

The effects of biotic and abiotic stressors on gene expression in chickens

by

Derrick Jamaal Coble

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

Program of Study Committee:
Susan J. Lamont, Major Professor
Max F. Rothschild
Michael E. Persia
Christopher K. Tuggle
Dan Nettleton

Iowa State University
Ames, Iowa
2013

Copyright © Derrick Jamaal Coble, 2013. All rights reserved.

DEDICATION

This dissertation is dedicated to my parents, my grandparents, and God for providing me with more love and care than any person could image. Thanks Mom for the care packages that you sent from time to time, they really helped me to make my home here in Iowa. Dad and Papa, thanks for all the time and work you've dedicated to my car. I'm sure sending my car back home has saved me thousands. I guess I will have to put gas in the boat now. Thanks Grandma Annie for the great meals that you prepare when I come back in North Carolina, highlighting the fact that there is no place like home. Thanks Grandma Jessie for the good conversations that we have when I come to your home. You always keep me in tune to what is going on at home. Finally, I would like to thank God for the guidance that you have provided me throughout life.

TABLE OF CONTENTS

	Page
LIST OF FIGURES	v
LIST OF TABLES	vii
NOMENCLATURE	ix
ACKNOWLEDGEMENTS	x
ABSTRACT	xi
CHAPTER 1. GENERAL INTRODUCTION	1
Introduction	1
Organization of Dissertation	2
Literature Review	3
References	29
 CHAPTER 2. DISTINCT LINES OF CHICKENS EXPRESS DIFFERENT SPLENIC CYTOKINE PROFILES IN RESPONSE TO <i>SALMONELLA</i> ENTERITIDIS	 50
Abstract	50
Introduction	51
Materials and Methods	53
Results	55

Discussion	56
Acknowledgements	59
References	59
 CHAPTER 3. IMPACTS OF <i>SALMONELLA</i> ENTERITIDIS INFECTION ON LIVER TRANSCRIPTOME IN BROILERS	 68
Summary	68
Introduction	69
Results	71
Discussion	73
Methods	78
Acknowledgements	81
Literature Cited	82
 CHAPTER 4. RNA-SEQ ANALYSIS OF BROILER LIVER TRANSCRIPTOME REVEALS NOVEL RESPONSES TO HEAT STRESS	 94
Abstract	94
Background	96
Results	97
Discussion	100
Conclusion	115
Methods	115

Acknowledgements	118
References	119
CHAPTER 5. GENERAL DISCUSSION, CONCLUSIONS, AND FUTURE DIRECTIONS	143
References	152

LIST OF FIGURES

CHAPTER 2.

Figure 1A. 66

Mean IL-18 mRNA expression levels by genetic line, in the spleen of hens either non-challenged or challenged with SE

Figure 1B 67

Means of adjusted Ct values for IL-6 mRNA expression by genetic line in the spleen of hens either non-challenged or challenged with SE

CHAPTER 3.

Figure 1. 92

Pathway analysis of cell cycle functions in broiler liver transcriptome in response to *S. Enteritidis*

Figure 2 93

Pathway analysis of metabolic functions in broiler liver transcriptome in response to *S. Enteritidis* infection

CHAPTER 4.

Figure 1. 140

Cell Signaling, Molecular Transport, Small Molecule Biochemistry Network

Figure 2. 141

Endocrine System Development and Function, Small Molecule

Biochemistry, Cell Signaling Network

Figure 3.

142

Significance levels of functions associated with DE genes in broiler
liver in response to chronic, cyclic heat stress

LIST OF TABLES

CHAPTER 2.

Table 1.	65
Genetic line, challenge, and line-by-challenge interaction effects on spleen mRNA expression	
Table 2.	65
Splenic IFN- γ and RANTES mRNA expression of hens from different genetic lines	

CHAPTER 3.

Table 1.	88
List of genes that belong to Cell Cycle and Metabolism networks	
Table 2.	89
Log ₂ fold change of hepatocyte gene expression between <i>S. Enteritidis</i> challenged and non-challenged birds	
Table 3.	89
Forward and reverse primer sequences used for qPCR	
Supporting Information Table 1.	90
List of genes, <i>P</i> -values, GO annotations, directions of differential expression and fold changes	

CHAPTER 4.

Table 1.	138
-----------------	-----

Forward and reverse primers used for qPCR

Table 2.	138
-----------------	-----

P-value of hepatocyte gene expression between heat stressed- and non-
heat stressed-broilers

Supporting Information Table 1.	139
--	-----

P-values, fold changes, and q-values for DE genes

NOMENCLATURE

B	Bursal
cDNA	Complementary Deoxyribonucleic Acid
Ct	Cycle Threshold
DE	Differential Expression
FDR	False Discovery Rate
IPA	Ingenuity Pathway Analysis
mRNA	Messenger Ribonucleic Acid
qPCR	Quantitative Polymerase Chain Reaction
RNA-seq	Ribonucleic Acid Sequencing
SE	<i>Salmonella</i> Enteritidis
T	Thymic
Th	Thymic helper

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Susan J. Lamont, and my committee members, Dr. Dan Nettleton, Dr. Max F. Rothschild, Dr. Michael E. Persia, and Dr. Christopher K. Tuggle, for their guidance and support throughout the course of this research.

From Chapter 2, I would like to thank SBR for participation in the animal trials, BH for participation in the qPCR analysis of genes, and SJL for participation in the animal trials and assisting in the drafting of the manuscript. From Chapter 3, I would like to thank EES and JA for participating in microarray hybridization, TJ for assisting with the statistical analysis of the data, DF for assisting with qPCR validation, HZ for supplying the chicken 44k Agilent microarrays, and SJL for assisting in the drafting of the manuscript. The acknowledgements for Chapter 4 are at the end of the Chapter.

I would like to re-iterate my thanks to Dr. Lamont for extending an invitation into your awesome lab group and even into your home on countless occasions. I would like to thank the Lamont Lab group, Michael, Ann, and the members of the Iowa State Poultry Farm Crew for making my time here a wonderful experience. I would like to thank all the BGSA family, but special thanks goes out to the group that was at ISU when I first entered my Ph.D. program. You guys provided a support network that I really leaned on throughout my years here. I would also like to thank my Café B friends for providing me with a perspective of an entirely different area of research. Finally, thanks to my family for their encouragement and hours of patience, respect, and love.

ABSTRACT

Poultry provides the largest livestock source of protein for human consumption. Due to this widespread consumption of poultry products, biotic and abiotic stressors of chickens can have negative impacts on the nutrition of humans, as well as the health and productivity of chickens. *Salmonella*-infected poultry products present a food safety threat to human consumers, while the exposure of poultry to hot environments raises animal welfare issues. Understanding the effects of *Salmonella* infection and heat stress on immunological and metabolic networks in chickens may help to develop strategies to circumvent these problems. Therefore, gene expression profiles of *Salmonella*-infected and heat-stressed chickens were characterized. The splenic profiles of selected cytokines in *S. Enteritidis*-infected broiler, Leghorn and Fayoumi hens were analyzed using Quantitative Polymerase Chain Reaction (qPCR). The results supported the concept that chickens from distinct genetic lines utilize different immune mechanisms in response to *S. Enteritidis* infection. Systemic metabolic effects were detected in the liver transcriptome of *S. Enteritidis*-infected broilers using microarrays. A predominant trend of down-regulation was observed among the 44 differentially expressed (DE) genes. Cell Cycle and Metabolism networks were created from Ingenuity Pathway Analysis (IPA) of the DE genes. Metabolic responses were also detected in the liver transcriptome in response to chronic, cyclic heat stress of chickens. Two networks were created from the 40 DE genes, “Cell Signaling, Molecular Transport, Small Molecule Biochemistry” and “Endocrine System Development and Function, Small Molecule Biochemistry Cell

Signaling”. Members of the Ras-Raf-MEK-ERK (MAPK) signal transduction cascade (*MAPK*, *P38 MAPK*, *ERK*, *ERK1/2*) were present in the broiler liver transcriptome in response to both *S. Enteritidis* infection and heat stress, suggesting a common response to a wide range of stressors. This dissertation provides novel insights into the effects of two important stressors, (*S. Enteritidis* infection and heat stress) on immunological and metabolic-related pathways in chickens, establishing a platform for further investigation into the genomics of the stress response of chickens. Ultimately, understanding the affected immune and metabolic-related pathways may lead to the commercial production of chickens resilient to the effects of *S. Enteritidis* infection and heat stress.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Stress response is the response of the body to stimuli that disturb its homeostasis. The stress response is a very complex and multifaceted mechanism; it involves a series of behavioral, physiological, metabolic, and immunological reactions that the body uses to redistribute the demands placed on it, adapt to stressors, and survive (Shini et al., 2010). Any stimulus that evokes this response is referred to as a stressor. Researchers have investigated the effects of a variety stressors (environmental, climatic, physiological, nutritional, microbial, viral) on bird well-being and performance (Al-Fataftah and Abu-Dieyeh, 2007; Mashalay et al., 2004; Mumma et al., 2006; Redmond et al., 2011; Sandercock et al., 2012; Sandford et al., 2011).

Commercial production of chickens has resulted in intensive selection for increased production efficiency. It has also resulted in crowded production environments, increasing the exposure of chickens to unintended stressors, such as microbial and heat stressors (Cheng et al., 2004). In some cases, selection for production traits has increased the line's susceptibility to stressors (Berrong and Washburn, 1998; De Smit et al., 2005; Olkowski et al., 1998). To simultaneously select for production traits and increased stress resistance, researchers must understand the genetic mechanisms that regulate them. If an immune trait and production trait are negatively correlated, selecting for one of these traits will result in poor performance in the other trait. In two White Leghorn lines divergently selected for antibody production, Zhou et

al. (2012) reported higher 4-week body weights in the line selected for lower antibody production when compared to the line selected for higher antibody production.

The characterization of gene expression profiles of host tissues in response to these stressors, have been used to improve the understanding of stress response. Quantitative Polymerase Chain Reaction (qPCR) allows researchers to analyze the expression of small numbers of candidate genes; microarray technology allows researchers to analyze thousands of genes simultaneously; and RNA-Seq technology allows researchers to detect the differential expression of unmapped genes, measure read abundance at each exon, locate splice events, and discover candidate genes. Gene expression profiles from different tissues and thousands of genes allow researchers to infer gene pathways that undely response to stress. The goal of the research summarized in this dissertation was to improve the understanding of chicken host response to *Salmonella* Enteritidis and heat stress. The transcriptomic response of the spleen of different chicken lines to *Salmonella* Enteritidis, the broiler liver to *Salmonella* Enteritidis, and the broiler liver to heat stress were assessed by qPCR, microarray, and RNA-Seq technologies, respectively.

Organization of Literature Review

This literature review examines the impacts of biotic and abiotic stressors on the immunological and metabolic responses of chickens, the performance of broilers and layers, and the efficiency of poultry production. The literature review also discusses the evolution of the molecular methods used to improve stress resistance in poultry. It has been organized into seven sections. The first section describes the stressors that affect

poultry, and their economic impact on the poultry industry. The second section discusses the effect of genetic background on a bird's immunological and metabolic responses to stressors. The third section reviews the host immune response of chickens to *Salmonella* infection as an example of a biotic stressor. The fourth section discusses the effects of heat as an abiotic stressor on production traits and immunity in chickens. The fifth section describes the effects of globalization on the poultry industry with implications on stress. The sixth section discusses the use of molecular markers to improve disease resistance in poultry. The seventh section reviews the different approaches that have been used to characterize the transcriptome of stressed chickens.

Literature Review

Stressors and their Impact on the Poultry Industry

A considerable portion of the production and financial losses that are incurred by poultry producers is due to environmental stress (Capua and Alexander, 2004; Kapczynski and King, 2005; St. Pierre et al., 2003). Stressors can include any components of the bird's surroundings that have negative impacts on the physical health, mental health, or productivity of the bird. Stressors can be biotic or abiotic. Stress associated with bacterial, viral, and fungal pathogens, and temperature is of particular interest for poultry production. *Salmonella* Enteritidis and *Salmonella* Typhimurium are major bacterial pathogens of interest to chicken producers because they can either produce clinical symptoms in young and immunocompromised birds or exist at subclinical levels (Zhang-Barber et al., 1999). As a result, they can lead to production costs due to mortality and culls or retail losses due to food safety concerns or recalls.

Salmonella Gallinarium is of particular importance to turkey producers because the production time to grow turkeys to market weight is sufficient for *S. Gallinarium* to cause Fowl Typhoid (Shivaprasad, 2000; Shivaprasad, 2003). In the United States, approximately 2 million cases of *Salmonella* infection occur in livestock annually, costing an average of \$1.4 billion (Comis, 2003).

Newcastle Disease Virus (NDV) and Avian Influenza Virus (AIV) are viruses that are of particular interest to the poultry industry. This interest stems from the widespread distribution of these viruses and total depopulation of the flock being required for eradication of these viruses (Marangon and Busani, 2006). NDV causes apoptosis of infected cells in birds, resulting in gross lesions of the respiratory and nervous systems, as well as the swelling of tissues around the eyes and neck, and mortality (Ravindra et al., 2008; Tang et al., 2012). The cost of eradicating the 2002-2003 NDV outbreak in California exceeded \$200 million (Kapczynski and King, 2005). AIV can cause the same symptoms as NDV but is zoonotic, highly contagious, and very deadly (Food and Agricultural Organization/World Organisation for Animal Health, 2005; Tang et al., 2012). The estimated losses to the U.S. poultry industry due to AIV are estimated between \$5-212 million (Capua and Alexander, 2004; Farnsworth et al., 2012). One outbreak in Canada in 2007 cost producers an estimated \$646 million U.S. dollars (Swayne, 2008).

Just as the bacterial and viral environments of production birds have impacts on the poultry industry, the temperature in which birds are reared has impacts. Heat stress is a stress associated with high temperature and is of particular interest to livestock

producers. Heat stress occurs when animals experience a negative balance between the net energy released by an animal into the surrounding environment, and the amount of heat energy the animal produces (Ajakaiye et al., 2011). Heat stress can be divided into “acute” and “chronic”. Acute heat stress is characterized by short, sudden periods of extremely high temperatures; chronic heat stress is characterized by long periods of high temperature (Emery, 2004). Heat stress is thoroughly being investigated by researchers because of the increase in temperatures in regions of poultry production and the expansion of the poultry industry into regions that have higher temperatures. The effects of heat stress can be inflated if the appropriate poultry house design isn’t used or if the poultry house is in poor condition. This can occur in poultry houses that don’t have appropriate ventilation during the summer or houses that have air flow from the outside during the winter, causing birds to crowd and overheat (Akyuz, 2009). In addition to region and housing temperatures contributing to heat stress in poultry, transport can also play a contributing role. Reports from the U.K. have indicated that as much as 40% of mortalities that result during transport are due to “transport stress” (Bayliss and Hinton, 1990). Exposure to ambient temperature and relative humidity are also thought to play roles in these mortalities (Hartung, 2003). The annual heat stress-related cost incurred by U.S. poultry producers was recently estimated at approximately \$125-165 million, with turkey producers incurring a total loss of \$14.4 million, and the broiler sector incurring \$51.8 million (St. Pierre et al., 2003). In 1995, Iowa experienced a 2 week period of extreme heat in which producers incurred \$9 million in losses due to layer mortality (Xin, 1998). Heat stress abatement has been shown to be economically beneficial.

Optimal heat abatement in the layer sector reduces total economic losses from \$98.1 to \$61.4 million annually (St. Pierre et al., 2003). In addition to the risk of production losses, heat stress conditions pose an animal welfare problem.

How Genetics Effects Immunological and Metabolic Response to Stressors

There are many factors that affect a bird's response to stressors. One of the most prominent factors is the genetic background of the bird. Genetic background plays a role in a bird's response to *Salmonella* infection (Berthelot-Hérault et al., 2003; Cheeseman et al., 2007; Redmond et al., 2011). Berthelot-Hérault et al. (2003) reported that IgG and IgA levels were significantly higher in a layer line (B13) and a Leghorn line (L2) compared to another Leghorn line (PA12) and a meat-type line (Y11). *S. Enteritidis* colonized the ceca of B13 and L2 lines at significantly higher levels than the PA12 and Y11 lines (Berthelot-Hérault et al., 2003). Upon evaluating cytokine mRNA expression in the spleen and cecum of young chickens infected with *S. Enteritidis*, Cheeseman et al. (2007) observed higher cytokine mRNA expression levels in the spleen and lower levels in the cecum of Leghorns versus broilers.

In addition to measuring cytokine mRNA expression levels in the organs of different breeds, cytokine mRNA expression levels of *S. Enteritidis*-stimulated heterophils have also been measured in different breeds. Redmond et al. (2011) reported that *S. Enteritidis*-stimulated heterophils from Leghorns expressed higher levels of *CXCLi2* mRNA than those from Fayoumis. Heterophils employ phagocytosis (Genovese et al., 1999), leukocyte degranulation (He et al., 2005), and extracellular traps to fight pathogens (Chuammitri et al., 2009), so they have been associated with livestock

pathogen resistance. Therefore, susceptibility of different genetic lines of chickens to *Salmonella* was evaluated by Swaggerty et al. (2004). The researchers found higher levels of heterophil migration to the abdominal cavity and higher levels of circulating *CXCLi2* mRNA in resistant versus susceptible lines.

Genetic background has also been shown to play a role in a bird's response to viral infection. In a study contrasting the immune response of a high cell-mediated immunity chicken line and a low cell-mediated immunity chicken line to NDV, Ahmed et al. (2007) observed higher levels of *iNOS*, *NO*, and *IFN- γ* mRNA in the high cell-mediated immunity line. Sacco et al. (1993) reported differences in antibody titers to NDV vaccination between a RBC2 (random-bred control) line of turkey and a line selected for higher 16-week body weight. The line selected for 16-week body weight, had higher serum antibody titers to NDV 3 weeks post vaccination at 9 and 12 weeks of age. A difference in susceptibility to HPAI (Highly Pathogenic Avian Influenza) H7N1 was observed between 3 commercial broiler lines (A, B, C), a White Leghorn line (E), and a New Hampshire line (D) (Sironi et al., 2008). Lines A, C, and D displayed high susceptibility to HPAI, while lines B and E displayed resistance to HPAI.

Genetic background also plays a role in a bird's response to heat stress. Heat stress is a major concern because it can affect the production traits of birds. Berrong and Washburn (1998) demonstrated that the Athens-Canadian Randombred (ACRB) chicken line had significantly lower plasma protein and body temperature than commercial broilers, in response to heat stress. The commercial broilers experienced a much greater decrease in body weight gain than the ACRB line. Felver-Gant et al. (2012) reported that

heat stress resulted in higher levels of heat shock protein 70 (*HSP70*) in the livers of White Leghorn hens selected for high productivity and survivability (KGB) than White Leghorn hens selected for high egg production (DXL). Heat stress can result in significantly different enzymatic concentrations in genetically distinct lines of chickens. In an experiment that compared egg quality traits in a LW (Lohman White) line, NH (New Hampshire) line, NN (Naked Neck) line, and the F₁ progeny of NN x LW and NN x NW crosses in response to heat stress, Melesse et al. (2010) reported that the NN line and the F₁ progeny displayed a greater level of resistance to heat stress than the commercial lines. Melesse et al. (2011) compared the levels of metabolic enzymes: lactate dehydrogenase, creatine kinase, glutamic pyruvic transaminase, and thyroid hormone (T3), in the blood of a WL-dw (dwarf White Leghorn) line, LW line, LB (Lohmann Brown) line, NW line, and a WL-FE (White Leghorn selected for feed efficiency) line. The WL-dw line was the most heat tolerant line (Melesse et al., 2011). The WL-dw line had significantly higher levels of lactose dehydrogenase compared to the LW, LB, and NH lines, significantly higher levels of creatine kinase compared to the LW, NH, and WL-FE lines, the largest glutamic pyruvic transaminase activity compared to the LB, NH, and WL-FE lines, and the highest level of thyroid hormone (T3) (Melesse et al., 2011).

In summary, distinct genetic lines respond differently to stressors, suggesting that immunological and metabolic stress response mechanisms of chickens vary by genetic background.

Host Immune Response of Chickens to *Salmonella*

The avian immune system contains two key branches, innate (non-specific) and adaptive (specific) immunity. The innate immune system is the first line of defense against pathogens and is composed of natural barriers, secretions, and leukocytes that have phagocytic functions. These leukocytes include macrophages, heterophils, thrombocytes, and natural killer (NK) cells (Erf, 2004). The first physical barrier of protection against *Salmonella* is the intestinal epithelium. *S. Enteritidis* has a high capability for colonization of the cecum compared to *S. Pullorum* and *S. Gallinarum* (Setta et al., 2010). The cecum is colonized within 12 hours of oral ingestion of *S. Enteritidis* (Van Immerseel, 2002). This colonization elicits a significant increase in the number of macrophages and heterophils that are present in the cecum. Tissue-specific colonization of macrophages involves the migration of monocytes from circulation in response to chemotactic signals (Kaspers et al., 2008). Withanage et al. (2004) detected the expression of *IL-8*, *MIP1- β* , and *K60* in the livers and spleens of newly hatched chicks shortly after *S. Typhimurium* infection. This gene expression pattern reflects the signaling of macrophages and heterophils to the site of infection. As mentioned earlier, macrophages along with heterophils are responsible for engulfing and removing pathogens (Erf, 2004). Along with performing phagocytosis, macrophages and heterophils can also produce oxygen derivatives that kill microorganisms through the process of oxidative burst (Lin, 1992). *S. Enteritidis*-exposed macrophages have been reported to stimulate IL-2 production, signaling NK (natural killer) cells to secrete IFN-

γ , activating other macrophages and leading to the production of nitric oxide (Eisenstein, 2001). NK cells are ready to kill through degranulation unless inhibited by signals such as TGF- β (Abbas et al., 2000). During the embryonic stage of development, the spleen is a key site of production of such granulocytes (Pope, 2001). Extracellular trap production and degranulation are two mechanisms by which heterophils kill microbial pathogens (He et al., 2005; Redmond et al., 2011). Once TLR-4 recognizes pathogen-associated molecular patterns (PAMP), the stimulation of heterophil phagocytosis, heterophil oxidative burst, and the expression of pro-inflammatory cytokines on the surface of heterophils occurs (Akashi et al., 2001; Farnell et al., 2003; Kogut et al., 2001; Kogut et al., 2005). The level of expression of pro-inflammatory cytokines on the surface of heterophils corresponds to the *S. Enteritidis* resistance level of chickens (Redmond et al., 2009; Swaggerty et al., 2004). Cytokine expression helps to regulate and signal transition from the innate and adaptive immune responses (Kaiser et al., 2006). For example, IL-6 is a pro-inflammatory cytokine that is important in the development of adaptive immune responses, leading to the differentiation and growth of B (Bursal) lymphocytes and cytotoxic T (Thymic) cells (Hirana et al. (1994).

The adaptive immune response is the branch of immunity that is specific. It is responsible for not only eliminating pathogens, but also for the memory of pathogens for a heightened immune response the second time the pathogen is encountered. The level of *Salmonella*-specific adaptive immune responses and cytokine production has been associated with the ability to eliminate systemic *Salmonella* infection (Beal, 2004). The adaptive immune response can be separated into the cell-mediated and humoral

responses. Cell-mediated immunity (CMI) is responsible for eliminating intracellular pathogens while humoral immunity is responsible for eliminating extracellular pathogens (Erf, 2004).

The antigen-specific cells of the CMI response are T cells (Chen et al., 1991). Avian T cells are classified into two groups ($\alpha\beta$ and $\gamma\delta$) based on their TCR (T cell receptor) (Berndt et al., 2006). Avian T cells with $\gamma\delta$ TCR are comprised of cells that contain $CD4^+$ and $CD8^+$ surface markers (Berndt et al., 2006). Avian T cells with $\alpha\beta$ TCR can be further divided into $V\beta_1$ and $V\beta_2$ subpopulations (Berndt et al., 2006). Avian $\alpha\beta$ T cells have been less characterized than the $\gamma\delta$ T cells. The $\gamma\delta$ T cells that bear the $CD4^+$ surface marker are referred to as Th (T-helper) cells. Th cells function in antigen recognition, the production of cytokines, and the expression of cell surface markers to activate other cells (Erf, 2004). The activation of either the cell-mediated or humoral immune responses is dependent upon the cytokines that Th cells produce (Arstila et al., 1994). Cell-mediated immunity is associated with the Th1 response and antibody production is associated with the Th2 response (Kaiser and Stäheli, 2008). The Th1 inflammatory response in chickens is driven by IL -12 (Kaiser and Stäheli, 2008). The production of Th1 cytokines IFN- γ , IL-1, IL-2, IL-6, IL-8, and MIP-1 β elicits the CMI response, whereas the production of Th2 cytokines IL-3, IL-4, IL-5, IL-13 and Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) produces the humoral response (Eckman and Kagnoff, 2001; Kaiser and Stäheli, 2008; Setta et al., 2012). In mammals, other members of the IL-12 family (IL-23 and IL-27) regulate the Th1/Th2 responses (Kaiser and Stäheli, 2008). Although IL-23 and IL-27 haven't been identified

in chickens, their receptors have, lending support that similar mechanisms may exist in chickens (Kaiser et al., 2005).

When T cells are screened for the antigen specificity of their TCR during T cell development, they also are screened for host MHC II (Major Histocompatibility Complex class II) (Erf, 2004). This process ensures that the T cell will have specificity for a particular antigen-peptide complex on APC (Antigen Presenting Cells) and be able to distinguish between foreign and host antigens (Erf, 2004). An antigen is either presented as endogenous or exogenous, this results in an antigen-peptide complex that is expressed on the surface of APC that are bound to either MHC class I or MHC II molecules (Kaspers et al., 2008). In the case of endogenous antigens, the APC presents the antigen-peptide complex to MHC I-associated molecules. In the case of exogenous antigens such as *Salmonella* infection, the APC presents the antigen-peptide complex to MHC II-associated molecules, leading to the proliferation of activated Th cells and the production of memory cells (Abbas et al., 2000). Upon a repeat *S. Enteritidis* encounter, these memory cells will result in the rapid elimination of the pathogen before it causes disease (Abbas et al., 2000).

The $\gamma\delta$ T cells that bear the $CD8^+$ surface marker are referred to as Tc (cytotoxic T) cells. Tc cells are effector cells of the CMI response that interacts with the endogenous antigen-peptide complex associated with the MHC I (Abbas et al., 2000). Because all nucleated cells express MHC I, no APC are needed for recognition. After $CD8^+$ T cells interact with the antigen-peptide complex, Th cells secrete IL-2 and IFN- γ cytokines that activate Tc cells (Erf, 2004). The production of specific cytokines at

particular times during the immune response to *Salmonella* controls the level and direction of that immune response. Using a murine model, Fahy et al. (2006) demonstrated that the chemotactic cytokine (chemokine) CXCL16 was involved in controlling the cell-mediated production of IFN- γ during primary *S. Enteritidis* infection and the bacterial burden of the liver and spleen during secondary infection. Upon activation, Tc cells differentiate into effector Tc cells or memory cells. The granules of effector Tc cells move to the site of Tc cell-antigen contact and release cytolytic products by exocytosis (Abbas et al., 2000).

The other component of adaptive immunity, the humoral response, involves the activity of B lymphocytes. Avian B cells originate and differentiate in an organ that is unique to avian species, the Bursa of Fabricius (Barnes, 2001; Glick, 1988). The Bursa of Fabricius contains between 8,000 and 12,000 follicles and a large amount of packed B cells in the cortex (Pope, 2001; Scott, 2004). The B cells mature in these follicles and develop antigenic diversity when exposed to external antigens by APC (Glick, 1977; Ratcliffe, 1989). Mature B cells migrate from the Bursa of Fabricius and reside in the spleen, mucosal-associated lymphoid tissue (MALT), lymph nodes, and the circulatory system (Pope, 2001). This allows the immune system to maintain a humoral response after the Bursa of Fabricius naturally degenerates at 14-20 weeks of age (Pope, 2001).

The chicken's humoral response heavily relies on the lymphoid tissues that line the gastrointestinal and respiratory tracts, MALT and gut-associated lymphoid tissue (GALT) (Olah et al., 2003). The cecal tonsil, the largest GALT tissue, plays a role in antibody production and is a designated location for B cell differentiation (Olah et al.,

2003). Humoral immunity in response to pathogens in the gastrointestinal tract is thought to be due to secretory antibodies, most notably IgA (Lee et al., 1981). Avian IgA and IgM are produced by plasma B cells that are located in the lamina propria (Bienenstock et al., 1973; Parry et al., 1977). The plasma B cells then secrete the antibodies into the gut lumen by transport through epithelial cells (Brandtzaeg and Baklien, 1977). Lee et al. (1981) detected high levels of specific antibody in the bile of *S. Typhimurium*-infected chickens and concluded that the liver-biliary system might be an important route for the delivery of specific antibody into the intestinal tract. There are various locations along the gastrointestinal tract (Peyer's patches) that are major sites for IgA responses to pathogens (Lillehoj, 1996). Mature B cells in the spleen help regulate the levels of antibody that enter the circulatory tract. Antibody levels in the serum are also indicative of the ability of a bird's immune system to respond to pathogenic bacterial infection (Hofacre et al., 1986; Leitner et al., 1992). To date, the most effective methods for monitoring humoral responses to *Salmonella* have been agglutination techniques or by ELISA assays (Zhang-Barber et al., 1999).

Effects of Heat Stress on Weight Gain, Egg Laying Efficiency, and Immunity in Chickens

As aforementioned, heat stress is a major stressor that affects the profitability of poultry producers (St. Pierre et al., 2003). In broilers, chronic heat stress has been shown to have detrimental effects on performance and immunity. Al-Fataftah and Abu-Dieyeh (2007) reported that long-term exposure to temperatures that exceed 25° C in 4-8 week old ISA Vedette broilers negatively affect performance. This reduction in performance is caused by a combination of factors including reduction in feed consumption, digestive

inefficiency, and impaired metabolism (Emmans and Charles, 1989; Farrell and Swain, 1978; Har et al., 2000). The metabolic utilization of nutrients that occurs after feed intake causes an increase in body temperature (Akyuz, 2009). Reduced feed intake in response to chronic heat stress is thought to be a mechanism by which the birds reduce their body temperatures (May and Lott, 1992). Geraert et al. (1996) reported a reduction in the metabolic utilization of nutrients in 2 and 4-week old Shaver broiler chickens under heat exposure in response to endocrine control. The altered metabolism from heat stress exposure has also been associated with poor carcass quality. Ain Baziz et al. (1996) reported that 4-week old broiler males had a decreased unsaturated: saturated fatty acid ratio in abdominal and subcutaneous tissues but not in intermuscular or intramuscular tissues, under heat stress conditions. Chronic heat stress significantly reduces the size of the gastrointestinal tract (Bonnet et al., 1997).

In layers, chronic heat stress has negative impacts on ovarian function, egg quality, and the immune response. Although the detrimental effect of heat stress on egg production has been well documented, the cause of this effect is less known (Rozenboim et al., 2007). Smith and Oliver (1972) demonstrated that the effect of high temperatures on egg production is largely unrelated to feed intake. Other researchers have reported that the reduction in egg production in response to heat stress is due to lowered reproductive performance (Etches et al., 1995). Heat reduces luteinizing hormone and hypothalamic gonadotropin-releasing hormone-I levels in Leghorns (Donoghue et al., 1989). It also results increased levels of prolactin which causes a reduction in gonadotropin levels and ovarian regression (Rozenboim et al., 1993; You et al., 1995;

Youngren et al., 1991). Wolfenson et al. (1981) suggests that reduction in ovarian function might be due to reduced blood flow to the ovaries. In concordance with reduced ovarian function, the qualities of eggs are negatively impacted by heat stress exposure (Mack et al., 2010). The weights of eggs produced by 30-week old White Leghorns significantly drop in response to heat stress (Rozenboim et al., 2007). In a study that examined characteristics of eggs from 28-week old White Leghorn and Dekalb layers randomly placed in heat stress or control treatments, Mack et al. (2010) reported a reduction in egg number, egg weight, and shell thickness in the birds that were exposed to heat stress. Some researchers have studied the effects of heat stress at specific times on particular egg characteristics. When mature laying hens were exposed to either 6-7 hour periods of 43°C for 8 days or 6-7 hour periods of 43°C for 5 nights, egg-shell quality was significantly reduced in eggs from the hens that experienced heat stress during the day (Wolfenson et al., 1978). Egg production was significantly decreased in hens that experienced nocturnal heat stress; there was no change in the egg production of hens that experienced heat stress during the day (Wolfenson et al., 1978).

In addition to having negative effects on the performance and physiological characteristics of poultry, heat stress also affects immune function. The first researchers to demonstrate that heat stress affected specific immunity were Thaxton et al. (1968). This study proved that the development of the specific immune response of young chickens was affected when they were exposed to temperatures that ranged from 44.4°C to 47.8°C (Thaxton et al., 1968). The suppression of white blood cells and the increase in the heterophil/lymphocyte ratio, an indicator of stress, are among the effects that heat

stress has on the immune function of poultry (Gross and Siegel, 1983; Heller et al., 1979; Mogenet and Youbicier-Simo, 1998). In addition to heterophil/lymphocyte ratio, Mashaly et al. (2004) reported that chronic heat stress in 26-week old layer hens resulted in a decrease in antibody titer to sheep red blood cells (SRBC) as well.

