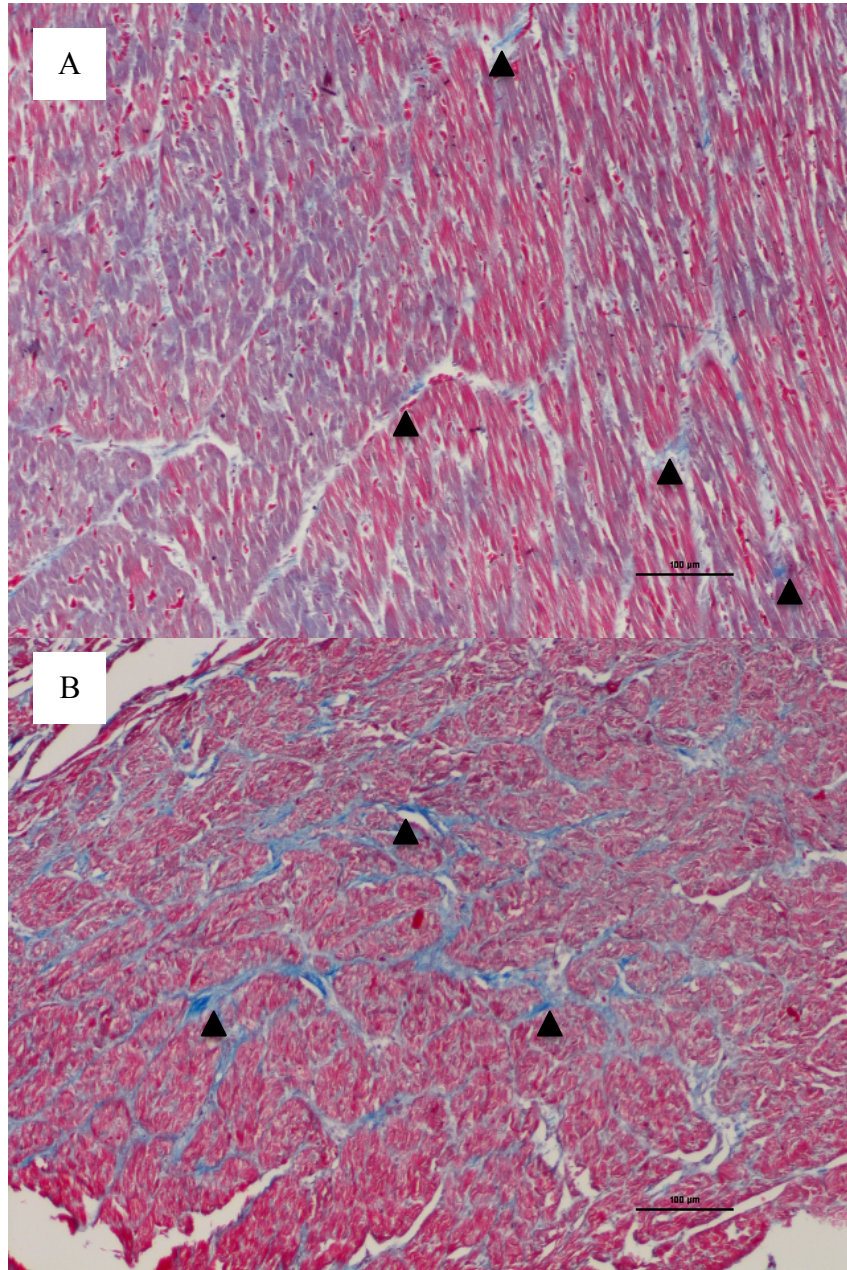


Figure 10 Heart, MT, 100x magnification, transverse; Representative images of thickened interstitial membrane (blue) of Ross 708 myocardium (B) when compared to Heritage myocardium (A) at 21 days post hatch.

