THE EFFECTS OF HEAT STRESS ON THE TRANSCRIPTOMICS OF DAY28 SPLEENS IN ROSS 708 AND ILLINOIS BROILERS

by

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ABSTRACT

Heat stress negatively affects the agricultural industry today. With global temperatures on the rise, evaluating the response of hyperthermia on broiler chickens is a vital application for poultry production. Heat stress can cause immunosuppression and we hypothesize that heat stress suppresses the immune response and negatively affects the splenic function. We predict there will be changes in gene expression seen in the transcriptomic profile comparisons of the D28 spleens from the Ross 708 and Illinois broiler chickens. Broiler eggs were obtained and following hatch, the males were divided into control and heat-stressed groups. The control groups were raised at a constant temperature of 25°C. The heat-stressed groups received eight hours of 39°C cyclic hyperthermia starting at D21 through D28 and maintained at 25°C for the remaining 16 hours of the day. Necropsies were performed approximately three to four hours into the heat cycle on D28 to obtain spleens. RNA extraction was performed and transcriptome libraries were constructed. Morphometric analysis revealed an impact on normalized spleen weights of both lines at D28 and a significant impact at D42. A minimal response was elicited by the spleen and the immune system in all control broiler groups suggesting normal physiological functions. The Ross 708 heat-stressed group highlighted possible occurrence of an intestinal epithelial barrier degradation resulting in the leakage of bacterial toxins into the systemic circulation, and eliciting an innate immune response from the spleen. Several genes products, IL-6, IL-17F, and...
TGF-β were found to require further investigation to definitively establish a connection with leaky gut syndrome. Overall, heat stress had an impact on the spleen as well as the immune system but, the extent of this effect is not completely understood.
Chapter 1

INTRODUCTION

The domestication of today’s chicken occurred between 7,000 and 10,000 years ago from a species known as the Red Jungle Fowl (RJF), *Gallus gallus*. Chickens originated from South East Asia with supporting evidence found in other parts of China, Asia and Europe [17, 26, 50]. The introgression of other species, like the gray jungle fowl has contributed to certain desirable traits in the poultry industry such as the golden color of the skin according to Schmidt, et al. Golden skin color was selected for in the meat industry of Ross 708 broilers [50]. The selection process began more than 50 years ago starting in the 1940’s. The separation of layers (egg type birds) and broilers (meat type birds) has also helped drive this selection process. Since the 1940’s, broilers were genetically selected for increased feed efficiency and body weight gain, allowing birds to reach market weight in about six weeks compared to the 16 weeks back in the 1940’s and 1950’s [20, 50].

In this study, commercial broilers are represented by the Ross 708 line which has undergone intense genetic selection. Birds modeled after the broilers from the 1950’s are known as the Illinois line and have not undergone intense genetic selection. Although high breast muscle yield, increased feed efficiency and, fast grow-out are desirable traits, they also have several implications in the poultry industry [50]. Faster growth has led to an increased metabolic rate, resulting in higher internal heat production. In addition, fast growth appears to have led to other issues including
immunosuppression which causes increases in susceptibility to disease, pathogens and increasing mortality rates in response to heat stress [54].

Many tissues in the avian body have been studied including the spleen, a vital organ to the immune system of a bird. The avian spleen is a small round, oval-shaped organ located “dorsally and to the left of the proventriculus” [23]. This organ does not begin to develop until embryonic day ten along with other immune organs such as the thymus and cloacal bursa. The endoderm germ layer is solely responsible for the emergence of the spleen and other immune related organs [1]. The main function is to filter the circulating blood that comes into the spleen which subsequently expands in size upon exposure to antigens. It contains both red pulp and white pulp, and the white pulp is comprised of the peri-arteriolar lymphoid sheath (PALS) and the Ellipsoids. The red and white pulp areas within the avian spleen are not clearly distinguished from one another, unlike the mammalian spleen [23]. Figure 1 illustrates the structural differences between the two.
Figure 1: Structural differences between Avian (A) and Mammalian spleens (B). The avian and mammalian spleen contains similar anatomical layouts; however, they also differ slightly in structural appearance. The avian spleen contains a closed microcirculation, without a well-defined marginal zone between the red and white pulps [52]. Germinal centers can be found in the PALS, a T lymphocyte dependent area. Germinal center formation can begin when foreign antigen is encountered and an immune response is elicited. The mammalian spleen differs in that it contains a very distinct marginal zone around the PALS and is more defined between the red and white pulp with an open microcirculation allowing blood to flow freely out of the penicillary capillaries [23, 52]. This figure was adopted from Kaiser, P. and Balic, A. 2014.
Lymphoid organs throughout the avian system are divided into two groups, primary and secondary. Maturation of immature lymphocytes occurs in primary lymphoid organs while secondary lymphoid organs allow lymphocytes to become activated when they interact with antigens. In the avian system, the Bursa of Fabricius (where B lymphocytes develop in birds) is an example of a primary lymphoid organ and the spleen is an example of a secondary lymphoid organ. Unlike the mammalian immune system, the avian immune system does not contain lymph nodes; instead, it contains lymph nodules which are aggregates of lymphoid tissue that are located throughout the bird’s body. Thus, the spleen is the predominant secondary lymphoid organ in birds and it is actively involved in antigen-specific immune responses, as evidenced by the presence of germinal centers [Emara, personal communication, 55]. This secondary lymphoid organ consists of many interactions of lymphoid and non-lymphoid cells, such as macrophages, dendritic cells, and heterophils (the avian equivalent to the mammalian neutrophil) [23].

The blood flow through the spleen is similar to that of mammals. However, in avian species, blood travels from the heart through the aorta into the celiac trunk, through the splenic artery, into the encapsulated trabecula and into the splenic pulp. Once in the splenic pulp, it moves through the central artery, surrounded by the peri-arteriolar lymphoid sheath (PALS) [23]. In the avian spleen the red pulp includes the blood-filled sinusoids and the white pulp is the lymphatic tissue. The PALS is a dense area of lymphatic tissue surrounding the central artery and it contains T lymphocytes along the central artery.

The blood continues through the smaller arterioles which arise from the central artery and into the ellipsoids and peri-ellipsoid white pulp. This area is where the first
exposure of antigens is detected and where B lymphocytes along with dendritic cells and macrophages (ellipsoid associated cells, EAC) reside. This interface is where the marginal zone is located and like many other features of the avian spleen, this zone is not well-defined, but separates the red and white pulp. These central arterioles divide further into the penicillary capillaries which contain no muscular layers and they are directly associated with the sinuses of the red pulp. In chickens, these penicillary capillaries are continuous with the venous sinuses, demonstrating a closed circulation through the spleen [9]. In the mammalian counterpart, the penicillary capillaries open in to the pulp cords allowing erythrocytes to migrate through the endothelial cells and into the venous sinuses [23]. In the chicken, the pulp cords are what separate the arterial and venous systems (See Figure 2).
Figure 2: Blood flow through the avian spleen. Blood circulating in the body flows out of the aorta into the celiac trunk and down into the trabecula of the spleen. From the trabecula, the blood moves into the central artery which divides and travels into the penicillary capillaries surrounded by the peri-ellipsoid white pulp where B lymphocytes are maintained [68]. Finally, the blood moves out of the capillaries and down through the pulp cords and out of the spleen through the venous sinuses. The avian splenic blood flow demonstrates a closed microcirculation because the blood is enclosed in an artery or vein at all times [9]. This figure was adapted from Kaiser, P. and Balic, A. 2014.
The red pulp in the spleen is mainly responsible for erythrocyte production from a common hematopoietic stem cell (HSC) progenitor but only after the spleen begins to mature [23]. The hematopoietic stem cells divide and differentiate to yield a variety of cell types including erythrocytes lymphoid/non-lymphoid cells, as well as platelets. Figure 3 illustrates the division from hematopoietic stem cells into a plethora of cells that will be used by the spleen and the immune system. Once erythrocytes have formed, the primary job of the red pulp is to filter and recycle the aged red blood cells. Other areas of the red pulp are also needed to regulate adhesion and/or migration of immune-related cells [23].

Figure 3: Recreated diagram representing the possible routes of differentiation into other immune related cells. Pluripotent stem cells (HSC) have the ability to differentiate into a variety of immune cells within the body. Many of these cell types will be used by the spleen to provide surveillance and detect foreign antigens. Others will migrate to other areas of the body when necessary. The image was adapted from https://en.wikipedia.org/wiki/Hematopoietic_stem_cell#/media/File:Hematopoiesis_simple.svg.
Chapter 2

LITERATURE REVIEW

Heat stress has many implications in the broiler chicken, including physiological, behavioral and molecular effects. By definition, “heat stress results from a negative balance between the net amount of energy flowing from the animal’s body to its surrounding environment and the amount of heat energy produced by the animal” [30, 54]. Heat stress can be divided into two main categories, acute and chronic. Acute is defined as 0-4 hours while chronic is five hours or more [14, 18]. Both acute and chronic heat stress have detrimental effects on the poultry industry [2, 14, 18, 30, 54].

Chickens are very sensitive to changes in temperature. They have a narrow core body temperature range of approximately 40.5-41.5°C [15, 62]. This sensitivity is due to a higher metabolic rate produced by broilers whose main purpose is to produce high quality skeletal muscle with a 42 day grow-out period. There are several factors that need to be taken into account for the thermoregulatory mechanisms of temperature homeostasis in chickens. The thermo-neutral zone is an important factor and by definition, it is when a bird at rest does not alter its oxygen consumption with temperature; meaning the metabolic rate is static. Temperatures above or below this zone are the upper critical temperature (UCT) and the lower critical temperature points (LCT). The UCT is a point at which a bird must relieve heat by evaporative cooling (through panting) because chickens do not contain sweat glands. However, if a chicken’s core body temperature is below the LCT, then birds will shiver to increase
their metabolic rate and produce more heat. Much of a broiler’s energy is spent producing skeletal muscle; i.e., breast muscle (BM) at a high turnover rate.

Birds initiate several behavioral and physiological changes in response to heat stress. A behavioral response to thermal challenge is panting (up to 300 times per minute). This permits chickens to utilize their air sacs and remove up to 20% of heat through evaporative cooling because they lack sweat glands. However, this method of evaporative cooling can also cause dehydration. Other responses include decreased feed intake, increased water intake, and lethargy [30]. A physiological response to heat stress comprises the interaction and relay of signals from the central nervous system (CNS). The CNS has been found to integrate three different systems within the body, nervous, endocrine, and immune. More importantly, the hypothalamic-pituitary-adrenal axis (HPA) and sympathetic-adrenal medullar (SAM) axes specifically control responses of the immune system providing an avenue to alter an immune response [30].

Immune cells such as lymphocytes and macrophages contain receptors that when bound by molecules or products from the HPA and SAM axes can modify various cellular activities (cell proliferation, secretion of cytokines etc). Many studies have also indicated a suppressed immune system in both broilers and layers when exposed to heat stress [41]. Wolowczuk, I., et al., 2008 indicated two main energy sources for immune cells, glucose and lipids. Lipids play a dual role as both an energy source and they also help maintain structural integrity of the immune cell membranes. They are supplied by the diet and by adipose tissue. Glucose on the other hand is considered to be the primary energy source for the cells of the immune system. It is required to prevent and recover from infection as well as eliciting an immune response.
for both innate and adaptive responses [61]. Activation of cells such as lymphocytes requires glycolysis to generate energy for cellular activities. Once an immune response is generated, the body has to change cellular activity shifting from homeostasis to a state of increased metabolic activity, demanding higher energy [61].

In response to high environmental temperatures, physical evidence has appeared in the form of reduced organ weights of the spleen, thymus, and liver [5, 30, 34]. In human models protein clumping has been observed within the spleen when exposed to chronic heat stress [33]. Many studies have also pointed out that thermal stress can increase the heterophil to lymphocyte blood ratio [51] and cause decreased intestinal integrity [30]. Loss of the intestinal permeability barrier integrity and function has been seen in both acute and chronic heat stress studies [41, 42]. Because of this permeability loss due to heat stress, the gut microflora can be altered and negatively affect body weight gain and other aspects of the chicken. This allows an influx of toxins or bacteria within the systemic circulation, contributing to inflammatory response and infection [41, 42].

Heat stress can be detrimental to the poultry industry through a variety of factors. In the poultry industry alone there has been an estimate of $128-165$ million of economic losses contributing to the overall economic loss of the U.S. livestock production totaling around $1.7-2.4$ billion [30, 54]. Some of these losses could be prevented by providing sprinkler systems to keep chickens cool during heat waves. If heat stress continues to cause high mortality rates in the poultry industry, there may be a decline in the total number of broilers available for human consumption. In 2015 the U.S. consumed an estimated 106lbs of poultry per capita (this number encompasses all poultry, turkey, chickens etc). The average person consumed about 89lbs in the broiler
category. In 2016, the U.S projected the average person will consume an estimated 90lbs of chicken (strictly broiler meat) and 108lbs across all categories of poultry [54].

This study exploits transcriptomics to understand the spleens response to heat stress in the chicken. A transcriptome is composed of DNA sequences that are transcribed into RNA transcripts found within the cells of an organism [36]. From this information one can identify those genes that respond to a particular environmental stress, such as heat stress. The transcriptome is important because external and internal factors can alter cellular activity, leading to detrimental outcomes. It identifies and quantifies every gene that has been annotated to the chicken genome (annotated in 2004, updated in 2015).

Transcriptomics are useful because it permits tissues to be analyzed and compile evidence as to what may be considered normal for a specific tissue in comparison to a diseased state. This in depth analysis would give scientists the opportunity to resolve problems across an array of disciplines. This is an excellent means for comprehending what is occurring at the cellular and molecular levels of a broiler and contributes to the overall understanding of the different chemical reactions and pathways [16, 19]. In the human and chicken genomes, as a whole, we do not know the function of every gene in the body and what they express [16, 19]. The chicken, like the human, is also used as a model organism for disease and vaccination research to maintain the highest health standards for meat processing and human consumption [10]. The more we comprehend the functionality of genes within model organisms, the better we can utilize them to our advantage for production purposes and overcome issues during the advancement of chicken genetics allowing farmers to raise healthy birds.
Several actions have been taken to counteract thermal stress including studying the effects of embryo thermal conditioning. Many studies have highlighted the possibility of improving a chicken’s ability to handle heat stress later in life [38, 60]. According to Moraes, V. M. B. et al., 2003 and Renaudeau, D. et al., 2012, pre-natal thermal conditioning may lead to improved thermo-tolerance. Thermoregulation and homeostasis is regulated by thyroid hormones triiodothyronine (T3) and thyroxine (T4) [53, 59]. Stojevic et al., 2000 found T3 to have a greater role in metabolic activity compared to that of T4 and indicating T3’s part in energy and heat production. The results of this study illustrated reduced levels of T3, to be correlated with stunted growth in deficient birds [53]. This was supported by evidence found in Moraes et al., 2003 which saw decreased T3 levels and triglycerides in blood plasma in response to heat stress. Overall lower levels of T3 would indicate a positive response to pre-natal thermo-conditioning and resistance to heat stress in older birds.

Although studies have been conducted on preventing the detrimental effects of heat stress, the consequences have also been reviewed to understand how heat stress can affect the broiler industry as a whole. Hyperthermia can cause significant damage to the body regardless of species. In a human study conducted by Lambert, G. P., 2008, intestinal permeability was observed in maintaining the balance of gut microbiota ensuring endotoxins (lipopolysaccharides) do not leak into systemic circulation. However, the study showed that a prolonged hyperthermia can erode the intestinal permeability barrier by disrupting tight junctions of enterocytes in the gut leading to local and systemic reactions. As a result of lipopolysaccharide leakage into the environment outside of the gut, cytokines can be released from monocytes or macrophages monitoring the area as endotoxins are encountered. Hyperthermia can
also cause a reduction in blood flow to core organs through vasoconstriction creating hypoxic conditions and oxidative stress. This further contributes to tissue damage within and outside of the gut [29]. The terminology used to describe this issue in the human model is known as leaky gut syndrome. The immune system responds by producing pro-inflammatory cytokines such as IL-6 and interferon gamma (IFNG) which can also be seen when high levels of LPS’s are found in systemic circulation. Sustaining elevated levels of LPS’s and pro-inflammatory cytokines can lead to multiple organ failures and a reaction similar to sepsis and heat stroke [29].

In the liver, heat stress has been known to cause oxidative stress leading to an increase in enzymatic activity (higher levels of glutathione and oxidized glutathione GSSH) and lower levels of the reduced glutathione GSH: GSSH ratio [32]. The result of this is a possible adaptive mechanism used by the body to cope and protect the liver. Similar results in a mouse study were also seen in response to chronic heat stress [4, 11]. Many studies have marked oxidative stress as a negative effect of heat stress in the liver however; it is unclear if the spleen also exhibits a similar response.

Comparative studies focusing on the differences between Ross 708 and Illinois broilers have shown the impact of human’s genetically altering broiler lines for increased production [50]. Illinois birds were produced from New Hampshire males crossed with females carrying the Columbian feather pattern which have been maintained and inbred at the University of Illinois since the 1940’s. This particular cross allows for easy male and female identification and represents a bird that has not been genetically selected for rapid growth [50]. This study evaluates the transcriptomic gene expression between Ross 708 and Illinois broilers (birds modeled after meat production types from the 1940’s). In this study, the first three weeks of life
(21 days) for both broiler lines were unstressed at 25°C. The heat-stressed groups were then introduced to a cyclic heat stress of 39°C starting at D21 and were maintained for eight hours each day until the birds reached D28.

