The genomics of heat stress and immune response in chickens

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The genomics of heat stress and immune response in chickens

by

Angelica Grace Van Goor

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:
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Iowa State University
Ames, Iowa
2016

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DEDICATION

This dissertation is dedicated to my grandmother, Irene, who taught me the meaning of sisu.
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NOMENCLATURE

AIL     Advanced Intercross Line
QTL     Quantitative Trait Loci
GWAS    Genome Wide Association Study
LPS     Lipopolysaccharide
BMDC    Bone Marrow Derived Dendritic Cells
BW      Body Weight
BT      Body Temperature
ACKNOWLEDGMENTS

I would like to thank my major professor, Sue Lamont, and my committee members, Jack Dekkers, Max Rothschild, Chris Tuggle, and Peng Liu, for their guidance, support, and flexibility throughout the course of this research. A special thank you to Max Rothschild for being a mentor to me since the 2010 REU program. Thank you for organizing the wonderful international research experience in Uganda. I will never forget how much that trip changed my outlook on the world. Also, thank you for helping promote me to the next level in my career by serving as a reference. A big thank you to Sue Lamont for your patience, encouragement, time and effort, excellent mentorship, the numerous invitations to your home for homemade meals, the summer lab socials, and for leading by example. Thank you for teaching me how to promote myself, a skill generally very difficult for women. I am forever grateful for the opportunity to train with you.

In addition, I would also like to thank the Lamont Lab group, past and present for all of the support. You all made my time at ISU truly a pleasure. Thank you to Michael Kaiser for the laboratory assistance, encouragement, and many insightful conversations. A special thank you to Damarius Fleming for being a great friend and colleague since the day we met during our interview at ISU in spring 2011. I will never forget encouraging each other before giving talks. Thank you to Linda Wild and Ann Shuey for making sure everything was in order and for being really great to work with. I would like to thank the Poultry Farm Staff for the excellent work with the animals.

I would like to thank the co-authors on my manuscripts; Anna Slawinska, Kevin Bolek, Chris Ashwell, Michael Persia, Max Rothschild, Carl Schmidt, and Sue Lamont. You have been great to work with. A special thanks to Anna Slawinska for being a great colleague
and for promoting me to my next position. Thank you to Melha Mellata for giving me the opportunity to work with you as a post-doctoral scholar. I’m very much looking forward to working together.

I would like to thank my friends for their encouragement during our graduate studies. Thank you to Andrew and Melanie Hess for the technical help, late night discussions, several holiday parties, and for being awesome friends through the journey of graduate school. Thanks to Emily and Adam Van Egmond for always being great people to spend time with. Emily, we sure have come a long way from where we started. Thanks to Junmarie and Johed for being really great friends to spend time with. A special thanks to Alex Walton for the encouragement, and generally great times during graduate school. Finally, to my dear friend Teresa Kasper for being such an incredible friend throughout the years. We deserve it.

Thank you to my family for supporting me over the years. Thanks to my dad, Kai, for working so hard to give us kids a good education. They sure won’t be able to “bad mouth” you for home schooling us now. To my mom, Caroline, for being a great role model for strength, compassion, and intelligence. To my dear sister, Kara, for being my best friend. Thank you for the support during my time in graduate school. Thanks for the numerous visits to Ames, and for the company in the U.K. during my training there. I greatly admire your drive, intelligence, and kindness. To my brother Johan, for all the well wishes, support, and frequent visits to Ames. Finally, to my oldest brother, Matt, for always believing in me. I will never forget when my high school teacher told me “you should be a nurse”, and you saying “no, you should be a doctor”. I wouldn’t be here without you. Also, thank you for sharing your love of books with me, which has influenced me greatly. Thank you to my mother-in-
law, Linda Van Goor, for the care packages, encouragement, and the visits to Ames. Thank you to the Van Goor family for the endless support. I am very lucky to be part of the family.

Finally, the biggest thank you is to my dearest husband, Justin, for supporting me through my graduate studies. You have been steady through the wonderful times, and the hard times. Thank you for the daily encouragement to push further and to be better. Thanks for the world travels, the procrastination car rides around rural Iowa, the fantastic dinners, the comic relief, your patience and understanding, and for the deepest love.
Climate change is expected to increase the average global temperature and negatively impact the food supply. Increasing population and economies increase demand for dietary animal protein. Heat stress in chicken decreases production and increases disease susceptibility. Understanding the genetic control of response to heat in chickens could enable breeding more climate-adaptable chickens that are heat and/or disease resistant. Therefore, we characterized the response to heat and/or immune stimulation using unique genetic lines as a discovery platform using population, tissue, and isolated cell population studies. At the population level, a highly advanced intercross line originating from broiler (heat susceptible) and Fayoumi (heat resistant) were exposed to heat stress; body temperature, growth, digestibility, and blood chemistry components were measured. Most traits were estimated to have low to moderate heritabilities. Using the 600K SNP array genotypes, 96 QTL were identified along with positional candidate genes. In another study, broiler and Fayoumi chickens were exposed to heat and/or the immune stimulus of lipopolysaccharide (LPS). Spleen transcriptional profiles were determined using RNA-sequencing technology. Differentially expressed genes were identified in all treatment contrasts, with the largest effect observed in response to LPS+heat in both breeds. Ingenuity pathway analysis revealed unique pathways in response to heat such as Remodeling of Epithelial Adherens Junctions. In response to treatment with LPS, many immune-related pathways were identified, such as Granulocyte Adhesion and Diapedesis. In response to LPS+heat the pathways were mostly immune-related, including Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis. We performed an in vitro study using cells collected from Fayoumi (disease resistant) and Leghorn (disease susceptible). Bone marrow derived dendritic cells
were stimulated with heat and/or LPS and functionally characterized. Fayoumi cells produced more nitric oxide, phagocyted more, and had higher MHCII surface expression compared to the Leghorn. Collectively, this research contributes essential knowledge that there is a genetic component to response to heat and/or immune stimulation in chickens, a foundation for further investigation and potential avenues for population improvement.
CHAPTER 1
GENERAL INTRODUCTION

Dissertation Organization

The dissertation is organized into six chapters. Chapter 1 is the general introduction to the dissertation and includes an extensive literature review, Chapters 2-5 are scientific journal manuscripts, and Chapter 6 is a general discussion of the dissertation topic.

Introduction

The increases in global population will require a 70-100% increase in food production by 2050 (Tilman et al. 2011). Increased urbanization is accompanied by an increase in consumption of animal protein, and chicken meat and eggs are expected to grow at the highest rate (FAO 2013). Hans Selye was the first to coin the term stress in 1936 as “the non-specific response of the body to any demand for change”, which can include abiotic environmental stressors such as heat or cold, and biotic stressors such as pathogen challenge. With the remarkable increase in livestock productivity and efficiency in the last 50 years, there is evidence for a decreased ability to thrive in stressful environments (Rauw et al. 1998). In the face of climate change, heat stress is a concern to the livestock industry. In the U.S. chicken industry, heat stress is estimated to result in an economic loss of $128 million dollars annually (St-Pierre et al. 2003). Heat stress results in physiological and behavioral changes and is a concern for animal health and welfare. Production losses due to heat stress may result from increased mortality, reduced body weight, reduced egg production, reduced feed intake, physiological changes, higher feed to gain ratio, and increased susceptibility to disease. Response to heat in chickens has been shown to have a genetic component. For instance, layer chickens divergently selected for heat tolerance have differences in
survivability (Wilson et al. 1975), indicating a heritable component exists. Yet, a deep understanding of the genetic regions and genes in chickens has yet to be elucidated. We propose that genetic selection is a feasible and sustainable option to improve response to heat stress and immune response in chickens. To select for chickens able to better withstand these stressors, researchers must first understand the genetic contribution and molecular responses. This dissertation begins to elucidate the relationship between genetics and response to heat stress and/or an immune stimulation using unique chicken lines as a discovery platform.