Globalization of the Poultry Industry

Poultry production has increasingly become a global industry over the last 4 decades. This globalization has been fueled by the creation of multinational corporations such as the E.W. Group, the Hendrix Group, and Brasil Foods. With technological advancements, global communication has drastically increased, facilitating the global exchange of resources and ideas. This globalization has had impacts on the poultry production landscape, international trade, public health, and biosecurity. The poultry industry has especially experienced remarkable growth over the past 4 decades. Poultry meat and egg production increased at a faster rate from 1970 to 2005 than that of beef and veal or swine production (Windhorst, 2006). Most of this growth occurred during the 1990s when the production of poultry meat and eggs in developed (North American and European) countries was surpassed by the production of developing (Asian and South American) countries. In 2005, developing countries contributed approximately 67% to the global egg production (Windhorst, 2006).

Accompanying shifts in the poultry production landscape, the import/export market of poultry products has also changed. Poultry meat exports from developing countries increased from only 2.9% in 1970 to 37.8% in 2004. During the 1990s and early 2000s, consumption in India and production of poultry products increased 11% as

compared to a global growth rate of 5% (Landes, 2002). In 2000, the top 3 exporters of chicken meat were the U.S. (37%), the E.U. (26%), and China (17%), while in 2005 the top 3 exporters of chicken meat were Brazil (34%), the U.S. (31%), and the E.U. (26%) (FAOSTAT, 2012). The U.S. market share decreased 7% during this time because of lower importation needs of the Russian Federation (Windhorst, 2006). The United States Department of Agriculture predicts that Brazil will continue to be the top exporter of chicken meat because of a higher demand for Brazilian products due to competitiveness and market promotion in new markets (USDA/ERS, 2007). Not only was there a change in the share of global exportation of poultry meat products from developing countries, there was also a change in the type of poultry meat that is exported. In 1970, chicken meat constituted 92% of the global poultry meat that was exported, this share of the global market had decreased to 75.4% in 2004 (Windhorst, 2006). This change was due to an increase in the consumption of turkey meat during this time.

With shifts in the export market of poultry meat and eggs from developed countries with well-established regulations and agencies to developing countries with less established infrastructure, public health and biosecurity has become a major area of focus. Generally trade is regarded as safe when in line with the World Organization for Animal Health's guidelines, but cross-border trade was identified as the cause for the spread of Avian Influenza (H5N1) in Africa and Asia (Kilpatrick et al., 2007). In developing countries most of the poultry production occurs in close proximity to human population centers (Otte et al., 2007). The risks of public safety and biosecurity are magnified by the fact that the human population has grown to approximately 700 million

people and most of this growth has occurred in developing countries (Otte et al., 2007). The production of large amounts of poultry products is commonly accompanied by large-scale swine operations in developing countries. The confinement of large populations of poultry alongside large populations of swine, increases the likelihood of development and evolution of zoonotic pathogens (Otte et al., 2007). The proximity of large urban centers near these operations further increases the risk of the development of a pandemic infectious disease. A common misconception is that better biosecurity measures are taken by larger facilities, when in fact the risk of infection and pathogen spread rises with the intensity of the operation (Otte et al., 2007). In the absence of proper infrastructure for intense poultry production, developing countries lack the ability to accommodate the large amounts of waste that are produced. The disposal of waste is important because the survival period of many enteric organisms ranges from a day to months in manure and waste water (Guan and Holley, 2003; Nicholson et al., 2005). According to Cole et al. (2000), pathogens such as *Campylobacter* spp, *Salmonella* spp, *Brucella* spp, *Clostridium perfringens* and *Listeria monocytogenes* can exist in high levels in fresh poultry manure; these organisms can also exist in infectious levels even after on-site holding. Biosecurity involves a combination of “bioexclusion” and “biocontainment” (Dargatz et al., 2002). Bioexclusion is defined as measures that prevent the introduction of a pathogen into members of a flock, whereas biocontainment addresses a pathogen’s ability to spread within a flock or escape containment (Dargatz et al., 2002). As large-scale poultry production continues to spread into new regions of the world, the ability to maintain biosecurity decreases. Sawabe et al. (2004) reported

that during a 2004 HPAI outbreak in Kyoto, Japan, flies that were captured near the broiler facilities carried the same strain (H5N1) as the chickens of the infected farm. Thus, with the globalization of the poultry industry into less developed regions of the world, the risk of the spread of infectious viral and bacterial diseases is heightened.

Improving Resistance Using Molecular Markers

Before the development of molecular markers, morphological markers were used to indirectly select for traits. Sax (1923) reported the co-segregation of seed size and seed coat color in *Phaseolus vulgaris*. The use of molecular markers to select for traits of interest is referred to as marker-assisted selection (MAS) (Beckmann and Soller, 1983). MAS was first proposed by Thoday (1961), but didn't become widespread until the development of molecular markers. The molecular markers that have been used in MAS include restriction fragment length polymorphisms (RFLPs), minisatellites, microsatellites, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs), and single nucleotide polymorphisms (SNPs). RFLPs were the earliest form of DNA markers used for MAS (Gholizadeh et al., 2008). The use of RFLPs for genotyping resulted in relatively low coverage of genomes and a high number of linkage groups (Siegel et al., 2006). This inability of RFLPs to be efficiently used in genotyping, led to the use of microsatellites. Prior to SNPs, microsatellites were the most widely used markers for quantitative trait loci (QTL) detection because they were easily transferred to resource populations that were segregating for QTLs of interest (Siegel et al., 2006). QTLs are loci that carry alleles that affect continuous traits

(Falconer and Mackay, 1996). To detect linkage between a marker and a QTL with low to moderate effects, a large number of animals are required (Darvasi et al., 1993).

Several QTL mapping experiments using microsatellites, have successfully identified disease resistance-associated regions in the chicken genome. Vallejo et al. (1998) used genetic mapping to identify 2 significant and 2 suggestive QTL that affected MDV susceptibility in an F₂ White Leghorn population. Upon fine mapping, Yonash et al. (1999) identified 8 new QTL that were associated with MDV susceptibility. Heifetz et al. (2007) used a reciprocal backcross population created by crossing two Leghorn lines that differed in their resistance levels to MDV to identify 15 QTL regions on 10 chromosomes that were associated with MDV resistance. Zhu et al. (2003) detected a QTL on chromosome 1 of coccidiosis-infected broilers that was associated with oocyst shedding. Subsequently, Kim et al. (2006) unsuccessfully attempted to use fine mapping techniques to determine the physical location of the QTL on chromosome 1. Lamont et al. (2002) reported that a positional candidate and 3 other candidate genes were associated with *S. Enteritidis* colonization in a novel genetic cross.

With the availability of the chicken genome sequence, the development of a genetic variation map containing 2.8 million SNPs, and the development of high density SNP panels, researchers were able to perform genome-wide association studies (GWAS) (International Chicken Genome Sequencing Consortium, 2004; International Chicken Polymorphism Map Consortium, 2004). GWAS is an approach that surveys the genome for genetic variants using panels that contain thousands of SNPs (Hirschhorn and Daly, 2005). It has been used as a powerful tool to provide insight into complex traits

(McCarthy et al., 2008). Using a panel of 3,000 SNPs to analyze abdominal fatness in two F₂ populations, Abasht and Lamont (2007) reported the first GWAS in chickens. The study identified 15 markers that were significantly associated with abdominal fatness in a broiler x Fayoumi cross and 24 markers that were associated with abdominal fatness in a broiler x Leghorn cross (Abasht and Lamont, 2007). Fife et al. (2010) utilized a high-density SNP panel containing 1255 SNPs covering the whole genome to identify 4 QTL that were associated with *S. Enteritidis* colonization. Once the GWAS approach became more common in poultry, more dense SNP panels were developed (Groenen et al., 2011). Using the 60K Illumina iSelect chicken array that was developed by the USDA Chicken Genome-Wide Marker Assisted Selection Consortium, Redmond et al. (2011) reported that the *SLC11A1* gene and the *SALI* locus were significantly associated with the phagocytosis of *S. Enteritidis*-stimulated heterophils. Li et al. (2012) also used the 60K Illumina iSelect chicken array to find two SNPs that were associated with MDV in Leghorn chickens.

Using high-density SNP panels, researchers are attempting to move from GWAS to genome-wide marker-assisted selection or genomic selection. Genomic selection was first proposed by Meuwissen et al. (2001) and involved performance prediction in a set of animals using their genotypes for a high density SNP panel. This prediction is based on LD between markers and genes of interest from a GWAS study using the same SNP panel (Wray et al., 2007). The availability of the 600K high-density SNP chip to provide very complete coverage of the chicken genome allows researchers to genotype animals at a much greater magnitude (Fulton, 2012). The combination of imputation of progeny

genotypes and parental high-density genotype information improves the accuracy of selection from 40% to 60% for production traits (Wang et al., 2011; Wolc et al., 2011). The use of genomic selection to select for disease resistance in chickens was shown to be promising in a preliminary genomic selection study for *Salmonella* carrier-state resistance (Calenge et al., 2011). Currently, the cost of high-density genotyping a single bird (approximately \$150-\$250), presents a challenge to wide spread adoption of genomic selection (Avendano et al., 2010). As the cost of high-density genotyping chips continues to decrease, the use of genomic selection by the poultry industry for disease resistance or other traits will become increasingly feasible.

Evaluating the Transcriptome of Stressed Chickens

The transcriptome represents the portion of the genome that is transcribed into mRNA and non-coding, regulator RNAs (Adams, 2008). The non-coding RNAs that have regulatory functions include tRNA (transfer RNA), rRNA (ribosomal RNA), snRNA (small nuclear RNA), snoRNA (small nucleolar RNA), and miRNA (microRNA) (Drenkow et al., 200). During transcription, the introns (non-coding sections of the genome that separate the coding sections) are spliced out of the transcript, leaving the exons (coding sections of the genome) to be transcribed into mRNA (Clancy, 2008). This splicing process creates the mRNA that will be translated into proteins. Alternative splicing, RNA editing, and termination sites, can produce many different variants or isoforms for one gene (Adams, 2008). The production or expression of these different variants can induce changes in disease-associated traits (Schadt et al., 2008). Researchers expect that, by characterizing the transcriptome of specific tissues, they can

determine what genes are up-regulated or down-regulated in response to different stimuli. In turn, they aim to associate different disease-associated phenotypes with the expression of particular genes or gene variants.

A common method used to analyze the expression of specific candidate genes is qPCR (Quantitative Polymerase Chain Reaction). This technique analyzes gene expression by monitoring the amplification level of a gene based on the initial amounts of template molecules (Wilhelm and Pingoud, 2003). This is done by using fluorescent dyes to generate fluorescence signals that are proportional to the PCR product that is produced (Wittwer et al., 1998). A major advantage of qPCR is its high accuracy compared to other methods that are used to quantify nucleic acids (Wilhelm and Pingoud, 2003).

The expressions of many of the disease-associated candidate genes in poultry that have been measured using qPCR include cytokines, toll-like receptors, and immunoglobulin. Parvizi et al. (2009) reported temporal differences in the splenic expression of *IFN- γ* , *IL-6*, *IL-10*, and *IL-18* in CD4⁺ and CD8⁺ T cells that were isolated from MDV-resistant and -susceptible chicken lines after MDV challenge. IgM and IgG were also differentially expressed in the spleen of MDV-infected chickens that varied in MDV resistance (Sarson et al., 2008). Cheeseman et al. (2007) reported that *IL-18* and *IFN- γ* mRNA levels were significantly more highly expressed in broilers, Fayoumis, and Leghorns after *S. Enteritidis* infection. Sadeyen et al. (2004) reported that *IFN- γ* mRNA levels in response to *S. Enteritidis* infection, were significantly lower in an *S. Enteritidis*-susceptible line compared to an *S. Enteritidis*-resistant line. In embryonic chicken

lymphoid cells, an up-regulation of *IL-6*, *IL-8* and *IFN- γ* mRNA levels was reported by Khatri and Sharma, (2009) in response to IBDV (Infectious Bursal Disease Virus).

Abasht et al. (2009) measured the expression of *TLR-2*, *TLR-4*, and *TLR-5* in the cecum and spleen of Fayoumis, Leghorns, and broilers in response to *S. Enteritidis* challenge and reported that Fayoumis expressed *TLR-2* and *TLR-4* mRNA at higher levels than Leghorns, while broilers expressed *TLR-5* at higher levels than Leghorns and Fayoumis.

With the advent of microarray technology, researchers were able to quantify the expression of thousands of genes simultaneously. Microarray technology provided relatively high specificity and gave researchers a snapshot of an organism's gene expression at a specific point in time (Karakach et al., 2010). This snapshot was a truer representative of the full transcriptome, not just the isolated elements that qPCR offered.

The two major types of microarrays are Affymetrix arrays and spotted arrays.

Affymetrix arrays are commonly used short oligonucleotide-based microarrays, which utilize multiple probes for each gene (Zakharkin et al., 2005). cDNA arrays are microarrays that contain a matrix of printed cDNA probes (Duggan et al., 1999). Both of these microarray platforms are selective in content, and require hybridization, image processing, and normalization techniques before any inferences can be made about differential expression (Karakach et al., 2010; Maskos and Southern, 1992; Schena, 2002).

Researchers have carried out many microarray experiments evaluating the effects of stressors on the transcriptome of poultry. Oligonucleotide and cDNA array technologies were used by van Hemert et al. (2006) to analyze early host response of

slow-growing and fast-growing lines to intestinal *S. Enteritidis* infection. Inflammatory genes were up-regulated in the fast-growing line after *S. Enteritidis* infection, while acute phase response genes, the fibrinogen system genes, and polymerization genes were up-regulated in the slow-growing line (van Hemert et al., 2006). Ciraci et al. (2010) used the 38,535-probeset Affymetrix GeneChip Chicken Genome array to demonstrate *in vitro* stimulation of HD11 cells with *S. Typhimurium* endotoxin causes an inflammatory response with the induction of *NFKBIA*, *IL-1B*, *IL-8*, and *CCL-4* consistently across all time points. Using the chicken 44k Agilent microarray to characterize response of the splenic transcriptome of commercial broilers to APEC (Avian Pathogenic *E. coli*), Sandford et al. (2011) reported that increases in gene expression levels were associated with more severe pathology. Upon gene ontology analysis, many of the overrepresented biological terms were white blood cell regulation, defense/immune response to bacteria, and metabolism (Sandford et al., 2011). According to Munira et al. (2005), stimulation of primary chicken embryo cells from specific pathogen free (SPF) chickens with NDV causes a pronounced suppression of the *IFN* genes, silencing of cytoskeletal genes, and down-regulation of the thioredoxin gene. Upon evaluating the effect of acute heat stress on spermatogenesis in the L2 strain of Taiwan country chickens, Wang et al. (2012) found that the differentially expressed genes were related to stress, transport, signal transduction, and metabolism.

Although microarray technology provided an improved view of the transcriptome, there are still limitations associated with microarrays. These limitations include cross hybridization artifacts, dye-based detection, and the difficulty of detecting

splice patterns and unmapped genes (Casneuf et al., 2007; Mortazavi et al., 2008).

Microarray technology can only detect the differential expression of the genes that are present on the array. A simpler, more effective technique to characterize the transcriptome is the RNA-seq approach. RNA-seq, the direct ultra-high-throughput sequencing of cDNA, yields sequence reads that are individually mapped to the genome and counted to obtain read abundance at each exon, locate splice events, and discover candidate genes (Casneuf et al., 2007). RNA-seq involves converting mRNA to a cDNA library with adapters at both ends, next each molecule with or without amplification is sequenced in a high-throughput process to obtain single- or paired-end reads, the sequences are then aligned to the genome to produce a transcriptional assembly of all reads across the genome (Wang et al., 2007).

In spite of RNA-seq being an emerging technology, researchers have used this technique to study the effects of stresses on the transcriptome of poultry. Connell et al. (2012) used the Illumina Genomone Analyzer II platform to compare the cecal transcriptome of two chicken lines differing in their resistance to *C. jejuni* colonization. After *C. jejuni* infection, higher mRNA expression levels of genes that participate in immune function, cytokine signaling, B cell and T cell activation, and angiotensin function were observed in birds from the resistant line, suggesting a suppression of these functions in the susceptible line (Connell et al., 2012). MacEachern et al. (2010) also used the Illumina Genome Analyzer II platform to characterize the splenic transcriptome of ADOL Line 6 (MDV-resistant) and ADOL Line 7 (MDV-susceptible) chickens in response to MDV. After analyzing the reads for allele-specific expression (ASE) using

the Illumina Golden Gate platform, the authors found 5,360 coding single nucleotide polymorphisms (cSNPs) in 3,773 genes displayed allelic imbalance and can be further studied to quantify the total effect of each cSNP on MDV resistance (MacEachern et al., 2010). Nie et al. (2012) used Solexa sequencing to compare the splenic transcriptome profiles of male broilers that were either challenged with APEC or non-challenged. The samples were grouped based on pathology severity (mild, severe, and non-challenged). There were 2,320 genes differently expressed between the mild and severe broilers, 7,764 genes differentially expressed between the severe and non-challenged broilers, and 6,844 genes that were differentially expressed between the non-challenged and mild broilers (Nie et al., 2012). Although the literature contains an abundance of reports using RNA-seq to characterize the transcriptome of viral- and bacterial-infected poultry, the literature using RNA-seq to characterize the effects of heat stress on the poultry transcriptome have been limited. Li et al. (2011) reported 110 DE genes in response to chronic, cyclic heat stress in broilers. Mitogen-associated protein kinase (*MAPK*) and *NFkB* pathways were elicited in response to the heat stress treatment. Wang et al. (2013) reported 169 DE genes in testis samples in response to acute heat stress in Taiwanese roosters. Response to stress, transport, signal transport, and metabolism were among the most represented functions of the DE genes.

In summary, researchers have moved from the ability to only analyze the genetic effects of stressors in poultry on a gene level to analyzing these effects on a transcriptome level. This will provide further insight in the gene expression and gene interactions that exist on a tissue level in response to stressors.

References

- Abasht, B., and S. J. Lamont. 2007. Genome-wide association analysis reveals cryptic alleles as an important factor in heterosis for fatness in chicken F2 population. *Anim. Genet.* 38:491-498.
- Abasht, B., M. G. Kaiser, J. van der Poel, and S. J. Lamont. 2009. Genetic lines differ in Toll-like receptor gene expression in spleens of chicks inoculated with *Salmonella enterica* serovar Enteritidis. *Poult. Sci.* 88:744-749.
- Abbas, A. K., A. H. Lichtman, and J. S. Pober. 2000. Cellular and Molecular Immunology, 4th Edition. W. B. Saunders, Philadelphia, PA.
- Adams, J. 2008. Transcriptome: connecting the genome to gene function. *Nature Education* 1:1.
- Ahmed, K.A., V. K. Saxena, A. Ara, K. B. Singh, N. R. Sundaresan, M. Saxena, and T. J. Rasool. 2007. Immune response to Newcastle disease virus in chicken lines divergently selected for cutaneous hypersensitivity. *Int. J. Immunogenet.* 34:445-55.
- Ain Baziz, H., P. A. Geraert, J. C. F. Padilha, and S. Guillaumin. 1996. Chronic heat exposure enhances fat deposition and modifies muscle and fat repartition in broiler carcasses. *Poult. Sci.* 75:505-513.
- Ajakaiye, J. J., A. Perez-Bello, and A. Mollineda-Trujillo. 2011. Impact of heat stress on egg quality in layer hens supplemented with l-ascorbic acid and dl-tocopherol acetate. *Vet. Arhiv.* 81:119-132.
- Akashi, S., Y. Nagai, H. Ogata, M. Oikawa, K. Fukase, S. Kusumoto, K. Kawasaki, M. Nishijama, S. Hayashi, M. Kimoto, and K. Miyake. 2001. Human MD-2 confers on

- mouse Toll-like receptor 4 specific lipopolysaccharide recognition. *Int. Immunol.* 13:1595-1599.
- Akyuz, A. 2009. Effects of some climates parameters of environmentally uncontrollable broiler houses on broiler performance. *J. Anim. Vet. Adv.* 8:2608-2612.
- Al-Fataftah, A. R. A., and Z. H. M. Abu-Dieyeh. 2007. Effect of chronic heat stress on broiler performance in Jordan. *Int. J. Poult. Sci.* 6:64-70.
- Arstila, P. T., O. Vainio, and O. Lassila. 1994. Central role of CD4+ T cells in avian immune response. *Poult. Sci.* 73:1019-1016.
- Avendano, S., K. A. Watson, A. Kranis. 2010. Genomics In Poultry Breeding – From Utopias To Deliverables. *Proc. 9th World Congress on Genetics Applied to Livestock Production, Leipzig, Germany.*
- Barnes, H. J. 2001. Hemic system, in C. Riddel (ed.), *Avian histopathology*, 2nd Edition. p. 18-34. American Association of Avian Pathologists, Kennett square, PA.
- Bayliss, P. A., and M. H. Hinton. 1990. Transportation of broilers with special reference to mortality rates. *Appl. Anim. Behav. Sci.* 28:93-118.
- Beal, R. K., C. Powers, P. Wigley, P. A. Barrow, and A. L. Smith. 2004. Temporal dynamics of the cellular, humoral and cytokine responses in chickens during primary and secondary infection with *Salmonella enterica* serovar Typhimurium. *Avian Pathol.* 33:25-33.
- Beckmann, J. S., and M. Soller. 1983. Restriction fragment length polymorphisms in genetic improvement: methodologies, mapping and costs. *Theor. Appl. Genet.* 67:35-43.

- Berndt, A., Pieper J., and Methner U. 2006. Circulating $\gamma\delta$ T cells in response to *Salmonella enterica* serovar Enteritidis exposure in chickens. *Infect. Immun.* 74:3967-3978.
- Berrong S. L., and K. W. Washburn. 1998. Effect of genetic variation on total plasma protein, body weight gains, and body temperature responses to heat stress. *Poult. Sci.* 77:379-385.
- Berthelot-Herault, F., F. Mompert, M. S. Zygmunt, G. Dubray, and M. Duchet-Suchaux. 2003. Antibody responses in the serum and gut of chicken lines differing in cecal carriage of *Salmonella enteritidis*. *Vet. Immunol. Immunopathol.* 96:43-52.
- Calenge, F., A. Legarra, and C. Beaumont. 2011. Genomic selection for carrier-state resistance in chicken commercial lines. *BMC Proceedings* 5:S24
- Capua I., and D. J. Alexander. 2004. Avian influenza: recent developments. *Avian Pathol.* 33:393-404.
- Carmen, A., M. France, G. Macleod and E. Julie. 1991. Alleviation of acute heat stress by food withdrawal or darkness. *Br. Poult. Sci.* 32:219-225.
- Casneuf, T., Y. Van de Peer, and W. Huber. 2007. In situ analysis of cross-hybridisation on microarrays and inference of expression correlation. *BMC Bioinformatics* 8:461.
- Cheeseman, J. H., M. G. Kaiser, C. Ciraci, P. Kaiser, and S. J. Lamont. 2007. Breed effect on early cytokine mRNA expression in spleen and cecum of chickens with and without *Salmonella enteritidis* infection. *Dev. Comp. Immunol.* 31:52-60.

- Chen, C.-L., J. M. Pickel, J. M. Lathi, and M. D. Cooper. 1991. Surface markers on avian immune cells, in J. M. Scharma (ed.), *Avian Cellular Immunology*, p. 1-22. CRC Press, Boca Raton, FL.
- Chuammitri, P., J. Ostojic', C. B. Andreasen, S. B. Redmond, S. J. Lamont, and D. Palic'. 2009. Chicken heterophil extracellular traps (HETs): novel defense mechanism of chicken heterophils. *Vet Immunol Immunopathol* 129:126-131.
- Ciraci, C., C. K. Tuggle, M. J. Wannemuehler, D. Nettleton, and S. J. Lamont. 2010. Unique genome-wide transcription profiles of chicken macrophages exposed to *Salmonella*-derived endotoxin. *BMC Genomics* 11:545-555.
- Clancy, S. 2008. RNA splicing: introns, exons and spliceosome. *Nature Education* 1:1.
- Comis, D. 2003. Seeing *Salmonella* move through pigs. USDA, Agric. Res. Serv., Washington, D. C. Available at: <http://www.ars.usda.gov/is/pr/2003/031118.htm> (accessed August. 16, 2012).
- Connell, S., K. G. Meade, B. Allan, A. T. Lloyd, E. Kenny, P. Cormican, D. W. Morris, D. G. Bradley, and C. O'Farrelly. 2012. Avian resistance to *Campylobacter jejuni* colonization is associated with an intestinal immunogene expression signature identified by mRNA sequencing. *PLoS ONE* 7:8.
- Dargatz, D. A., F. B. Garry, and J. L. Traub-Dargatz. 2002. An introduction to biosecurity of cattle operations. *Vet. Clin. North Am. Food Anim. Pract.* 18:1-5.
- Darvasi, A., A. Vinreb, V. Minke, J. I. Weller and M. Soller. 1993. Detecting marker-QTL linkage and estimating QTL gene effect and map location using a saturated genetic map. *Genetics* 134:M 943-951.

- Donoghue, D. J., B. F. Krueger, B. M. Hargis, A. M. Miller, and M. E. El Halawani. 1989. Thermal stress reduces serum luteinizing hormone and bioassayable hypothalamic content of luteinizing hormone-releasing hormone in hens. *Biol. Reprod.* 41:419-42.
- Duggan, D. J., M. Bittner, Y. Chen, P. Meltzer, and J. M. Trent. 1999. Expression profiling using cDNA microarrays. *Nature Genetics* 21:10-14.
- Eckmann, L. and Kagnoff, M.F. 2001. Cytokines in host defense against *Salmonella*. *Microbes Infect* 3:1191–200.
- Eisenstein, T. K. 2001. Implications of *Salmonella*-induced nitric oxide (NO) for host defense and vaccines: NO, an antimicrobial, antitumor, immunosuppressive and immunoregulatory molecule. *Microbes Infect.* 3:1223-1231.
- Emery, J. 2004. Heat stress in poultry-Solving the problem. Defra publications (ADAS). <http://www.alpharma.com/ahd>. (accessed on 26 October 2012)
- Emmans, G. C., and D. R. Charles. 1989. Climatic environment and poultry feeding in practice, 1st Edition. p. 212-222. Anchor Press Ltd, Essex.
- Erf, G. F. 2004. Cell-mediated immunity in poultry. *Poult. Sci.* 83:580-590.
- Etches, R. J., T. M. John, and A. M. Verrinder Gibbins. 1995. Behavioural, physiological, neuroendocrine and molecular responses to heat stress, in N. J. Daghir (ed.), *Poultry Production in Hot Climates*. p. 31-65. CAB Int., Wallingford, U.K.
- Fahy O. L., S. L. Townley, and S. R. McColl. 2006. CXCL16 Regulates cell-mediated immunity to *Salmonella enterica* serovar Enteritidis via promotion of gamma interferon production. *Infect. Immun.* 74:6885-6894.

- Falconer, D. S., and T. F. C. MacKay. 1996. *Introduction to Quantitative Genetics* (Fourth Edition). Longman Group Ltd., London, U.K.
- FAOStat. FAO statistical database. Available at: <http://faostat.fao.org/default.aspx>. (accessed on 2 November 2012).
- Farnell, M. B., H. He, and M. H. Kogut. 2003. Differential activation of signal transduction pathways mediating oxidative burst by chicken heterophils in response to stimulation with lipopolysaccharide and lipoteichoic acid. *Inflammation*. 27:3.
- Farnell M. B., T. L. Crippen, H. He, and C. L. Swaggerty. 2003. Oxidative burst mediated by toll like receptors (TLR) and CD14 on avian heterophils stimulated with bacterial toll agonists. *Dev. Comp. Immunol.* 27:423-429.
- Farnsworth, M. L., R. S. Miller, K. Pedersen, M. W. Lutman, S. R. Swafford, P. D. Riggs, and C. T. Webb. 2012. Environmental and Demographic Determinants of Avian Influenza Viruses in Waterfowl across the Contiguous United States. *PLoS ONE* 7:3.
- Felver-Gant J. N., L. D. Mack, R. L. Dennis, S. D. Eicher, and H. W. Cheng. 2012. Genetic variations alter physiological responses following heat stress in 2 strains of laying hens. *Poult. Sci.* 91:1542-51.
- Fife, M., J. Howell, N. Salmon, P. Hocking, P. van Diemen, M. Jones, M. Stevens, and P. Kaiser. 2011. Genome-wide SNP analysis identified major QTL for *Salmonella* colonization in the chicken. *Anim. Genet.* 42:134-140.
- Food and Agriculture Organization/World Organisation for Animal Health (FAO/OIE) (2005). A global strategy for the progressive control of highly pathogenic avian

influenza (HPAI). FAO, Rome and OIE, Paris. Available at:

[http://www.fao.org/ag/againfo/subjects/documents/ai/HPAIGlobalStrategy31Oct05.p](http://www.fao.org/ag/againfo/subjects/documents/ai/HPAIGlobalStrategy31Oct05.pdf)

df (accessed on 15 August 2012).

Fulton, J. E. 2012. Genomic selection for poultry breeding. *Animal Frontiers*. 2:30-36.

Genovese, L.L., V. K. Lowry, K. J. Genovese, J. R. DeLoach, and M. H. Kogut. 1999.

Enhancement of phagocytosis and bacterial killing by heterophils from neonatal

chicks after administration of *Salmonella enteritidis*-immune lymphokines. *Vet.*

Microbiol. 65:133-143.

Geraert, P. A., J. C. F. Padilha, and S. Guillaumin, 1996. Metabolic and endocrine

changes induced by chronic heat exposure in broiler chickens: biological and

endocrinological variables. *Br. J. Nutr.* 75:205-216.

Gholizadeh, M., G. R. Mianji and H. S. Zadeh. 2008. Potential use of molecular markers

in the genetic improvement of livestock. *Am. J. Anim. Vet. Adv.* 3:120-128.

Glick, B. 1977. The bursa of fabricius and immunoglobulin synthesis. *Int. Rev. Cytol.* 48:345-402.

Glick, B. 1988. Bursa of fabricius: development, growth, modulation, and endocrine

functions. *CrC Crit. Rev. Poult. Biol.* 1:107-132.

Groenen, M. A., H. J. Megens, Y. Zare, W. C. Warren, L. W. Hillier, R. P. Crooijmans,

A. Vereijken, R. Okimoto, W. M. Muir, and H. H. Cheng. 2011. The development

and characterization of a 60K SNP chip for chicken. *BMC Genomics* 12:274.

Gross W. B., and H. S. Siegel. 1983. Evaluation of the heterophil/lymphocyte ratio as a

measure of stress in chickens. *Avian Dis.* 27:972-978.

- Guan, T., and R. A. Holley. 2003. Pathogen survival in swine manure environments and transmission of human enteric illnesses – a review. *J. Environ. Qual.* 32:383-392.
- Har, L., D. Rong and Z. A. Zhang. 2000. The effect of thermal environment on the digestion of broilers. *Anim. Physiol.* 83:75-61.
- Hartung, J. 2003. Effects of transport on health of farm animals. *Vet. Res. Comm.* 27:525-527.
- He, H., V. K. Lowry, P. J. Ferro, and M. H. Kogut. 2005. CpG- oligodeoxynucleotide-stimulated chicken heterophil degranulation is serum cofactor and cell surface receptor dependent. *Dev. Comp. Immunol.* 29:255-264.
- Heifetz, E. M., J. E. Fulton, N. P. O'Sullivan, J. A. Arthur, J. Wang, J. C. M. Dekkers, and M. Soller. 2007. Mapping quantitative trait loci affecting susceptibility to Marek's disease virus in a backcross population of layer chickens. *Genetics.* 177:2417-2431.
- Heller, E. D., D. B. Nathan, and M. Perek. 1979. Short heat stress as an immunostimulant in chicks. *Avian Pathol.* 8:195-203.
- Hirschhorn, J. N., and M. J. Daly. 2005. Genome-wide association studies for common diseases and complex traits. *Nat. Rev. Genet.* 6:95-108.
- Hofacre, C., J. R. Glisson, and S. H. Kleven. 1986. Comparison of vaccination protocols of broiler breeder hens for *Pasteurella multocida* utilizing enzyme-linked immunosorbent assay and virulent challenge. *Avian Dis.* 31:260-263.