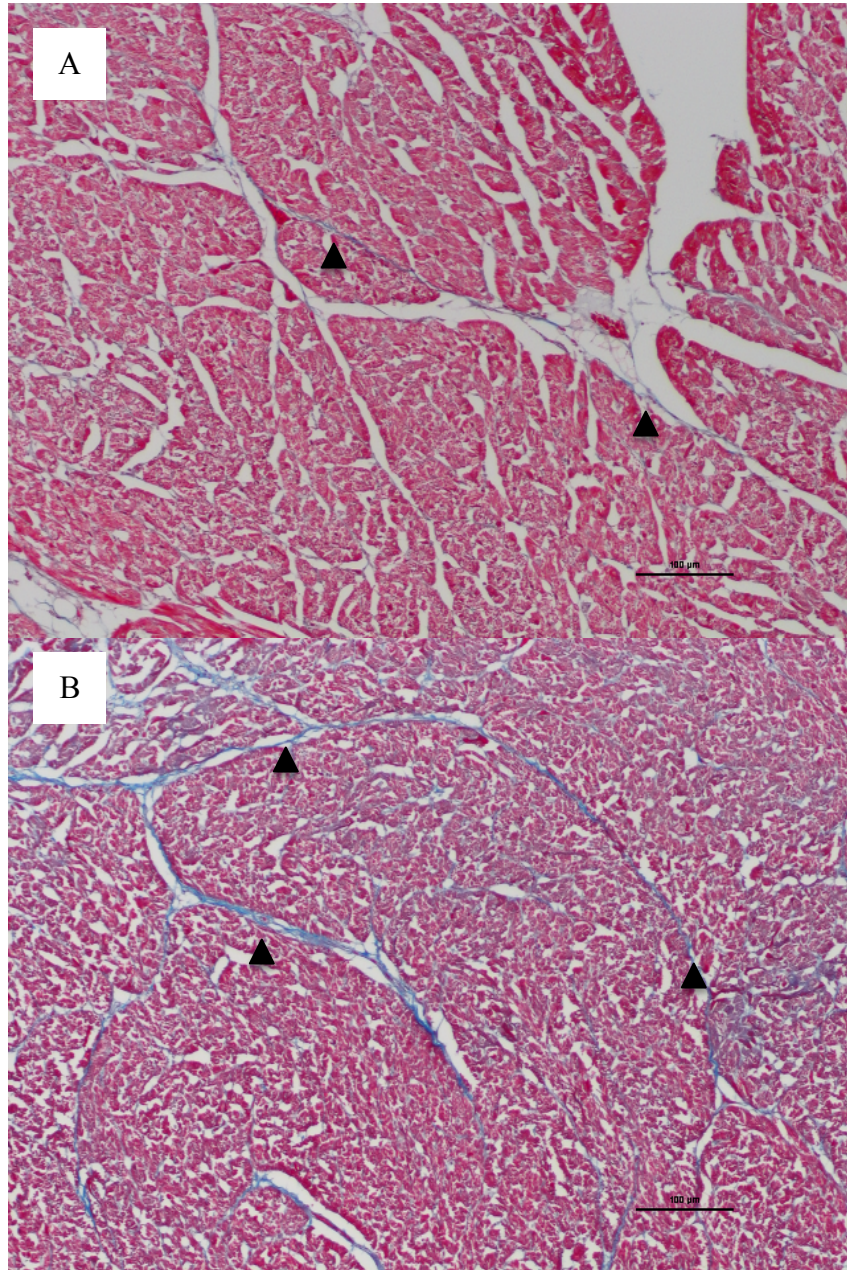


Figure 11 Heart, MT, 100x magnification, transverse; Representative images of thickened interstitial membrane (blue) of Ross 708 myocardium (B) when compared to Heritage myocardium (A) at 42 days post hatch.



## **Chapter 10**

### **CONCLUSIONS**

It is apparent that there are differences in gene expression patterns between the Ross 708 and Heritage lines at different stages. Transcription levels differ most at 21dph, with Ross 708 exhibiting a higher instance of extracellular matrix remodeling, developmental protein expression, hypertrophic stimuli and inflammation. Histology shows that although there is an individual with microscopic evidence of inflammation (epicarditis), most immune activity is in the form of leukocyte production (EMH) at all ages.