Analyses revealed activation of the innate immune system in response to heat stress in both broiler lines along with the suggestion of leaky gut syndrome. This study found more enriched gene transcripts in the Ross 708 broilers in response to heat stress compared to the Illinois broilers. One explanation as to why this may have occurred could be due to the fact that Ross 708 broilers may not be adapted to their fast growth rate. This growth rate may be acting as an additional stressor in combination with the heat stress within these birds. Future studies may provide a better explanation as to the mechanisms underlying the current findings in both broiler lines while also providing clarification as to why the spleen did not exhibit a robust response as hypothesized.
Chapter 3

HYPOTHESIS AND OBJECTIVES

Heat stress is one of many factors negatively affecting the broiler industry. Broiler chickens genetically selected for higher meat yield and faster body weight gain (Ross 708 line) are more susceptible to heat stress than broilers with slower growth rates and lower body weight gains (Illinois line). Qualities such as increased growth and weight gain have led to increases in metabolic rates allowing broilers to generate greater amounts of internal heat. The spleen being the main secondary lymphoid organ in a chicken is responsible for regulating immune responses. Studies have shown heat stress alone can cause immune suppression and disease susceptibility [5] however; the addition of increased metabolic rates in some broiler lines may exacerbate these types of responses. We believe that heat stress suppresses the immune response and negatively affects the spleen in various aspects. We predict there will be changes in gene expression seen in the transcriptomic profile comparisons of D28 spleens from the Ross 708 and Illinois broilers. The transcriptomic profile comparisons may reveal negative effects of thermal stress on the erythrocyte, as well as the B and T lymphocyte populations. The hypotheses on the effect of heat stress on gene expression in the chicken spleen include:

1. **Hypotheses 1:** The erythrocyte population will be damaged as a result of the thermal stress and express more genes responsible for blood clearance and hemoglobin production.
2. **Hypothesis$_{1.2}$**: B and T lymphocyte populations will be negatively affected by decreasing their total number of cells because of heat stress. As a result, there may be less gene expression from B and T lymphocytes along with antigen recognition and presentation cells because of immune suppression.

3. **Hypothesis$_{1.3}$**: There may be an increase in the expression of genes responsible for inducing a systemic response.

With these hypotheses in mind, this study was also established to explore the following three objectives:

1. Study the effects of heat stress on the spleen’s normalized growth rate in both Ross 708 and Illinois broilers.

2. Identify genes unique to the control and heat-stressed groups in both Ross 708 and Illinois broilers and understand the differences in gene expression by evaluating their relative tissue expression data. By understanding what genes respond to heat stress in the spleen, we can begin to describe the implications on the immune system.

3. To understand which broiler line demonstrates a larger overall response in the spleen and to identify immune-related genes affected by heat stress.
Chapter 4

MATERIALS AND METHODS

4.1 Animal Rearing, Experimental Design, and Tissue Collection

Broiler eggs were acquired from the University of Illinois (Illinois broilers) and Mountaire farms (Ross 708) in Millsboro, Delaware. All eggs were incubated at 37°C with 60% humidity and hatched out at the University of Delaware (UD). Once hatched, females were culled and the male chicks were divided evenly into control (C) and heat-stressed (HS) groups. They were then placed into separate housing on the UD Farm and given food and water ad libitum. Both groups remained at 37°C and decreased by 4°C each week until the temperature reached 25°C on day 21 (D21) post-hatch. After D21, the heat-stressed groups received 39°C for eight hours a day representing a heat wave condition while the control birds remained at 25°C until D42. The temperature of the heat stress houses was brought down to 25°C for the remaining sixteen hours of the day after receiving the eight hours of heat stress. For this study, splenic gene expression was evaluated on D28 (7 days of heat stress). On D28, the birds were euthanized by cervical dislocation and various tissues, including the spleen were collected for analysis. Each tissue was placed in a two milliliter tube, flash frozen in liquid nitrogen, and stored at -80°C until total RNA isolation. Figure 4 demonstrates the application of cyclic heat during the experiment.
Figure 4: Graph of cyclic heat stress during the Fall 2013 trial and time of necropsy. The graph illustrates the temperature applied (39°C) for eight hours per day to the heat-stressed broiler groups which decreased to 25°C for the remaining 16 hours of the day. The control birds remained at 25°C throughout the entire experiment, while all birds were raised under these conditions until 21 days of age. The red diamond displays the time of necropsy and tissue sample collection. The red circle marks the time frame, estimated to about three to four hours into the heat stress period when the necropsy was performed on D28 broilers.

4.2 Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Delaware (Permit Number: 2703-12-10).
4.3 RNA Extraction

Total RNA was extracted from approximately 45mg of spleen tissue using the MirVana Total RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s protocol. The total RNA was extracted from thirty six, D28 samples. The Ross 708 line had a total of 19 spleen samples. Nine of the samples were controls and the remaining ten were from heat-stressed birds. The remaining 17 samples were from the Illinois broilers. Eight of these samples represented the control birds and the remaining nine were from heat-stressed broilers. Table 1 displays the total number of tissues used in this experiment from both broiler lines in each condition after a RNA quality check was performed. After RNA extraction, each sample was treated with a DNA-free™ DNA Removal Kit following the manufacturer’s protocol (Thermo Fischer Scientific, Waltham, MA, USA) to remove any impurities and debris left behind from RNA isolation. Next, the concentration of each sample was determined using the Qubit 2.0 Fluorometer and sent to the Delaware Biotechnology Institute (DBI) in Newark, Delaware for a final quality check. All RNA samples from the 36 birds had a RNA Integrity Number of 3.1 or higher and they were used to construct transcriptomic libraries.
Table 1: The number of samples used in both control and heat-stressed groups for the Ross 708 and Illinois broiler lines. The total number of spleen samples used in this experiment is also provided and was established after a final RNA quality check.

<table>
<thead>
<tr>
<th>Day</th>
<th>Line</th>
<th>Number of Control Samples</th>
<th>Number of Heat-stressed Samples</th>
<th>Total Number of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>Ross 708</td>
<td>9</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td>28</td>
<td>Illinois</td>
<td>8</td>
<td>9</td>
<td>17</td>
</tr>
</tbody>
</table>

|       |        |                           |                                 | 36                      |

4.4 Library Preparation and Sequencing

After verifying the quality of each sample, a total of 4µg of RNA in conjunction with magnetic oligo-dt beads were used to purify the mRNA samples to generate cDNA constructs from reverse transcriptase. From these constructs, transcriptomic libraries were produced for each spleen sample and prepared using the Illumina TruSeq RNA Sample Preparation Kit (Illumina Inc., San Diego, CA, US) as per the manufacturer’s protocol. The 36 samples were then taken to DBI sequencing core facility in Newark, DE to be sequenced using the Illumina Highseq 2500 instrument. Each library was sequenced approximately at a depth of thirty million reads per library. Sequence reads were then mapped against the Galgal4 version (2011) of the chicken reference database.
4.5 Transcriptomic Analysis

Analysis was conducted after sequencing using an in house pipeline known as fRNAkenseq where Fragment per Kilobase of Exon per Million Fragment (FPKM) values were generated. The data was further analyzed using the statistical program JMP Pro 12 to determine the Differential Expression (Diff Express/DE) and Relative Tissue Expression (RTE) of each sample (See Appendix A).

To find differentially-expressed genes within the data, genes with mean values larger than 0.1 were identified as important because this would allow other methods such as qRT-PCR analysis, to confirm these results. Log$_2$ ratios of FPKM values for the control and heat-stressed chickens were established at D28 for both Illinois and Ross 708 broilers. Any genes with Log$_2$ values of $\geq 1$ and $\leq -1$ were further analyzed because they demonstrated a two-fold difference in expression. Genes were then recognized as differentially-expressed after a paired t-test was conducted and p-values of $< 0.05$ were established. This analysis compares the splenic tissues of both control and heat-stressed samples from both broiler lines. It identifies what genes are significant, if at all, in the 36 samples when compared to all of the spleen tissues analyzed by the lab (See Appendix A for protocol).

Relative tissue expression establishes what genes are enriched in the experimental spleen compared to the remaining tissues of the chicken (See Appendix A for protocol). The Maximum (Max), Median, and Log$_2$ ratio of the Max Tissue divided by the Median Tissue were calculated for each spleen sample from the two chicken lines. Then the Median of all other Tissues (those other than the eight control or heat-stressed spleen samples) was calculated along with the Log$_2$ ratio of Max Tissue divided by Median all Tissue. A means/anova/pooled T–test was performed and genes with a p-value of $< 0.05$ were examined in further detail. The control and
heat-stressed genes were separated at the end of the data transformation to assess the genes in each condition, individually. After completing the differential expression and relative tissue expression analyses, the transcriptomic data was then inserted into multiple bioinformatics tools including Path Rings, the gene ontology database AmiGO2 and WebGIVI. Figure 5 illustrates the different analyses conducted in this study.

**Figure 5:** A flow diagram that represents the different analyses used to identify the enriched genes in the transcriptomic data of the Ross and Illinois broilers. These same methods were also used to compare the enriched genes across broiler lines in each condition.

**4.6 Analysis Descriptions**

The bioinformatics tool Path Rings [67], reviews the pathways of genes found to be enriched in the relative tissue expression and differential expression analyses.
Once the expression data was uploaded, it was then visualized based on a color scale. From here, each pathway can be further expanded to evaluate the enriched genes with the Log$_2$ ratios and determine which genes are rate-limiting, along with their gene symbol and gene ID (entrez ID). This tool also allows for each gene to be described through the National Center for Biotechnology Information database (NCBI) by left clicking on the gene of interest and following the link to the NCBI database site.

AmiGO2 [8] is a gene ontology database that searches for gene annotations in relation to their molecular function, cellular components, and biological processes. For the purposes of this data, only the biological processes were examined. To utilize AmiGO2 the gene symbol associated with each gene from the JMP RTE and DE analysis was placed into the Term Enrichment Service box. The biological process was then selected along with *Gallus gallus* as the species. After launching the analysis, panther provided a list of phrases which can then be sorted based on the number of references found in relation to *Gallus Gallus*, number of genes identified, expected occurrence, fold-enrichment, or p-values. The Bonferroni correction was left marked because it helps to minimize errors and control for true p-values with a statistical significance of p <0.05. Panther also provided the number of mapped and unmapped ID’s. The purpose of this analysis was to grasp a better understanding of each gene’s functionality and determine their role in the avian spleen.

To utilize WebGIVI, the gene ID from Path Rings can be downloaded and inserted into this program to find informative terms (iTerms) and publications associated with those terms through eGIFT (a text mining tool). WebGIVI allows the viewer to manually remove any terms based on the p-value. This text mining tool also allows for deletion of any term that may not be associated with the tissue being
examined. For example, the iTerm spleen would not provide further knowledge on this specific tissue. This data inserted into this program can then be converted from a textual list to a visual aid through a concept map containing data on the inner and outer circles. The outer data is typically sorted based on frequency and the inner data is usually organized alphabetically. For the intents and purposes of this study, the present data was sorted by frequency on the outer circle and alphabetically on the inner circle. The middle of the circle contains a list of genes that can be selected and highlight associated terms on the outer part of the circle. Each gene can be described by right clicking on the gene of interest and following the link to the NCBI database. When a gene is selected in the middle of the inner data circle, a red highlight connects it to the terms on the outer circle. When these highlighted lines are selected, that particular term can then be explored using eGIFT.

An additional bioinformatics tool used was the program, Venn diagram. This program was beneficial because it allowed for transcriptomic data to be compared between different analyses. For example, the Ross 708 enriched genes in the control group could be inserted and compared to the enriched genes of the Illinois control group. Multiple gene lists can be inserted and compared at the same time. Venn diagram was also beneficial in finding the intersections and the complements of those intersections. This tool provides both a textual outcome, as well as a graphical representation of the data inserted.
Chapter 5

RESULTS AND DISCUSSION

5.1 Differential Expression Analysis Results

The differential expression analysis revealed very low numbers of enriched genes in the D28 spleen samples compared to other tissues that were not examined in the current study. A total of 44 differentially expressed genes were found in the Ross 708 broiler line with 18 differentially expressed genes in the control group and 26 enriched genes in the heat-stressed group. The Illinois broilers had a total of 52 differentially expressed genes with 31 genes enriched in the control group and 21 genes enriched in the heat-stressed. It was unclear why there were so few differentially expressed genes observed in the D28 spleen samples in both broiler lines. However, other tissues, such as the liver contained as many as 1,620 differentially-expressed genes with 1,389 genes enriched in the Ross 708 heat-stressed group and 231 genes enriched in the control group (Jastrebski, personal communication). The Ross 708 spleen and liver samples were comparable in this study because seven of the same birds were used. Tables 2 and 3 illustrate the bird identification numbers in the differential expression analysis for both control and heat-stressed D28 spleen tissue samples compared to the D28 liver samples. This finding demonstrates a robust response elicited by other organs which was not observed in the spleen. Other analyses, such as normalized spleen weights and RTE, however, did show a strong effect in the heat-stressed groups of both bird lines.
Table 2: Bird identification numbers that were used in Differential Expression analysis for Ross 708 D28 Control Liver samples compared to Ross 708 D28 Control Spleen samples. Each row represents a randomly selected broiler for this analysis with a total of nine samples in the spleen and seven in the liver.

<table>
<thead>
<tr>
<th>D28 Liver Bird Numbers</th>
<th>D28 Spleen Bird Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/A</td>
<td>204</td>
</tr>
<tr>
<td>212</td>
<td>212</td>
</tr>
<tr>
<td>223</td>
<td>223</td>
</tr>
<tr>
<td>228</td>
<td>228</td>
</tr>
<tr>
<td>235</td>
<td>235</td>
</tr>
<tr>
<td>302</td>
<td>302</td>
</tr>
<tr>
<td>318</td>
<td>318</td>
</tr>
<tr>
<td>N/A</td>
<td>328</td>
</tr>
<tr>
<td>360</td>
<td>360</td>
</tr>
</tbody>
</table>
Table 3: Bird identification numbers that were used in Differential Expression analysis for D28 Ross 708 D28 Heat-Stressed Liver samples compared to Ross 708 D28 Heat-Stressed Spleen samples. Each row represents a randomly selected broiler for this analysis with a total of ten samples in the spleen and seven samples in the liver.

<table>
<thead>
<tr>
<th>D28 Liver Bird Numbers</th>
<th>D28 Spleen Bird Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>207</td>
<td>207</td>
</tr>
<tr>
<td>208</td>
<td>208</td>
</tr>
<tr>
<td>219</td>
<td>219</td>
</tr>
<tr>
<td>N/A</td>
<td>234</td>
</tr>
<tr>
<td>246</td>
<td>246</td>
</tr>
<tr>
<td>331</td>
<td>331</td>
</tr>
<tr>
<td>335</td>
<td>335</td>
</tr>
<tr>
<td>341</td>
<td>341</td>
</tr>
<tr>
<td>N/A</td>
<td>357</td>
</tr>
</tbody>
</table>

Spleen weights were normalized (weight of spleen compared to the total weight of the bird) to account for weight differences between birds. This data encompasses every spleen that was collected during the fall 2013 heat stress trial conducted at the UD. A graph was constructed in JMP for each line to visualize the differences between heat-stressed and control. Figures 6 and 7 represent the average normalized weight of the spleen starting at day 7 (D7) post-hatch to day 42 post-hatch (D42). D28 and D42 were the only time points to receive heat stress (see materials and methods).
Figure 6: The graph represents the mean of the normalized spleen weights at D7, D21, D28 and D42 for control and heat-stressed Ross 708 broilers. The control group is represented by the blue bars and the heat-stressed group is represented by the red bars; each time point for both groups contains an error bar ±1 standard error from the mean. The graph illustrates the slight impact of heat stress on D28 spleens, with a statistically significant impact of heat stress (p-value < 0.002) on D42 spleens. The asterisk indicates statistical significance of heat stress on D42 broilers.
Figure 7: The graph represents the Illinois control and heat-stressed broiler groups of the mean normalized spleen weights at D7, D21, D28 and D42. The control group is represented by the blue bars and the heat-stressed group is represented by the red bars; each time point contains an error bar ±1 standard error from the mean. The graph illustrates the slight impact of heat stress on D28 spleens, with a statistically significant impact of heat stress (p-value < 0.002) on D42 spleens. The asterisk indicates statistical significance of heat stress on D42 broilers.

Figure 6 illustrates the mean weight of the Ross 708 control spleen group for D7, D21, D28 and D42. At D7 the graph shows an average normalized spleen weight of 0.056 grams which increased by 0.0319 grams (63.7%) giving a total organ weight of 0.0879 grams for the D21 control group. D21 and D28 control were similar in weight, differing by 0.0019 grams (increased by ~2.1%). When D7 was compared to D28 the spleen increased in weight by 0.0338 grams. When the three previous time points were compared to a market ready bird, the graph also showed an increase in weight demonstrating a normal growth pattern for Ross 708 broilers. As seen in the
graph, D7, D21 and D28 compared to D42 normalized spleens increased by 0.127 grams, 0.0951 grams and 0.0932 grams (49.1%).

The Ross 708 birds had a steady increase in organ weight under control conditions. Although, there was not much progress occurring between D21 and D28 time points, growth is still happening. The highest growth percentage was seen in the first three weeks of life (from D7 to D21 at 63.7%) suggesting the spleen grows more quickly between D7 and D21 compared to D21 and D28 or D28 and D42. This observation is suggestive of how energy is partitioned to the spleen and the immune system during the first three weeks of life. It is possible that D14 may have been an indicator of fast growth; however, this time point was not sampled during the fall 2013 heat stress trial. The second greatest amount of growth transpired from D28 to D42 which can be seen in Figure 6 (49.1%).

Heat stress did impact the normalized spleen weights as shown in Figure 6 represented by the last two bars on the right side of the graph. The heat-stressed D28 broilers had normalized spleen values that were slightly smaller, differing by 0.0109 grams (12.1%) compared to the control group. After reviewing D42 control birds in relation to D42 heat-stressed birds, the heat-stressed group was 0.0862 grams (~52.9%) smaller and this difference was found statistically significant in JMP with a p-value < 0.002. Lastly, D21 and D28 control birds in comparison to D42 heat stress birds had similar differences.

Although Ross 708 broiler D28 spleens reveal a small difference and less of a robust response to heat stress, there is an indication that heat stress had an impact on the spleen. The birds most likely were not sufficiently heat-stressed at D28 for the spleen or the immune system to manifest a vigorous response. D42 provides the most
evidence, demonstrating the greatest impact on the spleen in response to heat and most likely one of statistical significance. As shown in Figure 6, one can visualize the impact of heat stress on market weight bird spleens because they are half the size of the controls. Heat stress, in this case significantly impacts the overall growth, and growth rate and it suggests the possibility of impacting other activities of the spleen at both cellular and molecular levels. A similar result was also illustrated in the Ross 708 broiler graph when contrasting D21 control birds and D42 heat-stressed birds. This comparison was done to show that heat-stressed D42 normalized spleens were only slightly bigger than healthy D21 control spleens that are not fully developed.