- International Chicken Genome Sequencing Consortium. 2004. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. *Nature* 432:695-716.
- International Chicken Polymorphism Map Consortium. 2004. A genetic variation map for chicken with 2.8 million single-nucleotide polymorphisms. *Nature* 432:717-722.
- Jankovic D., Z. Liu, and W. C. Gause. 2001. Th1- and Th2-cell commitment during infectious disease: Asymmetry in divergent pathways. *Trends Immunol.* 22:450-457.
- Kaiser M. G., J. H. Cheeseman, P. Kaiser, and S. J. Lamont. 2006. Cytokine expression in chicken peripheral blood mononuclear cells after in vitro exposure to *Salmonella enterica* serovar Enteritidis. *Poult. Sci.* 85:1907-1911.
- Kaiser, P., Stäheli, P. 2008. Avian cytokines and chemokines, in F. Davison, B. Kaspers, K. A. Schat (ed.) *Avian Immunology*, p. 183–202. Amsterdam Academic Press, Amsterdam, Netherlands.
- Kapczynski D. R., and D. J. King. 2005. Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. *Vaccine* 23:3424-3433.
- Karakach, T. K., R. M. Flight, S. E. Douglas, and P. E. Wentzell. 2010. An introduction to DNA microarrays for gene expression analysis. *Chemometr. Intell. Lab.* 104:28-52.

- Kaspers, B., Kothlow, S., and Butter C. 2008. Avian antigen presenting cells, in F. Davison, B. Kaspers, K. A. Schat (ed.) *Avian Immunology*, p. 183–202. Amsterdam Academic Press, Amsterdam, Netherlands.
- Khatri, M., and J. M. Sharma. 2009. Response of embryonic chicken lymphoid cells to infectious bursal disease virus. *Vet. Immunol. Immunopath.* 127:316-324.
- Kilpatrick, A.M., A. A. Chmura, D. W. Gibbons, R. C. Fleischer, P. P. Marra, and P. Daszak. 2006. Predicting the global spread of H5N1 avian influenza. *Proc. Natl. Acad. Sci. USA* 103:19368-19373.
- Kim, B. S., Y. H. Tiong, W. Min, and H. S. Lillehoj. 2006. Finemapping of coccidia-resistant quantitative trait loci in chickens. *Poult. Sci.* 35:2028-2030.
- King, D.J. 1996. Influence of chicken breed on pathogenicity evaluation of velogenic neurotropic Newcastle disease virus isolates from cormorants and turkeys. *Avian Dis.* 40:210-217.
- Kogut M. H., M. Iqbal, H. He, V. Philbin, P. Kaiser, and A. Smith. 2005. Expression and function of Toll-like receptors in chicken heterophils. *Dev. Comp. Immunol.* 29:791-807.
- Lamont, S. J., M. G. Kaiser, and W. Liu. 2002. Candidate genes for resistance to *Salmonella enteritidis* colonization in chickens as detected in a novel genetic cross. *Vet. Immunol. Immunopathol.* 87:423-8.
- Landes, R. 2002. “Prospects for India’s Poultry Sector”. US Department of Agriculture.

- Lee, G. M., G. D. F. Jackson, and G. N. Cooper. 1981. The role of serum and biliary antibodies and cell-mediated immunity in the clearance of *S. Typhimurium* from chickens. *Vet. Immunol. Immunopathol.* 2:233-252.
- Leitner, G., Z. Uni, A. Cahaner, M. Gutman, and E. D. Heller. 1992. Replicated divergent selection of broiler chickens for high or low early antibody response to *Escherichia coli* vaccination. *Poult. Sci.* 71:27-37.
- Li, D. F., L. Lian, L. J. Qu, Y. M. Chen, W. B. Liu, S. R. Chen, J. X. Zheng, G. Y. Xu, and N. Yang. 2012. A genome-wide SNP scan reveals two loci associated with the chicken resistance to Marek's disease. *Anim. Genet.* doi: 10.1111/j.1365-2052.2012.02395.x.
- Liu, W., D. Li, J. Liu, S. Chen, L. Qu, J. Zheng, G. Xu, and N. Yang. 2011. A genome-wide SNP scan reveals novel loci for egg production and quality traits in White Leghorn and brown-egg dwarf layers. *PLoS ONE* 6:12.
- Lillehoj, H. S., and J. S. Trout. 1996. Avian gut-associate lymphoid tissues and intestinal immune responses to eimeria species. *Clin. Micro. Rev.* 9:349-360.
- Lin H.-K., S. E. Bloom, and R. R. Dietert. 1992. Macrophage antimicrobial functions in a chicken MHC chromosome dosage model. *J. Leukoc. Biol.* 52:307-14.
- MacEachern, S., W. M. Muir, S. Crosby, and H. H. Cheng. 2011. Genome-wide identification of allele-specific expression (ASE) in response to Marek's disease virus infection using next generation sequencing. *BMC Proc.* 5:S14.

- Mack, L. A., J. N. Felver-Gant, R. L. Dennis, and H. Cheng. 2010. Effects of heat stress on egg production and quality in two strains of layers. *Poult. Sci.* 89:M311(E-Suppl. 1).
- Marangon, S., and L. Busani. 2006. The use of vaccination in poultry production. *Rev. Sci. Tech. Off. Int. Epiz.* 26: 265-274.
- Mashaly, M. M., G. L. Hendricks, M. A. Kalama, A. E. Gehad, A. O. Abbas, and P. H. Patterson. 2004. Effect of heat stress on production parameters and immune responses of commercial laying hens. *Poult. Sci.* 83:889-894.
- Maskos, U., and E.M. Southern. 1992. Oligonucleotide hybridizations on glass supports: a novel linker for oligonucleotide synthesis and hybridization properties of oligonucleotides synthesised in situ. *Nucleic Acids Res.* 20:1679-1684.
- May, J. D., and B. D. Lott. 1992. Feed and water consumption patterns of broilers at high environmental temperatures. *Poult. Sci.* 71:331-336.
- Melesse, A., S. Maak, and G. von Lengerken. 2010. Effect of long-term heat stress on egg quality traits of Ethiopian naked neck chickens and their F1 crosses with Lohmann White and New Hampshire chicken breeds. *Livest. Res. Rural Dev.*, 22, Art. # 71. <http://www.lrrd.org/lrrd22/4/mele22071.htm> (accessed on 1 November 2012).
- Melesse A., S. Maak, R. Schmidt, and G. von Lengerken. 2011. Effect of long-term heat stress on key enzyme activities and T3 levels in commercial layer hens. *Int. J. Livestock Prod.* 2:107-116.

- Meuwissen, T. H. E., B. J. Hayes, and M. E. Goddard. 2001. Prediction of total genetic value using genome-wide dense marker maps. *Genetics* 157:1819-1829.
- Mogenet, L. Y., and B. J. Youbicier-Simo. 1998. Determination of reliable biochemical parameters of heat stress, and application to the evaluation of medications: example of erythromycin E. p. 538-541 in *Proceedings of 10th European Poultry Conference*, Jerusalem, Israel.
- Mortazavi, A., B. A. Williams, K. McCue, L. Schaeffer, and B. Wold. 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat. Methods* 5:621-628.
- Munira, S., J. M. Sharmab, and V. Kapur. 2005. Transcriptional response of avian cells to infection with Newcastle disease virus. *Virus Res.* 107:103-108.
- Nicholson, F. A., S. J. Groves and B. J. Chambers. 2005. Pathogen survival during livestock manure storage and following land application. *Bioresour. Technol.* 96:135-143.
- Nie, Q., E. E. Sandford, X. Zhang, L. K. Nolan, and S. J. Lamont. 2012. Deep sequencing-based transcriptome analysis of chicken spleen in response to Avian Pathogenic *Escherichia coli* (APEC) infection. *PLoS ONE* 7:7.
- Okamura, M., H. S. Lillehoj, R. B. Raybourne, U. S. Babu, and R. A. Heckert. 2004. Cell-mediated immune responses to a killed *Salmonella enteritidis* vaccine: lymphocyte proliferation, T-cell changes and interleukin-6 (IL-6), IL-1, IL-2, and IFN-g production. *Comp. Immunol. Microbiol. Infect. Dis.* 27:255-272.
- Olah, I., N. Nagy, A. Magyar, and V. Palya. 2003. Esophageal tonsil: a novel gut-associated lymphoid organ. *Poult. Sci.* 82:767-770.

- Otte, J., D. Roland-Holst, D. Pfeiffer, R. Soares-Magalhaes, J. Rushton, J. Graham , and E. Silbergeld. 2007. Industrial livestock production and global health risks. Pro-Poor Livestock Policy Initiative Research Report. Rome, Italy: FAO.
- Parvizia, P., L. R. Read, M. F. Abdul-Careema, A. J. Sarson, C. Lusty, M. Lambourne, N. Thanthrige-Don, S. C. Burgess, and S. Sharif. 2009. Cytokine gene expression in splenic CD4⁺ and CD8⁺ T cell subsets of genetically resistant and susceptible chickens infected with Marek's disease virus. *Vet. Immunol. Immunopath.* 132:209-217.
- Pedersen, L. D., A. C. Sørensen, and P. Berg. 2009. Marker-assisted selection can reduce true as well as pedigree-estimated inbreeding. *J. Dairy Sci.* 92:2214-2223.
- Pope, C. R. 2001. Lymphoid system, in C. Riddel (ed.), *Avian histopathology*, 2nd Edition. p. 18-34. American Association of Avian Pathologists, Kennett square, PA.
- Ratcliffe, M. J. H. 1989. Development of the avian B-lymphocyte lineage. *CrC Crit. Rev. Poult. Biol.* 2:207-234.
- Ravindra, P.V., A. K. Tiwari, B. Ratta, U. Chaturvedi, S. K. Palia, P. K. Subudhi, R. Kumar, B. Sharma, A. Rai, and R. S. Chauhan. 2008. Induction of apoptosis in Vero cells by Newcastle disease virus requires viral replication, de-novo protein synthesis and caspase activation. *Virus Res.* 133:285-290.
- Redmond, S. B., C. Phongsakorn, C. B. Andreasen, D. Palić, and S. J. Lamont. 2011. Genetic control of chicken heterophil function in advanced intercross lines: Associations with novel and with known *Salmonella* resistance loci and a likely

mechanism for cell death in extracellular trap production. *Immunogenetics* 63:449-458.

Rozenboim, I., C. Tabibzadeh, J. L. Silsby, and M. E. El Halawani. 1993. The effect of ovine prolactin (oPRL) administration on hypothalamic vasoactive intestinal peptide (VIP), gonadotropin-releasing hormone I and II (GnRH-I, II) content and anterior pituitary VIP receptors in laying turkey hens. *Biol. Reprod.* 48:1246-1250.

Rozenboim, I., E. Tako, O. Gal-Garber, J. A. Proudman and Z. Uni, 2007. The effect of heat stress on ovarian function of laying hens. *Poult.Sci.* 86:1760-1765.

Sacco R. E., K. E. Nestor, Y. M. Saif, H. J. Tsai, and R. A. Patterson. 1994. Effect of genetic selection for increased body weight and sex of poult on antibody response of turkeys to Newcastle disease virus and *Pasteurella multocida* vaccines. *Avian Dis.* 38:33-36.

Sandford, E. E., M. Orr, E. Balfanz, N. Bowerman, X. Li, H. Zhou, T. J. Johnson, S. Kariyawasam, P. Liu, L. K. Nolan, and S. J. Lamont. 2011. Spleen transcriptome response to infection with avian pathogenic *Escherichia coli* in broiler chickens. *BMC Genomics* 12:469.

Sarson, A. J., P. Parvizi, D. Lepp, M. Quinton, and S. Sharif. 2008. Transcriptional analysis of host responses to Marek's disease virus infection in genetically resistant and susceptible chickens. *Anim. Genet.* 39:232-240.

Sax K. 1923. The association of size differences with seed-coat pattern and pigmentation in *Phaseolus vulgaris*. *Genetics* 8:552-60.

- Sawabe K., K. Hoshino, H. Isawa, T. Sasaki, T. Hayashi, Y. Tsuda, H. Kurahashi, K. Tanabayashi, A. Hotta, T. Saito, A. Yamada, and M. Kobayashi. 2006. Detection and isolation of highly pathogenic H5N1 avian influenza A viruses from blow flies collected in the vicinity of an infected poultry farm in Kyoto, Japan, 2004. *Am. J. Trop. Med. Hyg.* 75:327-322.
- Schadt, E. E., C. Molony, E. Chudin, K. Hao, and X. Yang. 2008. Mapping the genetic architecture of gene expression in human liver. *PLoS Biol* 6:5.
- Schena, M. 2003. *Microarray Analysis*, 1st Edition. p. 648. J. Wiley & Sons, Hoboken, NJ.
- Scott, T. R. 2004. Our current understanding of humoral immunity in poultry. *Poult. Sci.* 83:574-579.
- Seal, B. S., D. J. King, and H. S. Sellers. 2000. The avian response to Newcastle disease virus. *Dev. Comp. Immun.* 24:257-268.
- Siegel, P. B., J. B. Dodgson, and L. Anderson. 2006. Progress from chicken genetics to the chicken genome. *Poult. Sci.* 85:2050-2060.
- Setta, P., P. A. Barrow, P. Kaiser, and M. A. Jones. 2012. Immune dynamics following infection of avian macrophages and epithelial cells with typhoidal and non-typhoidal *Salmonella enterica* serovars; bacterial invasion and persistence, nitric oxide and oxygen production, differential host gene expression, NF- κ B signalling and cell cytotoxicity. *Vet. Immunol. Immunopath.* 146:212-224.
- Shivaprasad, H.L. 2000. Fowl typhoid and pullorum disease. *Rev. Sci. Tech. Off. Int. Epiz.* 19: 405-424.

- Shivaprasad, H.L. 2003. Pullorum disease and fowl typhoid, in Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, and D. E. Swayne (ed.), Diseases of Poultry, 11th Edition, p. 568-582. Iowa State Press, Iowa, USA.
- Sironi L., J. L. Williams, A. M. Moreno-Martin, P. Ramelli, A. Stella, H. Jianlin, S. Weigend, G. Lombardi, P. Cordioli, and P. Mariani. 2008. Susceptibility of different chicken lines to H7N1 highly pathogenic avian influenza virus and the role of Mx gene polymorphism coding amino acid position 631. *Virology* 380:152-156.
- Smith, A. J., and L. Oliver. 1972. Some nutritional problems associated with egg production at high environmental temperatures. I. The effect of environmental temperature and rationing treatments on the productivity of pullets fed on diets of differing energy content. *Rhod. J. Agric. Sci.* 10:3-10.
- St. Pierre, N.R., B. Cobanov and G. Schnitkey. 2003. Economic losses from heat stress by US livestock industries. *J. Dairy Sci.* 86:E52-E77.
- Swaggerty C. L., M. H. Kogut, P. J. Ferro, L. Rothwell, I. Y. Pevzner, and P. Kaiser. 2004. Differential cytokine mRNA expression in heterophils isolated from *Salmonella*-resistant and -susceptible chickens. *Immunology* 113:139-148.
- Swaggerty, C. L., P. J. Ferro, I. Y. Pevzner, and M. H. Kogut. 2005. Heterophils are associated with resistance to systemic *Salmonella enteritidis* infections in genetically distinct chicken lines. *FEMS Immunol. Med. Microbiol.* 43:149-154.
- Swayne D. E. 2008. Avian Influenza. John Wiley and Sons, New York, New York.

- Tang, Q., J. Wang, J. Bao, H. Sun, Y. Sun, J. Liu, and J. Pu. 2012. A multiplex RT-PCR assay for detection and differentiation of avian H3, H5, and H9 subtype. *J. Virol. Methods* 181:164-169.
- Teeter, R. G., M. O. Smith, and C. J. Wiernusz. 1992. Effect of broiler acclimation to heat stress and feed intake on body temperature in birds exposed to thermoneutral and high ambient temperatures. *Poult. Sci.* 71:1101-1104.
- Temim, S., A. Chagneau, M. Peresson and S. Tesseraud. 2000. Chronic heat exposure alters protein turnover of three different skeletal muscles in finishing broiler chickens fed 20 or 25% protein diets. *J. Nutr.* 130: 813-819.
- Thaxton, P., C. R. Sadler, and B. Glick. 1968. Immune response of chickens following heat exposure or injections with ACTH. *Poult. Sci.* 47:264–266.
- Thoday J. M. 1961. Location of polygenes. *Nature* 191:368-370.
- United States Department of Agriculture. 2003. Watching Salmonella Move Through Pigs. USDA Animal Research Service Healthy Animals Newsletter, Issue 32, January 2008.
- Vallejo, R. L., L. D. Bacon, H. C. Liu, R. L. Witter, M. A. M. Groenen, J. Hillel, and H. H. Cheng. 1998. Genetic mapping of quantitative trait loci affecting susceptibility to Marek's disease virus induced tumors in F2 intercross chickens. *Genetics* 148:349-360.
- van Hemert, S., A. J. Hoekman, M. A. Smits, and J. M. Rebel. 2006. Early host gene expression responses to a *Salmonella* infection in the intestine of chickens with

- different genetic background examined with cDNA and oligonucleotide microarrays. *Comp. Biochem. and Physiol. Part D Genomics Proteomics*. 1:292–299.
- van Hemert, S., A. J. Hoekman, M. A. Smits, and J. M. Rebel. 2006. Gene expression responses to a *Salmonella* infection in the chicken intestine differ between lines. *Vet. Immunol. Immunopath.* 114:247-58.
- Van Immerseel F., J. De Buck, I. De Smet, J. Mast, F. Haesebrouck, and R. Ducatelle. 2002. Dynamics of immune cell infiltration in the caecal lamina propria of chickens after neonatal infection with a *Salmonella* Enteritidis strain. *Dev. Comp. Immunol.* 26:355-364.
- Wang, C., D. Habier, A. Kranis, K. A. Watson, S. Avendano, D. J. Garrick, R. Fernando, S. J. Lamont, and J. C. M. Dekkers. 2011. Accuracy of genomic EBV using an evenly spaced, low-density SNP panel in broiler chickens. *J. Anim. Sci.* 89:58.
- Wang S.-H., C.-Y. Cheng, P.-C. Tang, C.-F. Chen, H.-H. Chen, Y.-P. Lee, and S.-Y. Huang. 2013. Differential gene expressions in testes of L2 strain Taiwan country chicken in response to acute heat stress. *Theriogenology*. 79:374-382.
- Wang, Z., M. Gerstein, and M. Snyder. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* 10:57-63.
- Washietl, S., J. S. Pedersen, J. O. Korb, C. Stocsits, A. R. Gruber, J. Hackermüller, J. Hertel, M. Lindemeyer, K. Reiche, A. Tanzer, C. Ucla, C. Wyss, S. E. Antonarakis, F. Denoeud, J. Lagarde, J. Drenkow, P. Kapranov, T. R. Gingeras, R. Guigó, M. Snyder, M. B. Gerstein, A. Reymond, I. L. Hofacker, and P. F. Stadler PF.

2007. Structured RNAs in the ENCODE selected regions of the human genome. *Genome Res.* 17:852–864.
- Wilhelm, J., and A. Pingoud. 2003. Real-time polymerase chain reaction. *Chembiochem.* 4:1120-1128.
- Windhorst, H.-W. 2006. Changes in poultry production and trade worldwide. *World's Poultry Sci. J.* 62: 585-602.
- Wittwer, C. T., M. G. Herrmann, A. A. Moss, and R. P. Rasmussen. 1997. Continuous fluorescence monitoring of rapid cycle DNA amplification. *BioTechniques.* 22:130-131.
- Wolc, A., C. Stricker, J. Arango, P. Settar, J. E. Fulton, N. P. O'Sullivan, R. Preisinger, D. Habier, R. Fernando, D. J. Garrick, S. J. Lamont, and J. C. M. Dekkers. 2011. Breeding value prediction for production traits in layer chickens using pedigree or genomic relationships in a reduced animal model. *Genet. Sel. Evol.* 43:5.
- Wolfenson, D., Y. F. Frei, N. Snapir, and A. Berman. 1979. Effect of diurnal or nocturnal heat stress on egg formation. *Br. Poult. Sci.* 20:167-174.
- Wray, N. R., M. E. Goddard, and P. M. Visscher. 2007. Prediction of individual genetic risk to disease from genome-wide association studies. *Genome Res.* 17:1520-1528.
- Yahav, S., A. Straschnow, I. Plavnik and S. Hurwitz. 1996. Effects of diurnal cycling versus constant temperatures on chicken growth and food intake. *Br. Poult. Sci.* 37:43-54.

- You, S., L. K. Foster, J. L. Silsby, M. E. El Halawani, and D. N. Foster. 1995. Sequence analysis of the turkey LH subunit and its regulation by gonadotropin-releasing hormone and prolactin in cultured pituitary cells. *J. Mol. Endocrinol.* 14:117-129.
- Youngren, O. M., M. E. El Halawani, J. L. Silsby, and R. E. Phillips. 1991. Intracranial prolactin perfusion induces incubation behavior in turkey hens. *Biol. Reprod.* 44:425-431.
- Zakharkin S, K. Kim, T. Mehta, L. Chen, S. Barnes, K. E. Scheirer, R. S. Parrish, D. B. Allison, and G. P. Page. 2005. Sources of variation in Affymetrix microarray experiments. *BMC Bioinformatics* 6:214.
- Zhang-Barber, L., A. K. Turnerb, and P. A. Barrow. 1999. Vaccination for control of *Salmonella* in poultry. *Vaccine* 17:2538-2545.
- Zhu, J. J., H. S. Lillehoj, P. C. Allen, C. P. Van Tassell, T. S. Sonstegard, H. H. Cheng, D. Pollock, M. Sadjadi, W. Min, and M. G. Emara. 2003. Mapping quantitative trait loci associated with resistance to coccidiosis and growth. *Poult. Sci.* 82:9-16.

CHAPTER 2. DISTINCT LINES OF CHICKENS EXPRESS DIFFERENT SPLENIC CYTOKINE PROFILES IN RESPONSE TO *SALMONELLA* ENTERITIDIS CHALLENGE

A paper published in *Poultry Science*¹

D. J. Coble², S. B. Redmond³, B. Hale³, and S. J. Lamont^{2,4}

Abstract

Chicken meat and eggs contaminated with *Salmonella* result in economic losses in the poultry industry and potential human infection. Intestinal parasites have been shown to lead to a reduction in the utilization of nutrients and performance in poultry. This study provides insight into the immune responses used by hens of 3 genetically distinct chicken lines (broiler, Leghorn, and Fayoumi) in the presence and absence of *Salmonella* Enteritidis infection. Understanding the range of immune responses used by different lines in response to *Salmonella* Enteritidis may help the poultry industry genetically select birds that are more pathogen resistant. The splenic mRNA levels of several immune-related genes [IL-6, IL-8, IL-10, IL-18, macrophage inflammatory protein 1 β , interferon (IFN)- γ , transforming growth factor β 1, and regulated upon activation, normal T cell expressed, and secreted (RANTES)] were analyzed by quantitative PCR. Line, challenge, and their interaction were considered fixed effects.

¹ Reprinted with permission of *Poultry Science*, 2011, 90:1659-1663.

² Graduate student and Professor, respectively, Department of Animal Science, Iowa State University.

³ Collaborator, Department of Animal Science, Iowa State University.

⁴ Author for correspondence.

Line had a significant effect on the mRNA expression of RANTES ($P < 0.02$) and IFN- γ ($P < 0.03$). Broilers expressed significantly more splenic RANTES mRNA than Fayoumis, and significantly more splenic IFN- γ mRNA than Leghorns ($P < 0.05$). There was a significant interaction of genetic line and challenge on IL-18 ($P < 0.02$) and IL-6 ($P < 0.01$) mRNA expression. Although there was a significant interaction of genetic line and challenge for IL-18, Tukey's test analysis only showed differences at a suggestive level ($P < 0.1$). Bacterial challenge had a significant effect on IL-6 mRNA expression only within the Fayoumi line. Challenged Fayoumis expressed significantly less IL-6 mRNA than nonchallenged Fayoumis ($P < 0.05$). The observed differences in mRNA expression of selected cytokines support the concept that these distinct genetic lines utilize different immune responses at homeostasis and in response to *Salmonella* Enteritidis infection.

Introduction

Salmonella is an enteric, zoonotic bacterium that can infect poultry, swine, rodents, sheep, cattle, and humans. The economic impact of *Salmonella* infections in the United States, in relation to human health, is \$0.5 to \$2.3 billion dollars per year (Frenzen et al., 1999). There are about 1.4 million cases of *Salmonella* infection in the United States annually (Voetsch et al., 2004). *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most common serotypes of *Salmonella* isolated from humans (Patrick et al., 2004). Poultry is a major source of *Salmonella* Enteritidis infection in humans (Frenzen et al., 1999). Under-cooked chicken eggs are a common vector for *Salmonella* Enteritidis infections. Upon infection, *Salmonella* Enteritidis can colonize

the intestines and spread systemically throughout the chicken (Gast, 1994). The colonization of *Salmonella* Enteritidis in the liver and spleen has been reported to occur 1 d after inoculation (Van Immerseel et al., 2002). The poultry industry in the United States incurs financial losses of \$64 to \$114 million per year attributed to *Salmonella* infections in chickens (Bryan and Doyle, 1995). Recently, more than half a billion eggs from 2 US farms were recalled because the environments tested positive for *Salmonella* Enteritidis (US Food and Drug Administration, 2010).

There has been considerable research on the effects of host immune response and genetics on the response to *Salmonella* in poultry. *Salmonella* Enteritidis has been shown to affect the expression of many genes associated with innate and acquired immune responses. Eckmann and Kagnoff (2001) demonstrated that cytokines are a key component of the host's response to *Salmonella*. A microarray study performed using 2 meat-type chicken lines that differed in their response to systemic infection resulted in differential gene expression between the 2 lines after *Salmonella* Enteritidis infection (Van Hermert et al., 2006). Heterophils isolated from *Salmonella* Enteritidis-resistant broiler lines expressed significantly less transforming growth factor (TGF) β 1 mRNA than heterophils isolated from *Salmonella* Enteritidis-susceptible lines (Swaggerty et al., 2004).

Recent studies have evaluated several aspects of *Salmonella* response in the same genetic lines as used in the current study. Heterophils isolated from Leghorns that were fed corticosterone expressed significantly higher CXCLi2 mRNA than those isolated from Fayoumis that were fed corticosterone, after in vitro *Salmonella* Enteritidis

stimulation (Redmond et al., 2011). A study using birds at a younger age from the same genetic lines and hatches as those used in the current study showed that in vitro stimulation with *Salmonella* Enteritidis resulted in decreased expression of IL-6 and IL-10 mRNA in broiler and Leghorn heterophils, whereas IL-6 mRNA expression increased in Fayoumi heterophils in response to in vitro *Salmonella* Enteritidis stimulation (Redmond et al., 2009). The goal of the current study was to generate additional insight into the systemic immune mechanisms being employed by hens of these 3 distinct genetic lines by quantifying the splenic mRNA levels of specific cytokines.

Materials and Methods

***Salmonella* Enteritidis**

The *Salmonella* Enteritidis isolate used in this study was a virulent *Salmonella enterica* serovar Enteritidis Phage Type 8 obtained from the USDA-Animal and Plant Health Inspection Service National Veterinary Services Laboratory, Ames, Iowa (ID #SALM-08-2762). The isolate was grown on Luria-Bertani agar plates, and the colonies were cultured in LB broth at 37°C and grown under continuous agitation until reaching an exponential growth phase. The final concentration of the *Salmonella* Enteritidis inoculums was 2×10^8 cfu/mL after being diluted with sterile Hank's balanced salt solution. The bacterial concentration was determined spectrophotometrically at a wavelength of $\lambda = 595$ nm.

Birds and Tissues

Before the experiment, all birds were determined to be free of *Salmonella* Enteritidis by swabbing and plating cloacal samples (Iowa State University, Veterinary

Diagnostic Laboratory). Twelve broiler, 12 Leghorn line (Ghs-6), and 12 Fayoumi line (M15.2) hens approximately 6 mo of age were used. Six were challenged orally with 1 mL of 2×10^8 cfu/mL of *Salmonella* Enteritidis on 3 consecutive days and 6 were mock-challenged with 1 mL of Hank's balanced salt solution on 3 consecutive days in each line. Fecal samples were obtained from each bird, tested for presence of *Salmonella* Enteritidis, and confirmed that all challenged birds were shedding *Salmonella* Enteritidis (Iowa State University, Veterinary Diagnostic Laboratory). The spleens were removed at 10 d after initial inoculation, preserved in RNAlater (Ambion, Austin, TX), and stored at -20°C until RNA isolation. The Fayoumi is a hardy line of chicken that is native to Egypt and has been shown to have a high level of resistance to infection (Lakshmanan et al., 1996). The Leghorn line originated from commercial layer lines in the United States (Lamont and Chen, 1992). Both the Leghorn and Fayoumi lines are highly inbred. The broiler line of chickens has been specifically bred for meat production (Cheeseman et al., 2007).

RNA Isolation and Real-Time PCR

Ribonucleic acid was isolated and quantitative PCR was performed according to previously described procedures (Cheeseman et al., 2007). The primers used were described previously (IL-18, Kogut et al., 2003; IL-10, Rothwell et al., 2004; IL-6, IL-8, macrophage inflammatory protein (MIP) 1 β , Withanage et al., 2004; regulated upon activation, normal T cell expressed, and secreted (RANTES), Mohammed et al., 2007; interferon (IFN)- γ ; and TGF β 1, Shaughnessy et al., 2009). All reactions were run in duplicate, and a standard curve was generated to estimate reaction efficiency (slope).

Expression of the 28S ribosomal RNA was measured using the same procedure and used to account for differences in the amount of RNA included in each reaction. Adjusted cycle threshold (Ct) values for statistical analysis were calculated as follows:

$$40 - [(\text{sample mean Ct}) + (\text{median 28S Ct} - \text{mean 28S Ct}) \times (\text{sample gene slope}/28\text{S slope})].$$

Statistical Analysis

The adjusted Ct value of each sample run in duplicate was analyzed using JMP 8.0.2 software (SAS Institute Inc., 2004). The mRNA expression levels were analyzed using the ANOVA analysis of JMP 8.0.2 on combined data (*Salmonella* Enteritidis-challenged and mock-challenged chickens) for each gene separately, using the following model: $Y = \mu + \text{line} + \text{challenge} + \text{line} \times \text{challenge}$. Line, challenge, and interaction of genetic line and challenge were all considered fixed effects. Tukey's test of JMP 8.0.2 was used to determine rankings and significant differences ($P < 0.05$) among classes within the fixed effects.

Results

Genetic line had a significant effect on the mRNA expression of RANTES ($P < 0.02$) and IFN- γ ($P < 0.03$, Table 1). Broilers expressed significantly more RANTES mRNA than Fayoumis and significantly more IFN- γ mRNA than Leghorns ($P < 0.05$, Table 2). There was a significant interaction of genetic line and challenge for IL-18 ($P < 0.02$) and IL-6 mRNA expression ($P < 0.01$, Table 1). Although there was a significant interaction of genetic line and challenge for IL-18 mRNA levels by ANOVA analysis, Tukey's test only showed suggestive differences ($P < 0.1$). Challenged Leghorns and

nonchallenged Fayoumis expressed more IL-18 mRNA than nonchallenged broilers ($P < 0.1$, Figure 1a). Nonchallenged Fayoumis expressed significantly more IL-6 mRNA than challenged Fayoumis ($P < 0.05$, Figure 1b).

Discussion

The splenic cytokine and chemokine mRNA expression levels observed in the current study support the concept that distinct lines of chickens utilize different systemic immune mechanisms. The broiler hens expressed IFN- γ mRNA, which is involved in macrophage and natural killer thymic (T) cell activation, more highly than the Leghorn line. The IFN- γ has been shown to play a role in both innate and acquired immune functions. In innate immunity, IFN- γ is secreted by antigen presenting cells; this secretion is controlled by cytokines that are also secreted by antigen-presenting cells, IL-12, and IL-18 (Otani et al., 1999; Golab et al., 2000). In acquired immunity, IFN- γ is produced by T cells in response to an antigen binding to a major histocompatibility complex molecule (Ellis and Beaman, 2004). The IFN- γ then activates and enhances the oxidative burst of macrophages (Ellis and Beaman, 2004). Clearance of *Salmonella* from the chicken spleen has been shown to correlate directly with the level of IFN- γ expression (Beal et al., 2004). As a member of the CC family of chemokines, RANTES functions as a pro-inflammatory chemokine that attracts T cells and basophils to sites of inflammation and also induces proliferation and activation of natural killer cells (Maghazachi et al., 1996). In the current study, broilers had the highest level of RANTES mRNA. The genetic line effect on IFN- γ and RANTES suggests that broiler hens, both at homeostasis and after challenge, utilize immune mechanisms that favor

activation of macrophages and T cells, and enhance oxidative burst to a much higher degree than Leghorns.

The cytokine IL-18 is involved in the thymic-helper (Th)1 immune response by inducing the release of IFN- γ , a key cytokine involved in maximizing the efficacy and proliferation of cytotoxic T cells (Schroder et al., 2004). Because of the high levels of IFN- γ mRNA present in the spleens of broilers, they may not require the levels of splenic IL-18 mRNA expression that the Fayoumi line requires to achieve sufficient IFN- γ levels. Examining the within-line effect of challenge shows that *Salmonella* Enteritidis-challenged broilers have higher, and challenged Fayoumis have lower, IL-18 levels than their nonchallenged counterparts, though not statistically significant.