We characterize response using a population, a tissue, and an isolated cell population. For the studies reported in Chapters 2 and 3, a highly advanced intercross line (AIL) of chickens created from crossing a commercial broiler (heat susceptible) with highly inbred Fayoumis (heat and disease resistant) was used. This population was subjected to a heat stressor and a variety of traits were measured including body temperature, body weight, breast yield, feed digestibility, and thirteen blood chemistry components. These animals were genotyped using a high density 600K SNP array. We then estimated heritabilities, and identified and quantified quantitative trait loci (QTL) associated with response to heat stress. The AIL is an extremely useful line to fine map QTL due to the breakdown of linkage disequilibrium between loci after many generations (Darvasi and Soller 1995). In the study reported in Chapter 4, we used the broiler and Fayoumi to determine response to heat stress and/or lipopolysaccharide (LPS), an immune stimulus. The transcriptome of the spleen, an important immune tissue, was assayed using RNA-sequencing technology. Chapter 5 reports a study in which we characterized cells in vitro from Fayoumi and Leghorn (disease susceptible) for response to stressors of heat and/or LPS. The response to these stressors was characterized by functional assays and mRNA expression using Fluidigm technology. The
results of this dissertation serve as a platform for further investigation into the response to stress, and have identified biomarkers with potential to be used for selection of heat and/or disease resistance.

**Organization of the Literature Review**

The literature review discusses the response of chickens to heat stress and the various technologies utilized to quantify these responses. The first section describes the global demand for animal products and the effects of climate change on poultry production. The second section examines the literature for the effects of heat stress on production parameters and physiology in chicken. The third section is about the effect of heat stress on immunity in chicken. The fourth section describes the genetic component to thermal stressors and immunity in chickens. The fifth section describes the use of genetic markers in chicken. The sixth section discusses transcriptomic analyses in chicken. The seventh section discusses in vitro analyses of chicken cells. The final section contains conclusions from the literature review.

**Literature Review**

**The global demand for animal products and the effects of climate change on poultry production**

Chickens were domesticated in Southeast Asia approximately 10,000 years ago (Hui 2013), and are thought to have been domesticated from the Red Jungle Fowl (Darwin 1868). Chickens were not initially eaten by humans, but rather utilized for ceremonial purposes and fighting. A recent report suggested the first evidence of humans using chicken for food was 2,000 years ago. The modern day, commercial *Gallus gallus* is primarily used for meat and egg production and these breeds are referred to as broiler and layer, respectively. The human population continues to grow at an exponential rate. In 2016 it is estimated that the human
The global population is 7.4 billion and it is predicted that by 2050 this will increase to 9.6 billion, according to a report by the United Nations (Nations 2013). With the urbanization and development of countries, the dietary preference for animal based proteins also increases (Regmi and Dyck 2001). With increases in the population it is projected that food production will have to increase by 70-100% by 2050 (Tilman et al. 2011). This increase in production however, is not accompanied by the availability of more land or water. Thus, to meet the global demand for food production we must become more efficient at our current production strategies.

According to a report by the Food and Agriculture Organization of the United Nations, the global meat consumption estimates pork as the highest (100 million tons), and poultry as the second highest (82 million tons) and poultry is projected to have the largest percentage increase in demand, rising to 181 million tons by 2050 (FAO 2013). With increasing global average temperatures, and increase in demand for poultry products, it is clearly apparent that we must increase production of poultry in the face of increasing temperatures.

Climate change is defined as increased severity and variability of weather patterns. This includes increased the severity and incidence of droughts, flooding, cold bouts, and heat waves. Weather variability is the overarching theme, but it is expected that the global average temperature will increase by 4°C by 2100 (New et al. 2011). The changing environmental temperatures requires adaptation from all forms of life.

Both heat and cold stress in poultry decreases productivity and increases mortality. It is estimated that heat stress results in economic losses in the poultry industry of $128 million annually in the U.S. (St-Pierre et al. 2003). Production losses due to heat stress in particular
may result from increased mortality, reduced body weight, reduced egg production, reduced feed intake, physiological changes, and increased susceptibility to disease and higher feed to gain ratio.

Various methods to ameliorate effects of heat stress have been proposed and is reviewed by Lin et al. (Lin et al. 2006). Management techniques such as increased use of cooling mechanisms such as fans, air conditioners, and misting machines. Decreasing the stocking density of chicken houses, or moving chicken houses to regions with a more temperate climate could ameliorate effects of heat stress. Although management techniques would certainly improve production in the face of heat, it is not an economically viable option for producers. A permanent and sustainable option is to utilize genetics to breed birds that are more robust in the face of environmental challenges.

In summary, climate change is impacting global food production, the population is increasing exponentially, and there is increased global demand for animal protein sources, particularly poultry. To meet the global demand for poultry, we propose that breeding chickens for heat tolerance is a sustainable method.

**Effect of heat stress on production parameters and physiology in chicken**

The progress using genetic selection in chickens has greatly increased size and growth rate (McKay et al. 2000). However, this progress has been speculated to come at the cost of robustness, where the animals are not able to maintain production in the presence of stressors such as high temperatures. Generally, when comparing growth- or reproduction-selected birds to non-selected birds, the selected birds are more impacted by stressors and produce less. Growth in meat-type chickens is the most economically important trait. Under stress from biotic and abiotic sources, resources are diverted by the body from production
and resources are used to return to homeostasis. Several studies have quantified growth reduction due to heat stress using many different temperature treatments and chicken lines. Commercial male broilers heat stressed from 1 to 42 days of age have a 33% reduction in BW (Sohail et al. 2012). Heat stress has been shown to reduce growth in female broilers to the same extent as that in males. For example, male and female broilers originating from a cross between a fast and slow growing strain heat stressed from 8-22 days of age both had a 22% reduction in BW (Han and Baker 1993). Male broilers exposed to increased temperature from 28-49 days of age reduced BW by 35% (Cooper and Washburn 1998). Numerous other studies show consistent results in the reduction of BW during periods of heat stress (Berrong and Washburn 1998; Dale and Fuller 1980; Deeb and Cahaner 2002; Geraert et al. 1996; Niu et al. 2009; ). Growth reduction results in significant production losses, but meat quality is also negatively affected by heat stress. Male broilers at 35 and 63 days of age exposed to 2 hours of heat stress significantly reduced muscle membrane integrity, lower pH, increased water loss, and increased incidence of breast muscle haemorrhages (Sandercock et al. 2001). In male broilers heat stressed from 10-22 days of age breast muscle color and composition was negatively affected (Dai et al. 2012). In turkeys, heat stress has been implicated to increase incidence of a pale, soft and exudative (PSE) phenotype (Malila et al. 2013). This phenotype is becoming a major concern in the chicken industry and it has been estimated that 47% of broiler fillets tested a single commercial broiler plant display signs of PSE (Owens et al. 2000). Heat stress has been shown to alter body composition observed in a reduction of percentage breast muscle yield and increases in fat deposition and thigh muscle percentage (Baziz et al. 1996; Lu et al. 2007; Zhang et al. 2012).
Similar reduction in production due to heat stress is observed in laying hens. Egg laying birds have been intensely selection for egg production and currently a single layer can produce more than 300 eggs per year, whereas indigenous chickens may lay only 40-60 eggs per year (Sørensen 2010). Egg production was estimated to be reduced by 28.8% in 60 week old layers heat stressed for 12 days (Deng et al. 2012). The highest impact on egg production that has been reported was a 57% reduction in egg production of 20 week old layers that were heat stressed for 13 weeks (Farnell et al. 2001). High temperatures have been associated with not only reduced number but also decreased egg quality. Egg quality traits are commonly quantified using egg shell thickness and weight, egg white weight, egg shape, and weight of albumin and yolk (Rath et al. 2015). Numerous studies have documented negative effects of heat stress on egg quality (Mashaly et al. 2004; Sahin et al. 2002; Ciftci et al. 2005; Ajakaiye et al. 2010; Bollengier-Lee 1999; Deaton et al. 1982; Kirunda and Scheideler 2001; Lin et al. 2004).