Figure 7 represents the growth of the control and heat-stressed groups in the Illinois line. D7 had an average spleen weight of 0.0454 grams and grew by 0.0786 grams over seven days to reach a total organ weight of 0.124 grams at D21. This growth represents a 36.6% increase in weight and most likely size, of the spleen. However, when comparing D21 control birds to D28 control birds, they differed by 0.024 grams which approximated to a 16.2% increase in weight. Even though there was a small difference in weight between these two time points, there was a larger gap between D28 controls and D42 controls. These two time points differed by 0.145 grams estimating to be a 50.5% increase in weight (total of 0.293 grams at market age). A similar result was seen when comparing D21 to D42 control birds which showed a difference of 0.169 grams or about 42.3%.

Figure 7 also represents the implications heat stress had on the overall mean of normalized weight of the spleen for the Illinois broilers. The D28 heat-stressed spleen weight in comparison to the control D28 spleen weight is 0.02 grams or 13.5% smaller than the control birds. With these numbers in mind, it can be seen that D28 heat-
stressed spleens are very similar to the size of D21 control broilers. There was a larger
difference in normalized organ weight when D28 heat-stressed broilers were
contrasted with D42 control broilers. This comparison differs by 0.165 grams or about
43.7%. Similar results were seen with D42 heat-stressed birds paralleled with D42
control birds. The heat stress reduced the spleen weight by 0.138 grams which
estimated to be about 52.9% lighter than the control spleens. This comparison was also
found to be statistically significant when evaluated in JMP with a p-value < 0.002.
Finally when looking at the graph it can also be seen that heat challenged market
weight birds were only 0.031 grams (~20%) heavier than D21 control. This provides
evidence that heat stress does have an impact on normalized spleen weight. However,
the Illinois broilers just like the Ross 708 line may not have been exposed to adequate
heat stress at D28 to see a strong response.

As seen in Figure 8 overall, Ross control spleens were smaller in size
compared to that of the Illinois except at D7. A normalized spleen weight at D7 in the
Ross 708 line was 0.0106 grams or 18.9% larger than the Illinois D7 spleens. However,
for the remaining time points, Ross 708 broiler normalized spleen weights were
smaller. The D21 Ross 708 broilers spleen was 0.0361 grams (29.1%) smaller than that
of the Illinois; 0.0582 grams (39.3%) lighter at D28; and at D42, the normalized
spleen weight of the Ross 708 broiler was smaller by 0.11 grams (37.5%) compared to
the Illinois broiler. Table 4 demonstrates the average and normalized spleen weights
among both Illinois and Ross 708 broilers from D7 until D42. When a T-test was
performed in JMP pro 12, there was no statistical significance found in the spleen
weight between or within the two broiler lines looking at the control and heat stressed
groups.
Figure 8: The graph represents both broiler lines for the average normalized spleen weights at D7, D21, D28, D42. The control group is represented by the blue bars and the heat-stressed group is represented by the red bars; each time point contains an error bar ±1 standard error from the mean. The graph shows the proportional impacts of heat stress on both broiler lines when compared to one another. The line with asterisks indicates statistical significance found in both broiler lines at D42 when comparing control groups to heat-stressed groups.
Table 4: The average spleen weight and body mass under control and heat-stressed conditions measured in grams for both Ross 708 and Illinois broilers at D7, D21, D28 and D42. There was no statistical significance found between either line under control or heat-stressed conditions when the means of the absolute spleen weight and body mass were evaluated.

<table>
<thead>
<tr>
<th>Day &amp; Line</th>
<th>Absolute Spleen Control Mean (g)</th>
<th>Absolute Spleen Heat Stress Mean (g)</th>
<th>Normalized Spleen Control Mean (g)</th>
<th>Normalized Spleen Heat Stress Mean (g)</th>
<th>Control Body Mass Average (g)</th>
<th>Heat Stress Body Mass Average (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D7 Illinois</td>
<td>0.054545455</td>
<td>0.041666667</td>
<td>0.052090909</td>
<td>0.039416667</td>
<td>103.2090909</td>
<td>105.1833333</td>
</tr>
<tr>
<td>D7 Ross</td>
<td>0.090909091</td>
<td>0.083333333</td>
<td>0.056272727</td>
<td>0.055833333</td>
<td>158.2727273</td>
<td>146.2166667</td>
</tr>
<tr>
<td>D21 Illinois</td>
<td>0.6</td>
<td>0.56</td>
<td>0.124454545</td>
<td>0.1245</td>
<td>473.9</td>
<td>448.77</td>
</tr>
<tr>
<td>D21 Ross</td>
<td>0.791666667</td>
<td>0.85</td>
<td>0.079666667</td>
<td>0.098666667</td>
<td>995.975</td>
<td>859.5888889</td>
</tr>
<tr>
<td>D28 Illinois</td>
<td>1.058333333</td>
<td>1.111111111</td>
<td>0.133090909</td>
<td>0.14625</td>
<td>767.0727273</td>
<td>765.5555556</td>
</tr>
<tr>
<td>D28 Ross</td>
<td>1.408333333</td>
<td>1.236363636</td>
<td>0.084166667</td>
<td>0.083727273</td>
<td>1669.416667</td>
<td>1448.245455</td>
</tr>
<tr>
<td>D42 Illinois</td>
<td>2.6</td>
<td>3.666666667</td>
<td>0.189636364</td>
<td>0.272166667</td>
<td>1364.372727</td>
<td>1327.6</td>
</tr>
<tr>
<td>D42 Ross</td>
<td>4.641666667</td>
<td>2.533333333</td>
<td>0.144083333</td>
<td>0.088333333</td>
<td>3151.208333</td>
<td>2863.083333</td>
</tr>
</tbody>
</table>

When reviewing the heat stress impact within and between both lines it can be seen that D28 Illinois bird’s heat stress decreased the size of the spleen by 0.02 grams (~13.5%) in comparison to the control group. The heat-stressed Ross 708 birds at D28 only differed from the C by 0.0109 grams (12.1%). D42 also showed a similar result in each graph. Illinois D42 control bird’s spleens were 0.138 grams (52.9%) heavier than the heat challenged spleens, while the spleens of Ross 708 control market weight birds were 0.0862 grams (52.8%) heavier than the heat challenged spleens.
The above results suggest that both lines are taking a comparable decrease in the normalized spleen weight by heat stress at D28 and D42. Originally it was thought that the Illinois broilers would be more affected because they have a larger overall normalized organ mass compared to the Ross 708 broilers. The entirety of heat stress and its implications on the spleen is not seen at D28, but as shown in Figure 8, the effects were greater at market weight. It may be that the spleen is avoiding or dampening down a vigorous response at earlier time points and only beginning an inflammatory reaction. This could be due to less proliferation of B and T lymphocytes or a decreased blood flow through the spleen. However, the exact cause for a smaller normalized spleen is still unknown. These graphs demonstrate a turning point at D28 for broilers who have been subjected to heat stress. Overall in both broiler lines, the spleen grew more quickly between D7 and D21 compared to the rest of the body as reflected by both graphs. This suggests that the first three weeks of life may be more vital for growth and immune expansion than in the fourth week of life.

5.2 RTE Analysis using Gene Ontology (GO) and WebGIVI

Gene ontology (GO) analysis was conducted using AmiGO2 to find genes in the RTE analysis (17,493 genes were analyzed for broiler lines) for both Illinois and Ross 708 broilers. For each line, the genes specific to control and heat-stressed groups were inserted into AmiGO2 separately followed by WebGIVI. For further information on the functionality of the genes found in both bioinformatics tools, the genes were then examined by the text mining tool eGIFT, as well as NCBI if sufficient information was not found (Not all genes were found in each tool). After this analysis was performed, the informative term (iTerm) spleen was evaluated for the number of
genes associated with it in eGIFT and compared to the list of genes found in both chicken lines for each condition.

5.3 RTE Analysis of Ross 708 Control genes with the iTerm Spleen

A total of 1,212 genes were enriched in the Ross 708 D28 RTE analysis comprising the control and heat-stressed data. Of those genes, 751 were in common between both conditions while the control birds contained 235 genes; this data was found using Venn diagram. This list of 235 genes found in the control birds was then compared to the iTerm spleen list which contained 485 genes. The genes in common between the two lists were determined using JMP and checked by Venn diagram. From this, only three genes were highlighted by this analysis and assessed by AmiGO2. Upon GO evaluation, there were no biological processes found with Bonferroni correction.

A few biological processes were found when the Bonferroni correction was disabled (See Table 5). The top 10 GO processes were sorted, based on p-values with a statistical significance of <0.05 (smallest to largest) and the genes within each process were investigated further. The CD83 gene was the only genetic factor linked with each process during this analysis. However, CD83, tumor necrosis factor receptor superfamily, member 18 (TNFRSF18) and retinol binding protein 3, interstitial (RBP3) were all recognized by WebGIVI and their roles were determined through eGIFT.
Table 5: The top 10 biological processes found in the gene ontology database, AmiGO2 without the Bonferroni correction for the comparison of Ross 708 broilers Control RTE genes with the iTerm Spleen. The biological processes are sorted based on p-value of <0.05.

<table>
<thead>
<tr>
<th>GO biological process complete</th>
<th>#</th>
<th>#2</th>
<th>expected</th>
<th>Fold</th>
<th>Enrichment</th>
<th>+/-</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative regulation of interleukin-4 production</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>&gt; 100</td>
<td>+</td>
<td>1.90E-04</td>
<td></td>
</tr>
<tr>
<td>positive regulation of interleukin-10 production</td>
<td>13</td>
<td>1</td>
<td>0</td>
<td>&gt; 100</td>
<td>+</td>
<td>8.23E-04</td>
<td></td>
</tr>
<tr>
<td>regulation of interleukin-4 production</td>
<td>13</td>
<td>1</td>
<td>0</td>
<td>&gt; 100</td>
<td>+</td>
<td>8.23E-04</td>
<td></td>
</tr>
<tr>
<td>positive regulation of CD4-positive, alpha-beta T cell differentiation</td>
<td>14</td>
<td>1</td>
<td>0</td>
<td>&gt; 100</td>
<td>+</td>
<td>8.87E-04</td>
<td></td>
</tr>
<tr>
<td>positive regulation of CD4-positive, alpha-beta T cell activation</td>
<td>16</td>
<td>1</td>
<td>0</td>
<td>&gt; 100</td>
<td>+</td>
<td>1.01E-03</td>
<td></td>
</tr>
<tr>
<td>regulation of CD4-positive, alpha-beta T cell differentiation</td>
<td>17</td>
<td>1</td>
<td>0</td>
<td>&gt; 100</td>
<td>+</td>
<td>1.08E-03</td>
<td></td>
</tr>
<tr>
<td>regulation of interleukin-10 production</td>
<td>18</td>
<td>1</td>
<td>0</td>
<td>&gt; 100</td>
<td>+</td>
<td>1.14E-03</td>
<td></td>
</tr>
<tr>
<td>regulation of CD4-positive, alpha-beta T cell activation</td>
<td>20</td>
<td>1</td>
<td>0</td>
<td>&gt; 100</td>
<td>+</td>
<td>1.27E-03</td>
<td></td>
</tr>
<tr>
<td>positive regulation of interleukin-2 production</td>
<td>24</td>
<td>1</td>
<td>0</td>
<td>&gt; 100</td>
<td>+</td>
<td>1.52E-03</td>
<td></td>
</tr>
<tr>
<td>positive regulation of alpha-beta T cell differentiation</td>
<td>24</td>
<td>1</td>
<td>0</td>
<td>&gt; 100</td>
<td>+</td>
<td>1.52E-03</td>
<td></td>
</tr>
</tbody>
</table>
In the control birds normal expression levels of CD83 regulates B and T cells which produces CD4 + cells. TNFRSF18 (also known as glucocorticoid inducible tumor necrosis factor receptor related protein, GITR) also affects T and B cells, macrophages and regulatory T cells (Tregs). RBP, nonetheless, can be found everywhere in the body. It can be found in areas including, but not limited to, blood, (plasma and serum) digestive tract, and organs, such as the liver (hepatocytes). These three factors are expected in the control birds because of their basic functions and properties. The cytokines were inserted into the NCBI database to obtain more information and understand their role in the spleen.

Expression of CD83 in control broilers could indicate B and T lymphocytes encountering antigen from the environment and removing it. CD83 is typically expressed on various immune cells including, B cells, T cells and dendritic cells. At D28, these control broilers would express low levels of CD83 on resting lymphocytes to generate tolerance. If CD83 was detected in the heat-stressed broilers, it would be a good marker of activation of the immune system. If this occurred, the spleen could increase or decrease a response depending on what type of cytokines were present. Several anti-inflammatory and pro-inflammatory cytokines that could be secreted include interleukins 6, 10 (IL-6, IL-10) and tumor necrosis factor (TNF), all of which are recognized by the CD83 receptor. With invading pathogens, the spleen could dampen down the immune response or exacerbate a response to foreign pathogens.

5.4 RTE Analysis of Ross 708 Control genes with AmiGO2 and WebGIVI

The 235 genes enriched in the control RTE Ross 708 broiler line were evaluated through gene ontology with the Bonferroni correction. The major biological
process represented by the data set was sensory perception and unclassified. The 10 genes observed in sensory perception contained information related to auditory and visual perception with the exception of RBP. The gene list was then evaluated by WebGIVI which found visual perception genes that were strictly related to the retina and photoreceptors. There was no evidence linking the sensory perception genes to the spleen or immune response. The list of 235 enriched genes was then re-evaluated in AmiGO2 without the Bonferroni correction and sorted based on a p-value <0.05. The top ten processes were then examined and found to have biological processes related to regulation of T cell differentiation. Numerous processes relating to lymphocyte differentiation was also observed within the data (See Table 6).
Table 6: 235 enriched genes found in the Ross 708 control RTE analysis were inserted into AmiGO2 and the top 10 biological processes found without the Bonferroni correction. The biological processes were sorted based on a p-value of <0.05. The red boxes signify similar processes containing identical cytokines and differing by one factor found in the regulation of T cell differentiation process.

<table>
<thead>
<tr>
<th>GO biological process complete</th>
<th>#</th>
<th># expected</th>
<th>Fold Enrichment</th>
<th>+/-</th>
<th>P -value</th>
</tr>
</thead>
<tbody>
<tr>
<td>sensory perception</td>
<td>284</td>
<td>10</td>
<td>1.58</td>
<td>6.32</td>
<td>+</td>
</tr>
<tr>
<td>visual perception</td>
<td>95</td>
<td>6</td>
<td>0.53</td>
<td>11.33</td>
<td>+</td>
</tr>
<tr>
<td>sensory perception of light stimulus</td>
<td>100</td>
<td>6</td>
<td>0.56</td>
<td>10.77</td>
<td>+</td>
</tr>
<tr>
<td>positive regulation of CD4-positive, alpha-beta T cell differentiation</td>
<td>14</td>
<td>3</td>
<td>0.08</td>
<td>38.45</td>
<td>+</td>
</tr>
<tr>
<td>positive regulation of CD4-positive, alpha-beta T cell activation</td>
<td>16</td>
<td>3</td>
<td>0.09</td>
<td>33.64</td>
<td>+</td>
</tr>
<tr>
<td>regulation of CD4-positive, alpha-beta T cell differentiation</td>
<td>17</td>
<td>3</td>
<td>0.09</td>
<td>31.66</td>
<td>+</td>
</tr>
<tr>
<td>regulation of CD4-positive, alpha-beta T cell activation</td>
<td>20</td>
<td>3</td>
<td>0.11</td>
<td>26.91</td>
<td>+</td>
</tr>
<tr>
<td>neurological system process</td>
<td>478</td>
<td>10</td>
<td>2.66</td>
<td>3.75</td>
<td>+</td>
</tr>
<tr>
<td>positive regulation of alpha-beta T cell differentiation</td>
<td>24</td>
<td>3</td>
<td>0.13</td>
<td>22.43</td>
<td>+</td>
</tr>
<tr>
<td>regulation of T cell differentiation</td>
<td>59</td>
<td>4</td>
<td>0.33</td>
<td>12.16</td>
<td>+</td>
</tr>
</tbody>
</table>
Four processes within Table 6 had three identical gene products; IL-6, sterile alpha motif (SAM) and Src homology-3 domain containing (SH3) (SASH3), and CD83. IL-6 is a cytokine involved with maturing B cells, as well as acute and chronic inflammation. However, this cytokine can be anti-inflammatory or pro-inflammatory, based on the physiological state of the bird [49] (IL-6 is typically pro-inflammatory, in most cases) and SASH3 encodes a protein involved in cell signaling which may also be involved with lymphocytes. Several of the biological processes signified by the red boxes contained three identical gene products as the regulation of T cell differentiation process in Table 6. The regulation of T cell differentiation contained an extra gene known as Forkhead box N1 (FOXN1). CD83, FOXN1, SASH3, and IL-6 were inserted into WebGIVI for additional information and four terms were established as follows; differentiate, immune, lymphoid and mature. However; they were only related to CD83 and FOXN1 as illustrated in Figure 9.
Figure 9: Simplified version of the concept map from WebGIVI. The concept map converted the initial gene list (CD83, FOXN1, SASH3, and IL-6) from the Ross 708 control RTE data, into a visual representation. It illustrates the four terms associated with two of the four genes inserted for the analysis. Differentiate, immune, mature and lymphoid were the four terms found by this analysis and only CD83 and FOXN1 were found with an association.