The presence of enriched terms pertaining to oxidative stress and peroxisomes in both lines when genes are compared within a line and across time suggests that the birds are experiencing stress related to diminished oxygenation of cardiac tissues. It has been found that both ascites-inflicted and non-afflicted modern broilers both exhibit hypoxic states in the heart, which could be due to the high oxygen demand that the heart naturally exhibits during growth. The lack of differential gene expression at 42 DPH could be due to a state of equilibrium in growth. The initial point of the stress, based on previous reports, is most likely to occur at 14dph, where there is a sharp decline in normalized heart mass in the Ross 708 line. After this decline, the normalized heart mass in Ross 708 stays low. In contrast, the normalized heart mass in the Heritage line has a small decline at 14dph, but in the end is comparable to the starting ratio. The high instance of arrhythmia in broiler chickens could also be caused

by fibrosis, because fibrotic tissue can interfere with the electrical signals coming from the sinoatrial node and the atrioventricular node.

Differences in myofiber diameter were expected, but did not fit the original hypothesis that Ross 708 would have thicker myofibers than Heritage. It does, however support the theory that growth in hearts of Ross may be stunted overall. Upon observation at necropsy, although the hearts of the Ross 708 line appear bigger when compared to Heritage, they are smaller when normalized to body weight. Although Ross 708 myofibers are larger at 21DPH, their size does not change over time whereas in Heritage, myofiber size increases. One reason could be a loss of synergy in energy uptake between skeletal muscle and the heart in order to improve feed efficiency. The disability of Ross 708 birds to acquire adequate myofiber size and subsequent myocardial degeneration could be contributing to the incidence of clinically evident heart failure and ascites as they age.

## **Chapter 11**

### **FURTHER STUDIES**

In order to expand upon the analysis presented in this study and further explain the complete mechanism by which heart failure manifests in the modern broiler, it would be necessary to grow birds to higher age points to see if the changes detected at 21dph translate into an increased incidence of heart necrosis (infarction) and ascites. Monitoring oxygen consumption by heart tissue harvested from modern broilers in situ when presented with differing levels of energy substrate availability could also prove to be insightful.

Blood pressure and electrocardiogram (ECG) measurements could also contribute to our understanding of the effects of external stimuli such as high ambient temperature and low environmental oxygen and selective breeding on the heart in modern broilers [151]. For instance, a large QRS wave on the ECG could be indicative of left ventricular hypertrophy, the length of the QT interval could be indicative of lethal arrhythmias, a dip in the Q segments can indicate prior myocardial infarction, and a hump at the ST segment can indicate present myocardial infarction. Since myocardial hypertrophy and the deposition of collagenous matrix material can be caused by both pathological and physiologic reasons, ECG could be the basis of finding which type of hypertrophy is affecting the modern broilers when paired with histologic analysis of myofiber length and diameter.

Since heart size can also be mediated by energy availability, it would be a logical next step to monitor blood glucose levels in the Ross 708 and correlate heart

size to glucose uptake in the skeletal pectoral muscle. Since chickens are typically insulin resistant but do have a regulatory mechanism for blood glucose, it would be useful to perform a transcriptome analysis of glucose transporter levels in the skeletal muscle. It is possible that low blood glucose levels could be hindering heart growth by disrupting synergy between the skeletal and cardiac muscle.



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**Appendix A**

**AGRICULTURAL ANIMAL CARE AND USE COMMITTEE APPROVAL  
FORM**

**UNIVERSITY OF DELAWARE**

**COLLEGE OF AGRICULTURE AND NATURAL RESOURCES**

**AGRICULTURAL ANIMAL CARE AND USE COMMITTEE**

**Application for Use of Agricultural Animals**

**In Teaching or Research**

**AACUC Protocol Number: (27) 12-22-10R**

**TITLE OF PROJECT:** Scientific Investigation into the response of  
Broiler Chickens to heat stress by transcriptome analysis