The significant losses in production and quality is largely caused by reduced feed intake and feed utilization during periods of heat stress. Reducing feed intake is a mechanism to depress production of metabolic heat. Feed intake affects growth rate and was demonstrated by a paired feed study where genetically lean broilers that were exposed to chronic heat stress, from hatch to 9 weeks of age, showed increased weight gain and feed efficiency compared to less lean counterparts, although not to the same extent as in thermoneutral conditions (Geraert et al. 1993). Male broilers heat stressed from 1-42 days of age reduced their feed intake 16 % and increased feed conversion ratio by 26 % (Sohail et al. 2012). In another study using birds heat stressed from 2 to 4 weeks of age showed a reduced feed intake by 14 % (Geraert et al. 1996). A similar trend in reduced feed intake and poorer
feed conversion is observed in laying hens. It has been estimated that feed intake is reduced by 35% during a 5 week heat stress period (Mashaly et al. 2004), and feed conversion ratio increases by 32% for an acute heat (24 hours) exposure (Star et al. 2009). McDaniel et al. demonstrated a reduction in fertility from 74% to 46% due to heat stress in broilers (McDaniel et al. 1995). The production losses in chickens due to heat stress include reduced body weight, egg production, product quality, feed utilization, and death. The changes to production traits are well characterized but, ultimately, the losses in production are a result of physiological changes. However, reduced feed intake is not solely responsible for reduced production. This has been demonstrated in a paired feeding study in broilers, where birds in heat stress conditions gained less BW compared to pair fed birds in thermoneutral conditions (Lin et al. 2004), indicating that physiological changes other than feed intake are partially responsible for reduction in growth during heat stress in chickens.

The negative effects of heat stress in chickens have been discussed and feed intake/utilization contribute largely to this reduction. Yet, the physiological response is of great importance to understanding the negative effects of heat. The mean normal body temperature of chickens is 41°C and they have the highest productivity within the thermoneutral temperature zone, which has been estimated between 20-26°C for adult broilers and layers (Deaton et al. 1978; Meltzer 1983 Meltzer et al. 1982). The upper critical temperature is defined as the ambient temperature in which the animal continually increases body temperature until mortality results, and this is estimated between 36-37°C for broilers (Van Der Hel et al. 1991). When body temperature rises to 47°C mortality results (Moreng and Shaffner 1951). A consequence of selection for rapid growth is increased incidence of heart failure and increased blood pressure, often resulting in ascites (Julian 1998). The most
common cause of mortality in chickens during heat stress is heart attack (Aengwanich and Simaraks 2004).

Several methods are used to reduce core body temperature during periods of heat stress in chickens such as redirecting blood flow, evaporative cooling, excessive water intake, and behaviours such as wing spreading. Chickens redirect blood flow from the internal organs and to the combs and skin (Wolfenson et al. 1981). The redistribution of blood flow may contribute to reduced feed efficiency observed during high ambient temperatures because the digestive tract is not receiving the same concentration of nutrients to properly digest feed. Body temperature has been shown to be negatively correlated with body weight, feed intake, and feed conversion ratio after 7 days of heat stress in 3 week old male broiler chickens (Cooper and Washburn 1998). The ability of an animal to maintain normal body temperature under high environmental temperature would be a desirable characteristic. The main way that humans regulate body temperature is by evaporative cooling using the sweat glands, which chickens lack completely. The thermoregulatory capacity of sweat glands is replaced by evaporative cooling by respiration in the chicken. Chickens have a unique anatomy of the respiratory system that contributes to extensive ability to dissipate heat by respiration. Attached to the lungs are air sacs that serve as reservoirs for air exchange and result in increased efficiency in gas exchange compared to human (Brackenbury 1972). During periods of heat stress, chickens increase the depth and frequency of breaths (Zhou and Yamamoto 1997). The ability of a bird to dissipate heat using respiration is dependent upon the relative humidity (Lin et al. 2006). Two studies by Lin et al. (2005) identified that in four week old broilers exposed to 35°C heat stress that humidity above 60% impairs heat transmission from the body core to the peripheries, while using one
week old broilers heat stressed at 35°C the relative humidity had no effect on heat transmission (Lin et al. 2005). The disagreement between these two studies indicates that the age of the chicken is of great importance to interpretation of the results. These changes in respiration may result in respiratory alkalosis, which is a disturbance in the acid base balance characterized by an increase in blood pH accompanied by a decrease in pCO₂. Respiratory alkalosis occurs in broilers during heat stress and is associated with decreased growth (Teeter et al. 1985). Broilers 35 days of age that were heat stressed for 2 hours had significantly increased blood pH and decreased pCO₂ (Sandercock et al. 2001) and, in another study using broilers heat stress for 2 weeks at 28 days of age showed birds that were panting had similar responses (Teeter et al. 1985). In a study using 35 day old broilers, blood pO₂ significantly increased from after cyclical heat stress for 10 days at 35°C (Deyhim and Teeter 1991). These studies clearly demonstrate that respiratory alkalosis occurs during heat stress as a likely mechanism to reduce body temperature.

Metabolic alkalosis occurs when there is a disturbance in the fixed acids and bases in the extracellular fluid (Galla 2000). Imbalance of dietary Na, K, or Ca can result in metabolic alkalosis (Daghir 2008), which is characterized by an increase in blood pH, HCO₃⁻, and base excess, and can be induced in growing layers by high levels of calcium in feed (Guoa et al. 2008).

Base excess is considered a comprehensive measure of the metabolic components of bases, which reflects the nonrespiratory contribution to changes in acid-base disturbances (Chin et al. 2012). Base excess can be altered by changing the cation: anion ratio in the diet of broiler chickens and is associated with body weight and bone density (Halley et al. 1987). Furthermore, heat stress has been shown to increase base excess (Borges et al. 2004),
consistent with the hypothesis that chickens experience metabolic alkalosis under heat stress. Bicarbonate (HCO$_3$) is the most abundant buffer in the blood, is primarily regulated by the kidneys, and is a metabolic component of acid-base balance (Chin et al. 2012). Broilers at 28 days of age in which blood HCO$_3$ significantly decreased in panting birds under acute heat stress (Teeter et al. 1985), and another study using male broilers reported a decrease in HCO$_3$ after a heat stress at 32°C for 10 hours (Borges et al. 2003). This decrease in HCO$_3$ is consistent with increased respiration used to decrease body temperature because a large amount of CO$_2$ is expelled from the lungs. Additionally, during heat stress conditions water output in feces and urine is increased, causing further acid base imbalance (Belay and Teeter 1996).