FOXN1 is responsible for differentiation and development of the CNS and it may signify normal maintenance and upkeep within the Ross 708 broiler. However, mutations in this gene could lead to immunodeficiency in T cells and ultimately, causing immune diseases. This idea was confirmed by gene knockout studies completed on mice [66]. CD83, although described above, is found to be expressed on several cells including macrophages, dendritic cells and lymphocytes. It may be an indicator of the activation of the immune system because at D28 these broilers are still quite young. These broilers may be generating tolerance to an antigen picked up in their environment. This may be the reason why CD83 was enriched in the Ross 708 control RTE. With this in mind these enriched genes may be indicators of a properly functioning immune system and spleen. Further studies would provide additional
information on these specific genes and gene products as well as others possibly involved in later stages of broiler development. These enriched genes offer evidence of normal physiological processes taking place in the Ross 708 control broilers.

5.5 RTE Analysis of Ross 708 Heat-Stressed genes with the iTerm Spleen

Out of the 1,212 genes found to be enriched in the Ross 708 RTE data, 225 genes were identified in the Ross 708 heat-stressed condition by Venn diagram and then re-analyzed with the iTerm Spleen (485 genes). Figure 10 represents a typical result from the Venn diagram program. This comparison found ten genes in common which were then inserted into AmiGO2 to find the biological process, immune system. This process had seven genes in association with it (See Table 7). Three of the ten genes, acid phosphate, prostate (ACPP), colony stimulating factor 3 (CSF3) and tyrosine aminotransferase (TAT) were not found by AmiGO2. Two of the three genes, ACCP and TAT, were unclear as to what their role may be in the spleen, while CSF3 was found to have a relation to granulocytes and therefore, further examined. The relevance of these three genes to the current data was determined through utilization of the NCBI database. Table 7 contains the seven genes along with their entrez ID, gene symbol and gene names observed in the gene ontology analysis.
Figure 10: Recreated Venn diagram results. Venn diagram gives a visual representation of the genes enriched in the Ross 708 broiler line RTE data. There were a total of 1,212 genes with 751 enriched genes in common to both conditions with 235 specifically enriched genes in the control broilers and 225 genes enriched in the heat-stressed broilers.
Table 7: Seven of the ten genes found by the gene ontology analysis and relevant to D28 spleen data from the Ross 708 heat-stressed RTE in comparison with the iTerm Spleen (485 genes).

<table>
<thead>
<tr>
<th>Entrez ID</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>395733</td>
<td>BLNK</td>
<td>B cell linker protein</td>
</tr>
<tr>
<td>374075</td>
<td>BTK</td>
<td>Tyrosine-protein kinase BTK</td>
</tr>
<tr>
<td>420129</td>
<td>IFI30</td>
<td>Lysosomal thiol reductase</td>
</tr>
<tr>
<td>430409</td>
<td>IRF5</td>
<td>Interferon Regulatory Factor 5</td>
</tr>
<tr>
<td>396460</td>
<td>LCK</td>
<td>Proto-oncogene tyrosine protein kinase LCK</td>
</tr>
<tr>
<td>423165</td>
<td>RAG2</td>
<td>V(D)J recombination – activating protein 2</td>
</tr>
<tr>
<td>418638</td>
<td>TLR7</td>
<td>Toll Like Receptor 7</td>
</tr>
</tbody>
</table>

Three of the seven genes, B-cell linker (BLNK), Bruton tyrosine kinase (BTK) and Recombination activating gene 2 (RAG2), all are involved with BCR signaling pathways, B cell development and a small overlap with B and T cell rearrangement. Interferon regulatory transcription factor 5 (IRF5) and Toll like receptor 7 (TLR7) were both observed to have relations with pro-inflammatory cytokine processes, while Non-receptor tyrosine kinase (LCK/P56LCK) and Interferon gamma-inducible protein 30 (IFI30/GILT) were unrelated to either of the previous topics. They were shown to be involved with the innate immune system when challenged with bacteria.
Several of these genes were found to be involved in the innate immune system (BTK, IFI30, IRF-5 and TLR-7) although; most of them also have a role in the adaptive immune system (BTK, BLNK, and TLR-7). IRF5, TLR-7, (Within literature, the chicken TLR7 has been identified to be or have similar functionality compared to its mammalian counterpart [24] and IFI30 are particularly important because they interact with one another when they are challenged with bacteria. IRF-5 for example, is a transcription factor that can be expressed from various types of cells (e.g. dendritic cells and B cells). It typically has anti-viral containing properties to disrupt viral production; however, recent studies have demonstrated its role in “cell cycle, apoptosis, microbial infection and inflammation” [46]. Interestingly, studies have shown IRF-5 deficient mice cannot mount a response to bacteria (e.g lipopolysaccharides) and therefore, would not elicit TH1/TH17 responses; similar effects can be seen in autoimmune diseases [46]. Myeloid differentiation factor 88 (MyD88) can interact with IRF5 to secret pro-inflammatory cytokines and type 1 interferons; without such interaction, IL-6 production could be reduced [13]. These genes and their secreted products are important for eliciting a proper innate immune response.

According to several studies, heat stress can cause immunosuppressive activity through a variety of factors, such as oxidative stress [13, 35]. Oxidative stress is typically indicated by heat shock proteins (HSP); however, there were no HSP’s found in the D28 Ross 708 heat-stressed data. Another study conducted at UD observed oxidative stress occurring in D28 livers. This information can be supported by avian literature. Literature also indicates the liver and the intestines to be the first organs affected by hyperthermia and oxidative stress [21]. Another reason why this is
important is due to the numerous genes found at D28 for Ross 708 heat-stressed broilers regarding bacterial challenge. G. P. Lambert, 2008 identified how hyperthermia can be a source of leaky gut syndrome synergistically with oxidative stress or independently, and cause an increase in intestinal permeability to endotoxins, for example, lipopolysaccharides (LPS). Although these endotoxins normally inhabit the gut of a broiler, they can diffuse through damaged enterocytes and leak into the general circulation, causing a robust inflammatory response by the body [29]. The genes found in this analysis may imply an active innate immune response, while beginning to mount an adaptive immune response. Evidence can be seen in the immunoglobulin gene rearrangement expression of RAG2, along with the presence of TLR7. The interaction of these genes may provide an explanation on how chronic heat challenge negatively affects the spleen and immune response. The extent of this challenge on the broiler’s immune system is still unknown.

5.6 RTE Analysis of Ross 708 Heat-Stressed genes with AmiGO2 and WebGIVI

Another analysis was conducted with the 225 genes found to be enriched in D28 Ross 708 heat-stressed RTE using AmiGO2. Once analyzed, (without the Bonferroni correction), the major biological process of greatest interest was found to be cell communication. The remaining processes were very broad and provided minimal information on how heat stress affects the spleen. Thirty eight genes were associated with this process; however, two of the genes identified, LOC101748451 and LOC101749876 had very little information available pertaining to their function when reviewed by NCBI and WebGIVI. The thirty six genes were then inserted into WebGIVI of which nineteen were identified and described (See Table 8). The major
concepts found within those nineteen genes consisted of serotonin, LPS signaling, energy homeostasis, pro-inflammatory cytokines and relation to B cell activity.

Table 8: 225 genes found enriched in D28 Ross 708 heat-stressed RTE were inserted into AmiGO2. The top 10 biological processes were sorted based on a p-value of <0.05 with a disabled Bonferroni correction. The process, cell communication was found to have thirty eight genes containing pertinent information to the present study.

<table>
<thead>
<tr>
<th>GO biological process complete</th>
<th>#</th>
<th>#</th>
<th>expected</th>
<th>Fold Enrichment</th>
<th>+/-</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>single-organism process</td>
<td>7871</td>
<td>79</td>
<td>55.33</td>
<td>1.43</td>
<td>+</td>
<td>4.09E-06</td>
</tr>
<tr>
<td>single organism signaling</td>
<td>2872</td>
<td>38</td>
<td>20.19</td>
<td>1.88</td>
<td>+</td>
<td>4.09E-05</td>
</tr>
<tr>
<td>signaling</td>
<td>2875</td>
<td>38</td>
<td>20.21</td>
<td>1.88</td>
<td>+</td>
<td>4.19E-05</td>
</tr>
<tr>
<td>cell communication</td>
<td>2933</td>
<td>38</td>
<td>20.62</td>
<td>1.84</td>
<td>+</td>
<td>6.55E-05</td>
</tr>
<tr>
<td>signal transduction</td>
<td>2680</td>
<td>35</td>
<td>18.84</td>
<td>1.86</td>
<td>+</td>
<td>1.25E-04</td>
</tr>
<tr>
<td>immune response-activating signal transduction</td>
<td>114</td>
<td>6</td>
<td>0.8</td>
<td>7.49</td>
<td>+</td>
<td>1.68E-04</td>
</tr>
<tr>
<td>immune response-regulating signaling pathway</td>
<td>122</td>
<td>6</td>
<td>0.86</td>
<td>7</td>
<td>+</td>
<td>2.41E-04</td>
</tr>
<tr>
<td>activation of immune response</td>
<td>133</td>
<td>6</td>
<td>0.94</td>
<td>6.42</td>
<td>+</td>
<td>3.80E-04</td>
</tr>
</tbody>
</table>
Table 8 continued

<table>
<thead>
<tr>
<th>synaptic transmission, cholinergic</th>
<th>30</th>
<th>3</th>
<th>0.21</th>
<th>14.22</th>
<th>+</th>
<th>1.31E-03</th>
</tr>
</thead>
<tbody>
<tr>
<td>regulation of cellular process</td>
<td>6374</td>
<td>61</td>
<td>44.81</td>
<td>1.36</td>
<td>+</td>
<td>1.33E-03</td>
</tr>
</tbody>
</table>

Two genes in this data were identified with a connection to serotonin. One was recognized in eGIFT as Arrestin 3, retinal (ARR3) and the other gene, 5-hydroxytryptamine (serotonin) receptor 1F, G protein-coupled receptor (HTR1F) was found in the NCBI database. The three factors containing information related to lipopolysaccharides were Antigen receptor (CD14), Lymphocyte antigen (LY96) and, a C-X-C3 motif chemokine receptor (CX3CR1), while Angiopoietin-like 3 (ANGPTL3), Galanin receptor 3 (GALR3), Hypocretin (orexin) neuropeptide precursor (HCRT) and Prolactin releasing hormone receptor (PRLHR) were found to have an established connection to energy homeostasis. Each of these genes and/or gene products was found using the gene ontology database and text mining tool eGIFT.

The remaining ten components in this analysis contained two involved with pro-inflammatory cytokines; Interleukin 17F (IL-17F) and Interferon regulatory factor 5 (IRF-5). Three other elements within this group were found to be involved with BCR signaling; BLNK, BTK and CD24. The remaining five genes that were detected in WebGIVI were unrelated to the spleen and the five concepts recognized by the gene list.
The remaining seventeen genes (out of the thirty six analyzed) were then reviewed using the NCBI database, and they were grouped based on their descriptions and relativity to the spleen and immune system. Three genes out of this group were found with a relationship to the CNS; BR serine/threonine kinase 2 (BRSK2), gamma-aminobutyric acid type A receptor rho 3 subunit (GABRR3/GABA) and reticulon 4 receptor (RTN4R). BRSK2 was involved in polarizing neurons through the use of serine threonine kinase which also stimulates axonogenesis, cell cycle and the secretion of insulin when glucose levels are high. GABRR3 is a neurotransmitter (NT) receptor in the CNS regulating neurotransmitter passage across the synapses of neurons while RTN4R inhibits axon growth, but may be involved with axonal regeneration. Genes that were unrelated to either the spleen or the immune system was not mentioned in the paragraphs to follow.

The genes enriched in this data were found to interact with one another and were all related to serotonin, LPS and pro-inflammatory cytokines [25]. It was previously shown that serotonin is derived from the gut and transported into the blood (main function of the spleen is to filter the blood) which is why ARR3 and HTR1F may have shown up in the spleen tissue. However, according to this article, the recognition of serotonin is due to heat stress which can cause the aggregation of platelets when thermal challenge is sustained for long periods of time. The serotonin can be “released by agitation and lysis of platelets” [25]. Figure 11 which was taken from the Leon and Helwig 2010 study demonstrates the multiple effects of heat stress on different aspects of the body. Heat stress can cause coagulation and increased gut permeability, leading to LPS leakage into the systemic circulation. These endotoxins
as mentioned previously can cause inflammation when they are recognized by the innate immune system resulting in the release of several cytokines [33].

Figure 11: This figure was taken from Leon and Helwig, 2010 illustrating the numerous aspects heat stress can effect in broiler chickens. Heat stress has the ability to cause vasoconstriction in various areas of the body (brain and gut) and initiate a cascade of effects, activating various cytokines. Activation of cytokines and other factors can result in activation of the immune system and in severe cases cause organ failures [33].
Chronic heat stress may be causing the broilers to produce anti-inflammatory factors preventing them from eliciting a vigorous inflammatory immune response. Since the birds received seven days of heat stress by D28, their bodies may have begun to acclimate to an increase in ambient temperature. Factors such as cytokines would help dampen down an immune response in response to a thermal challenge. Observing IL-17F in this data suggests the broilers are mounting an immune response. Korn et al., 2009 showed that, IL-17 and IL-17F are produced by T helper 17 cells (TH17). TH17 cells are a newly discovered subset of TH cells which are beneficial in removing pathogens during immunogenic responses. IL-17 and IL-17F could also be an indicator of shifts in the gut microbiome as well as germinal center formation. Korn et al., 2009 concluded that TGF-β and IL-6 are required for TH17 cells to manufacture IL-17 along with IL-21 because it synergizes with IL-6 to turn the TGF-β driven Treg response into a TH17 response. Figure 12 shows the relationship between these genes and their products within a mouse model. Although TGF-β was not detected by the RTE analysis, TGF-β was found in the preliminary spleen data for the Ross 708 heat-stressed broilers. TGF-β is expressed everywhere in the body which may be the reason it was not enriched in the RTE analysis.
Figure 12: An example of cell differentiation in a mouse model adopted from Korn et al., 2009. This diagram demonstrates the relationship between innate immune cells, naïve T cells, TH17 cells in combination with TGF-β, IL-6 and the production of cytokines, such as IL-17 [27].

Heat stress alone can reduce blood flow, shunting the blood towards the skin by initiating vasoconstriction in splanchnic tissues for evaporative cooling purposes. If vital organs such as the spleen, liver, and gut do not receive proper blood flow, then the effects could be detrimental to overall tissue health. With vasoconstriction, a reduced blood flow can create a hypoxic environment and oxidative stress. In turn, the oxidative stress can cause a cascade of effects, permitting the passage of endotoxins out of the gut, through damaged enterocytes and, into systemic circulation. These endotoxins can then enter other organs and initiate an immune response. (LPS’s was not evaluated in the spleen in the present study). Figure 11 demonstrates the effects of a decreased blood flow in the gut [33]. With this in mind, energy is spent repairing the damage caused initially by an increased ambient temperature and not on the bird’s
growth and development. This may be why ANGPTL3, GALR3, HCRT and PRLHR are enriched in the heat-stressed group of Ross 708 broilers which help to maintain energy balance.

CD247, TLR7, IRF5 and IL-17F may be enriched in the Ross 708 heat-stressed data because they tend to be expressed when there is an innate, innate/adaptive, or an adaptive immune response occurring. Although the term autoimmune and autoimmune disease was found in the WebGIVI analysis, these genes are most likely indicative of an innate immune response. In humans, literature has shown how hyperthermia can occur when core temperatures reach between 41°C-47°C and cause certain diseases, such as warm hemolytic anemia. Although this can occur when temperatures are hot enough, in the chicken, the heat stress may cause damage to the erythrocyte population instead of inducing warm hemolytic anemia.

Warm antibody hemolytic anemia occurs when temperatures rise above the average body temperature (~41°C for chickens) causing hemolysis of RBC’s at a rate faster than they are produced. The national organization for rare disorders (NORD) stated that this anemia could be produced by low enzymatic levels of pyruvate kinase or glucose-6-phosphate dehydrogenase (G6PDH) [3]. The red blood cells were not analyzed in this current study but, it would be interesting to evaluate them at D42 to understand how heat stress (39°C) affected erythrocyte morphology and function. According to the study conducted by Mahmoud and Edens 2003 basal diets (diets without selenium supplementation) demonstrated a reduction in G6PDH in conjunction with heat stress in the liver. The same concept may be taking place in the spleen however, further experiments are needed in order to accept or reject this theory.
Finally, it would be interesting to see if there were more genes found to be enriched in the RTE analysis of D42 heat-stressed spleens of the Ross 708 broiler with a relationship to CNS. GABA, BRSK2 and RTN4R as stated earlier were found in this data. According to the article by Lara, L., and Rostagno, M., 2013 the HPA axis can be activated when high environmental temperatures are reached \[7, 42\]. (See literature review for the relationship of HPA and SAM axes with the CNS). Many lymphoid cells such as macrophages and granulocytes for example, contain receptors that can bind to neuroendocrine products such as cortisol. The result of these products binding macrophage surface receptors can alter various cellular activities such as proliferation and the secretion of cytokines. Many studies have shown how heat can alter these different pathways and ultimately affect the CNS \[30\]. Figure 11 illustrates how heat stress can directly affect the CNS through decreased cerebral blood flow.

5.7 RTE Analysis of Illinois Control genes with AmiGO2 and WebGIVI

A total of 991 genes were enriched in Illinois RTE data, among those 571 genes were shared among the control and heat-stressed groups. 249 genes were enriched in the control birds and 170 genes enriched in the heat-stressed birds. The 249 enriched genes in the control Illinois RTE data were evaluated in AmiGO2 with the Bonferroni correction and resulted in the biological phrase unclassified. This biological process contained 17 genes, many of which were uncharacterized and a possible indicator of an unknown function based on the current annotated chicken genome. Out of the 17 genes in the analysis with the Bonferroni correction, Growth hormone releasing hormone (GHRH) and Inducible T-cell co-stimulator (ICOS) were the only genes found by WebGIVI and eGIFT. ICOS helps stimulate T cells within the body to proliferate and secret other immune cells while aiding the secretion of
antibodies from B lymphocytes against foreign antigens. GHRH allows other immune
cells to produce and secret hormones such as Insulin like growth factor 1 (IGF-1) to
protect the spleen from foreign pathogens. These genes suggest the Illinois control
birds are performing normal immune and spleen functions.