Interleukin-6 is a pro-inflammatory cytokine involved in the transition from innate to acquired immunity and plays a key role in the recruitment of immune cells to sites of infection (Kaiser et al., 2000). An in vitro study using chicken kidney cells showed a significant downregulation in IL-6 mRNA levels in response to *Salmonella* Gallinarum and a significant downregulation in IL-2 mRNA levels in response to *Salmonella* Enteritidis (Kaiser et al., 2000). Using the same genetic lines as the current study, Kaiser et al. (2006) reported significant downregulation in IL-6 and CXCLi2 mRNA levels in *Salmonella* Enteritidis-infected peripheral mononuclear blood cells across all lines. The significantly lower expression of IL-6 mRNA in challenged Fayoumis compared with nonchallenged Fayoumis is an inverse of the response observed in the Redmond et al. (2009) in vitro *Salmonella* Enteritidis challenge of heterophils isolated from 2-mo-old birds of the same lines as the current study, which

resulted in a significantly higher expression of IL-6 mRNA in challenged compared with nonchallenged Fayoumis. The Redmond et al. (2009) paper suggests that Fayoumis have a rapid pro-inflammatory response, as measured in isolated heterophils, which may be a factor in effective resistance to disease. Several components of the experimental designs may have been attributed to IL-6 mRNA expression differences, including tissue/cell assayed, route of exposure to bacteria, and age.

The lack of differential expression of IL-8, IL-10, MIP1 β , and TGF β 1 in relation to line and *Salmonella* challenge in the current study differs from some other reports. In a previous study using the same genetic lines, Leghorns expressed significantly more splenic IL-10 mRNA than broilers (Cheeseman et al., 2007). The IL-8 mRNA and MIP1 β mRNA were expressed at significantly higher levels following *Salmonella* Typhimurium infection in the ilea of Rhode Island Red chickens (Withanage et al., 2004). Splenic TGF β 1 (formerly referred to as TGF β 4) mRNA was shown to be significantly higher expressed following *Salmonella* Typhimurium infection of Rhode Island Red chickens (Withanage et al., 2005). The differences in the expression of genes in the present experiment and previous studies may be due to differences in the experimental designs that alter gene expression, such as the age of chickens at infection, the *Salmonella enterica* serovar used, the number of days after challenge in which the spleen samples were collected, or the genetic background of the chickens used. During development there are differences in the proportions of CD3 $^{+}$ intraepithelial lymphocytes expressing TCR1 $^{+}$ and TCR2 $^{+}$ in chickens 1 to 8 wk of age and those 9 to 12 wk of age (Lillehoj and Chung, 1992). In a study of primary and secondary challenge

of *Salmonella* Typhimurium, older chickens produced a higher specific T cell response to secondary infection compared with younger chickens (Beal et al., 2004). In summary, the results of the current study support the concept that distinct genetic lines of chickens utilize immune mechanisms that differ by time, type, or magnitude in the presence and absence of *Salmonella* Enteritidis infection.

Acknowledgements

Supported by USDA National Institute of Food and Agriculture grant number 2007-35604-17866, a George Washington Carver Fellowship, and an Integrative Graduate Education and Research Fellowship awarded to D. J. Coble.

References

- Beal, R. K., P. Wigley, C. Powers, S. D. Hulme, P. A. Barrow, and A. L. Smith. 2004. Age at primary infection with *Salmonella enterica* serovar Typhimurium in the chicken influences persistence of infection and subsequent immunity to re-challenge. Vet. Immunol. Immunopathol. 100:151-164.
- Bryan, F. L., and M. P. Doyle. 1995. Health risks and consequences of *Salmonella* and *Campylobacter jejuni* in raw poultry. J. Food Prot. 58:326-344.
- Cheeseman, J. H., M. G. Kaiser, C. Ciraci, P. Kaiser, and S. J. Lamont. 2007. Breed effect on early cytokine mRNA expression in spleen and cecum of chickens with and without *Salmonella enteritidis* infection. Dev. Comp. Immunol. 31:52-60.
- Eckmann, L., and M. F. Kagnoff. 2001. Cytokines in host defense against *Salmonella*. Microbes Infect. 3:1191-1200.

- Ellis, T. N., and B. L. Beaman. 2004. Interferon-gamma activation of polymorph nuclear neutrophil function. *Immunology* 112:2-12.
- Frenzen P., T. Riggs, T. B. J. Buzby, T. Roberts, D. Voetsch, S. Reddy, and the FoodNet Working Group. 1999. *Salmonella* cost estimate update using FoodNet data. *Food Review* 22:10-15.
- Gast, R. K. 1994. Understanding *Salmonella* Enteritidis in laying chickens: The contributions of experimental infections. *Int. J. Food Microbiol.* 21:107-116.
- Golab, J., Zagozdzon, T. Stoklosal, R. Kaminski, K. Kozar, and M. Jakóbsiak. 2000. Direct stimulation of macrophages by IL-12 and IL-18—A bridge too far? *Immunol. Lett.* 72:153–157.
- Kaiser, M. G., J. H. Cheeseman, P. Kaiser, and S. J. Lamont. 2006. Cytokine expression in chicken peripheral blood mononuclear cells after in vitro exposure to *Salmonella enterica* serovar Enteritidis. *Poult. Sci.* 85:1907–1911.
- Kaiser, P., L. Rothwell, E. E. Galyov, P. A. Barrow, J. Burnside, and P. Wigley. 2000. Differential cytokine expression in avian cells in response to invasion by *Salmonella typhimurium*, *Salmonella enteritidis*, and *Salmonella gallinarum*. *Microbiology* 146:3217–3226.
- Kogut, M., L. Rothwell, and P. Kaiser. 2003. Priming by recombinant chicken interleukin-2 induces selective expression of IL-8 and IL-18 mRNA in chicken heterophils during receptor-mediated phagocytosis of opsonized and nonopsonized *Salmonella enteric* serovar enteritidis. *J. Mol. Immunol.* 40:603–610.

- Lakshmanan, N., M. G. Kaiser, and S. J. Lamont. 1996. Marek's disease (MD) resistance in MHC-congenic lines from Leghorn and Fayoumi breeds. Pages 57–62 in Current Research on Marek's Disease. R. F. Silva, H. H. Cheng, P. M. Coussens, L. F. Lee, and L. F. Velicer, ed. Am. Assoc. Avian Pathol., Kennett Square, PA.
- Lamont, S. J., and Y. Chen. 1992. Endogenous viral genes in thirteen highly inbred chicken lines and in lines selected for immune response traits. *Poult. Sci.* 71:530–538.
- Lillehoj, H. S., and K. S. Chung. 1992. Postnatal development of T-lymphocyte subpopulations in the intestinal intraepithelial and lamina propria in chickens. *Vet. Immunol. Immunopathol.* 33:347–360.
- Maghazachi, A. A., A. Al-Aoukaty, and T. J. Schall. 1996. CC chemokines induce the generation of killer cells from CD56+ cells. *Eur. J. Immunol.* 26:315–319.
- Mohammed, J., S. Frasca Jr., K. Cecchini, D. Rood, A. C. Nyaoke, S. J. Geary, and L. K. Silbart. 2007. Chemokine and cytokine gene expression profiles in chickens inoculated with *Mycoplasma gallisepticum* strains Rlow or GT5. *Vaccine* 25:8611–8621.
- Otani, T., S. Nakamura, M. Toki, R. Motoda, M. Kurimoto, and K. Orita. 1999. Identification of IFN-gamma-producing cells in IL-12/IL-18-treated mice. *Cell. Immunol.* 198:111–119.
- Patrick, M. E., P. M. Adcock, T. M. Gomez, S. F. Altekruse, B. H. Holland, R. V. Tauxe, and D. L. Swerdlow. 2004. *Salmonella* Enteritidis infection, United States, 1985–1999. *Emerg. Infect. Dis.* 10:1–7.

- Redmond, S. B., P. Chuammitri, C. B. Andreasen, D. Palić, and S. J. Lamont. 2011. Proportion of circulating chicken heterophils and CXCLi2 expression in response to *Salmonella* Enteritidis are affected by genetic line and immune modulating diet. *Vet. Immunol. Immunopathol.* doi:10.1016/j.vetimm.2011.01.006.
- Redmond, S. B., P. Chuammitri, D. Palić, C. B. Andreasen, and S. J. Lamont. 2009. Chicken heterophils from commercially selected and non-selected genetic lines express cytokines differently after in vitro exposure to *Salmonella enteritidis*. *Vet. Immunol. Immunopathol.* 132:129-134.
- Rothwell, L., J. R. Young, R. Zoorob, C. A. Whittaker, P. Hesketh, A. Archer, A. L. Smith, and P. Kaiser. 2004. Cloning and characterization of chicken IL-10 and its role in the immune response to *Eimeria maxima*. *J. Immunol.* 173:2675-2682. SAS Institute Inc. 2004. JMP Statistics and Graphics Guide, Ver. 5.1.1. SAS Institute Inc., Cary, NC.
- Schroder, K., P. Hertzog, T. Ravasi, and D. Hume. 2004. Interferon- γ : An overview of signals, mechanisms and functions. *J. Leukoc. Biol.* 75:163-189.
- Shaughnessy, R. G., K. G. Meade, S. Cahalane, B. Allan, C. Reiman, J. J. Callanan, and C. O' Farrelly. 2009. Innate immune gene expression differentiates the early avian intestinal response between *Salmonella* and *Campylobacter*. *Vet. Immunol. Immunopathol.* 132:191-198.

- Swaggerty, C. L., M. H. Kogut, P. J. Ferro, L. Rothwell, I. Pevzner, and P. Kaiser. 2004. Differential cytokine mRNA expression in heterophils isolated from *Salmonella*-resistant and -susceptible chickens. *Immunology* 113:139-148.
- US Food and Drug Administration. 2010. The outbreak of *Salmonella* in egg. Accessed Nov. 2010. <http://www.fda.gov/NewsEvents/Testimony/ucm226554.htm>.
- Van Hermert, S., A. J. Hoekman, M. A. Smits, and J. M. Rebel. 2006. Early host gene expression responses to a *Salmonella* infection in the intestine of chickens with different genetic background examined with cDNA and oligonucleotide microarrays. *Comp. Biochem. Physiol. D* 3:292-299.
- Van Immerseel, F., J. De Buck, I. De Smet, J. Mast, F. Haesebrouck, and R. Ducatelle. 2002. Dynamics of immune cell infiltration in the caecal lamina propria of chickens after neonatal infection with a *Salmonella enteritidis* strain. *Dev. Comp. Immunol.* 26:355-364.
- Voetsch, A. C., T. J. Van Gilder, F. J. Angulo, M. M. Farley, S. Shallow, R. Marcus, P. R. Cieslak, V. C. Deneen, and R. V. Tauxe. 2004. FoodNet estimate of the burden of illness caused by nontyphoidal *Salmonella* infections in the United States. *Clin. Infect. Dis.* 38:S127-S134.
- Withanage, G. S., P. Kaiser, P. Wigley, C. Powers, P. Mastroeni, H. Brooks, P. Barrow, A. Smith, D. Maskell, and I. McConnell. 2004. Rapid expression of chemokines and pro-inflammatory cytokines in newly hatched chickens infected with *Salmonella enterica* serovar Typhimurium. *Infect. Immun.* 72:2152-2159.

Withanage, G. S., P. Wigley, P. Kaiser, P. Mastroeni, H. Brooks, C. Powers, R. Beal, P. Barrow, D. Maskell, and I. McConnell. 2005. Cytokine and chemokine responses associated with clearance of a primary *Salmonella enterica* serovar Typhimurium infection in the chicken and in protective immunity to rechallenge. *Infect. Immun.* 73:5173-5182.

Table 1 - Genetic line, challenge, and line-by-challenge interaction effects on spleen mRNA expression

Numbers in bold represent significant effects. **Tukeys Test, ($P < 0.05$); *ANOVA Analysis, ($P < 0.05$)

Variable	Gene							
	IL-6	IL-8	IL-10	IL-18	IFN- γ	MIP1 β	RANTES	TGF β 1
Genetic Line	0.38	0.76	0.68	0.25	0.03**	0.95	0.02**	0.12
Challenge	0.22	0.69	0.33	0.42	0.39	0.40	0.63	0.33
Interaction	0.01**	0.46	0.68	0.02*	0.10	0.12	0.45	0.82

Table 2 - Splenic IFN- γ and RANTES mRNA expression of hens from different genetic lines

SE-challenged and non-challenged combined (Least Square Means of adjusted Ct values \pm SEM). ^{a,b} Different letters within a row indicate a significant difference (Tukeys, $P < 0.05$).

	Genetic Line		
	Broiler	Leghorn	Fayoumi
IFN- γ	6.40 \pm 0.56 ^a	4.20 \pm 0.56 ^b	5.64 \pm 0.56 ^{ab}
RANTES	17.72 \pm 0.49 ^a	17.23 \pm 0.49 ^{ab}	15.79 \pm 0.49 ^b

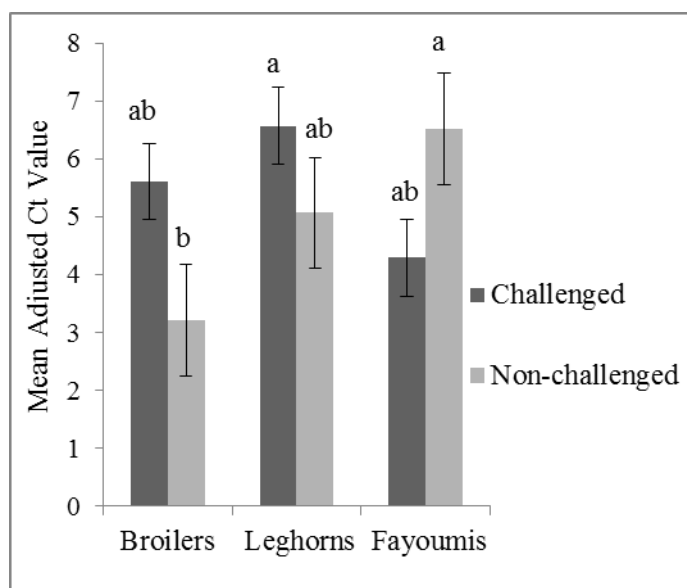


Figure 1A - Mean IL-18 mRNA expression levels by genetic line, in the spleen of hens either non-challenged or challenged with SE

^{a,b} Bars not sharing a letter indicate a suggestive difference shown by Tukey's test ($P < 0.1$).

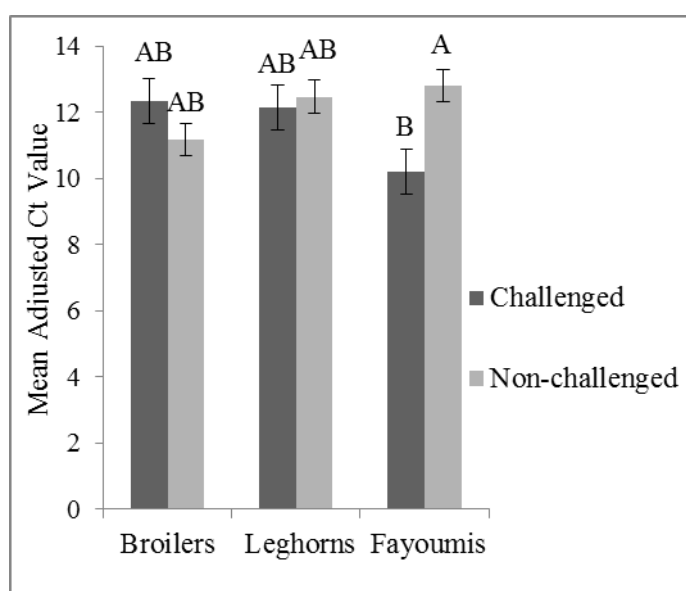


Figure 1B - Means of adjusted Ct values for IL-6 mRNA expression by genetic line in the spleen of hens either non-challenged or challenged with SE

^{A,B} Bars not sharing a letter are shown to be significantly different by Tukey's test ($P < 0.05$).

CHAPTER 3. IMPACTS OF *SALMONELLA* ENTERITIDIS INFECTION ON LIVER TRANSCRIPTOME IN BROILERS

A paper published in *genesis*⁵

D. J. Coble⁶, E. E. Sandford⁷, T. Ji⁸, J. Abermathy⁹, D. Fleming⁷, H. Zhou⁹, and S. J.
Lamont^{6,10}

Summary

Salmonella enterica serovar Enteritidis is an enteric bacterium that can contaminate chicken eggs and meat, resulting in production losses and consumer illness. To provide insight into the systemic metabolic effects of *S. Enteritidis* infection, liver samples were harvested 10-days postinfection from broiler hens. Hepatic global gene expression levels were assessed using a chicken 44K Agilent microarray. Forty-four genes were differentially expressed at a significance level of q value < 0.05 . One hundred eighty-three genes were differentially expressed at a suggestive significance level of q value < 0.1 . A predominance of down-regulation existed among significantly differentially expressed genes. Cell cycle and metabolism networks were created from the differentially expressed genes. Mitochondria-mediated apoptosis, electron transport, peptidase activity, vein constriction, cell differentiation, *IL-2* signaling, Jak-Stat signaling, B-cell receptor signaling, GDP/GTP exchange, and protein recycling were

⁵ Reprinted with permission of *genesis*, 2012, 51:357-364.

⁶ Graduate student and Professor, respectively, Department of Animal Science, Iowa State University.

⁷ Collaborator, Department of Animal Science, Iowa State University.

⁸ Collaborator, Department of Statistics, Iowa State University.

⁹ Collaborator, Department of Animal Science, University of California Davis.

¹⁰ Author for Correspondence.

among the functions of the differentially expressed genes that were down-regulated in response to *S. Enteritidis*. The effects of *S. Enteritidis* infection on the liver transcriptome profiles of broilers reflect a predominance of down-regulation of genes with cell cycle and metabolic functions. The most pronounced response was the down-regulation of genes that function in metabolic pathways, inflammation, and mitochondria-mediated apoptosis. These results provide insight into important systemic metabolic mechanisms that are active in the chicken liver in response to *S. Enteritidis* infection at 10-days postinfection.

Introduction

Salmonella enterica serovar Enteritidis is an enteric bacterium that can colonize chickens, contaminating meat and eggs, which results in production losses and illness in consumers (Frenzen et al., 1999; Voetsch et al., 2004). Eggs can be vertically contaminated through transovarian *Salmonella* exposure or hen cloacal infection (Lahellec and Colin, 1985). Horizontal transmission in chickens can occur through environmental factors such as infected fecal material, air, litter, unclean facilities, and vectors, such as rodents and humans (Amick-Morris, 1998; Hoover et al., 1997; Jones et al., 1991). After the chicken is infected, *S. Enteritidis* colonizes the intestines and can spread systemically throughout the chicken (Gast and Beard, 1990). Gast and Beard (1990) reported that after oral inoculation of adult Leghorn hens (20–88 weeks of age) with doses of 10^9 cells of *S. Enteritidis*, they recovered *S. Enteritidis* from 60% of cecal samples, 53% of livers, 49% of spleens, 19% of ovaries, and 17% of oviducts sampled

during the first 5-weeks post inoculation. Thus, the spread of *Salmonella* infection after oral inoculation can be systemic and often includes infection of the liver.

Several recent studies have evaluated the effects of *Salmonella* on the global gene expression of chicken immune tissues or cells (Chiang et al., 2008; Ciraci et al., 2010; Zhou and Lamont, 2007). Zhou and Lamont, (2007) using a 13k chicken array to analyze gene expression in splenic cells, reported that host genetics and time postinfection play important roles in *S. Enteritidis* infection in chickens. In a transcriptome study involving lines that differ in resistance to *S. Enteritidis*, immune-related genes were down-regulated in heterophils of the *S. Enteritidis*- susceptible line and up-regulated in the heterophils of the resistant lines (Chiang et al., 2008). In an in vitro study of macrophages, Ciraci et al., (2010) reported that 98% of the differentially expressed inflammatory genes in response to *S. Typhimurium* endotoxin were significant at 4-h poststimulation.

Although several studies have evaluated the effects of *S. Enteritidis* on the transcriptome profiles of chickens from an immune tissue perspective, little is known about the metabolic transcriptome profiles of other tissues in *Salmonella*-infected chickens. Analyzing the transcriptomic profiles of the liver of *Salmonella*-infected chickens may highlight interactions between metabolic and immunological pathways and identify novel changes in gene expression in nonimmune tissues. The objective of this study is to provide insight into the systemic metabolic effects of *S. Enteritidis* infection in broilers by evaluating transcriptomic profiles from the livers of *S. Enteritidis*-infected broilers.

Results

Microarray

There were 44 genes differentially expressed at the significance level of q value < 0.05 , using the procedure developed by Storey and Tibshirani (2003) to control the false discovery rate (FDR). In addition, there were 183 genes differentially expressed when controlling the FDR at the significance level of q value < 0.10 . Among these genes, only 12 were up-regulated, and the other 171 were down-regulated. Of the 30 annotated genes that displayed significant differential expression (q value < 0.05), there was a predominance of down-regulation of gene expression in response to *S. Enteritidis* (29 genes down-regulated, 1 up-regulated). Among all differentiated genes, the Log2 fold changes ranged from -1.57 to 3.9.

Pathway Analysis

Upon ingenuity pathway analysis (IPA), two gene networks were created. The first network consisted of genes with cell cycle functions and the second network involved metabolism-related genes (Fig. 1). The cell cycle network included *AIFM1* (Apoptosis-Inducing Factor, Mitochondrion-Associated 1), *BRCC3* (*BRCA1/BRCA2*-containing complex, subunit 3), *CTDSPL2* (Carboxylterminal domain of Ribonucleic Acid POL II, polypeptide A), *CYP27A1* (Cytochrome P450, family 27, subfamily A, polypeptide 1), *GRPEL1* (GrpE-like 1, mitochondrial (E.coli)), *HELLS* (Helicase, lymphoid-specific), *HTATSF1* (HIV-1 Tat specific factor 1), *LUC7L2* (LUC7-like 2 (*Saccharomyces cerevisiae*)), *MAD2L1* (MAD2 mitotic arrest deficient-like 1 (yeast)), *PAN2* (*USP52*) (*PAN2* poly(A) specific ribonuclease subunit homolog

(*Saccharomyces cerevisiae*), *PKM2* (Pyruvate kinase, muscle), *POLH* (Polymerase, DNA directed eta), *POLDIP3* (Polymerase, DNA directed, delta interacting protein 3), *RBM12* (RNA binding motif protein 12), *SAMM50* (Sorting and assembly machinery component 50 homolog (*Saccharomyces cerevisiae*), *SERPINE2* (Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1 member 2), *TMEM57* (Transmembrane protein 57), *USP7* (Ubiquitin specific peptidase 7, herpes virus-associated), and *WDR36* (WD repeat domain 36).

The metabolism network included *AVP* (Arginine vasopressin), *C14orf45* (Chromosome 14 open reading frame 45), *DPYSL2* (dihydropyrimidinase-like 2), *ESRRG* (Estrogen-related receptor gamma), *FURIN* (paired basic amino acid cleaving enzyme), *MYEF2* (Myelin expression factor 2), *PTPN6* (Protein tyrosine phosphatase, nonreceptor type 6), *RABGEF1* (RAB guanine nucleotide exchange factor (GEF) 1), *SLC25A37* (Solute carrier family 25, member 37), *SOCS6* (Suppressor of cytokine signaling 6), and *STEAP1* (Six transmembrane epithelial antigen of the prostate 1) (Fig. 2).

In summary, *S. Enteritidis* infection resulted in the down-regulation of genes involved in mitochondriamediated apoptosis, electron transport, peptidase activity, blood pressure increase, cell differentiation, *IL-2* signaling, Jak-Stat signaling, B-cell receptor signaling, GDP/GTP exchange, and protein recycling (Table 1, Supporting Information Table 1).

qPCR (Quantitative Polymerase Chain Reaction) Validation

The differential expression levels of 10 genes that were differentially expressed at a (q value < 0.05) significance level were all assayed using qPCR (P value < 0.05). The fold changes from the qPCR and microarray analysis of *C14orf45*, *AVP*, *SOCS6*, *PKM2*, *USP7*, *SLC25A37*, *POLH*, *PTPN6*, and *AIFM1* were all in the same direction and significant. Although suggestive, the fold change for *USP52* was nonsignificant when assayed using qPCR (Table 2).

Discussion

The down-regulation of four genes (*CYP27A1*, *BRCC3*, *PKM2*, and *GRPEL1*) from the cell cycle network and five genes (*AVP*, *DPYSL*, *FURIN*, *SLC25A37*, and *STEAP1*) from the metabolism network reflects a disruption of metabolic enzymes and pathways in the liver by *S. Enteritidis* at 10-days postinfection. *CYP27A1* is an electron carrier involved in oxidation-reduction reactions; *BRCC3* is responsible for binding damaged DNA, metal ions, and zinc ions; *PKM2* is involved in glycolysis and is responsible for binding ATP, magnesium ions, potassium ions, and transferase activity; *GRPEL1* is an adenylyl- nucleotide exchange factor involved in chaperone binding and binding unfolded proteins; *AVP* is responsible for vasoconstriction; *DPYSL2* is involved in cell differentiation and cell development; *FURIN* is an amino acid cleavage enzyme involved in cell proliferation; *SLC25A37*, a solute carrier and *STEAP1* are involved in transmembrane transport. The liver produces enzymes such as pyruvate kinase along with hepatic hormones that are involved in the homeostasis of host metabolic functions (Caetano et al., 2011). For example, eicosanoids regulate vasoconstriction, immune

responses, and platelet aggregation (Funk, 2001). *AVP* is a member of the eicosanoid pathway that is involved in renal water retention and vasoconstriction which in turns results in the elevation of blood pressure (Matsukawa and Miyamoto, 2011; Nielsen et al., 1995). *Salmonella* infection has been shown to severely disrupt the eicosanoid metabolic pathway (Caetano et al., 2011). The down-regulation of *AVP* expression may be a reflection of the disruptive effects that *Salmonella* infection has on the eicosanoid pathway. The alteration of *PKM2* expression by *Salmonella* infection may be another mechanism by which systemic metabolic effects are induced by the pathogen. The down-regulation of pyruvate kinase correlates with the reduction in hepatic pyruvate and oxaloacetate levels in response to *Salmonella* infection that was observed in Freeman and Chubb (1968). Although *Salmonella* infection resulted in a reduction of pyruvate and oxaloacetate levels in the liver, pyruvate levels in the blood remained the same, indicating that alternative pathways were not affected.

The down-regulation of three genes (*MAD2L1*, *USP52*, and *USP7*) from the cell cycle network, as well as *PTPN6* from the metabolism network, likely reflects a decrease in the expression of genes associated with the destruction and degradation of liver cells by macrophages and ubiquitin-specific peptidases at 10-days postinfection with *S. Enteritidis*. *MAD2L1* is a mitotic spindle checkpoint; *USP52* and *USP7* encode ubiquitin-specific peptidases. *PTPN6* is involved with hydrolase, phosphatase, B-cell Receptor signaling, Jak-STAT signaling, and *IL-2* signaling functions. The action of tagging proteins for proteolysis with ubiquitin is directly related to inflammation, apoptosis, and the immune response to *S. Enteritidis* (Te Pas et al., 2012). When

regulated, the programmed death of infected cells and the degrading action of ubiquitin-specific peptidases facilitate the clearance of systemic *Salmonella* infection (Jiang and Zhijian, 2012; Redmond et al., 2011). Excessive inflammatory actions, however, can result in epithelial damage that enhances the ability of *Salmonella* to further invade the area of infection (Kum et al., 2011). Using the murine model, Parent and Eichacker (1999) reported that a disproportionate accumulation of neutrophils and monocytes results in the release of toxic levels of proteases and reactive oxygen intermediates. The increased expression of inflammatory genes and the influx of heterophils in response to *S. Enteritidis* have been associated with tissue damage (Kaiser et al., 2000). Zinkernagel et al. (2007) explained that the expression of inflammatory mediators is an effective method to eliminate bacterial infection but can lead to the inflammatory damage of healthy cells. Therefore, the down-regulation of these genes may reflect a host protective mechanism to prevent tissue damage due to an excessive or prolonged inflammatory response.

The down-regulation of *SOCS6* from the metabolism network and *AIFM1* from the cell cycle network reflects a host response to suppress mitochondria-mediated apoptosis at 10 days post *S. Enteritidis* infection. Although *SOCS6* belongs to the *SOCS* family of proteins, which are generally characterized as negative feedback regulators for cytokine receptor signaling, *SOCS6* does not interact with cytokine signaling intermediates nor does *SOCS6* inhibit cytokine receptor signaling (Masuhara et al., 1997; Nicholson et al., 1999). This is in agreement with the lack of differential expression of proinflammatory genes in response to the down-regulation of *SOCS6*. Coble et al. (2011)

reported the lack of differential expression of splenic *IL-6* in response to *S. Enteritidis* infection. Lin et al. (2012) demonstrated that ectopic expression of *SOCS6* in a mammalian cell culture resulted in an increase in apoptotic activity through an intrinsic mitochondrial pathway that was inhibited by the knockdown of *SOCS6*. Therefore, *SOCS6* has proapoptotic properties and employs a mitochondrial pathway that is separate from the *TNF- α* (tumor necrosis factor- α) apoptotic pathway. Interestingly, the down-regulation of *SOCS6*, a gene associated with the initiation of a mitochondrial apoptotic pathway, corresponds in the present study with the down-regulation of *AIFM1*, a mitochondria- associated apoptosis inducing factor. Mitochondria execute cell death and initiate apoptosis through the release of cytochrome c and other proapoptotic factors (Garrido et al., 2006; Wang, 2001). During apoptosis, mitochondria undergo morphological changes from filamentous networks to fragments (Hu et al., 2012). *SOCS6* encodes for a novel outer mitochondrial membrane targeting protein that regulates mitochondrial dynamics and promotes DRP1 translocation from the cytoplasm to mitochondria, resulting in organelle constriction (Lin et al., 2012; Smirnova et al., 2001; Wasilewski and Scorrano, 2009). This process results in apoptosis (Lin et al., 2012). Therefore, the down-regulation of *SOCS6* and *AIFM1* reflects a host response that suppresses the mitochondria-mediated apoptosis pathway.

Although the number (44) of genes differentially expressed at a significance level of q value <0.05 in this study was low, it is similar to that found in some related studies on response to bacteria or bacterial components in chicken tissues or cells. In an in vitro study in which chicken macrophage cells were stimulated with *Salmonella* endotoxin,

Ciraci et al. (2010) reported that only 13 genes were differentially expressed at a significance level of q value < 0.05 at 1-h poststimulation and that 33 genes were differentially expressed at 2-h poststimulation. In the same study, 1,761 genes were differentially expressed at 4-h poststimulation. In a study that evaluated the chicken spleen transcriptome in response to avian pathogenic *E. coli* infection, Sandford et al. (2011) reported that there were only two differentially expressed genes between birds exhibiting mild and severe pathology on Day-1 postinfection. There were 799 genes differentially expressed between birds with mild and severe pathology on Day 5 postinfection. The transcriptomic response of the chicken or chicken cells to *Salmonella* depends upon the genetic background of the chicken, the *Salmonella* serovar, the tissue sampled, and the time point in which the samples are harvested. Therefore, the genes that were differentially expressed in the present study represent a focused analysis of the broiler liver transcriptome at 10-days postinfection with *S. Enteritidis*.

In summary, the effect of *S. Enteritidis* 10 days postinfection on the liver transcriptome profiles of broilers reflects the disruption of metabolic pathways, the down-regulation of genes involved in the destruction and recycling of proteins, and the down-regulation of genes involved in mitochondria-mediated apoptosis. These results provide insight into important systemic metabolic mechanisms in the chicken in response to *S. Enteritidis* infection.

Methods

Animals and Tissues

All birds were determined to be free of *S. Enteritidis* by swabbing and plating cloacal samples prior to the experiment (Iowa State University, Veterinary Diagnostic Lab). Sixteen broiler hens of ~ 5 months (eight challenged orally with 2×10^8 *S. Enteritidis* on each of three sequential days and eight mock-challenged with phosphate buffered saline) were used. To allow sufficient time for the infection to be established, livers were harvested at 10-days postinfection. Tissue was stored in RNALater (Ambion). All animal protocols were approved by the Iowa State University Institutional Animal Care and Use Committee.

Salmonella Enteritidis

The *S. Enteritidis* isolate used in this study was a virulent *Salmonella enterica* serovar Enteritidis Phage Type 8 obtained from the USDA-Animal and Plant Health Inspection Service National Veterinary Services Laboratory, Ames Iowa (ID #SALM-08-2762). This isolate is a publicly accessible virulent *S. Enteritidis* field strain. The isolate was grown on Luria-Bertani agar plates, and the colonies were cultured in LB broth at 37°C and grown under continuous agitation until reaching an exponential growth phase. The final concentration of the *S. Enteritidis* inocula was 2×10^8 cfu mL⁻¹ after being diluted with sterile Hank's balanced salt solution. This represents a high dose of *S. Enteritidis*, as defined by Asheg et al. (2003). The bacterial concentration was determined using a spectrophotometer at a wavelength of $\lambda = 595$ nm.