During periods of heat stress, the blood volume and oxygen carrying capacity are altered (Yahav et al. 1997) and dehydration, caused by increased respiration, can increase hematocrit values (Borges et al. 2004). Heat stress results in increased excretion of water and losses through respiration, and although water intake increases, it may not compensate for losses and this results in dehydration (Belay and Teeter 1996). Changes in blood volume and oxygen carrying capacity occur in chickens during periods of heat stress (Yahav et al. 1997). A study using male broilers observed a decrease in both hemoglobin and hematocrit after an acute heat stress at 32°C for 10 hours (Borges et al. 2004). Another measure of disturbances to blood volume and oxygen carrying capacity is blood sO$_2$, which is the proportion of oxyhemoglobin to total hemoglobin that is able to bind oxygen (Chin et al. 2012).

Glucose is the primary energy source for all metabolic reactions. The plasma glucose concentration is primarily regulated by hormones secreted by the pancreas. The hormones glucagon and insulin function to increase and decrease glucose levels, respectively.
Disturbances to this balance can result in damage to nerves, blood vessels, and even death. Plasma glucose level is increased in broilers exposed to heat stress at different ages and for different temperatures and durations (Borges et al. 2004; Garriga et al. 2006; Khan et al. 2002). It has been speculated that the increase in glucose could be caused by increased output of glucose from the liver without compensatory utilization of the excess glucose in the blood stream (Hargreaves et al. 1996). In chicken lines divergently selected for blood glucose concentration, the low glucose line was less efficient at food utilization compared to the high glucose line (Leclercq et al. 1987), indicating that basal lower glucose levels potentially increase feed efficiency. However, as previously stated, feed efficiency is decreased in chickens during heat stress.

Changes in physiology may occur by alterations in electrolyte balance which is essential for acid-base balance, maintenance of cellular homeostasis, synthesis of tissue protein, electrical potential of cell membranes, enzymatic reactions, and maintaining osmotic pressure (Borges et al. 2007). Increased fluid excretion in the urine is accompanied by increased concentrations of electrolytes in the urine (Belay and Teeter 1996). Altering electrolyte amounts in feed partially ameliorates the negative impacts of heat stress in broiler chickens (Borges et al. 2003). Previous studies report decreased concentrations of both K and Na in response to heat stress, likely due to increased water intake and higher excretion, which results in decreased concentrations of electrolytes within the blood (Ait-Boulahsen et al. 1989; Borges et al. 2004; Borges et al. 2003). The studies that have identified that the appropriate amount of electrolytes added to feed and/or water are of great importance to our understanding of methods to ameliorate heat stress in chickens. Breeding for a desirable
response to heat stress might be complimentary to, or more economical than, using alternate feed additives.

Advances in veterinary medicine have resulted in a very fast and efficient method to measure blood chemistry components using an iSTAT machine (i-STAT®1 2006). This machine is handheld and requires a blood sample of 100 μl and analyses the sample within 3 minutes. Blood chemistry components can be analysed using one of nineteen available cartridges. The CG8+ cartridge measures blood components representative of chemistries/electrolytes, hematology, and blood gases (i-STAT®1 2006). Using the CG8+ cartridge thirteen different components can be measured including pH, pCO₂, pO₂, base excess, HCO₃, TCO₂, K, Na, ionized Ca, hematocrit, hemoglobin, sO₂, and glucose. The iSTAT machine with the CG8+ cartridge is relatively easy to use, and has potential to identify biomarkers for response to treatment in animals. Advantages include the ability to sample over time and to use the same animal for breeding purposes.

**Effect of heat stress on immunity in chicken**

Selye defined stress and stressors as “the nonspecific response of the body to any demand. A stressor is an agent that produced stress at any time” (Selye 1976). Temperatures outside of the thermoneutral zone and immune stimulants (or pathogens) are both considered stressors. St. Pierre estimated $158 million in production losses in poultry in in 2003 within the U.S. alone due to heat stress (St-Pierre et al. 2003). Disease is estimated to result in 20% of production losses in the poultry industry (Biggs 1982), and is a concern for animal welfare and human health. According to the CDC, 48 million people (1 of 6) get ill from food eaten in the U.S. alone (Painter et al. 2013). Although over half of all human illnesses were caused
by produce, poultry accounted for the largest proportion (19%) of fatal cases of any type of food source (Painter et al. 2013).

The immune system is comprised of two main arms; innate and adaptive. The innate immune response is a non-specific response to pathogens and is initiated immediately upon pathogen invasion. Some examples of the innate immune system include skin and mucosa linings, saliva and mucus that trap and kill pathogens, and a variety of immune cells such as macrophages and dendritic cells that recognize and kill pathogens (Murphy 2011). The adaptive immune system typically requires seven days to become activated and effective. This immune arm functions to recognize specific non-host antigens, generate a large number of cells to efficiently remove pathogens, and develop B-cells and T-cells that elicit long term immunity (Murphy 2011).

Heat stress results in extreme stress, and if an animal is faced with an immune challenge simultaneously generally immune response is hampered. Several studies have identified decreases in adaptive immunity in chickens during heat stress conditions. In layers heat stressed for 5 weeks, the total white blood cell count decreased, antibody production to sheep red blood cells (SRBC) decreased, and lymphocyte activity decreased (Mashaly et al. 2004). A commonly used method to induce adaptive immune response to a foreign antigen is inoculation with SRBC. Another study identified a reduction in the relative weight of liver and increases the expression of heat shock protein 70 in layers due to heat stress (Felver-Gant et al. 2012). Examination of the gut epithelial cells in heat-stressed laying hens identified a decrease in the number of immune cells (Deng et al. 2012). The reduction in adaptive immunity during periods of heat stress has been studied in broiler chickens as well. Heat stressed broilers have lower weights of immune organs including the bursa of Fabricius,
thymus, and spleen (Niu et al. 2009; Quinteiro-Filho et al. 2010; Smith 2003). Lower relative weight in lymphoid organs is undesirable, and has been associated with decreased immune response to Newcastle Disease in chickens (Sijtsma et al. 1991). Total circulating antibody and specificity to SRBC are decreased in broilers under heat stress (Smith 2003). Broiler chicken lines selected for high and low antibody titre to SRBC were subjected to heat stress, and the highly responsive line had decreased antibody production to SRBC under heat stress conditions compared to thermoneutral conditions (Donker et al. 1990). Whereas, the broiler line selected for low antibody response in the previously mentioned study did not change antibody production during heat stress conditions. The authors attempted to confirm the results of decreased antibody in the high selection line, but the results were not consistent. Donker et al. concluded that the circulating antibody difference detected was relatively unimportant, and that likely the broiler lines assessed in the study were not in a state of heat stress (Donker et al. 1990). On the contrary to immunosuppressive effect of heat stress, short bouts (1-2 hours) have shown to increase antibody production to SRBC (Heller et al. 1979).