Another analysis was launched without the Bonferroni correction to find other
genes vital to normal spleen function. Out of the ten processes seen in Table 9 there
were only two to three genes found under each biological process. However, it was
interesting to find forelimb morphogenesis among the top ten processes. Within this
process were the genes, Homeobox D10 (HOXD10), T-box 5 (TBX5) and, short
stature homeobox2 (SHOX2). SHOX2 was not relevant to the Illinois data. However,
HOXD10 was found to be important for normal cell differentiation and TBX5 was
found to be involved with regulating development. At D28 broiler chickens are not
fully grown and are still undergoing development until D42 when they are market
ready. This may be the reason why these genes were enriched in this data set.

Table 9: 249 genes enriched in the Illinois Control RTE genes were assessed in
AmiGO2. The top 10 biological processes were selected based on p-
value <0.05 without the Bonferroni correction. Each category was then
evaluated using the text mining tool WebGIVI.

<table>
<thead>
<tr>
<th>GO biological process complete</th>
<th>#</th>
<th>#2</th>
<th>expected</th>
<th>Fold Enrichment</th>
<th>+/-</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>neutrophil apoptotic process</td>
<td>2</td>
<td>2</td>
<td>0.01</td>
<td>&gt; 100</td>
<td>+</td>
<td>4.67E-05</td>
</tr>
<tr>
<td>inflammatory cell apoptotic process</td>
<td>3</td>
<td>2</td>
<td>0.01</td>
<td>&gt; 100</td>
<td>+</td>
<td>1.05E-04</td>
</tr>
<tr>
<td>myeloid cell apoptotic process</td>
<td>3</td>
<td>2</td>
<td>0.01</td>
<td>&gt; 100</td>
<td>+</td>
<td>1.05E-04</td>
</tr>
</tbody>
</table>
## Table 9 continued

<table>
<thead>
<tr>
<th>Process</th>
<th>+Log2 Ratio</th>
<th>FDR</th>
<th>P-Value</th>
<th>Log2 Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>glycinergic import</td>
<td>3</td>
<td>2</td>
<td>0.01</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>L-amino acid import</td>
<td>5</td>
<td>2</td>
<td>0.02</td>
<td>82.02</td>
</tr>
<tr>
<td>regulation of cytolysis</td>
<td>6</td>
<td>2</td>
<td>0.03</td>
<td>68.35</td>
</tr>
<tr>
<td>neutrophil homeostasis</td>
<td>7</td>
<td>2</td>
<td>0.03</td>
<td>58.59</td>
</tr>
<tr>
<td>cellular protein metabolic process</td>
<td>2257</td>
<td>2</td>
<td>11.01</td>
<td>&lt; 0.2</td>
</tr>
<tr>
<td>forelimb morphogenesis</td>
<td>35</td>
<td>3</td>
<td>0.17</td>
<td>17.58</td>
</tr>
<tr>
<td>synaptic transmission, glycinergic</td>
<td>8</td>
<td>2</td>
<td>0.04</td>
<td>51.26</td>
</tr>
</tbody>
</table>

The other processes that are listed in Table 9 were expected in a properly functioning immune system. For example, two genes under neutrophil apoptotic process were IL-6 and Interferon gamma (IFNG). The IL-6 is involved in B cell maturation and it is produced in response to chronic stress, while IFNG is involved with immune responses in relation to bacterial and viral pathogens. If either of these genes were not regulated and they were produced unnecessarily, then, the homeostatic balance of housekeeping genes and products could cause damage to the surrounding tissue, depending on the other signals present.

### 5.8 RTE Analysis of Illinois Control genes with the iTerm Spleen

Next the 249 control genes enriched in the Illinois RTE were compared to the genes associated with the iTerm spleen (485 genes). Six genes were found in common between the two lists (See Table 10). Among these genes were ICOS and IFNG which...
were also seen in the AmiGO2, WebGIVI and eGIFT analyses. Four genes that were not seen in previous analyses include Lymphocyte activating gene 3 (LAG3), TAT, Aryl-hydrocarbon receptor repressor (AHRR) and Interleukin 22 (IL-22). In human studies, LAG-3 is a cell surface molecule displayed by stimulated T cells, natural killer cells, B cells, dendritic cells and IL-22. Most of this gene’s function is still undiscovered.

Table 10: 249 enriched Illinois Control RTE genes were compared to the iTerm Spleen (485 genes) using Venn diagram. Six genes were found in common between the two gene lists and further analyzed by AmiGO2 and WebGIVI.

<table>
<thead>
<tr>
<th>Entrez ID</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>420989</td>
<td>AHRR</td>
<td>aryl-hydrocarbon receptor repressor</td>
</tr>
<tr>
<td>424105</td>
<td>ICOS</td>
<td>inducible T-cell co-stimulator</td>
</tr>
<tr>
<td>396054</td>
<td>IFNG</td>
<td>interferon, gamma</td>
</tr>
<tr>
<td>417838</td>
<td>IL22</td>
<td>interleukin 22</td>
</tr>
<tr>
<td>418287</td>
<td>LAG3</td>
<td>lymphocyte-activation gene 3</td>
</tr>
<tr>
<td>415884</td>
<td>TAT</td>
<td>tyrosine aminotransferase</td>
</tr>
</tbody>
</table>
After a GO analysis, no biological processes were detected with the Bonferroni correction. However, without the Bonferroni correction, the biological processes were identified and sorted, based on p-value <0.05 and the top ten biological processes were investigated (See Table 11). Under the first four biological processes, the same two gene products (IFNG and IL-22) were detected. The remaining biological processes only contained IFNG with the tyrosine catabolic process containing TAT.

**Table 11:** The top 10 biological processes based on p-value <0.05 found during a GO analysis comparing the 249 enriched genes found in the Illinois Control RTE data and the iTerm spleen. This analysis was conducted without the Bonferroni correction. The first four processes contained the same two gene products, IL-22 and IFNG.

<table>
<thead>
<tr>
<th>GO biological process complete</th>
<th>#1</th>
<th>#2</th>
<th>expected</th>
<th>Fold Enrichment</th>
<th>+/-</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>positive regulation of STAT cascade</td>
<td>39</td>
<td>2</td>
<td>0.01</td>
<td>&gt; 100</td>
<td>+</td>
<td>3.65E-05</td>
</tr>
<tr>
<td>positive regulation of JAK-STAT cascade</td>
<td>39</td>
<td>2</td>
<td>0.01</td>
<td>&gt; 100</td>
<td>+</td>
<td>3.65E-05</td>
</tr>
<tr>
<td>regulation of STAT cascade</td>
<td>95</td>
<td>2</td>
<td>0.02</td>
<td>83.1</td>
<td>+</td>
<td>2.15E-04</td>
</tr>
<tr>
<td>regulation of JAK-STAT cascade</td>
<td>95</td>
<td>2</td>
<td>0.02</td>
<td>83.1</td>
<td>+</td>
<td>2.15E-04</td>
</tr>
<tr>
<td>positive regulation of CD4-positive, CD25-positive, alpha-beta regulatory T cell differentiation involved in immune response</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>&gt; 100</td>
<td>+</td>
<td>2.53E-04</td>
</tr>
<tr>
<td>regulation of CD4-positive, CD25-positive, alpha-beta regulatory T cell differentiation involved in immune response</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>&gt; 100</td>
<td>+</td>
<td>2.53E-04</td>
</tr>
</tbody>
</table>
Table 11 continued

| positive regulation of CD4-positive, CD25-positive, alpha-beta regulatory T cell differentiation | 1 | 1 | 0 | > 100 | + | 2.53E-04 |
| regulation of CD4-positive, CD25-positive, alpha-beta regulatory T cell differentiation | 1 | 1 | 0 | > 100 | + | 2.53E-04 |
| tyrosine catabolic process | 1 | 1 | 0 | > 100 | + | 2.53E-04 |
| regulation of carbohydrate phosphatase activity | 1 | 1 | 0 | > 100 | + | 2.53E-04 |

After reviewing the genes and gene products found in the Illinois control RTE broilers, it most likely demonstrates a fully functional spleen and immune system. As mentioned previously, IFNG is needed to respond to bacterial and viral pathogens acquired on a daily basis. IL-22 is also an indicator of normality, but studies have shown that it can play a role in chronic inflammatory conditions and it can stimulate production of anti-apoptotic and anti-microbial molecules [65]. This mechanism would help preserve the spleen if any tissue damage developed. The results of one study showed that IL-22 can provide a protective and inflammatory role, but both are duration and tissue-dependent. This study also exemplified the aryl hydrocarbon receptor as a necessary component for IL-22 production but it is unclear whether AHRR affects the expression of this gene [65].
5.9 RTE Analysis of Illinois Heat-Stressed genes with AmiGO2 and WebGIVI

170 genes were enriched in the Illinois HS RTE data and analyzed through multiple bioinformatics tools (AmiGO2, WebGIVI and eGIFT). From this exploration, the top ten biological processes were assessed based on p-value (<0.05). Table 12 illustrates the top ten processes that were explored in AmiGO2 with the Bonferroni correction. Although many of the processes within Table 12 were broad, leukocyte cell to cell adhesion provided insight as to what may be occurring in the heat-stressed Illinois broilers. Nine genes were explored in the leukocyte cell to cell adhesion process. The remaining processes contained the same genes drawing the conclusion that more research is needed to provide further evidence.
Table 12: 170 enriched genes in the heat-stressed Illinois RTE data were examined by AmiGO2 and the top ten biological processes were selected based on a p-value <0.05 with the Bonferroni correction. The biological process leukocyte cell to cell adhesion contained nine genes which were found to be markers of inflammation.

<table>
<thead>
<tr>
<th>GO biological process complete</th>
<th>#</th>
<th>#2</th>
<th>expected</th>
<th>Fold Enrichment</th>
<th>+/-</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unclassified</td>
<td>4299</td>
<td>21</td>
<td>22.6</td>
<td>0.93</td>
<td>-</td>
<td>0.00E+00</td>
</tr>
<tr>
<td>protein ADP-ribosylation</td>
<td>19</td>
<td>5</td>
<td>0.1</td>
<td>50.06</td>
<td>+</td>
<td>4.04E-04</td>
</tr>
<tr>
<td>leukocyte cell-cell adhesion</td>
<td>170</td>
<td>9</td>
<td>0.89</td>
<td>10.07</td>
<td>+</td>
<td>1.86E-03</td>
</tr>
<tr>
<td>T cell aggregation</td>
<td>147</td>
<td>8</td>
<td>0.77</td>
<td>10.35</td>
<td>+</td>
<td>7.14E-03</td>
</tr>
<tr>
<td>T cell activation</td>
<td>147</td>
<td>8</td>
<td>0.77</td>
<td>10.35</td>
<td>+</td>
<td>7.14E-03</td>
</tr>
<tr>
<td>lymphocyte aggregation</td>
<td>149</td>
<td>8</td>
<td>0.78</td>
<td>10.21</td>
<td>+</td>
<td>7.89E-03</td>
</tr>
<tr>
<td>leukocyte aggregation</td>
<td>153</td>
<td>8</td>
<td>0.8</td>
<td>9.95</td>
<td>+</td>
<td>9.59E-03</td>
</tr>
<tr>
<td>lymphocyte activation</td>
<td>217</td>
<td>9</td>
<td>1.14</td>
<td>7.89</td>
<td>+</td>
<td>1.37E-02</td>
</tr>
<tr>
<td>single organismal cell-cell adhesion</td>
<td>283</td>
<td>10</td>
<td>1.49</td>
<td>6.72</td>
<td>+</td>
<td>1.51E-02</td>
</tr>
<tr>
<td>positive regulation of T cell mediated immunity</td>
<td>18</td>
<td>4</td>
<td>0.09</td>
<td>42.27</td>
<td>+</td>
<td>1.72E-02</td>
</tr>
<tr>
<td>single organism cell adhesion</td>
<td>303</td>
<td>10</td>
<td>1.59</td>
<td>6.28</td>
<td>+</td>
<td>2.74E-02</td>
</tr>
</tbody>
</table>

Four of the nine genes were markers of inflammation: Selectin-P (SELP), radical S-adenosyl methionine domain containing 2 (RSAD2), purinergic receptor P2X, ligand gated ion channel, 7 (P2RX7), and CD8A. SELP is more involved with leukocytes and platelets when inflammation is detected, while RSAD2 is involved.
with DNA or RNA products that will stimulate the secretion of cytokines. P2RX7 is very similar to RSAD2 in that it will stimulate cytokines, but it promotes pro-inflammatory cytokines to be secreted from cells that are challenged with LPS. Lastly, CD8A has more of a role with an adaptive immune response. (Note: Adaptive immunity has two responses, cell-mediated and antibody-mediated. Cell-mediated is the predominate immune response in association with CD8A).

Several of the genes, SELP, RSAD2, P2RX7 and CD8A provide evidence that the immune system is responding to the chronic heat stress and exhibiting inflammation. Most of the genes are associated with the activation of the innate immune system. They could provide an explanation of the response seen in the Illinois heat-stressed broilers as the result of an elicited innate immune system. According to many studies involving the immune system, the adaptive immune system can take anywhere from ten days to two weeks to mount a response. Enriched CD8A expression could indicate an increased expression of cytotoxic T lymphocytes on conventional dendritic cells involved with antigen presentation and T lymphocyte activation. RTE analysis only identifies what genes are enriched and responded to heat stress. It does not identify genes that are up- or down-regulated. With this in mind, CD8a could suggest activation of the acquired immune response; however, more data is needed to confirm this theory. Inferences drawn from this data could be that a widespread response is developing in the Illinois broilers at D28 (Dyer, personal communication).

The remaining five genes were RAG2, protein tyrosine phosphatase, non-receptor type 22 (lymphoid) (PTPN22), GATA binding protein 3 (GATA3), basic leucine zipper transcription factor, ATF-like (BTAF) and, amyloid beta (A4) precursor
protein binding, family B, member 1 interacting protein (ApBB1IP). ApBB1IP and BTAF had very little information available however; in humans, BTAF is seen in hematopoietic cells when an allergic response is present.

These genes produce products that are typically involved with a TH2 lymphocyte response. This may suggest involvement of a B cell response that might be occurring in the splenic germinal centers (Dyer, personal communication). Although RTE only looks at what genes are enriched, enrichment of GATA3 could suggest TH2 lymphocytes are driving the development of B lymphocytes. This would then allow the B cells to differentiate into plasma cells and produce antibodies against an unknown encountered foreign antigen.

In humans, an allergic response can stimulate vast amounts of IgE which can bind to mast cells and basophils for example. A second contact with the allergen can then cause the degranulation of a mast cell or basophil, resulting in an inflammatory response (Examples of substances released: enzymes and cytokines). Evidence suggests that chickens express IgE antibody and therefore, it may be possible for heat stress to induce a similar reaction. However, whether this response can drive an autoimmune disease is unknown. The data in the current study do not suggest the involvement or development of an autoimmune disease (Emara, personal communication).

PTPN22, GATA3 and RAG2 were all identified with some relationship to autoimmune diseases in AmiGO2 and WebGIVI. Although it is possible for chickens to acquire autoimmune diseases within their lifetime, there is no evidence suggesting one is occurring in the present data. With the confirmation of GATA3 being enriched in the RTE data; it could cause naïve T cells to differentiate into mature TH2 cells.
These cells can then produce and secrete IL-6 (typically pro-inflammatory) and IL-10 (typically anti-inflammatory). This might suggest a humoral response to an unknown antigen that may have entered into the circulation and was identified by the spleen. RAG2 was also seen in the Ross 708 heat-stressed RTE data. This gene starts immunoglobulin rearrangement by breaking double-stranded DNA (dsDNA) bonds which could introduce mutations into immune cells and enhance the probability of recognizing a pathogen. PTPN22 had a close association in regulating TCR and BCR’s. It could identify increased expression of both TCR’s and BCR’s allowing them to recognize foreign antigen that was released in the systemic circulation. An increase in antigen recognition by both B and T lymphocytes may allow the body to initiate a systemic response as a result of heat stress.

5.10 RTE Analysis of Illinois Heat-stressed genes with the iTerm Spleen

Out of 485 genes with the iTerm Spleen only five genes were shared with the 170 genes enriched in the Illinois heat-stressed RTE data (See Table 13). The five genes, Paired box 5 (PAX5), RAG2, GATA3, Interleukin 2 receptor, gamma (IL2RG) and, IRF5 were examined by gene ontology without the Bonferroni correction. (No biological processes were found with the Bonferroni correction). The terms were sorted based on p-values with a statistical significance of <0.05 and the top ten were investigated further. Each process was found to have a relationship with one gene, GATA3. Table 14 shows the biological processes found in this analysis. The entrez ID’s of all five genes were then inserted into WebGIVI to obtain more information utilizing eGIFT.
Table 13: 170 enriched Illinois Heat-Stressed RTE genes were compared to the iTerm spleen (485 genes) using Venn diagram. The analysis established five genes in common between the two gene lists and was further investigated through AmiGO2 and WebGIVI.

<table>
<thead>
<tr>
<th>Entrez ID</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>419106</td>
<td>GATA3</td>
<td>GATA binding protein 3</td>
</tr>
<tr>
<td>395199</td>
<td>IL2RG</td>
<td>interleukin 2 receptor, gamma (severe combined immunodeficiency)</td>
</tr>
<tr>
<td>430409</td>
<td>IRF5</td>
<td>interferon regulatory factor 5</td>
</tr>
<tr>
<td>387330</td>
<td>PAX5</td>
<td>paired box 5</td>
</tr>
<tr>
<td>423165</td>
<td>RAG2</td>
<td>recombination activating gene 2</td>
</tr>
</tbody>
</table>

Table 14: The top 10 biological processes based on a p-value <0.05 found in the gene ontology database AmiGO2 without Bonferroni correction between the 170 genes enriched in the Illinois heat stress RTE data and the 485 genes found with the iTerm Spleen. Each biological process was associated with GATA3.