RNA Isolation

Liver samples of ~ 25 mg were homogenized in 0.6 mL⁻¹ of TRI Reagent Solution (Ambion) and total mRNA was isolated using the Ambion MagMax-96 kit protocol. To ensure high-quality RNA for microarray hybridization, samples were ethanol precipitated as follows. Samples were combined with 50 µL of RNase free water to 100µL. Ten µL of 3M sodium acetate, 300 µL of 100% ethanol, and 20 µg of glycogen were added to the water-RNA mixture. These samples were incubated at -80°C overnight and centrifuged at 4°C for 25 min at 14,000 rpm. The supernatant was aspirated and the pellets were washed with 500 µl of 70% ethanol. This mixture was centrifuged again at 7,600g for 5 min at 4°C. The supernatant was removed and the pellet was redissolved in RNase free water. The samples were stored at -80°C until usage.

Microarray

The cRNA was labelled and hybridized to the microarrays in accordance with the procedure of Chiang et al., (2008). The chicken 44K Agilent microarray was utilized in this study. Two microarray slides were used, and each microarray slide contained 4 arrays. Each array has 42,034 probes that were designed based on the whole chicken genome including autosomes, sex chromosomes, unlocalized chromosomes, and mitochondria, along with positive and negative controls (Li et al., 2008). A dye swap design was applied such that for each array in each slide, the Cy3 (Cyanide 3) and Cy5 (Cyanide 5) dyes were applied to the two treatment (control treatment and infected treatment) levels respectively, and the dye assignment to treatment levels was swapped

for each array in a slide. On each array, a *Salmonella*-infected and non-infected bird was represented.

Microarray Data Collection and Analysis

The microarray data were collected and analyzed as described in Chiang et al., (2008). The following linear model was used to contrast infection and control groups:

$$y_{ijkl(k)} = \mu + t_i + r_j + s_k + e_{ijkl(k)} \quad (1),$$

where y_{ijkl} represents the normalized signal contrast intensity with treatment level i ($i=1$ or 2 depending on the contrast direction), replicate j ($j=1,2,3$), array l ($l=1,2,3,4$) in slide k ($k=1,2$); μ is an overall mean gene expression; t_i is the fixed main effect of treatment (*S. Enteritidis* infection) i ; r_j is the fixed effect of replicate j ; s_k is the fixed effect of slide k ; and e_{ijkl} is the random error. The LIMMA (Smyth, 2004) package developed in R was used to analyze the data, which utilizes empirical Bayes method for improved statistical inferences. The data presented in this article have been deposited into NCBI's Gene Expression Omnibus (GEO) (Edgar et al., 2002) and can be accessed through GEO Platform number GPL6413 and Series accession number GSE39448.

Ingenuity Pathway Analysis

The chicken 44K Agilent array probe set was uploaded to Ingenuity Pathways Analysis (IPA; Ingenuity® Systems, <http://www.ingenuity.com>) software for the construction of gene networks. Upon selecting the *Gallus gallus* setting, statistically significant networks were constructed with a P -value threshold of 0.0001.

qPCR Validation

Total RNA was isolated and quantitative PCR was performed according to previously described procedures (Cheeseman et al., 2007; Eldaghayes et al., 2006). The forward and reverse primer sequences used for qPCR are listed in Table 1. All reactions were run in triplicate, and a standard curve was generated to estimate reaction efficiency (slope). Expression of the 28S ribosomal RNA was measured using the same procedure and used to account for differences in the amount of RNA included in each reaction. Adjusted Cycle threshold (Ct) values for statistical analysis were calculated as follows: $40 - [(sample\ mean\ Ct) + (median\ 28S\ Ct - mean\ 28S\ Ct) \times (sample\ gene\ slope / 28S\ slope)]$.

qPCR Statistical Analysis

The mRNA expression levels as mean adjusted Ct values of each sample were analyzed using the ANOVA analysis of JMP 8.0.2 software on combined data (S. Enteritidis-challenged and mock-challenged chickens) for each gene separately, using the following model:

$$Y = \mu + \text{challenge} + e.$$

Challenge was considered a fixed effect. Student's t test of JMP 8.0.2 software was used to determine significant differences (P -value < 0.05) between challenged and unchallenged samples.

Acknowledgements

The authors acknowledge the members of the Lamont Lab group for assistance with the animal study and tissue harvest. Funding for this project was provided by

USDA National Institute of Food and Agriculture grant number 2007-35604-17866, a George Washington Carver Fellowship (DJC), and a National Science Foundation Integrative Graduate Education and Research Traineeship Fellowship (DJC).

Literature Cited

- Amick-Morris J. 1998. Insects' contribution to *Salmonella* transmission in turkey flocks. MS Thesis West Virginia University, Morgantown, WV.
- Asheg AA, Levkut M, Revajová V, Sevcíková Z, Kolodzieyski L, Pistl J, Pilipcinec E. 2003. Spreading of *Salmonella enteritidis* in the cecum of chickens. *Folia Microbiol* 48:227-229.
- Caetano L, Antunes LC, Arena ET, Menendez A, Han J, Ferreira RB, Buckner MM, Lolic P, Madilao LL, Bohlmann J, Borchers CH, Finlay BB. 2011. Impact of *Salmonella* infection on host hormone metabolism revealed by metabolomics. *Infect Immun* 79:1759-1769.
- Cheeseman JH, Kaiser MG, Ciraci C, Kaiser P, Lamont SJ. 2007. Breed effect on early cytokine mRNA expression in spleen and cecum of chickens with and without *Salmonella enteritidis* infection. *Dev Comp Immunol* 31:52-60.
- Chiang HI, Swaggerty CL, Kogut MH, Dowd SE, Li X, Pevzner IY, Zhou H. 2008. Gene expression profiling in chicken heterophils with *Salmonella enteritidis* stimulation using a chicken 44 K Agilent microarray. *BMC Genomics* 9:526.
- Ciraci C, Tuggle CK, Wannemuehler MJ, Nettleton D, Lamont SJ. 2010. Unique genome-wide transcriptome profiles of chicken macrophages exposed to *Salmonella*-derived endotoxin. *BMC Genomics* 11:545.

- Coble DJ, Redmond SB, Hale B, Lamont SJ. 2011. Distinct lines of chickens express different splenic cytokine profiles in response to *Salmonella* Enteritidis challenge. Poult Sci 90:1659-1663.
- Edgar R, Domrachev M, Lash AE. 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 30:207-210.
- Eldaghayes I, Rothwell L, Williams A, Withers D, Balu S, Davison F, Kaiser P. 2006. Infectious bursal disease virus: strains that differ in virulence differentially modulate the innate immune response to infection in the chicken bursa. Viral Immunol 19:83-91.
- Freeman BM, Chubb LG. 1968. Effect of *Salmonella Gallinarium* on certain Krebs cycle intermediates of domestic fowl. J Bacteriol 88:93-95.
- Frenzen P, Riggs T, Buzby TBJ, Roberts T, Voetsch D, Reddy S, FoodNet Working Group. 1999. *Salmonella* cost estimate update using FoodNet data. Food Rev 22:10-15.
- Funk CD. 2001. Prostaglandins and leukotrienes: Advances in eicosanoid biology. Science 294:1871-1875.
- Garrido C, Galluzzi L, Brunet M, Puig P, Didelot C, Kroemer G. 2006. Mechanisms of cytochrome c release from mitochondria. Cell Death Differ 13:1423-1433.
- Gast RK, Beard CW. 1990. Isolation of *Salmonella enteritidis* from internal organs of experimentally infected hens. Avian Dis 34:991-993.
- Hoover NJ, Kenney PB, Amick JD, Hypes WA. 1997. Preharvest sources of *Salmonella* colonization in turkey production. Poult Sci 76:1232-1238.

- Hu J, Yao H, Gan F, Torkarski A, Wang Y. 2012. Interaction of OKL38 and p53 in regulating mitochondrial structure and function. PLOS ONE 7:8.
- Jiang X, Zhijian JC. 2012. The role of ubiquitylation in immune defence and pathogen evasion. Immunol 12:35-48.
- Jones FT, Axtell RC, Rives DV, Scheideler SE, Tarver J, Walker FR, Walker RL, Wineland MJ. 1991. A survey of *Salmonella* contamination in modern broiler production. J Food Prot 54:502-507.
- Kaiser P, Rothwell L, Galyov EE, Barrow PA, Burnside J, Wigley P. 2000. Differential cytokine expression in avian cells in response to invasion by *Salmonella typhimurium*, *Salmonella enteritidis*, and *Salmonella gallinarum*. Microbiology 146:3217-3226.
- Kum WW, Lo BC, Yu HB, Finlay BB. 2011. Protective role of Akt2 in *Salmonella enterica* serovar typhimurium-induced gastroenterocolitis. Infect Immun 79:2554-2566.
- Lahellec C, Colin P. 1985. Relationship between serotypes of *Salmonellae* from hatcheries and rearing farms and those from processed poultry carcasses. Br Poult Sci 26:179-186.
- Li X, Chiang H, Zhu J, Dowd SE, Zhou H. 2008. Characterization of a newly developed chicken 44K Agilent microarray. BMC Genomics 9:60.
- Lin HY, Lai RH, Lin ST, Lin RC, Wang MJ, Lin CC, Lee HC, Wang FF, Chen JY. 2012. Suppressor of cytokine signaling 6 (SOCS6) promotes mitochondrial fission via regulating DRP1 translocation. Cell Death Differ 19:1–15.

- Masuhara M, Sakamoto H, Matsumoto A, Suzuki R, Yasukawa H, Mitsui K. 1997. Cloning and characterization of novel CIS family genes. *Biochem Biophys Res Commun* 239: 439-446.
- Matsukawa T, Miyamoto T. 2011. Angiotensin II-stimulated secretion of arginine vasopressin is inhibited by atrial natriuretic peptide in humans. *Am J Physiol Regul Integr Comp Physiol* 300: R624-R629.
- Nicholson SE, Willson TA, Farley A, Starr R, Zhang JG, Baca M. 1999. Mutational analyses of the SOCS proteins suggest a dual domain requirement but distinct mechanisms for inhibition of LIF and IL-6 signal transduction. *EMBO J* 18:375-385.
- Nielsen S, Chou CL, Marples D, Christensen EI, Kishore BK, Knepper MA. 1995. Vasopressin increases water permeability of kidney collecting duct by inducing translocation of aquaporin-CD water channels to plasma membrane. *Proc Natl Acad Sci* 92:1013-1017.
- Parent C, Eichacker PQ. 1999. Neutrophil and endothelial cell interactions in sepsis. The role of adhesion molecules. *Infect Dis Clin North Am* 13:427-447.
- Redmond SB, Phongsakorn C, Andreassen CB, Dušan P, Lamont SJ. 2011. Genetic control of chicken heterophil function in advanced intercross lines: associations with novel and with known *Salmonella* resistance loci and a likely mechanism for cell death in extracellular trap production. *Immunogenetics* 63:449-458.
- Sandford EE, Orr M, Balfanz E, Bowerman N, Xian Yao Li X, Zhou H, Johnson TJ, Kariyawasam S, Liu P, Nolan LK, Lamont SJ 2011. Spleen transcriptome response

- to infection with avian pathogenic *Escherichia coli* in broiler chickens. *BMC Genomics* 12:469-481.
- Smirnova E, Griparic L, Shurland DL, van der Bliek AM. 2001. Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. *Mol Biol Cell* 12:2245–2256.
- Smyth GK. 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 1:3.
- Storey JD, Tibshirani R. 2003. Statistical significance for genomewide studies. *Proc Natl Acad Sci* 100:9440-9445.
- te Pas MFW, Hulsege I, Schokker D, Smits MA, Fife M, Zoorob R, Endale ML, Rebel JMJ. 2012. Meta-analysis of chicken - *Salmonella* infection experiments. *BMC Genomics* 13:146.
- Voetsch AC, Van Gilder TJ, Angulo FJ, Farley MM, Shallow S, Marcus R, Cieslak PR, Deneen VC, Tauxe RV. 2004. FoodNet estimate of the burden of illness caused by nontyphoidal *Salmonella* infections in the United States. *Clin Infect Dis* 38:S127-S134.
- Wang X. 2001. The expanding role of mitochondria in apoptosis. *Genes Dev* 15:2922-2933.
- Wasilewski M, Scorrano L. 2009. The changing shape of mitochondrial apoptosis. *Trends Endocrinol Metab* 20:287-294.

- Zhou H, Lamont SJ. 2007. Global gene expression profile after *Salmonella enterica* serovar *enteritidis* challenge in two F8 advanced intercross chicken lines. Cytogenet. Genome Res 117:131-138.
- Zinkernagel AS, Johnson RS, Nizet V. 2007. Hypoxia inducible factor (HIF) function in innate immunity and infection. J Mol Med 85:1339–1346.

Table 1 - List of genes that belong to Cell Cycle and Metabolism networks

Network	
Cell Cycle	Metabolism
AIFM1	AVP
BRCC3	C14orf45
CTDSPL3	DPYSL2
CYP27A1	ESRRG
GRPEL1	FURIN
HELLS	MYEF2
HTATSF1	PTPN6
LUC7L2	RABGEF1
MAD2L1	SLC25A37
PKM2	SOCS6
POLH	STEAP1
POLDIP3	
RBM12	
SAMM50	
SERPINE2	
TMEM57	
USP7	
USP52	
WDR36	

Table 2 - Log₂ fold change of hepatocyte gene expression between *S. Enteritidis* challenged and non-challenged birds

¹Positive values indicate higher expression in the first group. Negative values indicate lower expression in the challenged versus the non-challenged group. * P value < 0.05 in qPCR; ** P value < 0.01 in qPCR, q-value < 0.05 in microarray.

Gene	qPCR	Microarray
C14orf45	2.0*	3.9**
AVP	-1.9*	-1.5**
SOCS6	-2.0*	-1.5**
PKM2	-1.9*	-1.4**
USP7	-1.9*	-1.5**
SLC25A37	-2.0*	-1.5**
POLH	-1.9*	-1.5**
AIFM1	-1.9*	-1.5**
USP52	-1.2	-1.6**
PTPN6	-1.8*	-1.5**

Table 3 - Forward and reverse primer sequences used for qPCR

Gene	Forward Primer	Reverse Primer
28S	5' GGCGAAGCCAGAGGAAACT 3'	5' GACGACCGATTTGCACGTC 3'
C14orf45	5' CTGTTTTGAAATCACCGAGAG 3'	5' GGCCATCTCCAGTTTTTCC 3'
AVP	5' GTGTCTGCTGCAGTGCTGAC 3'	5' CAGCACCGTCAGGTTCTTCT 3'
SOCS6	5' ACTGGGAGATGGCTCTTGAA 3'	5' GATCAGCAGTGCTTGTGGTG 3'
PKM2	5' GGTTAAGGAGAAAGGCAAGG 3'	5' AGGTCCTGAATGTCCTTCTCTG 3'
USP7	5' CTTTATGGCCTGGAGTGAAG 3'	5' GCCAACATATCCTGTGTGCTT 3'
SLC25A37	5' GAATCCAGCTGAAGTGGTGA 3'	5' GGTGTAGCTGCGGTAGAAGG 3'
POLH	5' GATTTCGCACAACAAAATGC 3'	5' ACAGGCATCTGGCTGAAGAG 3'
AIFM1	5' TGGGAAGAAGAGCACAAACC 3'	5' CTGTGGTCCAGTTGCTCAGA 3'
USP52	5' TGCAGGAGACACAGAAGTACA 3'	5' AGGCATCAAACCTCGTGTTC 3'
PTPN6	5' CAAGAACATCTTGCCTTTTGA 3'	5' TAGGTCTTGGGGCACTCATC 3'

Supporting Information Table 1 - List of genes, *P*-values, GO annotations, directions of differential expression and fold changes

Gene	<i>P</i>-value	GO Annotation	Regulation	Fold Change
AIFM1	0.000076	Apoptosis inducing factor	Down	-1.5
AVP	0.000023	Responsible for vasoconstriction	Down	-1.5
BRCC3	0.000213	Binds damaged DNA, metal ions, and zinc ions	Down	-1.5
CTDSPL2	0.000365	Phosphatase-like hydrolase, phosphoprotein phosphatase	Down	-1.6
CYP27A1	0.000149	Electron carrier involved in oxidation-reduction reactions	Down	-1.5
C14orf45	0.000006	Chromosome 14 open reading frame 45, unknown	Up	3.9
DPYSL2	0.000367	Cell differentiation, cell development	Down	-1.6
ESRRG	0.000079	Steroid hormone receptor, metal ion and zinc ion binding	Down	-1.5
FURIN	0.000372	Amino acid cleavage enzyme involved in cell proliferation	Down	-3.0
GPREL1	0.000235	Adenyl exchange factor, chaperone and unfolded protein binding	Down	-1.6
HELLS	0.000203	Helicase activity, ATP and DNA binding	Down	-1.5
HTATSF1	0.000269	Nucleic acid and nucleotide binding	Down	-1.6
LUC7L2	0.000271	Negative regulator of striated muscle development, enzyme binding	Down	-1.5
MAD2L1	0.000098	Mitotic spindle checkpoint	Down	-1.5
MYEF2	0.000412	Part of Golgi apparatus, nucleic acid and nucleotide binding	Down	-1.6
PKM2	0.000029	Glycolysis, binding ATP, magnesium ions, and potassium ions	Down	-1.4
POLDIP3	0.000415	Positive regulator of translation, nucleic acid and nucleotide binding	Down	-3.1
POLH	0.000045	DNA synthesis, DNA repair, Post-replication repair	Down	-1.5
PTPN6	0.000171	Hydrolase, phosphatase, B-cell	Down	-1.5

		Receptor, Jak-STAT and IL-2 signalling		
RABGEF1	0.000128	GDP/GTP exchange factor	Down	-1.5
RBM12	0.000096	Nucleic acid and nucleotide binding	Down	-1.5
SAMM50	0.000200	Sorting and assembling machinery component	Down	-1.5
SERPINE2	0.000110	Serpin peptidase inhibitor	Down	-1.5
SLC25A37	0.000052	Solute carrier involved in transmembrane transport	Down	-1.5
SOCS 6	0.000027	Suppression of cytokine signalling	Down	-1.5
STEAP1	0.000181	Transmembrane epithelial antigen	Down	-1.5
TMEM57	0.000291	Component of neuron projection terminus	Down	-1.5
USP7	0.000036	Peptidase activity, ubiquitin thiolesterase	Down	-1.5
USP52	0.000371	Exonuclease and hydrolase activity, ubiquitin thiolesterase	Down	-1.6
WDR36	0.000127	RNA processing	Down	-1.5

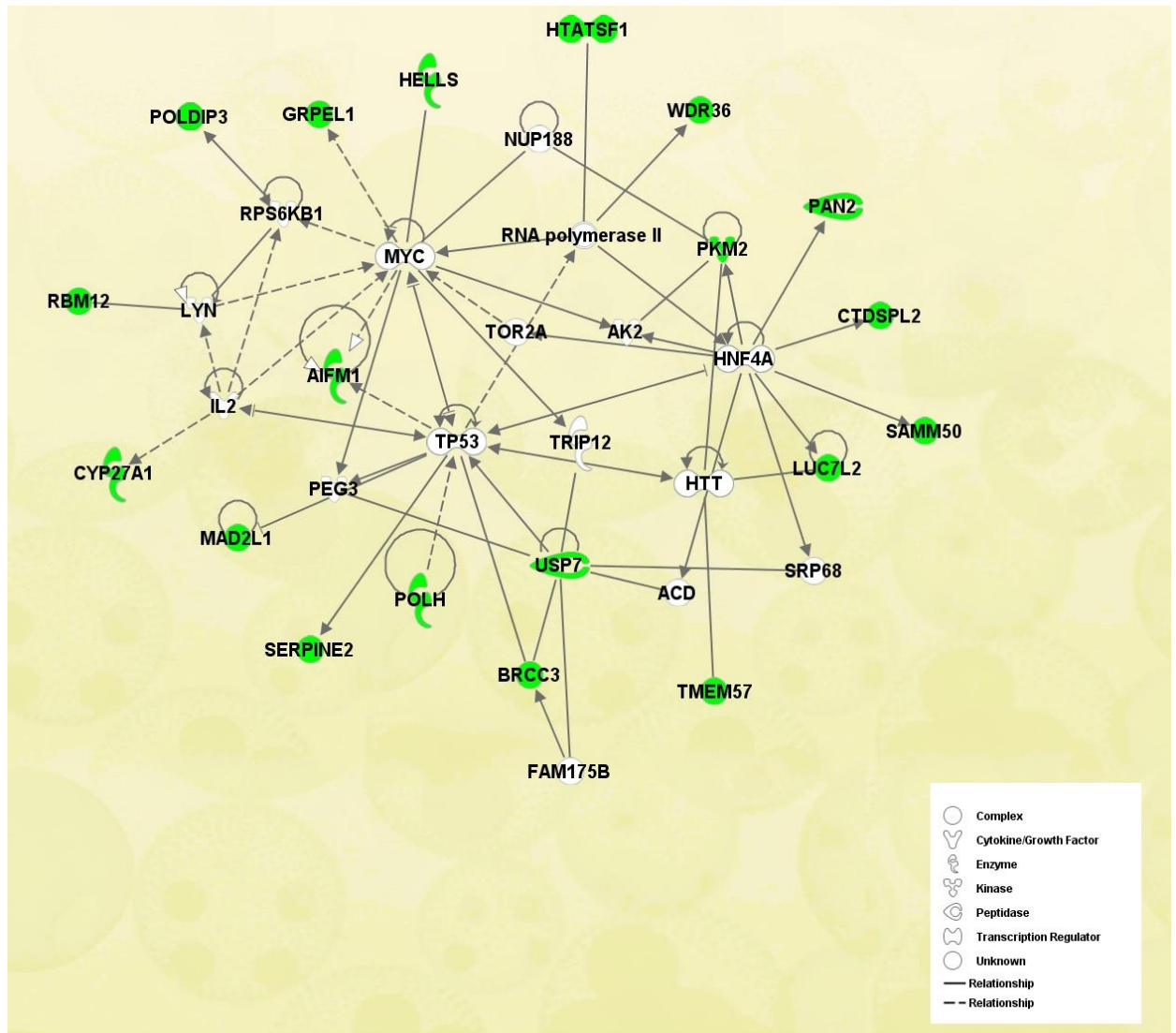


Figure 1 - Pathway analysis of cell cycle functions in broiler liver transcriptome in response to *S. Enteritidis*

Green color shows down-regulation (IPA). White molecules are not differentially expressed, but are included to illustrate association with significantly up-regulated and down-regulated genes.

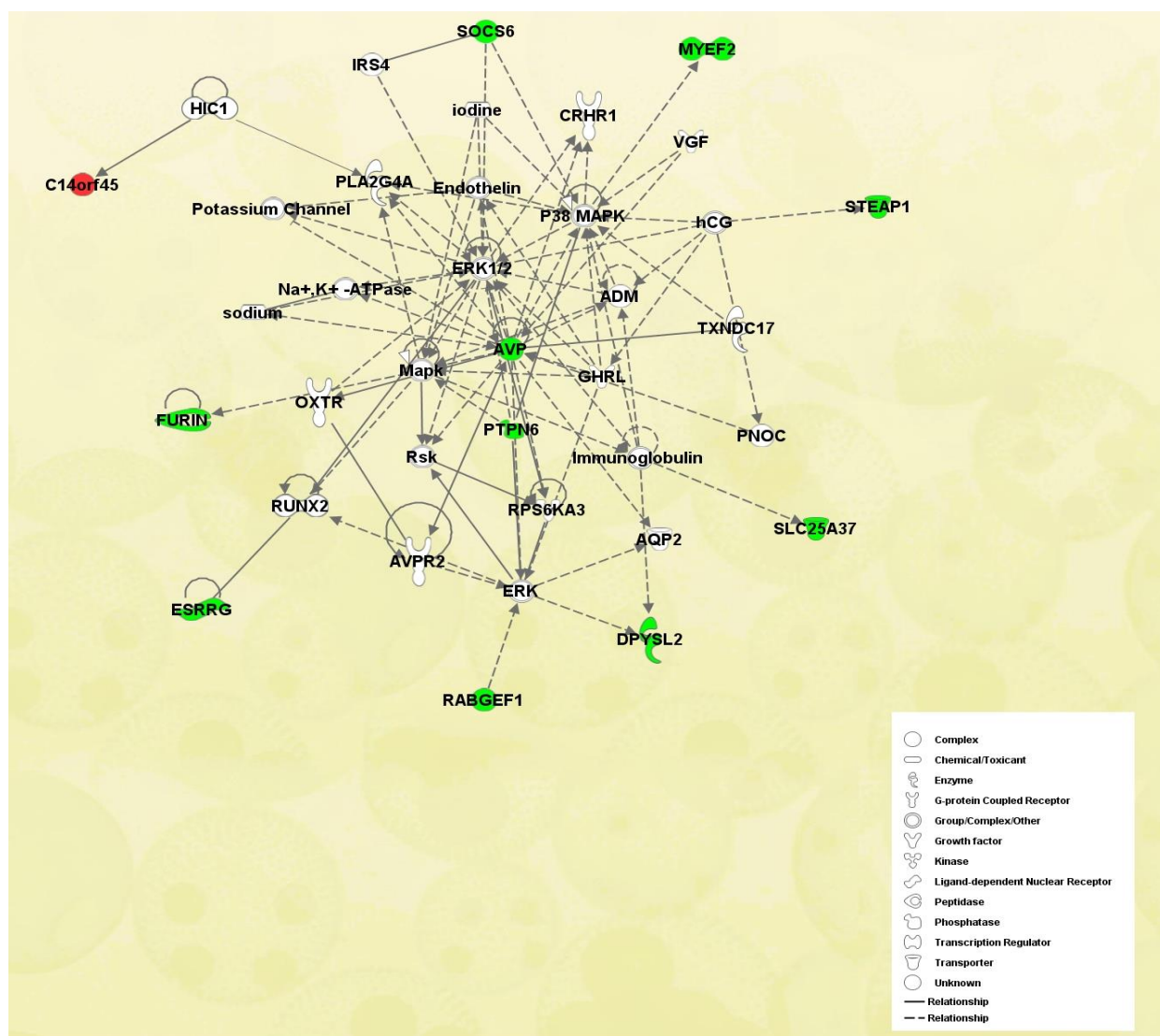


Figure 2 - Pathway analysis of metabolic functions in broiler liver transcriptome in response to *S. Enteritidis* infection

Red color shows up-regulation and green color shows down-regulation (IPA). White molecules are not differentially expressed, but are included to illustrate association with significantly up-regulated and down-regulated genes.

CHAPTER 4. RNA-SEQ ANALYSIS OF BROILER LIVER TRANSCRIPTOME REVEALS NOVEL RESPONSES TO HEAT STRESS

A paper prepared for *BMC Genomics*¹¹

D. J. Coble¹², D. Fleming¹³, M. E. Persia¹³, C. M. Ashwell¹⁴, M. F. Rothschild¹³, C. J.
Schmidt¹⁵, and S. J. Lamont^{12,16}

Abstract

Background

In broilers, heat stress can result in reduced feed consumption, digestion inefficiency, impaired metabolism, and even death. The broiler sector of the U.S. poultry industry incurs approximately \$51.8 million in heat stress-related losses annually. The objective of this study is to characterize the effects of chronic, cyclic heat stress on the transcriptome of a metabolically active organ, the liver. Characterizing the liver transcriptome of heat-stressed broilers will help clarify the effects of heat stress on broiler metabolism. This information will provide a platform for future investigations that further explore the broiler stress response to heat stress.

¹¹ For submission to *BMC Genomics*.

¹² Graduate student and Professor, respectively, Department of Animal Science, Iowa State University.

¹³ Collaborator, Department of Animal Science, Iowa State University.

¹⁴ Collaborator, Department of Poultry Science, North Carolina State University.

¹⁵ Collaborator, Department of Animal and Food Sciences, University of Delaware.

¹⁶ Author for Correspondence.

Results

Transcriptome sequencing of the livers of 8 broiler males using Illumina HiSeq 2000 technology, resulted in a total of 138 million, 100 base pair single end reads, yielding 13.8 gigabases of sequence. Forty genes were differentially expressed (DE) at a significance level of P -value < 0.05 and a fold change ≥ 2 in response to chronic, cyclic heat stress (mid-point of the last day of a 7-day cyclic heat stress of 7 hours per day), with 27 down-regulated and 13 up-regulated. Two gene networks were created from the function-based Ingenuity Pathway Analysis (IPA) of the DE genes; “Cell Signaling, Molecular Transport, Small Molecule Biochemistry” and “Endocrine System Development and Function, Small Molecule Biochemistry Cell Signaling”. Members of the MAPK signaling pathway and DE genes that are associated with MAPK-related functions were prominent in the networks. Cellular proliferation and differentiation, inflammation- and stress-related signaling, and apoptosis-associated genes were down-regulated in response to heat stress. Genes responsible for inhibiting feed intake and sphingolipid-related signaling were up-regulated. Genes involved with the regulation of inflammation, stress, thyroid hormone level, and body temperature were both up- and down-regulated.

Conclusions

In conclusion, chronic, cyclic heat stress of broilers results in metabolic changes that can be characterized through RNA-seq analysis of the liver transcriptome. The primary affected pathways included cell signaling, molecular transport, endocrine system development and signaling, and small molecule biochemistry.

Background

Heat stress is a key concern for the poultry industry, especially with the expansion of the poultry industry into regions of the world that experience extreme temperatures (Windhorst, 2006). Heat stress occurs when a negative balance exists between the net energy released by a chicken into the environment, and the amount of heat energy the chicken produces (Ajakaiye et al., 2011). Heat stress results in reduced feed consumption, digestion inefficiency, impaired metabolism, and death in broiler chickens (Emmans and Charles, 1989; Farrell and Swain, 1978; Hai et al., 2000). St. Pierre et al. (2003) estimated the annual heat stress-related economic loss incurred by broiler, layer, and turkey producers in the U.S. to be approximately \$125-165 million. The annual heat stress-related losses incurred in the broiler sector alone were estimated at \$51.8 million (St. Pierre et al., 2003). In the past few decades, genetic selection for broiler performance has resulted in remarkable improvements in growth rates (Deeb and Cahaner, 2002; McKay et al., 2000). The deleterious effects of heat stress on growth rate are greater in broilers selected for higher growth rates than those with lower growth rates (Cahaner and Leenstra, 1992). Thus, heat stress is a critical area of research for the broiler sector of the poultry industry.

Experiments investigating the effects of heat stress on the transcriptome of chicken tissues have been limited in number (Li et al., 2011; Wang et al., 2013). Li et al. (2011) investigated the transcriptome of broiler breast tissue in response to chronic, cyclic heat stress (6-hour daily cycles of 33°C, day 28 to 49 post-hatch). Of the 110 genes that were DE in response to chronic heat stress, 4 (*PM20/PM21*, *ASB2*, *USP45*,

and *TFG*) were novel heat stress-related genes. Gene ontology analysis, suggested involvement of the mitogen-associated protein kinase (MAPK), ubiquitin-proteasome, and nuclear factor kappa-light-chain-enhancer of activated B cells (*NFkB*) pathways in the broiler response to heat stress. Exposing L2 Taiwanese roosters to acute heat stress (4-hour heat stress at 38°C) resulted in the up-regulation of 169 genes and the down-regulation of 140 genes in testis samples (Wang et al., 2013). The DE genes were involved in response to stress, transport, signal transduction, and metabolism.

The objective of the current study is to characterize the effects of chronic, cyclic heat stress on the transcriptome of a metabolically active organ in broiler chicks, the liver. Characterizing the liver transcriptome of heat-stressed broilers will help clarify the effects of heat stress on broiler metabolism. This information will provide a platform for future investigation into the gene networks relevant to the production of commercial broilers resilient to heat stress.

Results

Sequencing the Transcriptome, Aligning and Mapping Reads to the Genome

Approximately 138 million, 100 base pair single-end reads were generated using Illumina HiSeq 2000 technology to sequence the cDNA libraries. All 8 samples were sequenced on 1 lane. This yielded 13.8 gigabases of total sequence and provided on average 17,249,597 reads per sample.

Using the Genomic Short-Read Nucleotide Alignment Program (GSNAP) (Wu and Nacu, 2010), 83% or more of the reads from each sample mapped back to the reference genome after alignment.

Counting Mapped Reads

On average, each sample had a total of 11,695,581 uniquely mapped reads. Approximately 2,375,095 of these reads were classified as “no feature” for each sample, meaning the reads couldn’t be assigned to any feature in the genome, which may be an effect of the sparse annotation that exists for the chicken genome. Approximately 332,309 reads per sample were classified as ambiguous, meaning the reads were assigned to multiple genomic features in the Generic Format File (GFF) file and couldn’t be assigned to a specific feature within the genome.