In addition to adaptive immune cell reaction to heat stress, broilers have been shown to have increased susceptibility to mild enteritis characterized by increased concentrations of white blood cells in lamina propria of the jejunum (Quinteiro-Filho et al. 2012).

The adaptive immune response to heat stress in chickens has been investigated more than the innate immune response. The few studies that have characterized innate response have reported heat-related decreases in activity. During a pathogen invasion, macrophages function to recognize the pathogens, eliminate them, and present foreign antigens to initiate the adaptive immune response (MacMicking et al. 1997). Heat stress decreases macrophage activity, as they have lower basal and bacterial induced oxidative burst activity (Quinteiro-
Filho et al. 2010). Broilers that are heat stressed and challenged with Salmonella Enteritidis show an increased amount of bacterial invasion of the spleen, and the authors speculate this may be due to gut barrier dysfunction during heat stress (Quinteiro-Filho et al. 2012).

The mucosa of the gastrointestinal tract protects the body from bacteria within the gut and has been shown to increase permeability of large and small molecules in response to heat stress in humans and has been extensively reviewed (Travis and Menzies 1992). The intestinal integrity has been studied in response to heat in chickens. Broilers under heat stress conditions increase intestinal permeability (Quinteiro-Filho et al. 2012; Quinteiro-Filho et al. 2010; Star et al. 2009), and layers have altered gut morphology of microvilli (Deng et al. 2012). A major concern of increased gut permeability is the infiltration of the gut microbiome to the blood stream. This rapid increase in bacteria within the blood can lead to endotoxic shock, sepsis, and death largely due to the pro-inflammatory cytokine storm (Maes 2008). The major contributing factor to the cytokine storm caused by disruption of the gut barrier is believed to be Lipopolysaccharide (LPS) (Mass et al. 2008). LPS is an essential component of gram negative bacteria and believed to be a major contributor to fatality of heat stroke in humans (Graber et al. 1971). The increased amount of circulating LPS during periods of heat stress could contribute to increased mortality and decreased production. LPS is a well-characterized inflammatory stimulus. The double stimulation of LPS and heat stress (as might occur in a typical poultry production environment) could increase body temperatures beyond the thermal comfort zone, resulting in increased mortality. Our experiment to identify the response of chickens to the double stimulus of LPS and heat stress, to investigate effects of heat stress on the immune response, will address an important gap in knowledge.
Genetic component of response to thermal stressors and immunity in chickens

To breed for a desirable trait, there must be a genetic component. The standard quantification of this is heritability, which is defined as the proportion of variance in a trait that is due to genetics. Evidence suggests that there is a genetic influence on response to heat stress. In a study on heat stress using Red Jungle Fowl, a village chicken strain, and a commercially selected broiler line, significant differences in response were quantified (Soleimani et al. 2011). The broiler strain exhibited a more negative response to heat treatment compared to the other two breeds, characterized by a higher body temperature, higher heterophili:lymphocyte ratio, and a higher plasma corticosterone concentration, which is a measure of stress response (Soleimani et al. 2011). The major conclusion from the aforementioned research is that commercial selection for production traits has altered the ability of modern chickens to withstand high temperatures. Previous studies in poultry have elucidated a genetic influence on response to heat. Layers that were divergently selected for heat tolerance displayed differences in survivability during increased heat conditions (Wilson et al. 1975). Significant differences in production traits have been found between a commercially fast growing chicken line and a local chicken breed from China during heat stress (Lu et al. 2007). Microsatellites were used to identify quantitative trait loci (QTL) for several traits measured during heat stress in a Japanese quail F2 intercross including body weight, feed intake, and body temperature (Minvielle et al. 2005). Some instances, however, have been reported of genetic lines not differing in response to heat stress. In a study using slow growing, fast growing, and heavy chicken lines, no difference was identified in corticosterone production, acid base balance, or electrolyte values in response to heat stress (Debut et al. 2005). Because, in poultry, the response to heat stress has been shown to have a
genetic component, it is possible to use genomic selection for heat tolerance, which will increase accuracies and response to selection (Goddard 2009). Several chicken lines created by selecting for differences in body weights and immune responses have been used to identify genetic component of response to heat stress. However, no study has evaluated a cross between divergent lines. Our large genetic study of advanced intercross chickens will be used to address a gap in knowledge by identifying the genomic regions associated with response to heat stress.

**Use of genetic markers in chicken**

A method to investigate the genetic components of a trait is to estimate the heritability. If the pedigree is known, then heritability can be computed using a sire model, an animal model, or a divergent selection experiment. If the genetic relationship between subjects is unknown, then a G matrix based on genetic markers can be used (Lynch and Walsh 1998). Once it is established that a trait is heritable, then investigation into the genetic regions is a logical next step to identify and quantify the effects of the underlying regions that influence the traits. An early technolog used to investigate the association between genetic loci and traits was the use of restriction length polymorphisms (RFLP) (Lander and Botstein 1989). The RFLP technology utilizes restriction enzymes derived from bacteria that recognize and cut specific nucleotide sequences. The differences in genetics can be identified by assaying the cut DNA products using a gel. The identification of variable regions within the DNA termed microsatellites and widespread use of PCR made microsatellites the preferred method to compare populations (Tautz 1989). As technologies to study genetic variation became more sophisticated, single nucleotide polymorphisms (SNPs) were introduced (Syvänen 2001) as a high throughput method to study genomic variation, and
largely replaced the formerly mentioned technologies. Over the past decade, SNP panels increased in density. Currently, there is an array for chicken which includes 600K SNPs (Kranis et al. 2013). As whole genome sequencing increases in accuracy and decreases in price, SNP panels will likely be replaced by low-coverage sequencing.

The experimental design of QTL mapping studies depends upon the specific research question. One method is to measure a quantitative trait on an outbred population and genotype the extreme phenotypes. Using an outbred population requires a dense SNP panel because on average, the linkage disequilibrium (LD) is short. Another design is to cross two divergent inbred lines, and genotype the F2 generation. This design requires a low density SNP panel because the LD has only been broken up by recombination one time, i.e. recombination within the F1. Alternatively, the F1 can be backcrossed to one of the original parental lines to identify QTL. This approach results in long stretches of LD and therefore a low density SNP panel is required. Another approach is to continue inter-mating the generations from crossing divergent lines to create an advanced intercross line (AIL). This type of mating results in very short stretches of LD in the population, and therefore requires a dense SNP panel to detect QTL near causative loci.

SNP panels are widely utilized for two main purposes 1) genome-wide association studies and 2) genomic prediction. Whereas the latter is based on a training and validation population to predict the performance of offspring based on SNPs in the population, genome-wide association studies (GWAS) are used to detect and quantify associations between SNPs and quantitative traits and these regions are termed QTL.

Many studies in chicken have focused on identifying QTL for production traits. For instance, 4,307 QTL have been identified in poultry for production traits, whereas the
reported numbers of QTL are 561 for health, 211 related to exterior, and only 117 for physiological traits ([www.animalgenome.org](http://www.animalgenome.org)). Currently, no QTL have been identified in chickens for responses to heat stress. Identifying the QTL associated with response to heat stress is very important to understanding the genomic architecture of the traits and to understanding the breeding potential of markers for genomic selection. Taken together, further investigation into the genomics of heat stress in chickens is warranted.