<table>
<thead>
<tr>
<th>GO biological process complete</th>
<th>#</th>
<th>#2</th>
<th>expected</th>
<th>Fold Enrichment</th>
<th>+/-</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pro-T cell differentiation</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>&gt; 100</td>
<td>+</td>
<td>6.33E-05</td>
</tr>
<tr>
<td>negative regulation of glial cell-derived neurotrophic factor receptor signaling pathway involved in ureteric bud formation</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>&gt; 100</td>
<td>+</td>
<td>6.33E-05</td>
</tr>
<tr>
<td>Pathway</td>
<td>p-value</td>
<td>q-value</td>
<td>Fold Change</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>---------</td>
<td>---------</td>
<td>-------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regulation of glial cell-derived neurotrophic factor receptor signaling</td>
<td>1</td>
<td>1</td>
<td>&gt;100</td>
<td>+ 6.33E-05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pathway involved in ureteric bud formation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative regulation of fibroblast growth factor receptor signaling</td>
<td>1</td>
<td>1</td>
<td>&gt;100</td>
<td>+ 6.33E-05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pathway involved in ureteric bud formation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regulation of fibroblast growth factor receptor signaling</td>
<td>1</td>
<td>1</td>
<td>&gt;100</td>
<td>+ 6.33E-05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pathway involved in ureteric bud formation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regulation of cellular response to X-ray</td>
<td>1</td>
<td>1</td>
<td>&gt;100</td>
<td>+ 6.33E-05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive regulation of thyroid hormone generation</td>
<td>1</td>
<td>1</td>
<td>&gt;100</td>
<td>+ 6.33E-05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regulation of thyroid hormone generation</td>
<td>1</td>
<td>1</td>
<td>&gt;100</td>
<td>+ 6.33E-05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative regulation of cell proliferation involved in mesonephros development</td>
<td>1</td>
<td>1</td>
<td>&gt;100</td>
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<td>Regulation of cell proliferation involved in mesonephros development</td>
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During this examination, PAX5 was highlighted as a rate-limiting gene. PAX5 and RAG2 were related to immunoglobulin rearrangement, while GATA3 and IL2RG were associated with T cell growth and expansion, and IRF5 was previously described (See Ross 708 heat-stressed RTE vs. iTerm spleen for description).

PAX5 (also known as ALL3 and BSAP) is a transcription factor that can work in conjunction with RAG1 and RAG2 to rearrange immunoglobulin genes. (Only RAG2 was found in this analysis). When IL-7 is expressed, this can affect the V (H) arrangement during B cell development. This gene is imperative for hematopoietic stem cell differentiation and it is vital to the expression of other genes that are needed for cell signaling and adhesion. With enriched genes in the data like PAX5 and RAG2, one can infer that a humoral, adaptive immune response could be occurring in the spleen. Further studies need to be completed to confirm up- or down-regulation of these genes and the adverse effects that they may cause in the broiler industry. (RAG2 was confirmed in this analysis as it was also seen in the GO and WebGIVI analyses of the Illinois heat-stressed RTE).

GATA3 extends beyond its function of regulating the immune system by also regulating immune cells such as T lymphocytes through every step of development. An increase or decrease in this gene’s expression may lead to mutations in T cells or over/under production. If this were to occur during the embryonic stages of broiler growth, then the overall development of the bird may not be up to industry standards. The same can be said for IL2RG which was also enriched in this analysis. IL2RG is also imperative in the growth of lymphoid tissue through different receptors for interleukins and can be seen during both innate and adaptive immune responses. This may infer as stated in the Ross 708 heat-stressed RTE in comparison to the iTerm
Spleen analysis that the innate immune system has mounted, while the adaptive response is just beginning.

There were a few indicators of oxidative stress in the Ross 708 broiler line, but none were observed in the Illinois data, except the basics of inflammation occurring in leukocytes and platelets (SELP gene found in Illinois heat-stressed RTE data). Brooker et al., 2011 and Kregel et al., 2002 stated that HSP’s are very common and can be found in all genomes, including bacteria, plants and animals [6, 28]. One interesting fact found in the article by Pockley, A. G., 2003 was that HSP’s could also be recognized by the body as autoantigens. This was also found in arthritis and diabetes studies in mammalian models. This finding would be an interesting point to evaluate for future data because no HSP’s were found in the current study. There may not have been enough stimulation of the immune system in the current experiment to understand the entirety of the spleens’ response in the RTE analysis for both broiler lines [22].

Previous studies have shown that tissues, such as the pituitary exhibit peak transcript levels of HSP’s at two hours post heat stress. These studies also showed HSP’s levels to drop approximately at four hours post heat stress with a very minimal response (Pritchett, personal communication). The cyclic heat that was applied during the experiment made the protocol reproducible, but it also gave the birds a chance to acclimate to the time frame in which the heat was applied. Differences in HSP transcript levels and the heat-stressed RTE data for both chicken lines may have been seen, if heat were applied over twenty four hours for seven days, without any time to recuperate. This method may have caused a bigger response to be exhibited, by the spleen and the immune system. It may also be possible that because the spleens were
extracted between four and six hours post-heat stress, that they were in a HSP “trough”.

Studies have determined that HSP’s can be released from both necrotic and non-necrotic cells, such as those undergoing oxidative stress [39]. Neither line demonstrated enriched hypoxia-inducible factors (HIF) or heat shock proteins in the RTE data. This result suggested normal oxygen levels but, with little to no evidence to support the absence of HIF or HSP’s, more data would need to be collected and analyzed to determine why this absence occurred. Both D28 Illinois and Ross 708 spleens had no physical evidence of the spleen cells undergoing necrosis when they were extracted and observed grossly. Microscopic and histological analysis could provide additional information on the state of the spleen cells at the time of extraction. To confirm these hypotheses, more research would need to be conducted on both the Illinois and Ross 708 broilers.

Several studies have recognized heat shock proteins as inducers of autoimmune diseases. Routsias, J. G., and Tzioufas, A. G., 2006 established a connection between stressors, like heat stress and heat shock proteins. The main purpose of a HSP is to assist in proper folding of polypeptides, ensuring proper functionality, as well as repairing or conducting protein degradation [28]. The HSP’s are divided into two main categories, major and minor. Major heat shock proteins reside in the cytosol (e.g HSP70 and HSP90), while minor HSP’s reside in the endoplasmic reticulum. If enough stress acts on a cell, it can cause the cell to lyse and release its internal contents. The contents would be recognized as damaged “self” proteins. If both T and B lymphocytes responded and antibodies were produced, an autoimmune response could be generated. This response is possible because of
conservation of these proteins across many species, including bacteria. Bacteria for example, have HSP’s that are similar in structure and function in comparison to HSP’s of avian species. They are sometimes not recognized as foreign by other species which is why autoantibodies could be generated against “self” HSP’s and bacterial HSP’s. This is also known as molecular mimicry [45]. These proteins also have the ability to elevate the number of pro-inflammatory cytokines which could exacerbate inflammation and start an immune response within five to seven days [39, 45].

As seen in the normalized spleen weight data for both bird lines, a possible explanation of a decrease in spleen size may be due to a response dampening down or down-regulation of genes being expressed. However, RTE analysis does not indicate if genes are up- or down-regulated; RTE illustrates what gene transcripts are enriched in the data. Other research conducted has shown that muscle catabolism occurs when there are high levels of cytokines to the extent of which they become toxic within the body. For example, macrophages and dendritic cells can secrete several of the same cytokines including IL-6, IL-1, TNF etc and act on various organs. The liver, bone marrow and hypothalamus are examples of a few areas that can be negatively impacted by high levels of these circulating cytokines. Accumulation of such cytokines could result in muscle wasting and be a possible avenue for smaller spleen size in the current study, as shown in the normalized data [57]. However, further research may provide insight as to what other cytokines are being expressed in both bird lines.

The only enriched gene transcript found in common between both control RTE analyses of the Ross and Illinois broilers was IFNG (This gene was not found by other analytical methods performed in this study). IFNG can be activated by a
multitude of factors such as MCH II antigen presentation, B lymphocytes or macrophages. Young and Hardy 1995, established that IFNG could be inhibited by glucocorticoids (Glucocorticoid levels cannot be detected through transcriptome analysis). Other results from the in vitro and in vivo studies also found IFNG to have possible feedback mechanisms to enhance its own mRNA expression in both human and mouse models. This cytokine can play a role in different aspects of the immune system and it can be manufactured by T cells and large granular lymphocytes in which the memory cells, have demonstrated to increase IFNG levels in the presence of IL-12 (pro-inflammatory) [64]. Although IL-12 was not found in spleen RTE control or heat-stressed data of either broiler line, it does not mean it could not be found by techniques other than transcriptomic comparison.

IFNG was observed in the control RTE Illinois data while Ross birds contained this cytokine in both control and heat-stressed groups. After reviewing the FPKM values between the Ross control and heat-stressed RTE preliminary data sets, a conclusion could not be made. No statistical tests were performed to evaluate the relationship before and after heat stress treatment. PCR methods [56] may suggest the presence or lack of specific gene expression markers. Evaluating D28 and later time points in both experimental groups of each chicken line could provide validation.

After reviewing the enriched genes in both broiler lines, it can be inferred that these two broiler lines regulate physiological processes in similar ways. However, the present data suggests the involvement of different genes and gene products. Ross birds are selected for high meat yield and quality, while Illinois birds are a representation of broilers from the 1950’s. From other studies conducted in this area of research, we can infer why certain genes and cytokines may be enriched in this data.
Calefi et al., 2016 and Quinteiro-Filho et al., 2015 established a connection between heat stress and the levels of corticosterone in the blood. Heat stress in both studies demonstrated increased corticosterone levels which activated the HPA axis as a response to stress. Increased levels led to a decline in body weight gain and feed intake. Both studies also reported an increase in gut inflammation, suggesting that gut inflammation also contributed to the decrease in feed intake. With changes in the gut microbiota, this could potentiate and further drive intestinal irritation (leaky gut) in response to heat stress. Overall, both studies seemed to stress the association of stress responses elicited by the spleen as a result of hyperthermia.

In the review by Sapolsky et al., 2000 glucocorticoids were identified to have immune depressive and enhancement effects. Other findings indicated the capability of glucocorticoids to induce apoptosis within naïve T and B cells and mature T cells, leading to atrophy on a smaller scale in lymphoid organs. This review documented the deterioration of the thymus as a response to the secretion of glucocorticoids [48]. Sapolsky et al., 2000 also found glucocorticoids capable of preventing antigen-major histocompatibility presentation to T lymphocytes, decreasing T and B cell expansion, activation and moving cellular responses from TH1 to TH2. Cvoro, A. et al., 1998 found that heat stress (41°C) can modify hormone receptors such as those needed for glucocorticoids, adding additional information on the theory of thermal stress suppressing the immune system. These findings may provide a possible explanation why heat stress caused a reduction in normalized spleen weight and why a robust response was not detected in the present study.

Post et al., 2003 suggested chronic heat stress in broiler chickens to be the primary reason for a rise in plasma corticosterone levels. These elevated levels
resulted in lower spleen and body weights, concluding immune organs are susceptible to high corticosterone levels [7, 40]. Studies have also shown these high levels to be correlated with increased counts of heterophils in the blood [40]. Studies suggest a feedback mechanism implemented by the body to control corticosterone levels in future responses to heat stress. This mechanism could also regulate the release of other glucocorticoids from the adrenal gland [63]. Calefi et al., 2016 demonstrated how heat stress did not have a large impact on the spleen. In conclusion, the spleen may have an underlying protective mechanism preventing it from exhibiting a robust response to thermal challenge in both broiler lines.

As mentioned previously, interleukins and cytokines like IL-17F were enriched in the Ross 708 heat-stressed RTE data and identified by other studies. Korn et al., 2009 and Sano et al., 2015 found cytokine production of IL-17F and IL-17 by Th17 cells (a subset of CD4+ T cells) in response to different stimuli. Sano et al., 2015 reviewed the effects of segmented filamentous bacteria (SFB) on the gut, determining a relationship with the gut microbiota. The conclusions of the study highlighted the importance of homeostasis and how stress can disrupt this state and activate the immune system in mice. The generation of TH17 in the gut could also migrate to the spleen and be the source of IL-17. IL-17 is an important cytokine in assisting germinal center formation where B cells can respond to antigens and start producing antibodies. Once antibody formation starts, the innate immune system can then be activated. In the present study, heat stress caused a decrease in the Ross 708 and Illinois broiler’s appetite, suggesting a change and disruption in the gut homeostatic balance. This may have been a contributing factor to the enriched genes found during transcriptomic analysis of D28 spleens.
McGonagle and McDermott, 2006 emphasized the idea of categorizing inflammation on a scale with autoimmune at one end and auto-inflammatory at the other. The results of the current study do not suggest an autoimmune or auto-inflammatory response. However, the data does suggest the activation of the innate immune response and possibly the start of the adaptive immune response. Using the concept described in McGonagle and McDermott, 2006 the broilers could be described on a spectrum of inflammation. At one end of the spectrum would be the normal physiological processes of a broiler with a gradual increase toward the involvement of the innate and the adaptive immune response as inflammation increases (See Figure 13). Further research on the present study may identify how the spleen elicits an immune response. It may also identify the degree of the response and determine if the gut is involved and how that may relate to a systemic reaction.
Figure 13: A spectrum describing the activation of different parts of the immune system as inflammation increases over time. When heat stress acts on a broiler, the bird will continue to perform normal physiological functions until an exogenous antigen is encountered and helps promote inflammation. As inflammation increases, the innate immune system will be activated followed by the adaptive immune response.
Chapter 6

CONCLUSION

In conclusion, this study highlighted the differences of heat stress in the transcriptomic gene expression of D28 spleens of Ross 708 and Illinois broilers. During this study it was clear that the Illinois broilers had a decreased growth rate compared to that of the Ross 708 birds. The reduction in body weight gain in the Illinois broilers most likely contributed to their ability to compensate and possibly adapt to the high ambient temperatures they were exposed to during this study. Ross 708 broilers, having a considerably faster growth rate and most likely had a very low ability to tolerate the thermal stress applied.

After reviewing the entirety of the results, Path Rings did not show any differences between the heat-stressed and control broilers within or across broiler lines. However, the normalized spleen weights for both lines illustrated proportional differences between the control and heat-stressed groups for both D28 and D42 spleens. Although these proportional differences were seen, D42 illustrated significant differences (p-value <0.002) between the control and heat stress groups in both broiler lines. The D42 normalized spleen weights were approximately 53% smaller than the controls. Even though both lines were proportional, these results provide evidence of heat stress having an impact on the spleen, but the extent of that is still unknown.

The transcriptomic analysis provided some insight on the differences in gene expression between the Ross 708 and Illinois broilers in the present study. The transcriptomic expression data from the Illinois control broilers suggested a competent immune system and spleen. There was no evidence found to suggest a deviation
within these birds, from normal physiological functions. However, data from the heat-stressed group did suggest a mounted innate immune response with the possibility of developing an adaptive immune response if thermal stress persisted or worsened.

The transcriptomic analysis of the Ross 708 control birds also revealed typical genes expected in broilers with a proficient immune system and spleen. Conversely, the heat-stressed data from these chickens provided greater awareness as to the possible consequences of hyperthermia. One of the consequences included possible HPA activation, which integrates the immune system and the central nervous system. Pro-inflammatory cytokines identified within the study such as IL-17F highly suggests that heat stress caused the breakdown of the intestinal epithelial barrier. The result of this is leakage of bacterial toxins into systemic circulation (leaky gut syndrome) causing the spleen to respond by eliciting an immune response. Literature proposed an idea of heat stroke with these pro-inflammatory cytokines and the addition of others only, if higher temperatures were achieved and sustained for long periods of time. However, ethical concerns may prevent such a study from occurring. Finally, according to mammalian studies, the after effects of heat stroke can take years to develop which would be difficult to evaluate with the short life span of a typical broiler.

The enriched genes found in the transcriptomic analysis could indicate a mechanism deployed by the spleen to cope with damaging external factors. Other studies have provided similar evidence demonstrating a small and insignificant response in the spleen to heat stress [7]. Reviewing the genes found in the present study along with other literature, we can hypothesize that the spleen may be protected from extrinsic factors by allowing other organs in the body to defend off pathogens.
Overall, it was unclear to the extent of damage heat stress caused on the immune system and the spleen in both broiler liners. The significance of this study was identifying that these birds experienced heat stress and responded by demonstrating a relationship between IL-6, IL-17 and LPS. D28 boiler immune systems may not be fully developed to see a large enough response to thermal stress. D42 however, may provide a better explanation on the effects of hyperthermia in broiler chickens and the detrimental effects it may have on the immune system as well as the spleen. Expanded studies of the present experiment may discover the link between the lack of response produced by the spleen and the immune system in both broiler lines.

Finally, it was evident that there were changes in the gene expression seen in the transcriptomic comparisons of the spleen in both broiler lines. Although changes were observed, there were no enriched transcripts in the current study suggesting a damaged erythrocyte population or an increase in genes responsible for erythrocyte clearance or hemoglobin production. There was also no evidence suggesting a decrease in the B and T lymphocyte populations or antigen recognition and presentation. The enriched gene transcripts found in the study were most likely indicative of an immune response with the possibility of inducing leaky gut syndrome and a systemic response. Lastly, the study illustrated more detectable differences in the Ross 708 broilers in response to heat stress compared the Illinois broilers.
Chapter 7

FUTURE WORK

After conducting this experiment, several limitations were discovered. One limitation found was tissue degradation by RNases. Certain tissues such as the spleen can easily be degraded by endogenous RNases (every tissue contains RNases) [31]. Flash freezing tissues immediately after extraction reduces this effect in combination with preservation solutions like RNAlater (this solution conserves the integrity of the RNA within a tissue). Other studies conducted at the University of Delaware using RNAlater demonstrated a greater level of RNA integrity after total RNA extraction and quality check was performed on spleen samples. For future work it would be highly recommended to use solutions such as RNAlater to preserve spleen tissue or any tissue susceptible to degradation by RNases [31].

Another limitation of this study was only collecting temperature data for liver samples. The liver was found to be nearly equivalent to the temperature of the cloaca in the heat-stressed birds (Schmidt, personal communication). Measuring the temperature of the spleen may have shown similar findings and provided an explanation to unanswered questions. It can be theorized that the spleen could have had an increased temperature similar to that of cloaca because it is located deep inside the bird, and it is surrounded by tissues superior and inferior to it. An optimal experiment would be to repeat the current study and collect temperature measurements on various tissues at D28 for comparison. This could provide an explanation as to why
heat stress did not present a vigorous response in the transcriptomic profiles of the spleen.