Testing for DE and Pathway Analysis

Forty genes were DE at a significance level of P -value < 0.05 and a fold change ≥ 2 in response to chronic, cyclic heat stress, with 27 down-regulated and 13 up-regulated. The fold changes ranged from -12.5 to 20.0 (Supplemental Table 1).

Two gene networks were created from the Ingenuity Pathway Analysis (IPA) of the DE genes; “Cell Signaling, Molecular Transport, Small Molecule Biochemistry” and “Endocrine System Development and Function, Small Molecule Biochemistry Cell Signaling”. Molecular transport and endocrine system development and function were the biological processes that distinguished the two networks. The genes were assigned to the networks based on their functions. IPA uses prior knowledge from the literature to populate the gene networks and infer relationships between the up-stream regulators and down-stream targets of the DE genes.

The DE genes in the “Cell Signaling, Molecular Transport, Small Molecule Biochemistry” network included *BRCA1/BRCA2*-containing complex, subunit 3

(*BRCC3*), *ELKS/RAB6*-interacting/CAST family member 2 (*ERC2*), fibroblast growth factor 14 (*FGF14*), *FMOD* (fibromodulin), G protein-coupled receptor 133 (*GPR133*), *LIM* and senescent cell antigen-like domains 2 (*LIMS2*), nidogen-1 (*NID1*), *ORM1*-like 3 (*Saccharomyces cerevisiae*) (*ORMDL3*), regulatory factor x-box binding family transcription factor member 6 (*RFX6*), ring finger protein 220 (*RNF220*), sodium channel, voltage-gated, type III, beta subunit (*SCN3B*), spondin 1, extracellular matrix protein (*SPON1*), splA/ryanodine receptor domain and SOCS box containing 4 (*SPSB4*), and tripartite motif containing 50 (*TRIM50*) (Fig. 1).

The DE genes in the “Endocrine System Development and Function, Small Molecule Biochemistry, Cell Signaling” network included aldo-keto reductase family 1, member C3 (*AKR1C3*), angiopoietin-like 4 (*ANGPTL4*), bradykinin receptor B1 (*BDKRB1*), basanuclin 1 (*BNC1*), cholecystokinin (*CCK*), deiodinase, iodothyronine, type II (*DIO2*), deiodinase, iodothyronine, type III (*DIO3*), keratin 14 (*KRT14*), myosin VIIA and Rab interacting protein (*MYRIP*), platelet derived growth factor D (*PDGFD*), *S100* calcium binding protein A1 (*S100A1*), *S100* calcium binding protein A4 (*S100A4*), and transient receptor potential cation channel, subfamily C, member 5 (*TRPC5*) (Fig. 2).

To determine the biological pathways elicited by heat stress in the broiler liver, the DE genes were categorized by function. Using IPA, the *P*-values of these biological functions were compared. The significance levels varied across the functional groups. Cell signaling, endocrine system development and function, molecular transport, small

molecule biochemistry, and vitamin and mineral metabolism were the most significant functions elicited in the broiler liver in response to chronic, cyclic heat stress (Fig. 3).

qPCR (Quantitative Polymerase Chain Reaction)

Five up-regulated genes and 5 down-regulated genes were selected for qPCR analysis (P -value ≤ 0.05 , Fold change ≥ 2) (Table 2). All samples represented in the RNA-seq analysis, and samples from 8 other broilers exposed to the same treatments were included in the qPCR analysis, resulting in 8 samples per group. Results from 9 of the 10 genes were in concordance between RNA-seq and qPCR analyses. For 4 genes, the qPCR differential expression between groups was in agreement with the results of the RNA-seq, and was significant. For an additional 5 genes, the relative ranking of the means was the same between heat-stress treatments for both qPCR and RNA-seq, but not significant for qPCR. There was discordance between RNA-seq and qPCR data in the relative expression levels between the heat-stress treatments for only one of the ten genes (*LIMS2*).

Discussion

Cell Signaling, Molecular Transport, Small Molecule Biochemistry Network

A centralized node in the “Cell Signaling, Molecular Transport, Small Molecule Biochemistry” network is tumor protein 53 (*TP53*) which is often referred to as the “cellular gatekeeper” because of its regulation of cell cycle arrest and apoptosis in response to cellular damage (Levine, 1997). *TP53* directly interacts in this network with amyloid β (A4) precursor protein (APP), nitric oxide synthase 2, inducible (*NOS2*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon

polypeptide (*YWHAE*), tumor necrosis factor receptor superfamily, member 1A (*TNFRSF1*), transforming growth factor beta 1 (*TGF β 1*), Finkel–Biskis–Jenkins murine osteosarcoma viral oncogene homolog (*FOS*), *SCN3B*, ubiquitin-conjugating enzyme E2D 1 (*UBE2D1*), ubiquitin-conjugating enzyme E2D 3 (*UBE2D3*), *BRCC3*, and cyclin K (*CCNK*).

Although not DE in response to the heat stress treatment in the current study, APP is a key node in the “Cell Signaling, Molecular Transport, Small Molecule Biochemistry” network. *SPON1* and *LIMS2* directly act on APP, while *ORMDL3* directly binds APP. The exact function of APP is unknown but it has been implicated as a regulator of synapse development, neural plasticity, and iron transport (Priller et al., 2006; Turner et al., 2003; Duce et al., 2010). The accumulation of amyloid β , a product of APP proteolysis, induces apoptosis through mitochondrial dysfunction in yeast (Sakono et al., 2013; Cha et al., 2012). Sakono et al. (2013) reported the cytotoxic formation of amyloid β fibrils by heat shock proteins on lipid membranes in response to heat stress. In the current study, *SPON1* was down-regulated in response to the heat stress treatment. *SPON1* (also known as F-spondin) encodes for a secreted adhesion molecule that attaches to the extracellular matrix (ECM) of human cells, resulting in neuronal development and repair, cartilage metabolism, and inhibition of APP cleavage into β -secretase (Kitagawa et al., 2012; Attur et al., 2009; Ho and Südhof, 2004). The down-regulation of *SPON1* leads to the cleavage of APP into amyloid β ; this directly relates to the accumulation of amyloid β fibrils and apoptosis.

Both *LIMS2* and *ORMDL3* were up-regulated in response to the heat stress treatment in the current study. *LIMS2* encodes for a protein that regulates physiological processes through localization at ECM contact sites in human gastric cancer cells (Kim et al., 2006). By forming a complex with integrin-linked kinase (ILK), *LIMS2* inhibits the formation of the *LIMS1*-ILK-parvin complex, thus reducing cell migration and spreading in human and rat embryonic cells (Zhang et al., 2002a; Zhang et al., 2002b). The up-regulation of *LIMS2* may reflect a response to reduce cell migration. *ORMDL3* belongs to a family of genes (ORM) that has been implicated in sphingolipid homeostasis through phosphorylation in yeast (Breslow et al., 2010). In yeast, heat stress has been reported to result in a transient accumulation of sphingolipid biosynthesis through ORM phosphorylation (Sun et al., 2012). Sphingolipid accumulation leads to cell growth, cell differentiation, cell senescence, apoptosis, and angiogenesis through the sphingolipid signaling pathways in yeast (Hannun and Obeid, 2008; Dickson, 2010; Nikolova-Karakashian and Rozenova, 2010). Further integrating the functions of *SPON1* and *ORMDL3*, Haughey et al. (2010) suggested that amyloidogenic APP proteolysis, APP processing that leads to amyloid β fibril accumulation, occurs within lipid rafts of human neuronal cells. Lipid rafts, specialized membrane domains, mainly consist of sphingolipids, cholesterol, and associated proteins. The up-regulation of *ORMDL3* may reflect an increase in phosphorylation, resulting in sphingolipid regulation and cell signaling through sphingolipid biosynthesis pathways.

Although not DE in response to the current heat stress treatment *NOS2*, *YWHAE*, *TNFRSF1*, *TGF β 1*, *FOS*, and *UBE2D1* are deemed important because they directly act

upon *TP53*; are directly acted upon by *TP53*, and directly interact with DE genes. *NOS2* is responsible for the production of nitric oxide, a potent source of reactive nitrogen species (RNS) that plays a key role in host intracellular defence (Kuang et al., 2010). *NOS2* is directly bound by *SPSB4*, which was down-regulated in response to the heat stress treatment in the current study. *SPSB4* negatively regulates nitric oxide (NO) production and limits cellular toxicity by linking *NOS2* and the Elongin B/C–Cullin-5-SOCS box protein (*ECS*)-*E3* ubiquitin ligase complex, resulting in ubiquitination and proteasomal degradation of *NOS2* (Nishiya et al., 2011). Chen et al. (2003) reported that NO plays a key role in maintaining endothelial integrity and protecting from apoptosis after liver damage. The down-regulation of *SPSB4* may represent an action responsible for promoting cell survival.

YWHAE encodes for tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide (*I4-3-3ε*), which is crucial for ribosomal S6 kinase (*Rsk*)-mediated apoptosome inhibition (Kim et al., 2012). *YWHAE* is directly bound by *ERC2*, which was up-regulated in response to the heat stress treatment in the current study. *ERC2* encodes for a protein that localizes at the voltage dependent calcium channels (VDCC) in mouse neuronal cells (Billings et al., 2012). Cellular amyloid β fibrils accumulate in response to heat stress (Sakono et al., 2013). Texel and Mattson (2011) reported that excess amyloid β fibril formation results in perturbed Ca^{2+} homeostasis in mouse neuronal cells and can possibly trigger apoptosis from Ca^{2+} overload. The up-regulation of *ERC2* may reflect a host response to return to homeostatic Ca^{2+} levels after a transient accumulation of amyloid β fibrils.

TNF- α encodes for a cell-death mediator that acts by binding TNF receptors and inducing apoptosis in mice (Imao et al., 2006). There are no reports of the *TNF- α* gene in chicken, but TNF receptors such as *TNFRSF1* have been previously reported in the chicken (Bae et al., 2008). *TNFRSF1* indirectly acts upon *NID1*, which was up-regulated in response to the heat stress treatment in the current study. As a basement membrane protein, *NID1* is responsible for anchoring epithelium to loose connective tissue via the cell matrix in mouse tumor cells (Ries et al., 2001). Chen et al. (2008) reported disruptions in cell-to-cell junctions of monkey Sertoli cells in response to heat stress. The up-regulation of *NID1* may be a mechanism to maintain hepatocyte integrity.

TGF β 1 is associated with tumor suppression by promoting both cell differentiation and growth inhibition (Ewart-Toland et al., 2004). *TGF β 1* is directly bound by *FMOD*, which was down-regulated in response to the heat stress treatment in the current study. As a liver proteoglycan, FMOD regulates ECM organization by fibrogenic stimuli in mouse liver cells and has been described as essential for tissue repair (Mormone et al., 2012). The down-regulation of *FMOD* may be a mechanism to regulate extracellular organization, thus allowing the dissociation of SPON1 from the ECM, the localization of LIMS2 to EMC contact sites, and the anchoring of epithelial tissue to connective tissue via NID1 and the cell matrix. *TGF β 1* indirectly acts on *RNF220*, which was up-regulated in response to the heat stress treatment in the current study. *RNF220* encodes for a RING E3-Ubiquitin ligase that targets Sin3B, a global co-repressor of gene transcription, for ubiquitination and proteasomal degradation in mice

(Kong et al., 2010). The up-regulation of *RNF220* represents an action to promote gene transcription.

The *FOS* transcription factor is responsible for cyclic adenosine monophosphate (cAMP)-mediated suppression of inflammatory cytokine production (Yoshida et al., 2012). *FOS* is indirectly bound by *FGF14*, which was up-regulated in response to the heat stress treatment in the current study. Fibroblast growth factors (FGF) are from a family of paracrine molecules that exert mitogenic, morphogenic, and angiogenic activities in various bovine cells and tissues throughout embryonic, fetal, and postnatal development (Fields et al., 2011). *FGF14* also activates signaling through the MAPK signal transduction pathway (Shakkottai et al., 2009; Verbeek et al., 2008). The up-regulation of *FGF14* reflects an increase in mitogenic signaling, cell morphogenesis, and angiogenesis.

UBE2D1 is an ubiquitin-conjugating enzyme that possesses ubiquitin-protein ligase activity (Hattori et al., 2007). Along with *UBE2D3*, *UBE2D1* directly binds to *TRIM50*, which was down-regulated in response to the heat stress treatment in the current study. As a RING E3-Ubiquitin ligase, *TRIM50* interacts with histone deacetylase (HDAC) 6, which participates in inflammation suppression, fibroblast migration, and lymphocyte chemotaxis in mouse embryo fibroblasts (Li et al., 2013; Fusco et al., 2012). The down-regulation of *TRIM50* may be a mode to suppress inflammation, promote fibroblast migration, and promote lymphocyte chemotaxis. *UBE2D1* also directly binds *BRCC3*, which in turn indirectly interacts with *CCNK*. *BRCC3* is a deubiquitinating enzyme that is a key regulator of angiogenesis in human

peripheral blood leukocytes (Miskinyte et al., 2011). *BRCC3* was down-regulated in response to the heat stress treatment in the current study. In conjunction with the up-regulation of *FGF14*, the down-regulation of *BRCC3* may reflect a mechanism responsible for regulating angiogenesis in response to the chronic heat stress treatment.

TP53 directly acts on *CCNK* and *SCN3B*. The action of *CCNK* includes restoring cell cycle progression, DNA damage response, and endoderm development in mouse hepatocytes, yeast cells, and human HeLa cells, respectively (Mori et al., 2002; Edwards et al., 1998; Blazek et al., 2011). *CCNK* directly binds *RFX6*, which was up-regulated in response to the heat stress treatment in the current study. *RFX6* is a gene that is expressed in the pancreas and liver and is involved in B cell maturation in human and mouse models (Aftab et al., 2008; Taleb and Polychronakos, 2011). Adachi et al. (2004) identified *SCN3B* as a *TP53*-inducible proapoptotic gene in mouse embryonic cells. *SCN3B* was down-regulated in response to the heat stress treatment in the current study. The down-regulation of *SCN3B* and *RFX6* may lead to a reduction of *TP53*-mediated apoptosis and B cell maturity, respectively.

The “Cell Signaling, Molecular Transport, Small Molecule Biochemistry” network includes a G protein-couple receptor (GPR) rich sub-network. In this sub-network, a central GPR directly interacts with another GPR (*GPR133*) that is down-regulated, 8 other GPRs that weren’t DE in the current study (*GPR111*, *GPR114*, *GPR144*, *GPR152*, *GPR157*, *GPR162*, *GPR171*, and *GPR174*), a trace amine associated receptor (*TAAR8*), and a vomeronasal type-1 receptor 2 (*VNIR2*) that connects the sub-network to the rest of the network through an indirect interaction with *FOS*. *TAAR* is a

novel member of the GPR family which trace amines have been shown to act upon (Maguire et al., 2009). *VNIR2* belongs to a family of receptors that transfers olfactory signals from the environment (Rodriguez and Mombaerts, 2002). Although associated with olfactory function, other studies have reported differential hepatic expression of *VNIR2*, which explains its representation in the network (Olender et al., 2008). As an adhesion GPR, *GPR133* is thought to participate in cell-to-cell and cell-matrix interactions (Yona et al., 2008; Kwakkenbos et al., 2004). Using mammalian pcDps expression vectors transfected with mouse and human *GPR133*, Bohnkamp and Schöneberg, (2011) reported that *GPR133* activates the Gs protein/adenylyl cyclase pathway. The Gs protein/adenylyl cyclase pathway is used to transfer signals from cell surface receptors to the nucleus of cells (Goldsmith and Dhanasekaran, 2007). The down-regulation of *GPR133* represents a decrease in the cellular stress response signaling through the adenylyl cyclase pathway.

Endocrine System Development and Function, Small Molecule Biochemistry, Cell Signaling Network

The “Endocrine System Development and Function, Small Molecule Biochemistry, Cell Signaling” network contains several central nodes. *NFκB* is a centralized node of focus because of its involvement in cellular stress. Expressed in almost all animal cells, *NFκB* is stimulated by stress, cytokines, free radicals, bacterial and viral agents (Gilmore, 2006; Perkins, 2007; Gilmore et al., 1999; Tian and Brasier, 2003; Tuggle et al., 2007). *NFκB* plays an important role in cytokine production and synaptic plasticity (Freudenthal et al., 1998; Albensi and Mattson, 2000). It directly acts upon *CCK*, representing one of only two direct interactions in the entire network. *CCK* is

also the only centralized node in the network that was DE in response to the heat stress treatment. *CCK* was up-regulated in response to the heat stress treatment in the current study. It is a potent inhibitor of feed intake in chickens (Rodriguez-Sinovas, 1997). This is accomplished by stimulating gastric emptying, stimulating the release of pancreatic digestive enzymes, and functioning as a signal to the brainstem to depress appetite (Richards and Proszkowiec-Weglarz, et al., 2007). Although the intestines are responsible for most of the body's CCK production, the liver is also a site of CCK secretion. During heat stress, animals experience a negative balance between the net energy released into the surrounding environment, and the amount of heat energy the animal produces (Ajakaiye et al., 2011). The inhibition of feed intake could be a mechanism to reduce the additional heat that is produced from digestive metabolism. Both *NFKB* and *CCK* indirectly act upon *Insulin*. Insulin has anabolic effects in the metabolism of carbohydrates, lipids, and proteins (Tesseraud et al., 2007). In the chicken liver, Insulin is responsible for glucose disposal and lipogenesis (Ji et al., 2012). It also promotes growth and cell division, while suppressing apoptosis (Dupont et al., 2009).

In this network, *Insulin* is subsequently acted upon by two GPRs (*GPR55* and *GPR119*), *MYRIP*, and *TPRC5*. *MYRIP* is up-regulated in response to the current heat stress treatment, while *TPRC* is down-regulated. *MYRIP* encodes for an actin motor that interacts with Rab GTPases, drives vesicle and organelle motility, and participates in endosome recycling in Madin-Darby canine kidney (MDCK) cells (Schollenberger, 2010). *TPRC5* belongs to a family of transient receptor potential channels (TRPC) that are non-selective cation channels that appear to be activated by both GPRs and growth

factor receptors coupled to phospholipase C in human embryonic cells (Blair et al., 2009). Wang et al. (2011) suggested that *TRPC5* is used in *CCK* cellular signaling. The up-regulation of *MYRIP* may be a mode to increase intracellular compartment motility and endosome recycling. The down-regulation of *TRPC5* may represent a mechanism to reduce cellular signaling through *TRPC5*, possibly *CCK* signaling. *NFKB* is also indirectly acted upon by two DE genes, *S100A1* and *S100A4*. *S100A1* is up-regulated in response to the current heat stress treatment, while *S100A4* was down-regulated. The S100 family of proteins have been implicated in the regulation of Ca^{2+} homeostasis, cell growth and differentiation, protein phosphorylation, and the inflammatory response in mouse brain cells, human macrophages, and mouse embryonic cells, respectively (Donato, 2001). The DE of an S100 family member (*S100A11*) and other genes involved in Ca^{2+} -related transport and signaling, was observed in catfish exposed to heat stress (Liu et al., 2013). The up-regulation of *S100A1* and the down-regulation of *S100A4* may reflect a response to regulate cell differentiation, Ca^{2+} - and phosphorylation-dependent signaling, and host response to inflammation.

In addition to the interaction with *CCK*, *S100A1*, *S100A4*, and *Insulin*, *NFKB* also indirectly signals to murine thymoma viral oncogene homolog (*Akt*), vascular endothelial growth factor (*VEGF*), extracellular-regulated signal kinases (*ERK*), *ERK1/2*, and mitogen-activated protein kinase p38 (*P38 MAPK*). *Akt* regulates cell survival, cell cycle, metabolism, and angiogenesis through interaction with downstream effectors (Lodish et al., 1999; Ramaswamy et al., 1999; Kandel et al., 2002; Whiteman et al., 2002; Chen et al., 2005). *VEGF* is a signaling molecule that plays an essential role in

embryonic vasculogenesis and angiogenesis (Carmeliet et al., 1996; Ferrara et al., 1996). *ERK* and *ERK1/2* are expressed in all tissues and participate in the Ras-Raf-MEK-ERK (MAPK) signal transduction cascade (Lu and Xu, 2006; Roskoski, 2012). In the absence of mitogens and stress, *ERK* is associated with *MEK* and is anchored in the cytoplasm (Fukuda et al., 1997). Mitogenic stimulation or stress induces biphasic *ERK* (*ERK1* and *ERK2*) activation, with an immediate burst of kinase activity and a second wave of lower kinase activity that last throughout the G1 phase of the cell cycle for up to 6 hours (Kahan et al., 1992; Meloche et al., 1992; Meloche et al., 1995). After phosphorylation of *ERK1* and *ERK2*, *MEK1* and *MEK2* dissociate from *ERK1* and *ERK2* (Khokhlatchev et al., 1998). *ERK1/2* then translocates to the nucleus and remains there throughout the G1 phase, and can be reversed upon removing the mitogenic stimulus (Adachi et al., 1999; Matsubayashi et al., 2001; Whitehurst et al., 2002; Kondoh et al., 2005). This cascade is directing cellular responses to a diverse array of stimuli such as mitogens, osmotic stress, heat stress, and proinflammatory cytokines (Cobb and Goldsmith, 1995). This signaling results in the regulation of cell proliferation, gene expression, cell differentiation, mitosis, cell survival, and apoptosis (Cobb and Goldsmith, 1995). *P38 MAPK* is a member of the MAPK signal transduction pathway and is a regulator of inflammatory cytokine biosynthesis (Lee et al., 1994; Rouse et al., 1994).

NFKB signals to nodes that interact with genes that are peripherally located in the network. *Akt* is indirectly acted upon by *CCK*, *KRT14*, and prokineticin receptor 1 (*PROKR1*). *KRT14* is down-regulated in response to the current heat stress treatment, whereas *PROKR1* is not DE. *KRT14* has cytoprotective function and modulates

phosphatidylinositol 3-kinase (PI3K)/*Akt*-mediated cell proliferation and/or Notch1-dependent cell differentiation in human epithelial cells (Alam et al., 2011). *PROKR1* is bound by prokineticins, which leads to calcium mobilization, stimulation of PI3K turnover, and the activation of the MAPK signal transduction cascade (Lin et al., 2002; Soga et al., 2002; Negri et al., 2007). The down-regulation of *KRT14* may represent a mechanism to regulate cellular proliferation and differentiation through certain cellular pathways.

VEGF indirectly acts upon *ANGPTL4*, *BNC1*, *GPR116*, *PDGFD*, and *TAAR5*, while being indirectly acted upon by *SUCNR1* and *S1004A*. The heat stress treatment in the current study resulted in the down regulation of *ANGPTL4*, *BNC1*, *PDGFD*, and *S1004A*. *ANGPTL4* functions in angiopoietin promotion, angiopoietin inhibition, and cholesterol synthesis in the mouse liver by lipase inhibition (Kersten, 2005; Lichtenstein et al., 2007). *BNC1* encodes for a polymerase I and II transcriptional factor that regulates ribosomal nucleic acid (rRNA) expression, thus increasing the potential for cellular proliferation and differentiation in squamous cell carcinomas (Boldrup et al., 2012). *GPR116* belongs to the G protein-couple receptor family and *TAAR5* belongs to the trace amine associated receptor family. *SUCNR1* participates in signaling to restore damage tissue, particularly in stress that is associated with energy balance (Ariza et al., 2012). *PDGFD* promotes cell proliferation, migration, survival, transformation, and differentiation through downstream messengers in MAPK and PI3K pathways in human cancer cells (Wang et al., 2010). The down-regulation of *ANGPTL4* reflects angiopoietin regulation and may reflect a response to reduce hepatic cholesterol synthesis. The down-

regulation of *BNC1* may represent a response to reduce cellular proliferation and differentiation. The down- regulation of *PDGFD* may reflect a mechanism to reduce cell proliferation, movement, and transformation.

ERK is indirectly acted upon by angiopoietin-like 1 (*ANGPTL4*), *PDGFD* and thymocyte selection associated family member 2(*THEMIS2*), while indirectly acting upon *BDKRB1*, *DIO2*, *DIO3*, *GPR126* and *CCK*, and sharing a two-way signaling relationship with *GPRC5C*. The heat stress treatment in the current study resulted in the up-regulation of *BDKRB1* and *DIO3*, while resulting in the down-regulation of *DIO2*. *ANGPTL4* inhibits the proliferation, migration, tube formation, and adhesion of endothelial cells as well as tumor growth in human lung cells (Kuo et al., 2013). *BDKRB1* encodes for a bradykinin receptor that belongs to the rhodopsin-like GPR that function in the regulation of inflammation (Leschner et al., 2011). The binding of *BDKRB1* leads to an increase in cytosolic Ca^{2+} and results in an inflammatory response in mouse embryo cells (Prado et al., 2002). Thyroid hormone plays roles in aerobic metabolism and thermal control (Silva, 2005). *DIO2* is involved in the preservation of thyroid hormone in rat brain tissue (Peeters et al., 2001). Upon early signs of liver damage in rats, it has been proposed that *DIO3* participates in maintaining energy equilibrium, thus avoiding global disruption of the hypothalamic-pituitary-thyroid axis (Dudek et al., 2013). *DIO3* is also associated with the inactivation and degradation of thyroid hormone (Friesema et al., 2006). *THEMIS2* has been suggested to transduce signals that are initiated from B cell receptors (Lesourne et al., 2012). *GPR126* and *GPRC5C* are members of the previously described GPR family. The up-regulation of

BDKRB1 may reflect an inflammatory response to the current heat stress. The combined down-regulation of *DIO2* and up-regulation of *DIO3* may reflect an action to inactivate and degrade thyroid hormone, thus regulating metabolism and body temperature. *P38 MAPK* indirectly acts upon *DIO2* and *DIO3*, while being acted upon by *THEMIS2* and N-arachidonylglycine, and being inhibited by activin-like kinase 3-bone morphogenetic protein receptor 2 (*ALK3-BMPR2*). Takenouchi et al. (2012) suggested that N-arachidonylglycine possesses anti-inflammatory properties that counteract the actions of pro-inflammatory macrophages. *ALK3-BMPR2* is a gene that functions in inflammation regulation in mice endothelial cells (Kim et al., 2013).

Another node of the “Endocrine System Development and Function, Small Molecule Biochemistry, Cell Signaling” network is *ERK1/2*. It indirectly acts upon *AKR1C3*, while indirectly being acted upon by *BDKRB1*, *CCK*, C-type lectin domain family 4 (*CLEC4A*), N-arachidonylglycine, *SI00A1*, and taste receptor, type 1, member 1 (*TAS1R1*). *ERK1/2* is also inhibited by *ALK3-BMPR2*. *AKR1C3* was down-regulated in response to the heat stress treatment in the current study. *AKR1C3* functions in inhibition of cell differentiation and the reduction of aldehydes and ketones into primary and secondary alcohols in human promyelocytic leukemia cells (Desmond et al., 2003; Jez and Penning, 2001). *CLEC4A* belongs to a family of Ca^{2+} -dependent glycan binding proteins (C-type lectins) that function as signaling receptors in inflammation, cellular tumor response, and cellular viral response (Varki et al., 2009). *TAS1R1* has been implicated in glucose sensing and energy uptake (Byerly et al., 2010). The up-regulation

of *AKR1C3* may reflect an increase in cell differentiation and alcohol metabolism in response to chronic heat stress.

Li et al. (2011), using gene ontology analysis, also suggested that members of the MAPK signal transduction cascade (*MAPK*, *P38 MAPK*, *ERK*, *ERK1/2*) were present in the broiler breast muscle transcriptome in response to chronic heat stress. The MAPK pathway transfers signals from cell surface receptors to the nucleus, regulating gene expression, mitosis, metabolism, motility, survival, apoptosis, and cellular differentiation (Cargnello and Roux, 2011). In the current study, the genes DE in the broiler liver transcriptome in response to heat stress included a polymerase I and II transcriptional factor (*BNC1*), thyroid hormone genes (*DIO2* and *DIO3*), a movement gene (*LIMS2*), cell survival genes (*SPSB4* and *NIDI*) an apoptosis-related gene (*SPON1*), and a gene involved with cellular differentiation, *PDGFD*. The presence of the MAPK pathway and DE genes associated with MAPK-related functions, strongly suggests that the MAPK pathway is elicited in a wide range of tissues in the broiler in response to chronic heat stress.

To summarize these results, the liver transcriptome of broilers in response to heat stress is characterized by a prominent down-regulation of genes involved with cellular proliferation and differentiation, a prominent down-regulation of genes involved with cellular inflammation- and stress-related signaling, DE in both directions of genes associated with regulating inflammation and stress, DE that reflects the breakdown of thyroid hormone, DE that reflects cell survival, and the up-regulation of genes

responsible for inhibiting feed intake and promoting sphingolipid-related signaling function.

Conclusions

In conclusion, chronic, cyclic heat stress results in metabolic changes that were observed through RNA-seq transcriptome analysis of the broiler liver. The primary affected pathways included cell signaling, molecular transport, endocrine system development and signaling, and small molecule biochemistry. The information from this study provides novel insight into the effects of heat stress on metabolic-related pathways; providing a platform for future investigations into the gene networks involved in the broiler stress response.

Methods

Tissue Collection

From 22 to 28 days of age, heat-stressed broilers were exposed to daily 7-hour cycles of 35° C, while a control group was kept at 25° C throughout this time. Liver samples were harvested from 4 sets of full-sibs at the midpoint of the last heat cycle on day 28, with one bird of each pair from the heat-stress treatment and one from the control group; these samples were used for transcriptome sequencing. Liver samples were also harvested from 8 more broilers (4 control and 4 heat-stressed), and included with the 8 samples used for RNA-seq for qPCR validation of the transcriptome sequencing.

Sequencing the Transcriptome

Total RNA was isolated from the liver samples following the procedures described in Cheeseman et al. (2007). The RNA was submitted to the Iowa State University DNA Facility, where the liver transcriptomes were sequenced using Illumina HiSeq 2000 technology (http://www.illumina.com/systems/hiseq_2000_1000.ilmn).

Quality Control of RNA-seq Reads

Read quality was controlled using the FastQC suite version 0.10.1. (<https://preview.iplantcollaborative.org/de/#workspace>). A Phred score of 28 was used to control for read quality.

Mapping Reads to Genome

The RNA-seq reads were aligned using GSNAP (Wu and Nacu, 2010). This program can be used to align single- or paired- end reads that are as short as 14 base pairs in length. The RNA-seq reads were mapped back to the genome based on the NCBI *Gallus gallus* Build 4.0 reference genome. The experiment was run using “32 worker threads” to optimize running efficiently on the server. The output was set to “split” and put into a SAM format for convenient downstream analysis. During the alignment, the “-m” setting for mismatches was set at default to allow GSNAP to auto set the number of allowed mismatches based on read length. This allowed for the best alignment across intron-exon boundaries.

Counting Mapped Reads

Raw reads were calculated and annotated using the HT-seq package (version 4.7) in Python (<http://www-huber.embl.de/users/anders/HTSeq/>), which is an open source

program that allows the input of raw counts from aligned reads to be annotated with gene names based on genomic features. The parameters “m<mode>”, and “--mode=<mode>” were set to “intersection non-empty”. These settings are less restrictive and allow HT-seq to identify as many genes as possible based on overlap resolution. The parameter “-stranded” was set to “no” because the cDNA library preparation was not strand specific. The parameter “-a <minqual>” was set to “default (0)” because the read quality threshold was cutoff at PHRED 28. The feature type was set at “-- type=exon” so that the reads would be counted based on exons. The GFF attribute used as the feature ID (“-idattr=<id attribute>”) was set to “gene_id”, allowing the NCBI GFF file to output gene names. In the case of unmapped reads, special categories were provided that explained why the reads were not mapped back to the reference genome.

Testing for DE

To account for the over-dispersion associated with RNA-seq data, the QuasiSeq (Lund et al., 2012) package developed in R (R Development Core Team, 2011) was used to analyze the data for DE. The QuasiSeq package is a quasi-likelihood approach based on a negative binomial distribution. The important benefit of using QuasiSeq is the ability to incorporate the uncertainty in the modeled variances using the estimated quasi-likelihood dispersion parameter (Lund et al., 2012).

qPCR and Statistical Analysis

Total RNA was isolated and quantitative PCR was performed according to previously described procedures (Cheeseman et al., 2007; Eldaghayes et al., 2006). All

reactions were run in triplicate. The forward and reverse primers used for qPCR are listed in Table 1. The mRNA expression levels as mean adjusted Ct values of each sample were analyzed using the ANOVA analysis of JMP 8.0.2 software on combined data (heat-stress challenged and control-temperature broilers) for each gene separately, using the following model:

$$Y = \mu + \text{challenge} + \text{replicate} + e.$$

Challenge and replicate were considered fixed effects. Student's t test of JMP 8.0.2 software was used to determine significant differences (P -value < 0.05) between heat-challenged and control treatments.