**INSTRUCTOR/PRINCIPAL INVESTIGATOR:** Carl Schmidt

**New or Three Year Review (mark one)**

**NEW**    ☒

**THREE YEAR**    ☐

**If this is a 3 year renewal, what is the assigned existing  
protocol number?**    \_\_\_\_\_

.....  
**(This section for Committee use only)**

**Application Approved (date): 1-5-2011**

**Application Rejected (date):** \_\_\_\_\_

**Reason for Rejection:**

\_\_\_\_\_



**1-5-2011**

**Signature, Animal Care and Use Committee  
Date**

**APPLICATION INFORMATION:**

Title: Scientific Investigation into the response of Broiler Chickens to heat stress by transcriptome analysis

Principal Investigator(Research): Carl J. Schmidt

Address: 107 Allen Lab, 601 Sincock Lane, University of Delaware, Newark, Delaware 19716

Telephone: (302)-831-1334 Email: [schmidt@c@udel.edu](mailto:schmidt@c@udel.edu)

Proposed start date: February 1 2011 End date: January 31, 2014

Teaching/Outreach ☐ Research ☒

If TEACHING box was checked, select from the following:

☐ Demonstration ☐ Laboratory ☐ Student Project

If student project, please define project:

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Have all participants listed above reviewed the application and is familiar with the proposed work?

YES    ☒

NO    ☐

If no, identify those needing to review application.

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Are all proposed animal care management procedures 1) defined as “pre-approved” by the Animal Care and Use Committee, or 2) part of the Standard Operating Procedures developed by the Animal Care and Use Committee for that particular species?

YES    ☒    NO    ☐    To be determined by AACUC    ☐

Have all participants been trained?    YES    ☒    NO    ☐

Which participants have not been trained?

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Name the person responsible for conducting the training.

---

If after hours participation is required by students, please describe how this is being handled. (e.g. supervisors, assistants, etc.)  
Please include the times and days that students may be on site.

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**ANIMAL INFORMATION:**

Common Name of the Animal Requested: Chickens

Amount Being Requested: 1600

Source of Animals: Allen Family Foods and Chet Utterback at the University of Illinois

Where are the animals being held: UD Poultry Farm

Briefly Describe the Goals or Objectives of this Application (use additional space as needed).

The goal of this study is to determine the ability of the modern broiler chicken to handle heat stress compared to the heritage variety. Following treatment, birds will be euthanized by cervical dislocation and organs harvested for transcriptome analysis.

**Rationale for scale of study:** This is a new area of research, using new genomic approaches to understand how birds respond to heat stress. The large numbers of birds are necessitated in order to achieve statistical significance in our gene mapping studies.

**Birds:** Heritage birds will be obtained from Chet Utterback at the University of Illinois and the Ross708 birds from a local supplier. Birds will be wing tagged and randomly placed into control and experimental groups as described below (Heat Shock Scheme). In each experiment 100 birds from each line will be included in each experimental group. The size of the facilities at the University of Delaware limit the number of birds per chamber, hence we anticipate multiple replicates over time to a total of 1600 birds per line. Blood will be taken from each bird for DNA extraction prior to heat stress. Also, 12 birds from each group will be removed on post hatch days 2, 7 and 21, euthanized (cervical dislocation) and tissues harvested. Blood biomarker data using the iSTAT will be collected from these birds prior to euthanasia. Chambers will be monitored on a daily basis to insure adequate feed and water and to remove

any sick or dead birds.

**Heat Shock Scheme:** Controls are hatched from eggs incubated at 37°C (99°F) while thermal conditioned embryos will be incubated at 39.6°C (103°F) from embryonic days 10-18, then returned to 37°C. Following hatch through day 21, they will be kept at ambient temperatures. At day 22, the original Control birds will be split into two populations (Control A and B) and the *In Ovo* Heat-conditioned bird also split into two groups (*In Ovo* Heat Conditioned A and B). The A populations will be kept at ambient temperatures while the B populations will be heat stressed at 35°C (95°F) or 7 hours per day for 21 days. There will be 20 birds per chamber. Multiple replicates (hatches) will be conducted. At the end of the trial (6 weeks from hatch), birds will be euthanized and tissues collected.