To fine map QTL and identify underlying genes, an intercross between divergent lines is a good resource to use. Darvasi and Soller first described the use of an AIL for fine genetic mapping (Darvasi and Soller 1995). When a population is created from two inbred or divergent lines, and the progeny subsequently inter-mated for several generations, there is a high probability of recombination between loci. This phenomenon allows accurate fine-mapping of QTL. The AIL mapping population used in the current study was created from two extremely divergent breeds, the broiler and the Fayoumi (Deeb and Lamont 2002). The broiler originated from a commercial broiler breeder male line, whereas the Fayoumi is more than 99% inbred (Zhou and Lamont 1999b). The broiler breed was commercially selected for muscle accretion, whereas the Fayoumi breed has not undergone commercial selection. The Fayoumi breed originated in the Fayoum region of Egypt and subsequently has undergone natural selection for tolerance to heat. Several studies have compared the parental lines of the AIL, mostly for immune response traits (Abasht et al. 2009; Cheeseman et al. 2007; Redmond et al. 2009; Sandford et al. 2011). This intercross line is therefore a good candidate to investigate the response to heat stress and identify QTL.
Transcriptomic response to heat stress in chicken

Not only is it desirable to determine the genetic component of a trait by estimating heritability and performing association studies, but another approach to understanding genetic control is to quantify the expression of transcripts. The most widely studied class of transcripts is mRNA which is transcribed from DNA, processed within the nucleus, and subsequently translated into amino acids on ribosomes within the cytoplasm. There are many technologies to quantify mRNA expression including northern blot, RT-PCR, and whole transcriptome profiling using RNA-sequencing methods. RNA-sequencing is currently the preferred method to assay transcriptional levels because it is an a priori approach that doesn’t require investigators to choose genes to assay expression but, rather, assays expression for all genes that are transcribed. RNA-sequencing can reveal genes and pathways that are responsive to treatment effects. The transcriptomic response to heat stress in chickens has been documented in several tissues and cell lines. The liver of heat stressed broilers revealed 43 differentially expressed genes using RNA-sequencing technology (Coble et al. 2014). In Silkie fowl, the breast muscle revealed 110 genes differentially expressed using microarray technology (Li et al. 2011). In a male white leghorn liver cancer cell line, RNA-sequencing revealed 812 transcripts that were responsive to in vitro heat treatment (Sun et al. 2015). In meat-type birds exposed to heat stress, the hypothalamus was evaluated using microarray, which showed 1,967 genes to be differentially expressed (Sun et al. 2015). Contrasting responses of livers from fast and slow growing broilers to heat stress resulted in differential expression of two heat-related genes analysed using RT-PCR (Rimoldi et al. 2015). Taken together, a variety of breeds of chickens and tissues have been investigated for response to
heat stress. No studies, however, have been done to investigate the global transcriptomic response in an immune tissue to the double stressor of heat stress and immune stimulation.

**In vitro analyses in chicken**

In vitro approaches can provide information that is useful for application in vivo. The benefits of in vitro experimentation are that it requires less monetary investment and, in most cases, allows one to study an isolated cell population in a reductionist approach. There are several immortalized chicken cell lines available, with one of the best characterized being HD11 macrophage-like (Beug et al. 1979). Macrophages are essential as effector cells in the innate immune response and to potentiate the acquired immune response. In vitro analysis of chicken peritoneal macrophages exposed to 45°C for 1 hour displayed reduced non-specific phagocytic ability (Miller and Qureshi 1992). Immune response is affected during periods of environmental stress, but whether the stress results in a positive (more resistant/tolerant) or negative (more susceptible) outcome depends on the stressor and the infectious agent (Griffin 1989). It is well documented that stress reduces the adaptive immune response in chickens (Davison et al. 1989; Donker et al. 1990; Donker and Beuving 1989; Hangalapura et al. 2003; Kiecolt-Glaser et al. 1996; Liew et al. 2003; Mashaly et al. 2004). Little is known about the consequences of stress on the innate immune response; however, some studies suggest that stress potentiates innate immunity and decreases acquired immunity (Coussons-Read et al. 1994; Fleshner et al. 1998; Quinteiro-Filho et al. 2010). On the contrary, chicken macrophage activity decreases during periods of heat stress (Quinteiro-Filho et al. 2010), suggesting impaired, rather than enhanced, and innate immunity. Clearly, more research is required to elucidate the complex interaction between environmental stress and innate immunity.
Until recently, methods to study chicken innate immune cells differentiated in vitro was impossible due to lack of immunological assays. The cloning of CSF-1 (Garceau et al. 2010), and GM-CSF and IL-4 (Avery et al. 2004), allowed differentiation of bone marrow (BM) cells into antigen presenting cells (APC). The purified recombinant cytokines for BM differentiation are now commercially available to researchers, enabling research which will surely improve our understanding of chicken innate immunity. Similar to their mammalian counterparts, chicken bone marrow derived dendritic cells (BMDC) can be differentiated in vitro (Wu et al. 2010; Garceau et al. 2010), and exhibit conserved signatures of morphology and phagocytic activity and the BMDC express the DEC-205 cell surface marker, highly express CCR6 and lowly express MHC II (Wu et al. 2010). Upon stimulation with LPS, chicken BMDC decrease phagocytic activity, shift expression from CCR6 to CCR7, and increase both DC-LAMP and MHC II expression (Wu et al. 2010).

There is an increasing body of evidence linking genetics with APC function in response to pathogens and autoimmune disorders. Curtis et al. (2015) determined that a single SNP in the human ASAP1 gene is associated with the level of the protein expression in the DC of the humans infected with Mycobacterium tuberculosis and, thus, the predisposition to the disease (Curtis et al. 2015). Cooney et al. (2010) demonstrated that individuals with Crohn’s disease express a risk variant of NOD2 that is associated with the defective autophagy and antigen presentation in DC (Cooney et al. 2010). Furthermore, a single SNP locus in the IRF7 gene modulates interferon production in DC in response to influenza virus (Gregersen 2014). Finally, a variant in the murine PTPN22 gene causes DC hyper responsiveness leading to increased risk of autoimmune disease (Zhang et al. 2011). These
results suggest that the innate immune response expressed by different DC can be strongly influenced by the genotype.

Chicken breeds with distinct genetic background, such as Leghorn and Fayoumi, represent different levels of adaptation to pathogenic and environmental stressors. Leghorn is a major commercial egg laying breed, whereas Fayoumi represents a wild-type strain of chicken, originated from Egypt. Multiple studies indicate that Fayoumi is a hardier genetic line than Leghorn, with a higher level of resistance to infections with Marek’s disease (Lakshmanan et al. 1996), coccidiosis (Pinard-Van Der Laan et al. 1998), Newcastle disease (S. Lamont, personal communication), avian influenza (Wang et al. 2014), and Salmonella (Redmond et al. 2011) compared to Leghorns. Both genetic lines used in this study, Leghorn (GhS 6 line) and Fayoumi (M 5.1 line), are highly inbred (99.9%) (Zhou and Lamont 1999a), and they have been used as an excellent discovery platform in numerous studies on avian immunogenetics and disease resistance (Kim et al. 2008; Coble et al. 2011; Redmond et al. 2009). Hereby, we hypothesize that the Fayoumi genetic line will be also superior to the Leghorn line in mounting a stronger innate immunity in response of BMDC to complex environmental stimuli.