Authors' contributions

DJC participated in the heat stress trials of the birds, participated in sample collection, carried out RNA isolation, prepared samples for RNA-seq, carried out DE analysis, carried out pathway analysis, and drafted the manuscript. DF participated in the heat stress trials of the birds, participated in sample collection, carried out RNA-seq read alignment and mapping, carried out RNA-seq read counting, and helped draft the manuscript. MEP participated in the heat stress trials of the birds and sample collection. CMA participated in the heat stress trials of the birds and sample collection. MFR participated in sample collection and manuscript preparation. CJS was involved in designing the heat stress trials of the birds. SJL designed the heat stress trials of the birds, participated in the heat stress trials of the birds, participated in sample collection, and helped draft the manuscript.

Acknowledgements

Supported by USDA Agriculture and Food Research Initiative Competitive Grant number 2011-67003-30228, a USDA National Needs Fellowship awarded to D. Fleming, a George Washington Carver Fellowship awarded to D.J. Coble, and Integrative Graduate Education Research and Training Fellowships awarded to D. Fleming and D. J. Coble.

References

- Adachi K, Toyota M, Sasaki Y, Yamashita T, Ishida S, Ohe-Toyota M, Maruyama R, Hinoda Y, Saito T, Imai K, Kudo R, Tokino T: **Identification of SCN3B as a novel p53-inducible proapoptotic gene.** *Oncogene* 2004, **23**(47):7791-7798.
- Adachi M, Fukuda M, Nishida E: **Two co-existing mechanisms for nuclear import of MAP kinase: passive diffusion of a monomer and active transport of a dimer.** *EMBO J* 1999, **18**(19):5347-5358.
- Aftab S, Semenec L, Chu JS, Chen N: **Identification and characterization of novel human tissue-specific RFX transcription factors.** *BMC Evol Biol* 2008, **8**:226.
- Ajakaiye J, Perez-Bello A, Mollineda-Trujillo A: **Impact of heat stress on egg quality in layer hens supplemented with I-ascorbic acid and dl-tocopherol acetate.** *Vet Archiv* 2011, **81**:119-132.
- Alam H, Sehgal L, Kundu ST, Dalal SN, Vaidya MM: **Novel function of keratins 5 and 14 in proliferation and differentiation of stratified epithelial cells.** *Mol Biol Cell* 2011, **F2**(21):4068-4078.
- Albensi BC A, Mattson MP: **Evidence for the involvement of TNF and NF- κ B in hippocampal synaptic plasticity.** *Synapse* 2000, **35**:151-159.
- Ariza AC, Deen PM, Robben JH: **The succinate receptor as a novel therapeutic target for oxidative and metabolic stress-related conditions.** *Front Endocrinol (Lausanne)* 2012, **3**:22.
- Attur MG, Palmer GD, Al-Mussawir HE, Dave M, Teixeira CC, Rifkin DB, Appleton CT, Beier F, Abramson SB: **F-spondin, a neuroregulatory protein, is up-**

regulated in osteoarthritis and regulates cartilage metabolism via TGF-beta activation. *FASEB J* 2009, **23**(1):79-89.

Bae WY, Kim LS, Hur DY, Jeong SW, Kim JR: **Secondary apoptosis of spiral ganglion cells induced by aminoglycoside: Fas-Fas ligand signaling pathway.** *Laryngoscope* 2008, **118**(9):1659-1668.

Billings SE, Clarke GL, Nishumune H : **ELKS1 and Ca(2+) channel subunit b4 interact and colocalize at cerebellar synapses.** *Neuroreport* 2012, **23**:49-54.

Blair N, Kaczmarek J, Clapham DE: **Intracellular calcium strongly potentiates agonist-activated TRPC5 channels.** *J Gen Physiol* 2009, **133**:525-546.

Blazek D, Kohoutek J, Bartholomeeusen K, Johansen E, Hulinkova P, Luo Z, Cimermancic P, Ule J, Peterlin BM: **The Cyclin K/Cdk12 complex maintains genomic stability via regulation of expression of DNA damage response genes.** *Genes Dev* 2011, **25**(20):2158-2172.

Bohnekamp J, Schöneberg T: **Cell adhesion receptor GPR133 couples to Gs protein.** *J Biol Chem* 2011, **286**(49):41912-41916.

Boldrup L, Coates PJ, Laurell G, Nylander K: **p63 Transcriptionally regulates BNC1, a Pol I and Pol II transcription factor that regulates ribosomal biogenesis and epithelial differentiation.** *Eur J Cancer* 2012, **48**(9):1401-1406.

Breslow DK, Collins SR, Bodenmiller B, Aebersold R, Simons K, Shevchenko A, Ejsing CS, Weissman JS: **Orm family proteins mediate sphingolipid homeostasis.** *Nature* 2010, **463**(7284):1048-1053.

- Byerly MS, Simon J, Cogburn LA, Le Bihan-Duval E, Duclos MJ, Aggrey SE, Porter TE: **Transcriptional profiling of hypothalamus during development of adiposity in genetically selected fat and lean chickens.** *Physiol Genomics* 2010, **42**(2):157-167.
- Cahaner A, Leenstra F: **Effects of high temperature on growth and efficiency of male and female broilers from lines selected for high weight gain, favorable feed conversion, and high or low fat content.** *Poultry Sci* 1992, **71**(8):1237-1250.
- Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A: **Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele.** *Nature* 1996, **380**(6573):435-439.
- Cha MY, Han SH, Son SM, Hong HS, Choi YJ, Byun J, Mook-Jung I: **Mitochondria-specific accumulation of amyloid β induces mitochondrial dysfunction leading to apoptotic cell death.** *PLoS One* 2012, **7**(4):e34929.
- Chen J, Somanath PR, Razorenova O, Chen WS, Hay N, Bornstein P, Byzova TV: **Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo.** *Nat Med* 2005, **11**(11):1188-1196.
- Chen M, Cai H, Yang JL, Lu CL, Liu T, Yang W, Guo J, Hu XQ, Fan CH, Hu ZY, Gao F, Liu YX: **Effect of heat stress on expression of junction-associated molecules and upstream factors androgen receptor and Wilms' tumor 1 in monkey sertoli cells.** *Endocrinology* 2008, **149**(10):4871-4882.

Chen T, Zamora R, Zuckerbraun B, Billiar TR: **Role of nitric oxide in liver injury.**

Curr Mol Med 2003, **3**(6):519-526.

Cheeseman JH, Kaiser MG, Ciraci C, Kaiser P, Lamont SJ: **Breed effect on early cytokine mRNA expression in spleen and cecum of chickens with and without**

***Salmonella enteritidis* infection.** *Dev Comp Immunol* 2007, **31**(1):52-60.

Cobb MH, Goldsmith EJ: **How MAP kinases are regulated.** *J Biol Chem* 1995,

270(25):14843-14846.

Deeb N, Cahaner A: **Genotype-by-environment interaction with broiler genotypes**

differing in growth rate 3. Growth rate and water consumption of broiler

progeny from weight-selected vs. non-selected parents under normal and high

ambient temperatures. *Poult Sci* 2002, **81**(3):293-301.

Desmond JC, Mountford JC, Drayson MT, Walker EA, Hewison M, Ride JP, Luong QT,

Hayden RE, Vanin EF, Bunce CM: **The aldo-keto reductase AKR1C3 is a novel**

suppressor of cell differentiation that provides a plausible target for the non-

cyclooxygenase-dependent antineoplastic actions of nonsteroidal anti-

inflammatory drugs. *Cancer Res* 2003, **63**(2):505-512.

Dickson RC: **Roles for sphingolipids in *Saccharomyces cerevisiae*.** *Adv Exp Med Biol*

2010, **688**:217-231.

Donato R: **S100: a multigenic family of calcium-modulated proteins of the EF-hand**

type with intracellular and extracellular functional roles. *Int J Biochem Cell Biol*

2001, **33**(7):637-668.

- Duce JA, Tsatsanis A, Cater MA, James SA, Robb E, Wikhe K, Leong SL, Perez K, Johanssen T, Greenough MA, Cho HH, Galatis D, Moir RD, Masters CL, McLean C, Tanzi RE, Cappai R, Barnham KJ, Ciccotosto GD, Rogers JT, Bush AI: **Iron-export ferroxidase activity of β -amyloid precursor protein is inhibited by zinc in Alzheimer's disease.** *Cell* 2010, **142**(6):857-867.
- Dudek KM, Suter L, Darras VM, Marczylo EL, Gant TW: **Decreased translation of Dio3 mRNA is associated with drug-induced hepatotoxicity.** *Biochem J* 2013 **453**(1):71-82.
- Dupont J, Tesseraud S, Simon J: **Insulin signaling in chicken liver and muscle.** *Gen Comp Endocrinol* 2009, **163**(1-2):52-57.
- Edwards MC, Wong C, Elledge SJ: **Human cyclin K, a novel RNA polymerase II-associated cyclin possessing both carboxy-terminal domain kinase and Cdk-activating kinase activity.** *Mol Cell Biol* 1998, **18**(7):4291-4300.
- Emmans GC, Charles DR: **Climatic environment and poultry feeding in practice, 1st Edition.** Essex: Anchor Press Ltd; 1989.
- Ewart-Toland A, Chan JM, Yuan J, Balmain A, Ma J: **A gain of function TGFB1 polymorphism may be associated with late stage prostate cancer.** *Cancer Epidemiol Biomarkers Prev* 2004, **13**(5):759-764.
- Farrell DS, Swain S: **Effects of temperature treatments on the energy and nitrogen metabolism of fed chickens.** *Br Poult Sci* 1978, **18**:735-748.

- Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW: **Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene.** *Nature* 1996, **380**(6573):439-442.
- Fields SD, Hansen PJ, Ealy AD: **Fibroblast growth factor requirements for in vitro development of bovine embryos.** *Theriogenology* 2011, **75**(8):1466-1475.
- Freudenthal R, Locatelli F, Hermitte G, Maldonado H, Lafourcade C, Delorenzi A, Romano A: **Kappa-B like DNA-binding activity is enhanced after spaced training that induces long-term memory in the crab *Chasmagnathus*.** *Neurosci Lett* 1998, **242**(3):143-146.
- Friesema EC, Kuiper GG, Jansen J, Visser TJ, Kester MH: **Thyroid hormone transport by the human monocarboxylate transporter 8 and its rate-limiting role in intracellular metabolism.** *Molecular Endocrinology* 2006, **20** (11):2761-2772.
- Fukuda M, Gotoh Y, Nishida E: **Interaction of MAP kinase with MAP kinase kinase: its possible role in the control of nucleocytoplasmic transport of MAP kinase.** *EMBO J.* 1997, **16**(8):1901-1908.
- Fusco C, Micale L, Egorov M, Monti M, D'Addetta EV, Augello B, Cozzolino F, Calcagnì A, Fontana A, Polishchuk RS, Didelot G, Reymond A, Pucci P, Merla G: **The E3-ubiquitin ligase TRIM50 interacts with HDAC6 and p62, and promotes the sequestration and clearance of ubiquitinated proteins into the aggresome.** *PLoS One* 2012, **7**(7):e40440.

Gilmore T: **The Rel/NF-B signal transduction pathway: introduction.** *Oncogene* 1999, **18**:6842-6844.

Gilmore T: **Introduction to NF-B: players, pathways, perspectives.** *Oncogene* 2006, **25**:6680-6684.

Goldsmith ZG, Dhanasekaran DN: **G protein regulation of MAPK networks.** *Oncogene* 2007, **26**(22):3122-3142.

Hai L, Rong D, Zhang Z-Y: **The effect of thermal environment on the digestion of broilers.** *Anim Physiol* 2000, **83**(2):57-64.

Hannun YA, Obeid LM: **Principles of bioactive lipid signalling: lessons from sphingolipids.** *Nat Rev Mol Cell Biol* 2008, **9**(2):139-150.

Hattori H, Zhang X, Jia Y, Subramanian KK, Jo H, Loison F, Newburger PE, Luo HR: **RNAi screen identifies UBE2D3 as a mediator of all-trans retinoic acid-induced cell growth arrest in human acute promyelocytic NB4 cells.** *Blood* 2007, **110**(2):640-650.

Haughey NJ, Bandaru VVR, Bae M, Mattson MP: **Roles for dysfunctional sphingolipid metabolism in Alzheimer's disease neuropathogenesis.** *Biochimica et Biophysica Acta* 2010, **1801**(8):878-886.

Ho A, Südhof TC: **Binding of F-spondin to amyloid-beta precursor protein: a candidate amyloid beta precursor protein ligand that modulates amyloid-beta precursor protein cleavage.** *Proc Natl Acad Sci* 2004, **101**(8):2549-2553.

- Imao M, Nagaki M, Imose M, Moriwaki H: **Differential caspase-9-dependent signaling pathway between tumor necrosis factor receptor- and Fas-mediated hepatocyte apoptosis in mice.** *Liver International* 2006, **26**(1):137-146.
- Jez JM, Penning TM: **The aldo-keto reductase (AKR) superfamily: an update.** *Chem Biol Interact* 2001, **130-132**(1-3):499-525.
- Ji B, Ernest B, Gooding JR, Das S, Saxton AM, Simon J, Dupont J, Métayer-Coustard S, Campagna SR, Voy BH: **Transcriptomic and metabolomic profiling of chicken adipose tissue in response to insulin neutralization and fasting.** *BMC Genomics* 2012, **13**:441.
- Kahan C, Seuwen K, Meloche S, Pouyssegur J: **Coordinate, biphasic activation of p44 mitogen-activated protein kinase and S6 kinase by growth factors in hamster fibroblasts. Evidence for thrombin-induced signals different from phosphoinositide turnover and adenylylcyclase inhibition.** *J Biol Chem* 1992, **267**(19):13369-13375.
- Kandel E, Skeen J, Majewski N, Di Cristofano A, Pandolfi P, Feliciano C, Gartel A, Hay A: **Activation of Akt/protein kinase B overcomes a G(2)/m cell cycle checkpoint induced by DNA damage.** *Mol Cell Biol* 2002, **22**:7831-7841.
- Kersten S: **Regulation of lipid metabolism via angiopoietin-like proteins.** *Biochem Soc Trans* 2005, **33**(Pt 5):1059-1062.
- Khokhlatchev AV, Canagarajah B, Wilsbacher J, Robinson M, Atkinson M, Goldsmith E, Cobb MH: **Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation.** *Cell* 1998, **93**(4):605-615.

- Kim CW, Song H, Kumar S, Nam D, Sang Kwon H, Hwa Chang K, Ju Son D, Kang DW, Brodie SA, Weiss D, Vega JD, Alberts-Grill N, Griendling K, Taylor WR, Jo H: **Anti-Inflammatory and Antiatherogenic Role of BMP Receptor II in Endothelial Cells.** *Arterioscler Thromb Vasc Biol* 2013, **33**(6):1350-1359.
- Kim J, Parrish AB, Kurokawa M, Matsuura K, Freel CD, Andersen JL, Johnson CE, Kornbluth S: **Rsk-mediated phosphorylation and 14-3-3 ϵ binding of Apaf-1 suppresses cytochrome c-induced apoptosis.** *EMBO J* 2012, **31**(5):1279-1292.
- Kim S-Y, Jang H-R, Kim J-H, Noh S-M, Song K-S, Kim M-R, Kim S-Y, Yeom, Y-I, Kim N-S, Yoo H-S, Kim Y-S: **The epigenetic silencing of LIMS2 in gastric cancer and its inhibitory effect on cell migration.** *Biochem Biophys Res Commun* 2006, **349**(3):1032-1040.
- Kitagawa M, AO M, Miyauchi M, Abiko Y, Takata T: **F-spondin regulates the differentiation of human cementoblast-like (HCEM) cells via BMP7.** *Biochem Biophys Res Commun* 2012, **418**(2):229-233.
- Kondoh K, Torii S, Nishida E: **Control of MAP kinase signaling to the nucleus.** *Chromosoma* 2005, **114**(2):86-91.
- Kong Q, Zeng W, Wu J, Hu W, Li C, Mao B: **RNF220, an E3 ubiquitin ligase that targets Sin3B for ubiquitination.** *Biochem Biophys Res Commun* 2010, **393**(4):708-713.
- Kuang Z, Lewis RS, Curtis JM, Zhan Y, Saunders BM, Babon JJ, Kolesnik TB, Low A, Masters SL, Willson TA Kedzierski L, Yao S, Handman E, Norton RS, Nicholson

- SE: **The SPRY domain-containing SOCS box protein SPSB2 targets iNOS for proteasomal degradation.** *J Cell Biol* 2010, **190**(1):129-141.
- Kuo TC, Tan CT, Chang YW, Hong CC, Lee WJ, Chen MW, Jeng YM, Chiou J, Yu P, Chen PS, Wang MY, Hsiao M, Su JL, Kuo ML: **Angiopoietin-like protein 1 suppresses SLUG to inhibit cancer cell motility.** *J Clin Invest* 2013, **123**(3):1082-1095.
- Kwakkenbos MJ, Kop EN, Stacey M, Matmati M, Gordon S, Lin HH, Hamann J: **The EGF-TM7 family: a postgenomic view.** *Immunogenetics* 2004, **55**(10):655-666.
- Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW: **A protein kinase involved in the regulation of inflammatory cytokine biosynthesis.** *Nature* 1994, **372**(6508):739-746.
- Leschner J, Ring L, Feierler J, Dinkel K, Jochum M, Faussner A: **Fever-like temperature modification differentially affects in vitro signaling of bradykinin B(1) and B(2) receptors.** *Biol Chem* 2011, **392**(11):1021-1029.
- Lesourne R, Zvezdova E, Song KD, El-Khoury D, Uehara S, Barr VA, Samelson LE, Love PE: **Interchangeability of Themis1 and Themis2 in thymocyte development reveals two related proteins with conserved molecular function.** *J Immunol* 2012, **189**(3):1154-1161.
- Levine A: **p53, the cellular gatekeeper for growth and division.** *Cell* 1997, **88**:323-331.

- Li C, Wang X, Wang G, Li N, Wu C: **Expression analysis of global gene response to chronic heat exposure in broiler chickens (*Gallus gallus*) reveals new reactive genes.** *Poult Sci* 2011, **90**(5):1028-1036.
- Li Y, Shin D, Kwon SH: **Histone deacetylase 6 plays a role as a distinct regulator of diverse cellular processes.** *FEBS J* 2013, **280**(3):775-793.
- Lichtenstein L, Berbée JF, van Dijk SJ, van Dijk KW, Bensadoun A, Kema IP, Voshol PJ, Müller M, Rensen PC, Kersten S: **Angptl4 upregulates cholesterol synthesis in liver via inhibition of LPL- and HL-dependent hepatic cholesterol uptake.** *Arterioscler Thromb Vasc Biol* 2007, **27**(11):2420-2427.
- Lin DC, Bullock CM, Ehlert FJ, Chen JL, Tian H, Zhou QY: **Identification and molecular characterization of two closely related G protein-coupled receptors activated by prokineticins/endocrine gland vascular endothelial growth factor.** *J Biol Chem* 2002, **277**(22):19276-19280.
- Liu S, Wang X, Sun F, Zhang J, Feng J, Liu H, Rajendran KV, Sun L, Zhang Y, Jiang Y, Peatman E, Kaltenboeck L, Kucuktas H, Liu Z: **RNA-Seq reveals expression signatures of genes involved in oxygen transport, protein synthesis, folding, and degradation in response to heat stress in catfish.** *Physiol. Genomics* 2013, **45**(12):462-476.
- Lodish H, Berk A, Zipursky LS, Matsudaira P, Baltimore D, Darnell J: **Figure 23-50: BAD interaction with Bcl-2.** In. *Molecular cell biology*. New York: Scientific American; 1999.

- Lund SP, Nettleton D, McCarthy DJ, Smyth GK: **Detecting differential expression in RNA-sequence data using quasi-likelihood with shrunken dispersion estimates.** *Stat Appl Gen Mol Biol* 2012, **11**:5.
- Lu Z, Xu S: **ERK1/2 MAP kinases in cell survival and apoptosis.** *IUBMB Life* 2006, **58**:621-631.
- Maguire JJ, Parker WA, Foord SM, Bonner TI, Neubig RR, Davenport AP: **International Union of Pharmacology. LXXII. Recommendations for trace amine receptor nomenclature.** *Pharmacol Rev* 2009, **61**(1):1-8.
- Matsubayashi Y, Fukuda M, Nishida E: **Evidence for existence of a nuclear pore complex-mediated, cytosol-independent pathway of nuclear translocation of ERK MAP kinase in permeabilized cells.** *J Biol Chem* 2001, **276**(45):41755-41760.
- McKay JC, Barton NF, Koerhuis ANM, McAdam J: **The challenge of genetic change in the broiler chicken.** In *The Challenge of Genetic Change in Animal Production*. Edited by Hill WG, Bishop SC, McKay JC. Edinburgh: Occasional Publishing; 2000:1-7.
- McKee JS, Harrison PC, Riskowski GL: **Effects of supplemental ascorbic acid on the energy conversion of broiler chicks during heat stress and feed withdrawal.** *Poult Sci* 1997, **76**(9):1278-1286.
- Meloche S: **Cell cycle reentry of mammalian fibroblasts is accompanied by the sustained activation of p44mapk and p42mapk isoforms in the G1 phase and their inactivation at the G1/S transition.** *J Cell Physiol* 1995, **163**(3):577-588.

- Meloche S, Seuwen K, Pages G, Pouyssegur J: **Biphasic and synergistic activation of p44mapk (ERK1) by growth factors: correlation between late phase activation and mitogenicity.** *Mol Endocrinol* 1992, **6**(5):845-854.
- Miskinyte S, Butler MG, Hervé D, Sarret C, Nicolino M, Petralia JD, Bergametti F, Arnould M, Pham VN, Gore AV, Spengos K, Gazal S, Woimant F, Steinberg GK, Weinstein BM, Tournier-Lasserre E: **Loss of BRCC3 deubiquitinating enzyme leads to abnormal angiogenesis and is associated with syndromic moyamoya.** *Am J Hum Genet* 2011, **88**(6):718-728.
- Moraes VMB, Malheiros RD, Bruggeman V, Collin A, Tona K, Van AS P, Onagbesan OM, Buyse J, Decuyper E, Macari M: **Effect of thermal conditioning during embryonic development on aspects of physiological responses of broilers to heat stress.** *J Therm Biol* 2003, **28**(2):133-140.
- Mori T, Anazawa Y, Matsui K, Fukuda S, Nakamura Y, Arakawa H: **Cyclin K as a direct transcriptional target of the p53 tumor suppressor.** *Neoplasia* 2002, **4**(3):268-274.
- Mormone E, Lu Y, Ge X, Fiel MI, Nieto N: **Fibromodulin, an oxidative stress-sensitive proteoglycan, regulates the fibrogenic response to liver injury in mice.** *Gastroenterology* 2012, **142**(3):612-621.
- Negri L, Lattanzi R, Giannini E, Melchiorri P: **Bv8/Prokineticin proteins and their receptors.** *Life Sci* 2007, **81**(14):1103-1116.
- Nikolova-Karakashian MN, Rozenova KA: **Ceramide in stress response.** *Adv Exp Med Biol* 2010, **688**:86-108.

- Nishiya T, Matsumoto K, Maekawa S, Kajita E, Horinouchi T, Fujimuro M, Ogasawara K, Uehara T, Miwa S: **Regulation of inducible nitric-oxide synthase by the SPRY domain- and SOCS box-containing proteins.** *J Biol Chem* 2011, **286**(11):9009-9019.
- Olender T, Lancet D, Nebert DW: **Update on the olfactory receptor (OR) gene superfamily.** *Hum Genomics* 2008, **3**(1):87-97.
- Prado GN, Taylor L, Zhou X, Ricupero D, Mierke DF, Polgar P: **Mechanisms regulating the expression, self-maintenance, and signaling-function of the bradykinin B2 and B1 receptors.** *J Cell Physiol* 2002, **193**(3):275-286.
- Peeters R, Fekete C, Goncalves C, Legradi G, Tu HM, Harney JW, Bianco AC, Lechan RM, Larsen PR: **Regional physiological adaptation of the central nervous system deiodinases to iodine deficiency.** *Am J Physiol Endocrinol Metab* 2001, **281**(1):E54-61.
- Perkins N: **Integrating cell signalling pathways with NF-B and IKK function.** *Nat Rev Mol Cell Biol* 2007, **8**:49-62.
- Priller C, Bauer T, Mitteregger G, Krebs B, Kretschmar HA, Herms J: **Synapse formation and function is modulated by the amyloid precursor protein.** *J Neurosci* 2006, **26**(27):7212-7221.
- R Development Core Team: **R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing.** [<http://www.R-project.org/>].
- Ramaswamy S, Nakamura N, Vazquez F, Batt DB, Perera S, Roberts T, Sellers W: **Regulation of G1 progression by the PTEN tumor suppressor protein is linked**

to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. In.: *Proc Natl Acad Sci USA* 96. 1999: 2110-2115.

Richards MP, Proszkowiec-Weglarz M: **Mechanisms regulating feed intake, energy expenditure, and body weight in poultry.** *Poult Sci* 2007, **86**(7):1478-1490.

Ries A, Göhring W, Fox JW, Timpl R, Sasaki T: **Recombinant domains of mouse nidogen-1 and their binding to basement membrane proteins and monoclonal antibodies.** *Eur J Biochem* 2001, **268**(19):5119-5128.

Rodriguez I, Mombaerts P: **Novel human vomeronasal receptor-like genes reveal species-specific families.** *Curr Biol* 2002, **12**(12):R409-411.

Rodriguez-Sinovas A, Fernández E, Manteca X, Fernández AG, Goñalons E: **CCK is involved in both peripheral and central mechanisms controlling food intake in chickens.** *Am J Physiol* 1997, **272**(1 Pt 2):R334-340.

Roskoski R: **ERK1/2 MAP kinases: structure, function, and regulation.** *Pharmacol Res* 2012, **66**(2):105-143.

Rouse J, Cohen P, Trigon S, Morange M, Alonso-Llamazares A, Zamanillo D, Hunt T, Nebreda AR: **A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins.** *Cell* 1994, **78**(6):1027-1037.

Sakono M, Utsumi A, Zako T, Abe T, Yohda M, Maeda M: **Formation of non-toxic A β fibrils by small heat shock protein under heat-stress conditions.** *Biochem Biophys Res Commun* 2013, **430**(4):1259-1264.

- Schollenberger L, Gronemeyer T, Huber CM, Lay D, Wiese S, Meyer HE, Warscheid B, Saffrich R, Peränen J, Gorgas K, Just WW: **RhoA regulates peroxisome association to microtubules and the actin cytoskeleton.** *PLoS One* 2010, **5**(11):e13886.
- Shakkottai VG, Xiao M, Xu L, Wong M, Nerbonne JM, Ornitz DM, Yamada KA: **FGF14 regulates the intrinsic excitability of cerebellar Purkinje neurons.** *Neurobiol Dis* 2009, **33**(1):81–88.
- Silva JE: **Thyroid hormone and the energetic cost of keeping body temperature.** *Biosci Rep* 2005, **25**:129-148.
- Soga T, Matsumoto S, Oda T, Saito T, Hiyama H, Takasaki J, Kamohara M, Ohishi T, Matsushime H, Furuichi K: **Molecular cloning and characterization of prokineticin receptors.** *Biochim Biophys Acta* 2002, **1579**(2-3):173-179.
- St. Pierre NR, Cobanov B, Schnitkey G: **Economic losses from heat stress by US livestock industries.** *J Dairy Sci* 2003, **86**:E52-E77.
- Sun Y, Miao Y, Yamane Y, Zhang C, Shokat KM, Takematsu H, Kozutsumi Y, Drubin DG: **Orm protein phosphoregulation mediates transient sphingolipid biosynthesis response to heat stress via the Pkh-Ypk and Cdc55-PP2A pathways.** *Mol Biol Cell* 2012, **23**(12):2388-2398.
- Takenouchi R, Inoue K, Kambe Y, Miyata A: **N-arachidonoyl glycine induces macrophage apoptosis via GPR18.** *Biochem Biophys Res Commun* 2012, **418**(2):366-371.

- Taleb N, Polychronakos C: **RFX6 is needed for the development and maintenance of the b-cell phenotype.** *Islets* 2011, **3**(5):291-293.
- Tesseraud S, Métayer S, Duchêne S, Bigot K, Grizard J, Dupont J: **Regulation of protein metabolism by insulin: value of different approaches and animal models.** *Domest Anim Endocrinol* 2007, **33**(2):123-142.
- Texel SJ, Mattson MP: **Impaired adaptive cellular responses to oxidative stress and the pathogenesis of Alzheimer's disease.** *Antioxid Redox Signal* 2011, **14**(8):1519-1534.
- Tian B, Brasier A: **Identification of a nuclear factor B-dependent gene network.** *Recent Prog Horm Res* 2003, 58:95-130.
- Tuggle TK, Wang Y, Couture O: **Advances in Swine Transcriptomics.** *Int J Biol Sci* 2007, **3**(3):132-152.
- Turner PR, O'Connor K, Tate WP, Abraham WC: **Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory.** *Prog Neurobiol* 2003, **70**(1):1-32.
- Varki, A, Cummings, RD, Esko, JD, Freeze, HH, Stanley, P, Marth, JD, Bertozzi, CR, Hart, GW, Etzler, ME: **Symbol Nomenclature for Glycan Representation (Viewpoint Article).** *Proteomics* 2009, **9**: 5398–5399.
- Verbeek DS, Goedhart J, Bruinsma L, Sinke RJ, Reits EA: **PKC γ mutations in spinocerebellar ataxia type 14 affect C1 domain accessibility and kinase activity leading to aberrant MAPK signaling.** *J Cell Sci* 2008, **121**(14):2339–2349.

- Wang S, Zhang AP, Kurada L, Matsui T, Lei S: **Cholecystokinin facilitates neuronal excitability in the entorhinal cortex via activation of TRPC- like channels.** *J Neurophysiol* 2011, **106**(3):1515-1524.
- Wang SH, Cheng CY, Tang PC, Chen CF, Chen HH, Lee YP, Huang SY: **Differential gene expressions in testes of L2 strain Taiwan countr chicken in response to acute heat stress.** *Theriogenology* 2013, **79**(2):374-382.
- Wang Z, Ahmad A, Li Y, Kong D, Azmi AS, Banerjee S, Sarkar FH: **Emerging roles of PDGF-D signaling pathway in tumor development and progression.** *Biochim Biophys Acta* 2010, **1806**(1):122-130.
- Wheelock JB, Rhoads RP, Vanbaale MJ, Sanders SR, Baumgard LH: **Effects of heat stress on energetic metabolism in lactating Holstein cows.** *J Dairy Sci* 2010, **93**(2):644-655.
- Whitehurst AW, Wilsbacher JL, You Y, Luby-Phelps K, Moore MS, Cobb MH: **ERK2 enters the nucleus by a carrier-independent mechanism.** *Proc Natl Acad Sci U S A* 2002, **99**(11):7496-7501.
- Whiteman E, Cho H, Birnbaum M: **Role of Akt/protein kinase B in metabolism.** *Trends in Endocrinology and Metabolism* 2002, **13**:444-451.
- Windhorst H-W: **Changes in poultry production and trade worldwide.** *World's Poultry Sci. J.* 2006, **62**(4):585-602.
- Wu TD, Nacu S: **Fast and SNP-tolerant detection of complex variants and splicing in short reads.** *Bioinformatics* 2010, **26**(7):873-881.

- Yona S, Lin HH, Siu WO, Gordon S, Stacey M: **Adhesion-GPCRs: emerging roles for novel receptors.** *Trends Biochem Sci* 2008, **33**(10):491-500.
- Yoshida R, Suzuki M, Sakaguchi R, Hasegawa E, Kimura A, Shichita T, Sekiya T, Shiraishi H, Shimoda K, Yoshimura A: **Forced expression of stabilized c-Fos in dendritic cells reduces cytokine production and immune responses in vivo.** *Biochem Biophys Res Commun* 2012, **423**(2):247-252.
- Zhang Y, Chen K, Guo L, Wu C: **Characterization of PINCH-2, a new focal adhesion protein that regulates the PINCH-1-ILK interaction, cell spreading, and migration.** *J Biol Chem* 2002, **277**(41):38328-38338.
- Zhang Y, Chen K, Tu Y, Velyvis A, Yang Y, Qin J, Wu C: **Assembly of the PINCH-ILK-CH-ILKBP complexes precedes and is essential for localization of each component to cell-matrix adhesion sites.** *Cell Sci* 2002, **115**(24):4777-4786.