Attached below is additional protocol information.

Does this procedure involve surgery? YES NO ☒

If yes, explain in detail the surgery.

Are drugs, vaccines and/or medications being used? YES ☐  
NO ☒

If yes, describe what is being used. Include dosages and routes of administration.

How often are animals monitored and how are sick or injured animals being handled?

The birds will be checked daily and given food and fresh water *ad libitum*. Sick or injured animals will be euthanized by cervical dislocation.

What is the method of euthanasia, if specified in the protocol?

Cervical dislocation as per AVMA Guidelines on Euthanasia 2007

List the veterinarian who is on-call:

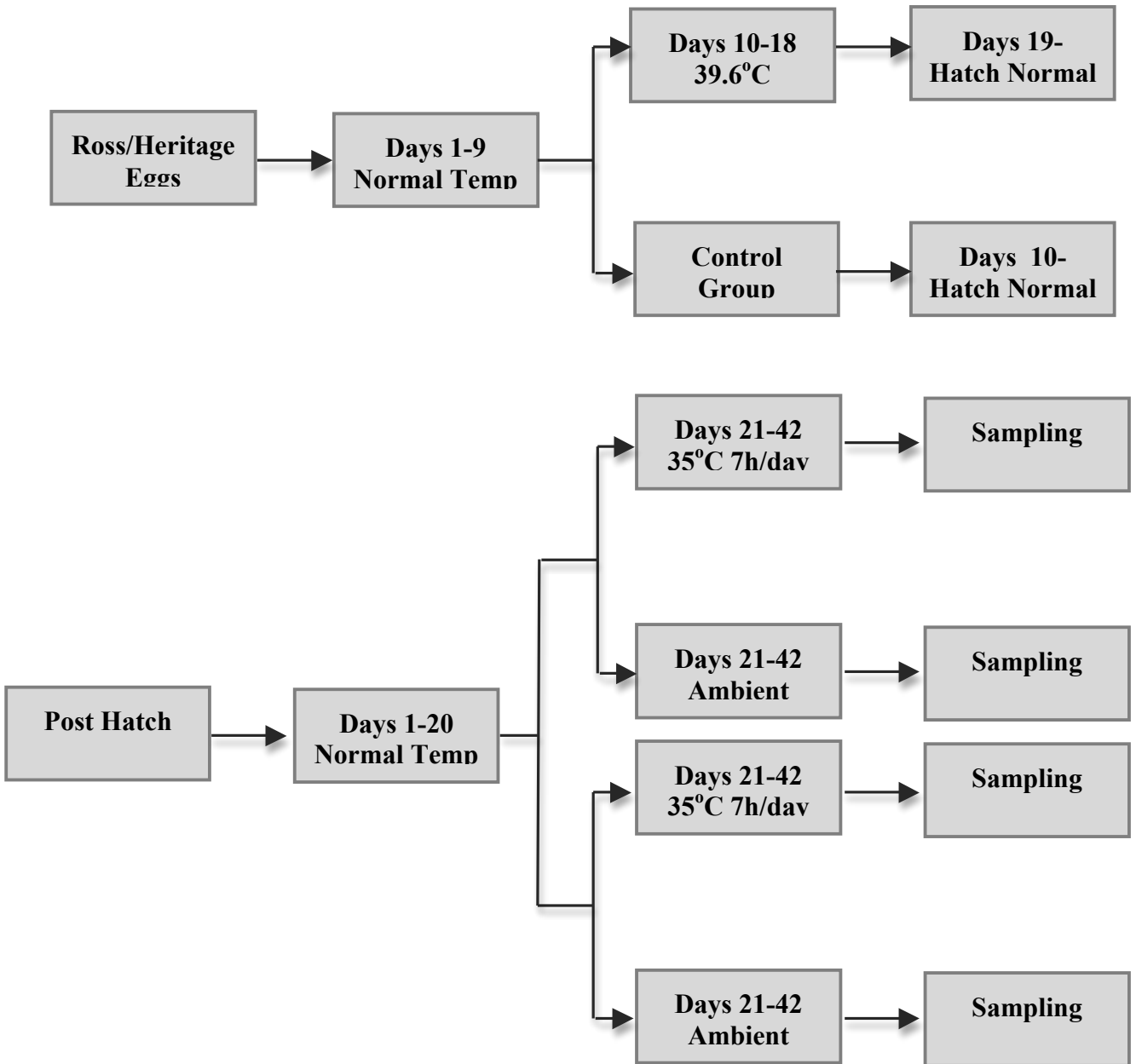
Name: Miguel Ruano

Telephone: 302-831-1539

Does this application require approval from Occupational Health & Safety (OHS)? YES ☐ NO ☒

If yes, what form(s) are attached? \_\_\_\_\_

NOTE: OHS approval is required for experiments involving the use of hazardous substances such as radioactive materials, highly toxic or carcinogenic materials, human reproductive hazards, or zoonotic or human pathogens.



**Ross Heritage heat stress experiment:** Eggs will be either heat stressed or maintained as controls from embryonic days 10-18, and then returned to normal temperatures. Subsequently, both heat stressed and control birds will be split into two groups each, with one group heat stressed from days 21-42 post-hatch, with the second group kept at ambient temperatures to function as a control. So, there will be a total of 8 groups at the end of each experiment.



Tissue Samples: Genomic DNA & RNA:

- Blood
- Brain
- Heart