The increasing knowledge on characterization and culturing of the BMDC in avian species, availability of chicken lines that differ in the genetics and disease phenotype, and the growing impact of environmental (increasing incidences of heat episodes) and epidemiological (infectious disease outbreaks) stressors in animal production make it timely to better understand the interaction of selected environmental stressors on the function of crucial immune cell types. We therefore conducted an in vitro study on chicken BMDC response to an immune stimulation and an environmental stressor. To our knowledge, there
has been no report to provide functional comparisons between cellular and molecular responses to LPS, heat, and LPS+heat in chicken BMDC derived from distinct genetic lines. This type of investigation has potential to validate the in vitro system to study immune and thermal stressors, and to potentially identify biomarkers for resistance to these.

**Dissertation study addresses gaps in knowledge**

Herein we propose to characterize the response of chickens to heat stress using state of the art technologies, including high density genotyping on the 600K SNP array, whole transcriptome profiling using RNA-sequencing, and in vitro methods to study bone marrow derived dendritic cells. The GWAS will give insight into regions and genes that control body weight, digestibility, and blood chemistry components. The objectives of the QTL mapping study were to estimate theheritabilities, and identify and quantify QTL. The objective of the RNA-sequencing study were to identify differentially expressed genes and pathways due to treatment. The objective of the in vitro studies were to identify functional differences between unique genetic lines due to treatment. The RNA-sequencing of the spleen will elucidate the transcriptomic response of a secondary lymphoid tissue and bone marrow derived antigen presenting cells to heat stress, LPS, and LPS+heat. Using genetic markers, identified genes that respond to treatment, and in vitro methods will allow us to identify potential biomarkers associated with a favorable response to heat stress in chickens. These biomarkers may be used to breed for more heat tolerant birds that respond well to the changing environment and hot climates.

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CHAPTER 2
IDENTIFICATION OF QUANTITATIVE TRAIT LOCI FOR BODY TEMPERATURE, BODY WEIGHT, BREAST YIELD, AND DIGESTIBILITY IN AN ADVANCED INTERCROSS LINE OF CHICKENS UNDER HEAT STRESS

Modified from a paper published in Genetics Selection and Evolution on December 17, 2015
DOI: 10.1186/s12711-015-0176-7

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Author’s contribution
AVG participated in sample collection, isolated DNA for genotyping, carried out all statistical analyses, and drafted the manuscript.

Abstract

Background

Losses in poultry production due to heat stress have considerable negative economic consequences. Previous studies in poultry have elucidated a genetic influence on response to heat. Using a unique chicken genetic resource, we identified genomic regions associated with body temperature (BT), body weight (BW), breast yield, and digestibility measured during heat stress. Identifying genes associated with a favorable response during high ambient temperature can facilitate genetic selection of heat-resilient chickens.

Methods

Generations F18 and F19 of a broiler (heat-susceptible) by Fayoumi (heat-resistant) advanced intercross line (AIL) were used to fine-map quantitative trait loci (QTL). Six hundred and thirty-one birds were exposed to daily heat cycles from 22 to 28 days of age, and phenotypes were measured before heat treatment, on the first day and after one week of
heat treatment. BT was measured at these three phases and BW at pre-heat treatment and after one week of heat treatment. Breast muscle yield was calculated as the percentage of body weight at day 28. Ileal feed digestibility was assayed from digesta collected from the ileum at day 28. Four hundred and sixty-eight AIL were genotyped using the 600K Affymetrix chicken SNP (single nucleotide polymorphism) array. Trait heritabilities were estimated using an animal model. A genome-wide association study (GWAS) for these traits and changes in BT and BW was conducted using Bayesian analyses. Candidate genes were identified within 200-kb regions around SNPs with significant association signals.

Results

Heritabilities were low to moderate (0.03 to 0.35). We identified QTL for BT on *Gallus gallus* chromosome (GGA)14, 15, 26, and 27; BW on GGA1 to 8, 10, 14, and 21; dry matter digestibility on GGA19, 20 and 21; and QTL of very large effect for breast muscle yield on GGA1, 15, and 22 with a single 1-Mb window on GGA1 explaining more than 15% of the genetic variation.

Conclusions

This is the first study to estimate heritabilities and perform GWAS using this AIL for traits measured during heat stress. Significant QTL as well as low to moderate heritabilities were found for each trait, and these QTL may facilitate selection for improved animal performance in hot climatic conditions.

Background

The climate is becoming increasingly warmer, according to the Intergovernmental Panel on Climate Change, and the global average temperature will continue to increase by 0.2°C per decade. Heat stress in poultry impacts animal production and welfare. Heat stress
in the poultry industry causes an estimated economic loss of $125 to 165 million in the U.S., with the broiler industry alone accounting for $58.1 million (St-Pierre et al. 2003). In 2007, an extreme heat wave in California resulted in more than 700,000 deaths in poultry (USAgNet 2006) and in 2009, over 1.5 million layer hens died during a summer heat wave (National Oceanic and Atmospheric Association).

Production losses due to heat stress may result from mortality, reduced body weight, reduced egg production, reduced feed intake, and higher feed to gain ratio. A recent study on broilers that were exposed to chronic heat stress from 1 to 42 days of age showed a reduced body weight (32.6 %), increased feed conversion ratio (25.6 %), and reduced feed intake (16.4 %) (Sohail et al. 2012), and another study using shorter periods of heat stress on younger birds, from 2 to 4 weeks of age showed a reduced feed intake by 14 % (Geraert et al. 1996). In a paired feed study, genetically lean broilers that were exposed to chronic heat stress, from hatch to 9 weeks of age showed increased weight gain and feed efficiency compared to less lean counterparts, which supports the hypothesis that increased fat accretion is inversely related to thermo-regulation (Geraert et al. 1993). Previous studies in poultry have elucidated a genetic influence on response to heat. Layers that were divergently selected for heat tolerance displayed differences in survivability during increased heat conditions (Wilson et al. 1975). Significant differences in production traits have been found between a commercially fast growing chicken line and a local chicken breed from China during heat stress (Lu et al. 2007).

Microsatellites were used to identify quantitative trait loci (QTL) for several traits measured during heat stress in a Japanese quail F2 intercross including body weight (BW), feed intake, and body temperature (BT) (Minvielle et al. 2005). Because in poultry, response
to heat stress involves a genetic component, it is possible to use genomic selection for heat tolerance, which will increase accuracies and response to selection (Goddard 2009). To increase our understanding of the genetic influence on response to heat stress in chickens, we used the F18 and F19 generations of a broiler (heat-susceptible) x Fayoumi (heat-resistant) advanced intercross line (AIL) and an environmentally-controlled experiment to identify genomic markers related to response to high ambient temperatures.

Chickens of this AIL were exposed to high ambient temperatures for seven days during which BW, BT, breast yield, and digestibility were measured. These traits, as well as the changes in BT and BW due to heat treatment, were used for genome-wide association studies (GWAS). The genes and markers associated with thermal tolerance can help elucidate the genetic architecture of traits involved in heat stress and, subsequently, be used to breed more heat-resilient chickens.