Other caveats of the present study included collecting transcriptome data from only D28 samples. It would be interesting to know if D42 had a more dynamic response to heat stress or if those spleens achieved thermo-tolerance. Setting up a future experiment to evaluate the signals present in the current study would be an ideal way to obtain this information. For example, a similar study with an increased environmental temperature, we might expect a more robust response from the spleen. A future study such as this may detect increases in expression of IL-6, IL-17, and LPS’s. We also might expect an increase in mortality and morbidity depending on the severity of the heat stress and the duration of the study. Cell culture work and other studies conducted on the pituitary have revealed peak levels of HSP’s to be detected approximately two hours into a heat stress cycle. During this parallel study it would be optimal to perform necropsies on broilers and extract tissues samples two hours into the thermal stress period. The expectation would be to observe increased HSP transcripts in the transcriptome data after relative tissue expression and differential expression analyses.

Investigating the blood of the chickens may also provide valuable information on the physiological state of the broilers on both a cellular and molecular level. By reviewing the levels of GSH in the blood, (reduce glutathione form) we would be able to cross confirm that the broilers were experiencing heat stress. Cui, Y. et al., 2016 concluded that the liver is a designated storage center for GSH and when heat stress acts on the liver, GSH becomes mobile and migrates into the blood stream. Based on these findings, if GSH was not detectable in the blood of a future study, it could
suggest removal by the spleen. Other useful information that could be evaluated in the blood includes collecting samples before, during and after a future experiment to assess the heterophil to lymphocyte ratio. This would permit a baseline to be established and conduct comparisons throughout the study. Mack et al., 2013 pointed out heat stress as the principal cause of an increased heterophil to lymphocyte ratio [51]. During the fall 2013 trial, blood samples and rectal temperatures were not collected but, they would have been a valuable asset based off the current results of both broiler lines and avian literature.

A follow up analysis utilizing PCR methods may shed light as to why a vigorous response by the spleen was not observed. From the current findings the data suggests, a systemic immune response and it also points to leaky gut syndrome. Several cytokines could be analyzed within spleen tissues such as IL-6, IL-17, TLR-2 and TLR-4, all of which are capable of inducing a pro-inflammatory response [45]. Establishing a significant number of markers in this analysis could confirm the theory of leaky gut syndrome. A future study could also comprise an exploration of B and T lymphocyte markers at both D28 and D42. Comparing these time points would provide evidence of an expected exacerbated response based on the current findings of the present study. This might only be feasible if the future study introduced an antigenic challenge in combination with heat stress.

A typical immunological response to antigens initiated by the spleen causes surveillance cells (e.g. dendritic cell and macrophages) to bind or phagocytize foreign antigens with the involvement of both T and B cells (Emara, personal communication). A response will only be generated if antibodies are produced against the foreign antigen. Only then, can a response be mounted and germinal centers
formed. PCR would be advantageous in identifying which T and B cells were stimulated in response to thermal stress with antigenic challenge [56]. It could potentially highlight the factors initiated by heat stress that caused immunosuppression or it might establish the underlying reason for a lack of a response observed in the D28 spleens. Assessing tissue samples for anti-inflammatory cytokines (e.g. TGF-B and IL-10) may also provide cross confirmation by demonstrating as inflammation increases, anti-inflammatory factors increase to dampen down and maintain hemostasis in the immune system. A lack of these factors may lead to the assumption of a systemic response throughout the body causing havoc and damaging tissues. Many questions remained unanswered until a study such as this is conducted.

A future experiment could expand the current study to explore tissue samples from D28 and D42 comparing the spleen, liver and intestine. Although there was minimal information supplied by the present study, D42 may attest to a more vigorous response elicited by the spleen due to a longer duration of thermal stress. In literature, the liver and gut have demonstrated robust responses to chronic heat stress [41, 44] however; only liver tissue at the University of Delaware has been analyzed for D28 and D42 in the Ross 708 broiler line. Evaluating the liver and intestines in both lines using both time points, could provide a definitive answer as to why these other tissues exhibit a far greater response compared to the spleen. Ideas of why this might be occurring could be that the spleen is protected in some manner but the answer to this remains unclear.

Finally, a future study may include challenging the immune system of either the Ross 708 or Illinois broilers by injecting the broilers with an infectious agent. A typical healthy bird would mount an appropriate immunological response and generate
germinal centers. However, if the birds were unable to mount an immune response, the result of death may indicate thermal stress increasing hormones such as glucocorticoids (e.g. corticosterone) and causing immunosuppression. If death was not observed, heat stress may not be solely responsible for the lack of a response elicited by the spleen and other factors may be influencing the immune response (Emara, personal communication).

Specifics of such a study would entail a similar experimental design and temperatures from the present study with a slight adaptation from the experiment conducted by Calefi et al., 2016. In this proposed study, only one bird line would be evaluated to control confounding variables and focus strictly on the effects of an immune challenge in combination with heat stress. Four groups would be established as follows, a control group, a heat-stressed group, a control group with the addition of an infectious agent, and finally, a heat-stressed group with the addition of an infectious agent. Male birds would be hatched out and reared at 25°C until D21. Then on D21 the infectious agent would be injected and cyclic heat stress would be applied for eight hours a day at 39°C until D42 and decrease to 25°C for the remaining sixteen hours.

On D22, broilers would be randomly selected from each group to collect various parameter measurements at two, four, six, and eight hours into the cyclic heat period and final necropsies would be performed on D28 and D42 broilers. Morphometric data such as spleen temperature, spleen weight, body weight, blood samples (to analyze corticosterone levels) and rectal temperatures would be collected during these various time points. These parameters would provide a baseline starting at D22 to compare later time points to. Only the control with heat stress and the heat stress with the infectious agent groups would experience the cyclic thermal stress.
Once analyzed, this information could reveal the answers to various questions about how acute and chronic heat stress affects the spleen at various developmental stages and the underlying mechanisms involved. Evaluating the same parameters at D28 and D42 for all four proposed groups, would be interesting none the less. It would provide clarification and help us understand the exact mechanisms in which the spleen responds to acute and chronic heat stress in conjunction with an immunological challenge. Possible questions to address during this proposed study may include those listed below.

1. Does acute heat stress and/or chronic heat stress dampen the immune response and/or the functionality of the spleen?
2. What type of mechanisms may be protecting the spleen?
3. Does the spleen respond by undergoing apoptosis and reduce its overall size?
4. How does acute heat stress response differ from that of chronic heat stress?
5. Does acute and chronic heat stress alter the gut microbiota and intensify the overall immune response of the bird?

Another way to evaluate functionality of the spleen in such a study would be to analyze the different populations of T lymphocytes present. Several ways to conduct this test could include, collecting heat-stressed broiler spleens and using a mesh separation technique to isolate the T lymphocytes. According to Trizio, D., and Cudkowicz, G., 1974, nylon wool will separate T lymphocytes from the remaining cell populations in spleens without causing cells to lyse [58]. Trizio, D., and Cudkowicz, G., 1974 proposed mixing different concentrations of B and T lymphocytes after
isolation to evaluate efficacy in vivo. The in vivo studies demonstrated an elicited immune response when the cell populations were injected back into mice. A similar test could be set up for the proposed study described above [58].

Other methods of obtaining similar results might be to collect blood samples and centrifuge the isolated concentration of cells to separate the plasma, buffy coat, and erythrocytes. Then the cells within the buffy coat could be plated for cell culture analysis. Different fluorescent markers could then be added to the cell plates to fluorescently mark various T cell populations. For example, adding a CD4+ marker and anti-sera would identify the T helper population while CD8+ with the addition of anti-sera would identify cytotoxic T cells. Using a wash to remove unbound T lymphocytes, may help in identifying the T cell population primarily present. In addition, complement could be added to deplete the remaining white blood cell populations on the plates. An in vitro study could be completed to identify functionality of the isolated cells and if they are efficient in producing antibodies (Emara, personal communication).

Calefi et al., 2016 suggested chronic heat stress in addition to intestinal inflammation could create an environment to manufacture a feedback mechanism through activation of the HPA axis. This negative feedback mechanism was proposed to decrease the total concentration of corticosterone in the blood resulting in reduction of adrenocorticotropic hormone (ACTH) from the pituitary. The results of this secretion would be a dampened down immune response to maintain homeostasis and allow the birds to live longer by generating a more immunologically equipped broiler. Rout et al., 2016, found HSP stimulation in response to chronic heat stress to “induce
acclimation to the stressor and involves reprogramming gene expression and metabolism” [44].

A study such as the one proposed above may provide definitive answers or strongly suggest what is causing the spleen to produce a weak response and how gene expression is altered. Future studies may illustrate specific changes in the erythrocyte, B and T lymphocyte populations and how signals are initiating leaky gut syndrome. Based off the current study’s results the answers to these questions are not well defined. It will be important to understand the mechanisms behind immune responses on a larger scale to benefit the poultry industry. It will also be vital to understand the effects of modifying broiler genetics with rising temperatures to ensure maximum production while maintaining the highest health standards for broilers.
REFERENCES


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Appendix A

DIFFERENTIAL EXPRESSION AND RELATIVE TISSUE EXPRESSION ANALYSIS PROTOCOLS

A.1 Differential Expression Analysis Protocol

Step 1: Add 4 new columns after locus of Library_(library number will be here). Go to columns add multiple then type 4. Now double click on each new column and change the names of each to the following categories Mean_Control, Mean_HS, HS/Control Ratio, and Log\textsubscript{2}HS/Cratio.

- Mean_Control
- Mean_HS
- HS/Control_Ratio
- Log2_of_Ratio_(HS/C)

To find the Mean, right click on a column, select formula, click statistical in the right handed section and then mean. Then select all of the control libraries, hit apply and ok. Now repeat this for heat stress. Then for HS/C ratio column right click on this column, select formula, then click HS / (this button on the keyboard and the control, apply and ok. Finally, for the Log\textsubscript{2} HS/C ratio right click this column, select formula, transcendental, LOG then click the small box and add a comma then double click to put the number 2. Now select the entire box and click the HS/C ratio. When you are finished click apply and ok.

Step 2: Now click on the log\textsubscript{2} ratio, tables (located at the top of screen), sort then click log\textsubscript{2} ratio HS/C and click by. Then apply and ok. Now press shift and select
all the dots and delete these rows in the log₂ HS/C ratio. Now go to analyze, distribution on the Log₂ ratio then click Y, column and ok. Then select all of the dots and it will remove the nonsignificant ones. Lastly, go to tables, subset, selected rows, and all columns.

**Step 3:** Stay in the Log₂ ratio and go to tables (located up at the top of the screen) click transpose. Then transpose columns (select all library numbers control and heat stress) label by gene short name and click ok. Then add a new column after label and rename this as condition. Then go to analyze, fit x by y. The genes are your Y and the condition is your X, select ok. While holding down the command key on a MAC or control on a PC, choose means/anova/pooledT. In the t-test area hold the command or control key and click test and click in the area with all the different numbers and combined data table. Now in the new table sort by ascending and remove everything in Column 4 that is not the probability of the absolute value of T. Then click the left side of the table and delete those rows (Delete Prob<t) then do the same for the opposite. You want to keep all Prob>|t|. Now delete everything except column Y and column 6. Save this table as p-value.

Once saved, sort by p-value and delete everything greater than or equal to 0.05 and save. Click tables, join, log₂ ratio then select gene name and gene short. Click the drop multiples with table and then ok. You can delete gene short name column but keep gene name. Finally, sort by the log₂ and choose ascending.

**A.2 Relative Tissue Expression Analysis Protocol**

Step 1: Start with RTE_TABLE_JUNE2016 in Google drive in folder titled 4RTE in Data section. Open the NCBI2016 file in the “4RTE” file in JMP Expression file for use in step 3.
Step 2: If gene names are in columns, Click tables, transpose, all genes in transpose columns, Column label if not, skip this step. Replace all zeros with 0.001. This is done to ensure genes of interest are not lost when performing log transformations.

Step 3: Click tables, join, and choose symbol under columns then select NCBI2016 file to join (in white box above. Note, this file must be open for it to appear in the white box and select match). **Recommended Action:** save this file (in a new file in the JMP expression folder with your initials) so you can begin with this table for future analysis. Lastly be sure to save all future files after this step in your corresponding folder.

Step 4: On the left side of the JMP table, find the libraries for the tissue of interest, select all and drag them to the top so these libraries are at the beginning of the library list.

Step 5: Now select the description column, select columns, add multiple columns and add three new columns. This will allow you to add new columns just after your selected the description column and label the columns as follows, maximum “tissue name”, median “tissue name”, and Log\(_2\) MaxTiss/MedTiss.

Step 6: To calculate maximum values, right click the column heading and click formula, statistical, maximum and the select all of the libraries for that tissue (or tissues if you are doing the maximum of all other tissues).

Step 7: To calculate median values, right click the column heading and click formula, statistical quantile, type 0.5 and present enter. Now click the ^ button (the JMP ^ button above your displayed formula next to the + and – and not on the
keyboard). To create a new empty box select the first tissue, hold shift, and select the last tissue library. This should include all libraries now press apply and ok

Step 8: To calculate $\log_2 \text{MaxTiss}/\text{MedTiss}$ values, right click column heading and click formula, select maximum tissue column on left and press Divide button. Then select median tissue column on left select the whole formula (red box around entire formula) and choose transcendental, log, select the formula again and press the comma key. Now change the ten to a two, select apply then ok. It is highly recommended to save this file.

Step 9: Select the $\log_2 \text{MaxTiss}/\text{MedTiss}$ column, analyze, distribution and put the $\log_2 \text{MaxTiss}/\text{MedTiss}$ in the Y column and choose ok. Now remove all rows and/or genes with increased ratios of maximum to median by selecting the outlier genes (black dots). To do so, simply click and drag a box to cover the black dots.

Step 10: Select rows, row selection and invert row selection.

Step 11: Click tables, subset, selected rows, all columns and hit ok.

Step 12: Click tables, sort and select $\log_2 \text{MaxTiss}/\text{MedTiss}$ and change the symbol from smallest to largest and choose ok.

Step 13: Delete all of the rows that contain a dot in the $\log_2 \text{MaxTiss}/\text{Med Tiss}$ column by selecting the first row and scrolling to last row. Now hold shift and click. Then right click and delete those selected rows.

Step 14: In the table just created, add two new columns and label them, Median of All Tissues (median of all tissues other than the tissue being analyzed) and $\log_2 \text{MaxTiss}/\text{MedAllTiss}$. Refer back to steps seven, eight and nine for these calculations. It is highly recommended to save this file.
Step 15: Select the Log2 MaxTiss/MedAllTiss column, analyze, distribution, and place the Log2 MaxTiss/MedAllTiss in the Y column, now hit ok.

Step 16: Now select the outlier genes (black dots). If there are outlier genes on both the positive and negative, select both by holding the shift key down.

Step 17: Click table, subset, selected rows, all columns and then ok. It is highly recommended to save this file.

Step 18: Click tables transpose and transpose all libraries and label by gene symbol.

Step 19: Click columns and add multiple columns, add 1 column and title it condition placing it after the library name column. Now label tissue as tissue name and all other tissues as other.

Step 20: Then click analyze, Fit X by Y and all genes should be included in the Y response and the X factor should be condition. Now hit ok. While holding down control, click the red arrow located next to the first graph and select “means/anova/pooled t.

Step 21: Hover the mouse over the t-test table under first graph. While holding down control on the keyboard, right click and select make into combined data table. Now select column four and sort (ascending or descending is fine)

Step 22: Highlight all the rows labeled prob>|T|. Then click tables, subset, selected rows and all columns. Now label column six as p-value and delete the remaining columns except for Y and p-value.