- Liver
- Duodenum
- Jejunum
- Ileum
- Large Intestine
- Ceca (and contents)
- Fat pad
- Breast muscle
- Spleen

Weekly Measurements:

- iSTAT metabolic measurements
- Weight

Day 21/42

- Shank length
- Shank Width

Morphometric:

- Liver
- Spleen
- Duodenum
- Jejunum
- Ileum
- Large Intestine
- Breast muscle
- Heart

Samples are needed for:

- RNAseq

- microRNA
- Genomic DNA
  - SNP
  - CVN
  - Epigenetics

## Appendix B

### BIRD AND RNA LIBRARY INFORMATION

Below is an outline of library samples synthesized that were used in this study.

Key:

V= nonspecific ventricular tissue

LV= left ventricle

Experimental Run #1- Summer 2011				
Line	Tag #	Lib #	Tissue	Age
Heritage	450	64	LV	42
Heritage	47	68	LV	42
Ross	982	65	LV	42
Ross	615	66	LV	42
Ross	610	67	LV	42

<b>Experimental Run #2- Fall 2011</b>				
<b>Line</b>	<b>Tag #</b>	<b>Lib #</b>	<b>Tissue</b>	<b>Age</b>
<b>Ross</b>	961	211	V	D6
<b>Ross</b>	990	214	V	D6
<b>Ross</b>	968	219	V	D6
<b>Heritage</b>	63	212	V	D6
<b>Heritage</b>	60	213	V	D6
<b>Heritage</b>	51	218	V	D6
<b>Ross</b>	988	225	V	D21
<b>Ross</b>	956	226	V	D21
<b>Ross</b>	961	227	V	D21
<b>Heritage</b>	73	234	V	D21
<b>Heritage</b>	58	235	V	D21
<b>Heritage</b>	46	236	V	D21

<b>Experimental Run #3- Spring 2012</b>				
<b>Line</b>	<b>Tag #</b>	<b>Lib #</b>	<b>Tissue</b>	<b>Age</b>
<b>Ross</b>	402	321	LV	D21
<b>Ross</b>	405	322	LV	D21
<b>Ross</b>	408	323	LV	D21
<b>Heritage</b>	431	328	LV	D21
<b>Heritage</b>	434	329	LV	D21
<b>Heritage</b>	435	330	LV	D21
<b>Ross</b>	403	350	LV	D42
<b>Ross</b>	407	351	LV	D42
<b>Ross</b>	418	352	LV	D42
<b>Heritage</b>	480	357	LV	D42
<b>Heritage</b>	491	358	LV	D42
<b>Heritage</b>	499	359	LV	D42