Table 1 – Forward and reverse primers used for qPCR validation of RNA-seq

Gene	Forward Primer	Reverse Primer
RFX6	5' GCCGGTTTAAACGGGAGAAGT 3'	5' CATCATCTGCGTGATGCTCT 3'
SPSB4	5' AATGGACTTGACCCGGAAC 3'	5' TTTCAGAGACAGAGGCAAAGG 3'
TRPC5	5' CTGCCCTGGGTTCTAGGTTT 3'	5' GGGAGTTCATTGCAAAATCC 3'
BNC1	5' TGGATATGTGCTGCAGGATG 3'	5' TGCCATTAACTCCACAATGG 3'
ANGPTL4	5' TGTGACATGACTGCAGAAGG 3'	5' CAGCCAGAAGTCACCATGAA 3'
LIMS2	5' CAGATGGGCTTTTCTATGAGTT 3'	5' GAAACATTCTGGGTGCCAGT 3'
ERC2	5' GTCTTGCCTCAACACAGCAA 3'	5' GGCAATGTTTGCATCCTTTT 3'
S100A1	5' AGCTGAGCAAGAAGGAGCTG 3'	5' GGTCTGTCATGATCTTCTCC 3'
FGF14	5' TACCCAAGCCATTGGAAGTT 3'	5' GTTTGCCGCCATTTCATTATT 3'
DIO3	5' GCTCTCTTCCTTCGGGATCT 3'	5' CCCATTTCAAATCGGTCAT 3'

Table 2 – *P*-value of hepatocyte gene expression between heat stressed- and non-heat stressed-broilers

Gene	RNA-seq	qPCR
RFX6	0.01	0.4
SPSB4	0.001	0.008
TRPC5	0.02	0.2
BNC1	0.004	0.1
ANGPTL4	0.0006	0.02
LIMS2	0.02	0.4
ERC2	0.02	0.8
S100A1	0.005	0.008
FGF14	0.008	0.7
DIO3	0.00009	0.02

Supporting Information Table 1 – *P*-values, fold changes, and q-values for DE genes

Gene	<i>P</i>-value	Fold Change	q-value
RFX6	0.01	-12	0.6
LOC100857	0.007	-11	0.6
SPSB4	0.001	-11	0.3
GPR133	0.0005	-11	0.2
TRPC5	0.02	-11	0.7
LOC427426	0.02	-11	0.7
BNC1	0.004	-11	0.5
LOC423425	0.02	-10	0.7
ANGPTL4	0.0006	-3	0.2
LOC100859	0.03	-2	0.7
LOC420770	0.04	-2	0.7
LOC100858	0.01	-2	0.7
KRT14	0.009	-2	0.6
FMOD	0.001	-2	0.3
LOC419500	0.004	-2	0.5
LOC100858	0.01	-2	0.7
SCN3B	0.03	-2	0.7
S100A4	0.005	-2	0.5
LOC100858	0.04	-2	0.7
TRIM50	0.05	-2	0.7
BRCC3	0.02	-2	0.7
LOC771141	0.08	-2	0.8
PDGFD	0.03	-2	0.7
AKR1C3	0.05	-2	0.7
SPON1	0.03	-2	0.7
DIO2	0.006	-1	0.5
NID1	0.01	1	0.6
HFM1	0.01	1	0.6
BDKRB1	0.04	2	0.7
CCK	0.04	2	0.7
MYRIP	0.007	2	0.5
ORMDL3	0.05	2	0.7
RNF220	0.03	2	0.7
LIMS2	0.02	2	0.7
ERC2	0.02	2	0.7
S100A1	0.005	2	0.5
FGF14	0.008	2	0.5
DIO3	0.00009	3	0.1
LOC395159	0.001	5	0.3
LOC100857	0.0008	20	0.3

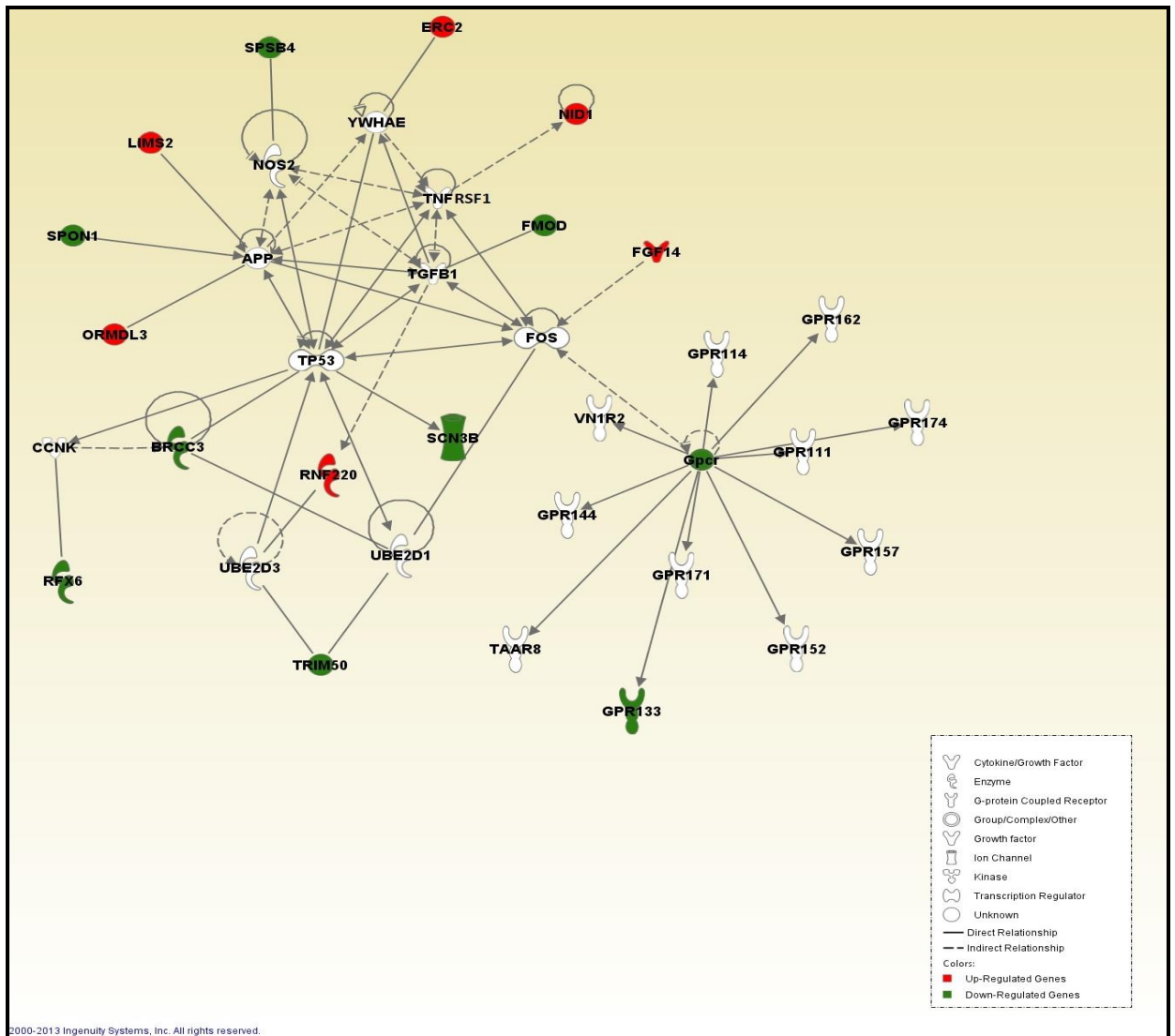


Figure 1 - Cell Signaling, Molecular Transport, Small Molecule Biochemistry Network

Pathway analysis of gene functions in broiler liver transcriptome in response to chronic, cyclic heat stress. Red color shows up-regulation and green color shows down-regulation (IPA). White molecules are not DE, but are included to illustrate association with significantly up-regulated and down-regulated genes.

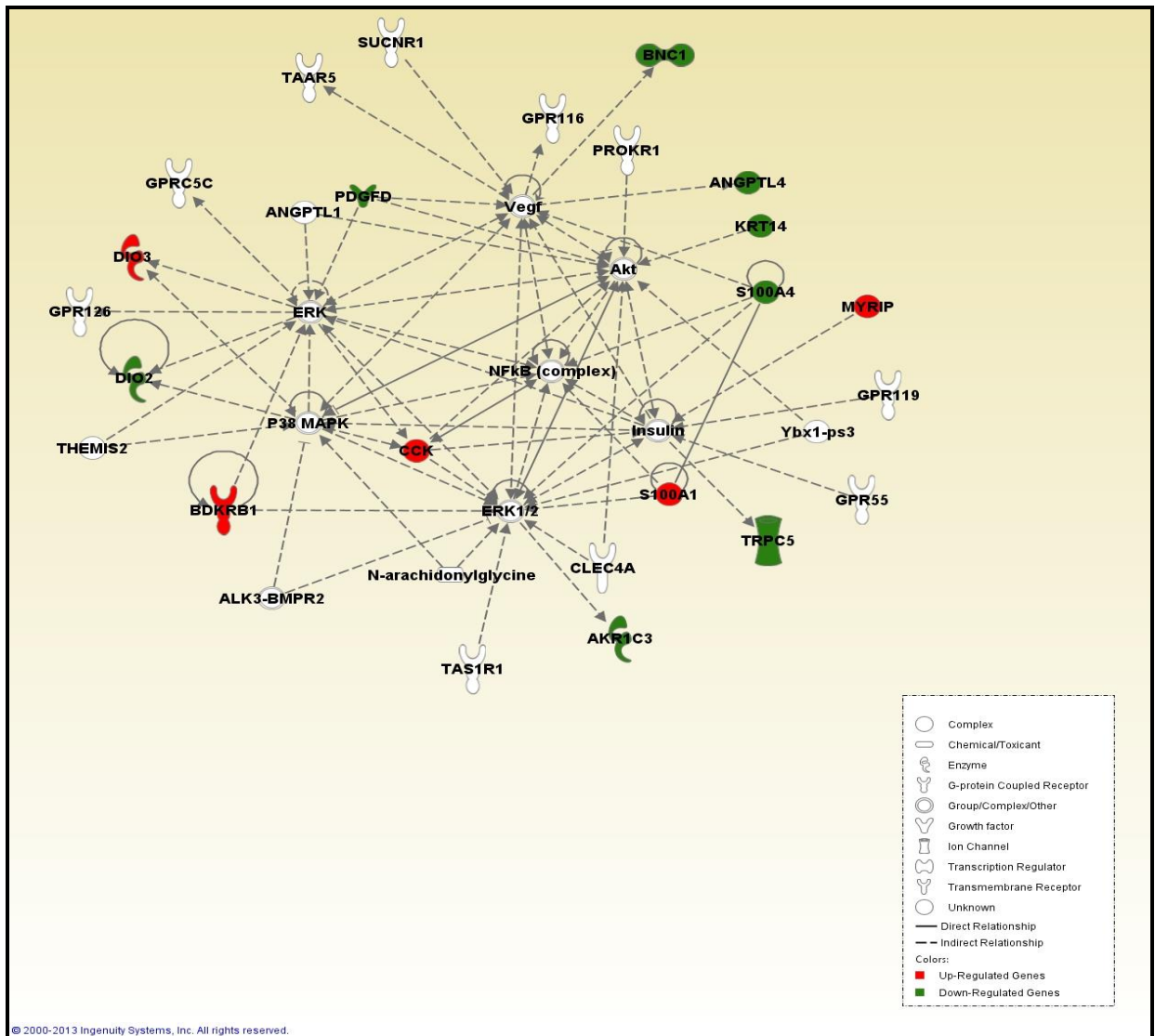


Figure 2 - Endocrine System Development and Function, Small Molecule Biochemistry
Cell Signaling Network

Pathway analysis of gene functions in broiler liver transcriptome in response to chronic, cyclic heat stress. Red color shows up-regulation and green color shows down-regulation (IPA). White molecules are not DE, but are included to illustrate association with significantly up-regulated and down-regulated genes.

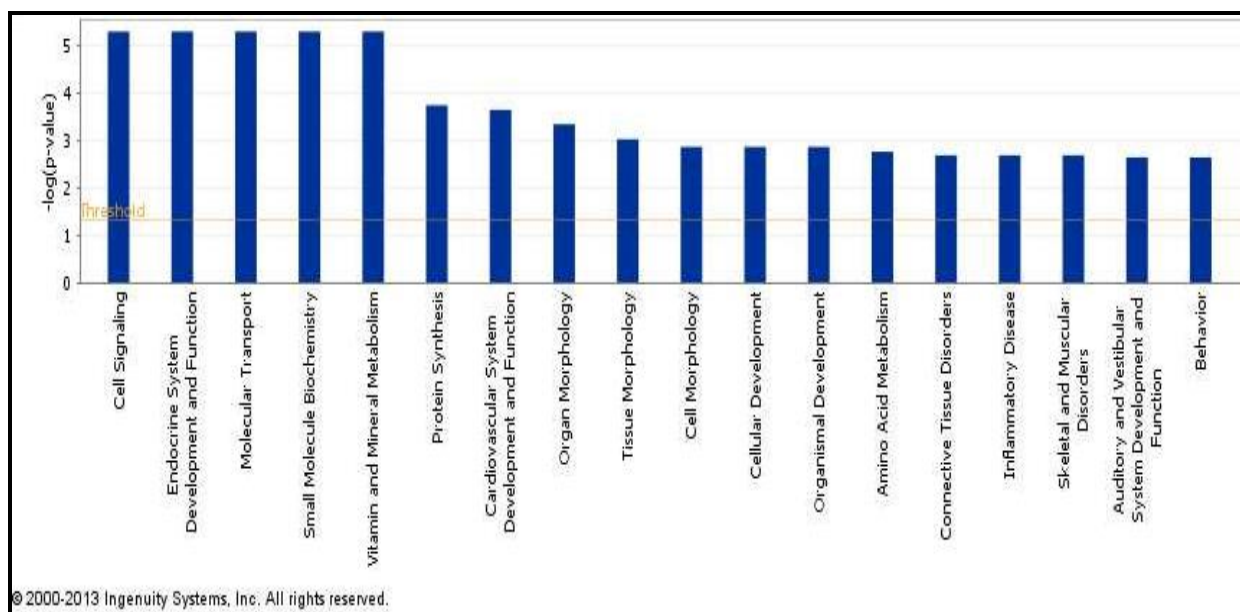


Figure 3 - Significance levels of functions associated with DE genes in broiler liver in response to chronic, cyclic heat stress

Threshold was set at $P = 0.05$ and indicated as $-\log(P\text{-value})$ on the Y-axis. X- axis shows functions of DE genes.

CHAPTER 5. GENERAL DISCUSSION, CONCLUSIONS, AND FUTURE DIRECTIONS

Progression through increasingly complex gene expression platforms

The current dissertation represents a progression through the technological platforms that have been used to analyze gene expression in poultry over the past decade. I begin my dissertation research in 2009, a pivotal period in poultry genetics. The draft genome sequence of the Red Jungle fowl had just been released 5 years earlier, the tools used for the statistical analysis of microarray data were being refined, data mining tools for gene network analysis were being enhanced, and the experimental designs for microarray experiments were being appropriately applied (Bhattacharya and Mariani, 2009; International Chicken Genome Sequencing Consortium, 2004). Upon entering the final study of my dissertation (Chapter 4), next-generation sequencing technology was being used to characterize the transcriptome of specific tissues in chickens (Li et al., 2011). Conducting my research during this dynamic period in poultry genetics, afforded me the opportunity to progress from using qPCR technology to analyze the expression of candidate genes in poultry, to using microarray technology to analyze the expression of tens of thousands of genes in the liver of *Salmonella*-infected broilers, to finally using RNA-seq technology to characterize the liver transcriptome of heat-stressed broilers.

These platforms were used to analyze the gene expression profiles of immunological and metabolic-related tissues of chickens in response to *S. Enteritidis* infection and heat stress. The information obtained from these studies added to our

understanding of the chicken response to stressors on both a candidate and global gene level. The following specific topics were explored: 1) splenic cytokine profiles of broiler, Leghorn, and Fayoumi hens in response to *S. Enteritidis* infection, 2) liver transcriptome of broilers in response to *S. Enteritidis* infection, and 3) liver transcriptome of broilers in response to chronic, cyclic heat stress.

The value of conserving genetic diversity

The cytokine profiles of 3 distinct genetic lines of chickens (broiler, Leghorn, and Fayoumi) were characterized in Chapter 3. This study highlighted a variety of immune responses and mechanisms that are employed by chickens from distinct genetic backgrounds. The difference in immune response to *S. Enteritidis* can be attributed to the genetic diversity that exists among the 3 lines. Highly inbred research lines such as the Fayoumi and Leghorn lines in Chapter 3 have rapidly disappeared (Fulton, 2012). Although these lines may not be suitable for introgression into commercial lines, they serve as highly effective discovery platforms for modern-day poultry production. The maintenance of such lines is crucial for the research of disease-related traits because of the high extent of linkage disequilibrium (LD) they possess.

The influence of genetic diversity on resistance traits in poultry has been well documented (Kramer et al., 2003; Tohidi et al., 2012). According to Cheng et al. (2013), the reduction of genetic diversity in chickens increases the possibility for disease outbreaks. Inbreeding depression, a reduction in the fitness traits of a population, may be the underlying mechanism responsible for reduced disease resistance in a population

with limited genetic variability. Bijlsma and Loeschcke (2012) stated that inbreeding practices under stressful conditions lowers a population's ability to adapt to the stress.

With the consolidation of commercial poultry production to just a few global companies, the conservation of genetic diversity in the public sector is more important than ever. The worldwide production of poultry is basically relegated to two groups, the EW group (layers, broilers, and turkeys) based in Germany and the Hendrix group (layers and turkeys) based in the Netherlands (Silversides and Liu, 2013). Poultry breeders maintain distinct grandparent genetic lines as a source of diversity. As commercial production leads to increasingly crowded environments, commercial chickens are experiencing higher temperatures and higher probabilities of microbial infection. This further highlights the need to maintain diverse grandparent stocks. The maintenance of grandparent stock using artificial insemination is the simplest method to conserve genetic diversity. Although this may be the easiest method to maintain genetic diversity at this point, the cost of feed, vaccination, and the housing of grandparent stock are expenses associated with this practice. Researchers are currently developing technologies that employ cryopreservation of avian gonads to conserve genetic diversity (Silversides and Liu, 2013).

Characterizing the metabolic stress response of broilers

The purpose of the studies reported in Chapters 3 and 4 was to characterize the liver transcriptomes of broilers in response to *S. Enteritidis* infection and chronic, cyclic heat stress. The major advantage of characterizing the transcriptome of broilers in response to both a biotic and an abiotic stressor is that we can determine common gene

expression signatures of both gene network analyses. By determining the common signatures in response to different types of stressors, we can better understand the consensus-type metabolic response of broilers to stressors.

Members of the MAPK signal transduction cascade (*MAPK*, *P38 MAPK*, *ERK*, *ERK1/2*) were present in the broiler liver transcriptome in response to both *S. Enteritidis* infection and heat stress. These results are consistent with another chronic, cyclic heat stress experiment in broilers, that of Li et al. (2011). The *MAPK* pathway transfers signals from cell surface receptors to the nucleus, this regulates gene expression, mitosis, metabolism, motility, survival, apoptosis, and cellular differentiation (Cargnello and Roux, 2011). Both network analyses have DE genes that represent these functions. The DE genes in the broiler liver transcriptome in response to *S. Enteritidis* included polymerases (*POLDIP3* and *POLH*), a mitotic spindle checkpoint (*MAD2L1*), a cholesterol oxidation gene (*CYP27A1*), pro-apoptotic genes (*AIFM1* and *SOCS6*), and a gene that promotes cellular differentiation gene, *DPYSL2*. The DE genes in the broiler liver transcriptome in response to heat stress included a polymerase I and II transcriptional factor (*BNC1*), thyroid hormone genes (*DIO2* and *DIO3*), a movement gene (*PDGFD*), an apoptosis-related gene (*SPON1*), and a gene involved with cellular movement, *LIMS2*. The presence of this pathway and DE genes with related functions in both analyses, strongly suggests that a wide range of stressors elicit the *MAPK* pathway in the broiler liver transcriptome.

The only specific gene that was DE in response to both *S. Enteritidis* and chronic, cyclic heat stress was *BRCC3*. It is a key regulator of angiogenesis and a loss of function

of this gene can result in artery constriction (Miskinyte et al., 2011). *BRCC3* was down-regulated in both network analyses suggesting a possible host mechanism to reduce inflammation in response to *S. Enteritidis* infection and heat stress. Overall, the comparison of the two network analyses provides us with insight into the genetic signature of metabolic stress response of broilers.

Novel nature of characterizing liver transcriptome

As mentioned in the previous section, the broiler liver transcriptome was characterized in the studies summarized in Chapters 3 and 4. All previous transcriptome studies of *Salmonella*-infected chickens have analyzed immune-related tissues (Chiang et al., 2008; Ciraci et al., 2010; Zhou and Lamont, 2007). In Chapter 3, the transcriptome of a metabolic-related tissue, the liver, in response to *S. Enteritidis* infection was characterized. This study provides the first report of a transcriptome analysis in a metabolic-related tissue in *Salmonella*-infected chickens. Furthermore, this is the first study to characterize the transcriptome of the liver in *Salmonella*-infected chickens.

In Chapter 4 the transcriptome of the broiler liver in response to chronic, cyclic heat stress was characterized. This was the first study to characterize the effects of heat stress on the liver transcriptome in chickens, the first RNA-seq study of metabolic-related tissue in chickens, and only the second RNA-seq study reported on heat-stressed broilers. RNA-seq experiments in heat-stressed chickens have been limited in number. The other two published RNA-seq experiments studying the effects of heat stress on the transcriptome of chicken tissue were Li et al. (2011) and Wang et al. (2013). Li et al. (2011) characterized the breast muscle of heat stressed broilers, reporting the

involvement of *MAPK* and *NFKB* pathways. Chapter 4 validates the presence of these same pathways in the metabolic stress response of liver of broilers to heat stress. Wang et al. (2013) characterized the transcriptome of sertoli cells in Taiwanese roosters to heat stress. Collectively, the studies summarized in Chapters 2, 3, and 4 provide a novel view into the host response to stress. They also provide a platform for other studies to investigate the effects of biotic and abiotic stressors on the transcriptome of metabolic-related chicken tissue.

Limitations of the research

Although the research reported in this dissertation provides the scientific community with many novel insights into the stress response of chickens, as with any research, there are limitations. One of these limitations is the time-specific nature of our gene-expression investigations. Thus, the gene expression profiles only represent a snapshot of the time point in which the tissues were sampled. Another limitation of the research was the tissue-specific nature of the investigations. Each tissue in an organism expresses its own specific population of RNAs, or transcriptome; thus the gene expression profiles only represent the sampled tissues. In the RNA-seq study (Chapter 4), splice site detection, the discovery of novel transcripts, and the quantification of transcript isoforms weren't performed. These analyses weren't performed because the sequencing depth of the samples didn't meet the recommended threshold.

The poor functional annotation of genes within the chicken genome was a major limitation of the research. One of the main journal reviewer comments of the Chapter 3 study was regarding the discrepancy between the expression of *SOCS6* and the

expression of *AIFM1*, *MAD2L1*, *USP7*, and *USP52*. Prior to Lin et al. (2012), *SOCS6* was thought to function as a member of the suppressor of cytokines (SOC) family. The down-regulation of a (SOCS) member would have been a contradiction to the down-regulation of genes associated with inflammation. Fortunately, the publishing of Lin et al. (2012) occurred during the journal manuscript revision period of the Chapter 3 study. Lin et al. (2012) reported that *SOCS6* causes apoptotic activity through an intrinsic mitochondrial pathway. This timely finding strongly supported the gene expression pattern observed in Chapter 3, which became a part of the successful version of the manuscript which is now published. Furthermore, another pro-apoptotic gene that functions in a mitochondria-associated pathway, *AIFM1*, was down-regulated in the broiler liver response to *S. Enteritidis* infection. The correct functional annotation of *SOCS6*, helped clarify the metabolic stress response of broilers to *S. Enteritidis* infection.

In addition to the limitations on gene-level analyses, the poor functional annotation limited the network analyses of DE genes. Although 44 genes were DE in the broiler liver in response to *S. Enteritidis* infection (Chapter 3), only 30 were annotated. This eliminates almost one-third of the genes DE in response to *S. Enteritidis* infection from network analysis; thus eliminating candidate genes and only partially characterizing the liver transcriptome. Although 40 genes were DE in the broiler liver in response to chronic, cyclic heat stress (Chapter 4), only 27 genes were annotated and included in network analysis. Manual functional annotation based on the literature is needed to resolve this limitation in function annotation.

Future research directions

The research described in this dissertation has contributed to the characterization of gene expression profiles of immunological and metabolic tissues in chickens in response to biotic and abiotic stressors. The characterization of these tissues adds to our understanding of the response to *S. Enteritidis* infection and heat stress on both candidate and global gene levels in chickens. These findings provide a platform for further investigation into the characterization of the mitochondria-associated apoptosis pathway, the characterization of transcriptomes of immune and non-immune tissue, and commercial application to produce broilers resilient to the effects of *S. Enteritidis* and heat stress.

Chapter 2 highlighted the need to fully understand the mitochondria-associated apoptosis pathway. Gene knockout approaches to investigate gene function are feasible in other models, such as mice, but have not been fully developed in chickens (Nishijima and Iijima, 2013; Sung et al., 2013). Comparative genomics can be used to explore gene function, but chicken orthologs may function differently than in other organisms because of phylogenetic distance. Researchers are currently exploring methods to incorporate transcriptional activation-like effector nucleases (TALENs) to knockout genes in chickens (Nishijima and Iijima, 2013). TALENs can be used to incorporate double strand breaks at specific sites in the DNA, which facilitates homologous recombination and errors in repair. The errors in repair that result from these breaks can be used as a method to knockout genes. To further understand the mitochondria-associated pathway in which *SOCS6* functions, TALENs could be used to knock out *SOCS6* and *DNM1L*

activity. *DNMIL* is a gene that encodes for DRP1, a protein that SOCS6 recruits to the mitochondria for organelle constriction. By knocking out *SOCS6* and *DNMIL*, researchers could determine the up-stream regulators of *SOCS6* and other down-stream targets of *DNMIL*.

Additional transcriptome experiments are needed to completely understand the immune and metabolic responses of broilers to *S. Enteritidis* and heat stress, and the interaction of genes across different tissues. Additional immune and non-immune tissues should be studied to investigate the response of broilers to *S. Enteritidis*. Characterizing the transcriptome of only immune tissues doesn't highlight gene interactions that exist between immune and non-immune tissues. These studies should also be done in a temporal manner to clarify these responses over time.

Genotyping technologies can be used to enhance selection for the commercial production of broilers that are resilient to *S. Enteritidis* and heat stress. Although broilers have a lower extent of LD compared to layers, the 600k Chicken SNP array developed by the Roslin Institute, Affymetrix Ltd., Aviagen Ltd., Hy-Line International, and the German Synbreed consortium is equipped to capture variance in broilers (Kranis et al., 2013). According to Megens et al. (2009), 100 SNPs is the threshold for an array to be effective in whole-genome marker assay in chickens. The 600k Chicken SNP array incorporates a (3:2) ratio of broiler and layer SNPs, providing similar capture power of the genetic diversity from both breeds (Qanbari et al., 2010). Poultry breeders currently use SNP data to select for desired traits. Thus, these SNPs can be used for genomic selection to select for disease and heat resistance in broilers. Although genotyping the

entire population will not be feasible for broiler producers, genotyping the parent stock with the 600k Chicken SNP array, genotyping the progeny with 60k SNP chips (Illumina Inc., San Diego, CA), and using imputation methods will eliminate the phase problems of MAS can be done (Fulton, 2012). As tools become more available and functional annotation of the chicken genome improves, using high-density arrays to select for commercial broilers that are resilient to *S. Enteritidis* and heat stress will become increasingly plausible.

References

- Bhattachary, S., and T. J. Mariani. 2013. Systems biology approaches to identify developmental bases for lung diseases. *Pediatr. Res.* 73:514-522.
- Bijlsma, R., and V. Loeschcke. 2012. Genetic erosion impedes adaptive responses to stressful environments. *Evol. Appl.* 5: 117-129.
- Cargnello, M., and P. P. Roux. 2011. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol. Mol. Biol. Rev.* 75:50-83.
- Cheng, H. H., P. Kaiser, and S. J. Lamont. 2013. Integrated genomic approaches to enhance genetic resistance in chickens. *Rev. Anim. Biosci.* 1:239-260.
- Chiang, H. I., C. L. Swaggerty, M. H. Kogut, S. E. Dowd, X. Li, I. Y. Pevzner, and H. Zhou. 2008. Gene expression profiling in chicken heterophils with *Salmonella enteritidis* stimulation using a chicken 44 K Agilent microarray. *BMC Genom.* 9:526.

- Ciraci, C., C. K. Tuggle, M. J. Wannemuehler, D. Nettleton, and S. J. Lamont. 2010. Unique genome-wide transcription profiles of chicken macrophages exposed to *Salmonella*-derived endotoxin. *BMC Genomics* 11:545-555.
- Fulton, J. E. 2012. Genomic selection for poultry breeding. *Animal Frontiers*. 2:30-36.
- International Chicken Genome Sequencing Consortium. 2004. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. *Nature* 432:695-716.
- Kramer, J., M. Malek, and S. J. Lamont. 2003. Association of twelve candidate gene polymorphisms and response to challenge with *Salmonella enteritidis* in poultry. *Anim. Genet.* 34:339-348.
- Kranis, A., A. A. Gheyas, C. Boschiero, F. Turner, L. Yu, S. Smith, R. Talbot, A. Pirani, F. Brew, P. Kaiser, P. M. Hocking, M. Fife, N. Salmon, J. Fulton, T. M. Strom, G. Haberer, S. Weigend, R. Preisinger, M. Gholami, S. Qanbari, H. Simianer, K. A. Watson, J. A. Woolliams, D. W. Burt. 2013. Development of a high density 600K SNP genotyping array for chicken. *BMC Genomics* 14:59.
- Li, C., X. Wang, G. Wang, N. Li, and C. Wu. 2011. Expression analysis of global gene response to chronic heat exposure in broiler chickens (*Gallus gallus*) reveals new reactive genes. *Poult. Sci.* 90:1028-1036.
- Lin, HY, R. H. Lai, S. T. Lin, R. C. Lin, M. J. Wang, C. C. Lin, H. C. Lee, F. F. Wang, and J. Y. Chen. 2012. Suppressor of cytokine signaling 6 (SOCS6) promotes mitochondrial fission via regulating DRP1 translocation. *Cell. Death Differ.* 19:1-15.

- Liu, J., Y. Song, K. M. Cheng, and F. G. Silversides. 2010. Production of donor-derived offspring from cryopreserved ovarian tissue in Japanese quail (*Coturnix japonica*). Biol. Reprod. 83:15-19.
- Megens, H.-J., R. P. M. A. Crooijmans, J. W. M. Bastiaansen, H. H. D. Kerstens, A. Coster, R. Jalving, A. Vereijken, P. Silva, W. M. Muir, H. H. Cheng, O. Hanotte, and M. A. M. Groenen. 2009. Comparison of linkage disequilibrium and haplotype diversity on macro- and microchromosomes in chicken. BMC Genetics 10:86.
- Miskinyte, S., M. G. Butler, D. Hervé, C. Sarret, M. Nicolino, J. D. Petralia, F. Bergametti, M. Arnould, V. N. Pham, A. V. Gore, K. Spengos, S. Gazal, F. Woimant, G. K. Steinberg, B. M. Weinstein, and E. Tournier-Lasserre. 2011. Loss of BRCC3 deubiquitinating enzyme leads to abnormal angiogenesis and is associated with syndromic moyamoya. Am. J. Hum. Genet. 88:718-728.
- Nishijima, K., and S. Iijima. 2013. Transgenic chickens. Dev. Growth Differ. 55:207-216.
- Qanbari, S., E. C. G. Pimentel, J. Tetens, G. Thaller, P. Lichtner, A. R. Sharifi, and H. Simianer. 2010. The pattern of linkage disequilibrium in German Holstein cattle. Anim. Genet. 41: 346-356.
- Silversides, F. G., and J. Liu. 2013. Novel techniques for preserving genetic diversity in poultry germplasm, in D. Hemming (ed.), Animal Science Reviews 2012, p. 207-214. CAB International, Oxfordshire, UK.

- Sung, Y. H., I.-J. Baek, D. H. Kim, J. Jeon, J. Lee, K. Lee, D. Jeong, J.-S. Kim and H.-W. Lee. 2013. Knockout mice created by TALEN-mediated gene targeting. *Nature Biotech.* 31:23-24.
- Tohidi, R., I. B. Idris , J. M. Panandam, and M. H. Bejo. 2012. The effects of polymorphisms in IL-2, IFN- γ , TGF- β 2, IgL, TLR-4, MD-2, and iNOS genes on resistance to *Salmonella* Enteritidis in indigenous chickens. *Avian Pathol.* 41:605-612.
- Wang, S.-H., C.-Y. Cheng, P.-C. Tang, C.-F. Chen, H.-H. Chen, Y.-P. Lee, and S.-Y. Huang. 2013. Differential gene expressions in testes of L2 strain Taiwan country chicken in response to acute heat stress. *Theriogenology.* 79:374-382.
- Zhou, H., and S. J. Lamont. 2007. Global gene expression profile after *Salmonella enterica* serovar *enteritidis* challenge in two F8 advanced intercross chicken lines. *Cytogenet. Genome Res.* 117:131-138.