Step 23: Click tables, join and join this table onto the last saved file. It is highly recommended to save the file.
Step 24: Select \( \log_2 \) MaxTiss/MedAllTiss and sort. Then select all genes with a positive \( \log_2 \) value and subset these genes. It is highly \textbf{recommended to save this file because this file will be used for further analysis}. 
**Appendix B**

**DIFFERENTIAL EXPRESSION RAW DATA TABLES FOR ROSS AND ILLINOIS CONTROL AND HEAT-STRESSED GROUPS**

Table 15: Raw data table for 44 differentially expressed genes found in the Ross Control and Heat-stressed groups. Control genes are illustrated with a negative Log\(_2\) HS/C ratio and Heat-stressed genes are illustrated by a positive Log\(_2\) HS/C ratio.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Description</th>
<th>Log(_2) RatioHS/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOC416500</td>
<td>uncharacterized LOC416500</td>
<td>-2.972153376</td>
</tr>
<tr>
<td>FKBP6</td>
<td>FK506 binding protein 6, 36kDa</td>
<td>-2.678017939</td>
</tr>
<tr>
<td>PI15</td>
<td>peptidase inhibitor 15</td>
<td>-1.714001352</td>
</tr>
<tr>
<td>PDK4</td>
<td>pyruvate dehydrogenase kinase, isozyme 4</td>
<td>-1.629398436</td>
</tr>
<tr>
<td>DPT</td>
<td>dermatopontin</td>
<td>-1.498553577</td>
</tr>
<tr>
<td>FIGF</td>
<td>c-fos induced growth factor (vascular endothelial growth factor D)</td>
<td>-1.319823567</td>
</tr>
<tr>
<td>CIDEA</td>
<td>cell death-inducing DFFA-like effector a</td>
<td>-1.31760101</td>
</tr>
<tr>
<td>HSD17B2</td>
<td>hydroxysteroid (17-beta) dehydrogenase 2</td>
<td>-1.25845163</td>
</tr>
<tr>
<td>P4HA3</td>
<td>prolyl 4-hydroxylase, alpha polypeptide III</td>
<td>-1.227338631</td>
</tr>
<tr>
<td>LOC417962</td>
<td>uncharacterized LOC417962</td>
<td>-1.225404802</td>
</tr>
<tr>
<td>TMEM2</td>
<td>transmembrane protein 2</td>
<td>-1.219374042</td>
</tr>
<tr>
<td>ELN</td>
<td>elastin (supravalvular aortic stenosis, Williams-Beuren syndrome)</td>
<td>-1.213016229</td>
</tr>
<tr>
<td>AGRP</td>
<td>agouti related protein homolog (mouse)</td>
<td>-1.065093368</td>
</tr>
<tr>
<td>RPL31</td>
<td>ribosomal protein L31</td>
<td>-0.996933487</td>
</tr>
<tr>
<td>CRISPLD2</td>
<td>cysteine-rich secretory protein LCCL domain containing 2</td>
<td>-0.995448506</td>
</tr>
<tr>
<td>KCNE3</td>
<td>potassium voltage-gated channel, lsk-related family, member 3</td>
<td>-0.990330128</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Score</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>EDAR</td>
<td>ectodysplasin A receptor</td>
<td>-0.906436841</td>
</tr>
<tr>
<td>IL1RL1</td>
<td>interleukin 1 receptor-like 1</td>
<td>-0.821938908</td>
</tr>
<tr>
<td>NR5A1</td>
<td>nuclear receptor subfamily 5, group A, member 1</td>
<td>0.809367772</td>
</tr>
<tr>
<td>RASL10B</td>
<td>RAS-like, family 10, member B</td>
<td>0.8263707</td>
</tr>
<tr>
<td>LAPTM4B</td>
<td>lysosomal protein transmembrane 4 beta</td>
<td>0.844854862</td>
</tr>
<tr>
<td>NELL2</td>
<td>NEL-like 2 (chicken)</td>
<td>0.8476245</td>
</tr>
<tr>
<td>GGT1</td>
<td>gamma-glutamyltransferase 1</td>
<td>0.862093566</td>
</tr>
<tr>
<td>SKA3</td>
<td>spindle and kinetochore associated complex subunit 3</td>
<td>0.865038645</td>
</tr>
<tr>
<td>LOC769174</td>
<td>C-type lectin-like receptor variant</td>
<td>0.906552922</td>
</tr>
<tr>
<td>CLDN5</td>
<td>claudin 5</td>
<td>0.907201453</td>
</tr>
<tr>
<td>ATP8A2</td>
<td>ATPase, aminophospholipid transporter, class I, type 8A, member 2</td>
<td>0.934420674</td>
</tr>
<tr>
<td>KIF9</td>
<td>kinesin family member 9</td>
<td>0.979619021</td>
</tr>
<tr>
<td>LGALS2</td>
<td>lectin, galactoside-binding, soluble, 2</td>
<td>1.001902507</td>
</tr>
<tr>
<td>LOC769421</td>
<td>heparan sulfate glucosamine 3-O-sulfotransferase 3B1-like</td>
<td>1.011920481</td>
</tr>
<tr>
<td>FAM40B</td>
<td>family with sequence similarity 40, member B</td>
<td>1.012093535</td>
</tr>
<tr>
<td>W5CD1</td>
<td>WSC domain containing 1</td>
<td>1.018402566</td>
</tr>
<tr>
<td>OLFM1</td>
<td>olfactomedin 1</td>
<td>1.042285014</td>
</tr>
<tr>
<td>FSTL4</td>
<td>follistatin-like 4</td>
<td>1.062433796</td>
</tr>
<tr>
<td>EMID2</td>
<td>EMI domain containing 2</td>
<td>1.096438084</td>
</tr>
<tr>
<td>GGT5</td>
<td>gamma-glutamyltransferase 5</td>
<td>1.123759115</td>
</tr>
<tr>
<td>SHISA2</td>
<td>shisa homolog 2 (Xenopus laevis)</td>
<td>1.125131535</td>
</tr>
<tr>
<td>HS3ST6</td>
<td>heparan sulfate (glucosamine) 3-O-sulfotransferase 6</td>
<td>1.205730758</td>
</tr>
<tr>
<td>ANKDD1A</td>
<td>ankyrin repeat and death domain containing 1A</td>
<td>1.209397975</td>
</tr>
<tr>
<td>CACNG3</td>
<td>calcium channel, voltage-dependent, gamma subunit 3</td>
<td>1.210029841</td>
</tr>
<tr>
<td>HS3ST3A1</td>
<td>heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1</td>
<td>1.307975896</td>
</tr>
</tbody>
</table>
Table 15 continued

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Description</th>
<th>Log₂ Ratio HS/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDPD4</td>
<td>glycerophosphodiester phosphodiesterase domain containing 4</td>
<td>1.499691217</td>
</tr>
<tr>
<td>RTN4R</td>
<td>reticulon 4 receptor</td>
<td>1.803914417</td>
</tr>
<tr>
<td>KCNA1</td>
<td>potassium voltage-gated channel, shaker-related subfamily, member 1 (episodic ataxia with myokymia)</td>
<td>2.295530035</td>
</tr>
</tbody>
</table>

Table 16: Raw data table for 52 differentially expressed genes found in the Illinois Control and Heat-stressed groups. Control genes are illustrated with a negative Log₂ HS/C ratio and Heat-stressed genes are illustrated by a positive Log₂ HS/C ratio.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Description</th>
<th>Log₂ Ratio HS/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOC101751242</td>
<td>uncharacterized LOC101751242</td>
<td>-5.209302225</td>
</tr>
<tr>
<td>TRNAA-AGC</td>
<td>transfer RNA alanine (anticodon AGC)</td>
<td>-4.746199087</td>
</tr>
<tr>
<td>LOC101751970</td>
<td>uncharacterized LOC101751970</td>
<td>-2.707595711</td>
</tr>
<tr>
<td>LOC769339</td>
<td>fatty acyl-CoA hydrolase precursor, medium chain-like</td>
<td>-2.21941452</td>
</tr>
<tr>
<td>TXNDC5</td>
<td>thioredoxin domain containing 5 (endoplasmic reticulum)</td>
<td>-2.102477296</td>
</tr>
<tr>
<td>C8ORF22</td>
<td>chromosome 2 open reading frame, human C8orf22</td>
<td>-1.933605283</td>
</tr>
<tr>
<td>MMP13</td>
<td>matrix metallopeptidase 13 (collagenase 3)</td>
<td>-1.860037345</td>
</tr>
<tr>
<td>LOC101747372</td>
<td>uncharacterized LOC101747372</td>
<td>-1.750561296</td>
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<tr>
<td>PRIMA1</td>
<td>proline rich membrane anchor 1</td>
<td>-1.73082845</td>
</tr>
<tr>
<td>GZMA</td>
<td>granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)</td>
<td>-1.706739092</td>
</tr>
<tr>
<td>FAM46C</td>
<td>family with sequence similarity 46, member C</td>
<td>-1.686212723</td>
</tr>
<tr>
<td>LOC100857334</td>
<td>60S ribosomal protein L17-like</td>
<td>-1.621733558</td>
</tr>
<tr>
<td>LOC100857546</td>
<td>uncharacterized LOC100857546</td>
<td>-1.524082995</td>
</tr>
<tr>
<td>BVES</td>
<td>blood vessel epicardial substance</td>
<td>-1.491193443</td>
</tr>
<tr>
<td>MINPP1</td>
<td>multiple inositol-polyphosphate phosphatase 1</td>
<td>-1.237389676</td>
</tr>
<tr>
<td>LOC101751282</td>
<td>RANBP2-like and GRIP domain-containing protein 2-like</td>
<td>-1.189103358</td>
</tr>
<tr>
<td>ASB5</td>
<td>ankyrin repeat and SOCS box containing 5</td>
<td>-1.186350909</td>
</tr>
<tr>
<td>EAF2</td>
<td>ELL associated factor 2</td>
<td>-1.166793669</td>
</tr>
<tr>
<td>CIDEA</td>
<td>cell death-inducing DFFA-like effector a</td>
<td>-1.163525573</td>
</tr>
<tr>
<td>Gene Symbol</td>
<td>Description</td>
<td>Expression Ratio</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>CEP97</td>
<td>centrosomal protein 97kDa</td>
<td>-1.159943231</td>
</tr>
<tr>
<td>LOC776577</td>
<td>T-cell receptor gamma chain V region V108A-like</td>
<td>-1.125103715</td>
</tr>
<tr>
<td>LOC101751594</td>
<td>uncharacterized LOC101751594</td>
<td>-1.115761592</td>
</tr>
<tr>
<td>CCR9</td>
<td>chemokine (C-C motif) receptor 9</td>
<td>-1.099090036</td>
</tr>
<tr>
<td>PPP2R3A</td>
<td>protein phosphatase 2, regulatory subunit B', alpha</td>
<td>-1.095514485</td>
</tr>
<tr>
<td>COL6A3</td>
<td>collagen, type VI, alpha</td>
<td>-1.062887815</td>
</tr>
<tr>
<td>LOC100859106</td>
<td>envelope glycoprotein gp95-like</td>
<td>-1.059285853</td>
</tr>
<tr>
<td>BMP6</td>
<td>bone morphogenetic protein 6</td>
<td>-1.053106622</td>
</tr>
<tr>
<td>GDA</td>
<td>guanine deaminase</td>
<td>-1.046552776</td>
</tr>
<tr>
<td>HPGDS</td>
<td>hematopoietic prostaglandin D synthase</td>
<td>-0.995023148</td>
</tr>
<tr>
<td>EDAR</td>
<td>ectodysplasin A receptor</td>
<td>-0.984323735</td>
</tr>
<tr>
<td>ANKRD26</td>
<td>ankyrin repeat domain 26</td>
<td>-0.983726905</td>
</tr>
<tr>
<td>WDR74</td>
<td>WD repeat domain 74</td>
<td>0.939443067</td>
</tr>
<tr>
<td>SARS2</td>
<td>seryl-tRNA synthetase 2, mitochondrial</td>
<td>0.96245558</td>
</tr>
<tr>
<td>LOC101749414</td>
<td>uncharacterized LOC101749414</td>
<td>0.964320429</td>
</tr>
<tr>
<td>MTG1</td>
<td>mitochondrial ribosome-associated GTPase 1</td>
<td>0.996154818</td>
</tr>
<tr>
<td>LOC101749974</td>
<td>uncharacterized LOC101749974</td>
<td>1.001649483</td>
</tr>
<tr>
<td>LOC424872</td>
<td>zinc finger protein DZIP1L-like</td>
<td>1.068418048</td>
</tr>
<tr>
<td>LOC101752009</td>
<td>kaptin-like</td>
<td>1.069424512</td>
</tr>
<tr>
<td>LOC101751192</td>
<td>protein timeless homolog</td>
<td>1.089566264</td>
</tr>
<tr>
<td>LOC429445</td>
<td>forkhead box protein I1-ema-like</td>
<td>1.089590544</td>
</tr>
<tr>
<td>RASAL1</td>
<td>RAS protein activator like 1 (GAP1 like)</td>
<td>1.109969659</td>
</tr>
<tr>
<td>COL16A1</td>
<td>collagen, type XVI, alpha</td>
<td>1.136194441</td>
</tr>
<tr>
<td>HS3ST6</td>
<td>heparan sulfate (glucosamine) 3-O-sulfotransferase 6</td>
<td>1.177699717</td>
</tr>
<tr>
<td>SRR</td>
<td>serine racemase</td>
<td>1.206049069</td>
</tr>
<tr>
<td>PAQR3</td>
<td>progestin and adipoQ receptor family member III</td>
<td>1.247349329</td>
</tr>
<tr>
<td>LOC101750092</td>
<td>battenin-like</td>
<td>1.287085408</td>
</tr>
<tr>
<td>G6PCE</td>
<td>glucose 6 phosphatase, catalytic, 3</td>
<td>1.29072614</td>
</tr>
<tr>
<td>C11ORF49</td>
<td>chromosome 5 open reading frame, human C11orf49</td>
<td>1.320214282</td>
</tr>
<tr>
<td>ZP1</td>
<td>zona pellucida glycoprotein 1 (sperm receptor)</td>
<td>1.486874975</td>
</tr>
<tr>
<td>ENGASE</td>
<td>endo-beta-N-acetylglucosaminidase</td>
<td>1.494900646</td>
</tr>
<tr>
<td>MFSD8</td>
<td>major facilitator superfamily domain containing 8</td>
<td>2.039350008</td>
</tr>
<tr>
<td>TMEM220</td>
<td>transmembrane protein 220</td>
<td>2.687208501</td>
</tr>
</tbody>
</table>
Appendix C

RAW DATA TABLES FOR THE RELATIVE TISSUE EXPRESSION ANALYSIS FOR THE ROSS AND ILLINOIS CONTROL AND HEAT-STRESSED GROUPS

Table 17: Raw data table for the enriched genes found in the relative tissue expression data for Ross Control and Heat-stressed groups. The genes enriched in the control group are unbolded and the enriched heat-stressed genes are illustrated in bold.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Description</th>
<th>Log2MaxSpleen/MedAllTiss</th>
</tr>
</thead>
<tbody>
<tr>
<td>LITAF</td>
<td>lipopolysaccharide-induced TNF factor</td>
<td>5.428884444</td>
</tr>
<tr>
<td>LOC101748229</td>
<td>nuclear factor interleukin-3-regulated protein-like</td>
<td>5.429411393</td>
</tr>
<tr>
<td>SAMD9L</td>
<td>sterile alpha motif domain containing 9-like</td>
<td>5.433580259</td>
</tr>
<tr>
<td>FUT5</td>
<td>fucosyltransferase 5 (alpha (1,3) fucosyltransferase)</td>
<td>5.433627167</td>
</tr>
<tr>
<td>TLR15</td>
<td>toll-like receptor 15</td>
<td>5.43535711</td>
</tr>
<tr>
<td>LOC101750425</td>
<td>uncharacterized LOC101750425</td>
<td>5.436961338</td>
</tr>
<tr>
<td>LOC101750207</td>
<td>uncharacterized LOC101750207</td>
<td>5.437960088</td>
</tr>
<tr>
<td>MBOAT1</td>
<td>membrane bound O-acyltransferase domain containing 1</td>
<td>5.43942454</td>
</tr>
<tr>
<td>LOC101749657</td>
<td>uncharacterized LOC101749657</td>
<td>5.441616269</td>
</tr>
<tr>
<td>RUFY4</td>
<td>RUN and FYVE domain containing 4</td>
<td>5.448854806</td>
</tr>
<tr>
<td>LOC101749391</td>
<td>uncharacterized LOC101749391</td>
<td>5.457134594</td>
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<tr>
<td>LOC101748449</td>
<td>uncharacterized LOC101748449</td>
<td>5.457134594</td>
</tr>
<tr>
<td>FAM132A</td>
<td>family with sequence similarity 132, member A</td>
<td>5.457199023</td>
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<tr>
<td>MYO7L1</td>
<td>myosin-7-like 1</td>
<td>5.465974465</td>
</tr>
<tr>
<td>HOXB5</td>
<td>homeobox B5</td>
<td>5.467933219</td>
</tr>
<tr>
<td>ICOS</td>
<td>inducible T-cell co-stimulator</td>
<td>5.475042571</td>
</tr>
<tr>
<td>BIN2</td>
<td>bridging integrator 2</td>
<td>5.477605949</td>
</tr>
<tr>
<td>RGS20</td>
<td>regulator of G-protein signaling 20</td>
<td>5.484742479</td>
</tr>
<tr>
<td>LOC101749208</td>
<td>GTPase IMAP family member 7-like</td>
<td>5.484946749</td>
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<tr>
<td>OR52R1</td>
<td>olfactory receptor, family 52, subfamily R, member 1</td>
<td>5.485426827</td>
</tr>
<tr>
<td>LOC101750917</td>
<td>uncharacterized LOC101750917</td>
<td>5.494008452</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>LogFoldChange</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>FUT7</td>
<td>Fucosyltransferase 7 (alpha (1,3) fucosyltransferase)</td>
<td>5.49959481</td>
</tr>
<tr>
<td>LCP2</td>
<td>Lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76kDa)</td>
<td>5.500846883</td>
</tr>
<tr>
<td>LOC768553</td>
<td>E3 SUMO-protein ligase RanBP2-like</td>
<td>5.504310335</td>
</tr>
<tr>
<td>LOC101750697</td>
<td>uncharacterized LOC101750697</td>
<td>5.509243069</td>
</tr>
<tr>
<td>MYH1G</td>
<td>Myosin, heavy chain 1G, skeletal muscle (similar to human myosin, heavy chain 1, skeletal muscle, adult)</td>
<td>5.512319402</td>
</tr>
<tr>
<td>BCL11A</td>
<td>B-cell CLL/lymphoma 11A (zinc finger protein)</td>
<td>5.52499576</td>
</tr>
<tr>
<td>DDX43</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 43</td>
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</tr>
<tr>
<td>LOC101749765</td>
<td>E3 ubiquitin-protein ligase ICP0-like</td>
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</tr>
<tr>
<td>LOC101747558</td>
<td>uncharacterized LOC101747558</td>
<td>5.547819957</td>
</tr>
<tr>
<td>LOC101748830</td>
<td>inositol 1,4,5-trisphosphate receptor-interacting protein-like 1-like</td>
<td>5.548128324</td>
</tr>
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Table 18: Raw data table for the enriched genes found in the relative tissue expression data for Illinois Control and Heat-stressed groups. The genes enriched in the control group are unbolded and the enriched genes in the heat-stressed group are illustrated in bold.

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Appendix D

PERMISSION LETTERS
Appendix E

AACUC 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) 03-12-14R

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  x□

If this is a 3 year renewal, what is the assigned existing protocol number?
_(27) 12-22-10R

-------------------------------------------------------------------------

(This section for Committee use only)

Application Approved (date): 01/05/2011

Application Rejected (date): _____________

Reason for Rejection: ____________________________
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: schmidtc@udel.edu

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

Teaching/Outreach □    Research  X

If TEACHING box was checked, select from the following:

   Demonstration □   Laboratory □   Student Project □

If student project, please define project: __________________________

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

   YES   X□   NO   □

If no, identify those needing to review application.

   __________________   __________________   ________________
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?

____________     ________________   _______________

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.

______________________________________________________________

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  X□

If yes, explain in detail the surgery.

Are drugs, vaccines and/or medications being used?  YES □  NO X□

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 libidum*. 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 X□

If yes, what form(s) are attached?  ________________________

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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