Methods

Chicken lines

All animal experiments were approved by the Institutional Animal Care and Use Committee at Iowa State University: Log #4-11-7128-G. We used the F18 and F19 generations of an AIL between two highly divergent chicken lines for thermo-tolerance, i.e. it was created by crossing a single broiler sire to six highly inbred Fayoumi dams (Deeb and Lamont 2002). Although this population has limited variability due to the initial mating, the broiler sire was characterized by the offsprings’ phenotypic means and variances of body composition phenotypes, which showed that it was representative of the entire broiler population (Deeb and Lamont 2002). We hypothesize that the highly inbred Fayoumi breed became fixed for alleles that had the highest frequency in the founder line. Thus, this
population is a powerful resource to identify QTL. The broiler breed has been commercially selected for muscle accretion, whereas the Fayoumi breed has not undergone commercial selection. The Fayoumi breed originated in the Fayoum region of Egypt that is characterized by a high-temperature climate and thus this breed has undergone natural selection for tolerance to heat. Birds were reared on floor pens with wood shavings and had ad libitum access to water and corn-soy feed that met all NRC requirements for the duration of the study (NRC 1994).

**Heat stress experimental design**

We used birds from two generations with each generation producing two hatches. Six hundred and thirty-one birds from the four hatches were used for independent heat stress experiments (four replicates). Six hundred animals from 17 sire families were phenotyped for BT, BW, and breast yield. Digestibility measurements were available for 461 animals from 14 sire families. At 17 days of age, birds were transferred to environmentally-controlled chambers and acclimated for five days. There were four chambers, each containing six pens, per replicate. From days 22 to 28 of age, the chambers were heated to 35°C for 7 hours per day and remained at 25°C at all other times.

**Phenotypic measurements**

Cloacal BT was measured by inserting a digital thermometer approximately 2.5 cm into the cloaca on days 20, 22, and 28 of age. The precision of the digital thermometer was 0.1°C. BW was measured using a digital scale on days 21 and 28 of age. Breast yield (%) was determined by weighing one half of the pectoralis major and minor muscle, multiplying this value by two, and then dividing it by the total BW on day 28. Dry matter digestibility was measured as described in (Bolek 2013). Briefly, dry matter was determined by drying
ileal and feed samples for 24 hours at 110° C. Titanium dioxide was used as a marker for both ileal and feed samples and was analysed as described in (Leone 1973). Dry matter digestibility was calculated by the following equation (Bolek 2013):

\[
\left[ \frac{\% \text{ Diet DM} - (\frac{\% \text{ Fecal DM} \times \% \text{ Diet Ti}}{\% \text{ Fecal Ti}})}{\% \text{ Diet DM}} \right] \times 100,
\]

where DM is dry matter and Ti is titanium dioxide. Dry matter digestibility was log-transformed to obtain a normal distribution of the data and the transformed data was used for all downstream analyses.

**DNA isolation and genotyping**

Blood was collected from the wing vein by using an EDTA-coated syringe and needle, and then stored at -20°C. DNA was extracted using a salting out method. Briefly, whole blood was incubated with lysis buffer containing proteinase K. Proteins were precipitated out using 5 M NaCl and the supernatant was recovered. 70 % ethanol was added to the supernatant to precipitate DNA. DNA isolated from 468 AIL, six broiler, and six Fayoumi chickens was genotyped using the Affymetrix 600K chicken SNP (single nucleotide polymorphism) axiom array (Kranis et al. 2013) by GeneSeek Inc., Lincoln, NE. SNP chromosomal locations were based on the Gallus_gallus_4.0 assembly through Ensembl.

**Statistical analyses**

Means, standard errors, fixed effects, and covariates for the GWAS analyses were calculated based on ANOVA (analysis of variance) estimates, and significant terms were included as fixed effects with a P value less than 0.05 using JMP statistical software (SAS Institute 2000). Heritability was estimated using a single-trait animal model in ASReml (Gilmour et al. 2009) using pedigree to estimate heritability. For all phenotypes, the model was sex fitted as a fixed effect, while chamber nested within replicate and within animal were
fitted as random effects. To estimate BW21 heritability, dam was fitted as a random effect. For the GWAS for BT, the closest BW measurement in days was fitted as a covariate.

Genotyping console (Affymetrix) software was used to obtain genotyping calls and to perform quality control based on whole animal DishQC score ≥ 0.7. The SNPolisher (Affymetrix) R package was used to perform quality control of individual SNPs for all the animals that passed the DishQC criterion. For SNP genotypes to be included in the analysis, SNP call rate had to be greater than 95 % and minor allele frequency (MAF) higher than 5 %.

GWAS for phenotypic traits with SNP genotypes was done using GenSel software (Fernando and Garrick 2008). Bayes B, which fits all SNPs simultaneously as random effects, was used for the analysis. The following mixed model was used for the GWAS:

$$y = Xb + \sum_{j} z_j \alpha_j \delta_j + \epsilon$$

where y is a vector of phenotypes, X is an incidence matrix to account for fixed effects on phenotypes, b is a vector of fixed effects, zj is a vector of genotypes for SNP j based on the number of B alleles (-10, 0, +10, or the average of the genotypes at SNP j), αj is the allele substitution effect for SNP j, δj is a parameter that indicates whether SNP j was included in the Markov chain Monte Carlo (MCMC) chain, and ε is the error associated with the analysis. For one analysis per trait, a total of 41,000 MCMC iterations were completed and the first 1000 iterations were discarded.

SNPs were split into 1001 non-overlapping 1-Mb windows across the genome. Thus, the windows that have the SNP, which is most frequently included in the MCMC iterations (post-burn-in), are predicted to have an effect on the phenotype. δj was set so that π = 0.9978 to avoid fitting more SNPs than the number of animals in a given iteration. Using a true
infinitesimal model, each window is expected to explain 0.1 % (100%/1001) of the genetic variation; therefore, a 1-Mb window was considered significant if it explained more than 0.5 % of the total genetic variation.

**Candidate genes**

For each trait, the window explaining the largest percentage of genetic variation was investigated, and, within this window, the SNP that was the most frequently included in the model was identified. Then, all annotated genes within 200 kb (100 kb upstream and 100 kb downstream) of that SNP were identified using ENSEMBL biomart (Cunningham et al. 2015). We chose a 200-kb window based on the average linkage disequilibrium (LD) in commercial broiler populations i.e., less than 1 cM on average (Andreescu et al. 2007), and on the fact that the chicken genome contains 250 kb per cM on average (Cheng 2011). In the F18 and F19 AIL chickens, LD was expected to cover a shorter distance than in the commercial broiler population because of their unique population structure and the large number of generations in which recombination occurred.

**Results**

**Phenotypic measurements and heritabilities**

Phenotypic means, standard errors, ranges, and heritabilities are in Table 1. BT measurements had low heritabilities that ranged from 0.03 to 0.11. Changes in BT after acute heat and chronic heat treatments had low heritabilities of 0.03 with large standard errors and were not statistically different from 0. BW measurements had moderate heritabilities that ranged from 0.15 to 0.35. Breast yield and dry matter digestibility, both measured on day 28, had moderate heritabilities of 0.15 and 0.33, respectively.
Genotypes

Of the 480 birds that were genotyped, 458 AIL and all 12 parental line birds passed the whole animal DishQC criterion. Of the 580,961 SNPs on the array, filtering based on a SNP call rate greater than 95% removed a small proportion, i.e. 59,789 SNPs, while filtering based on MAF removed a much larger proportion, i.e. 311,055 SNPs, thus 210,117 SNPs remained for subsequent analyses.

GWAS

The detailed results for each window that explained a significant percentage of the genetic variation (> 0.5%) and the SNP within each window that had the highest effect on the trait are in Table 2. To increase clarity, significant consecutive windows were designated as a single QTL. In total, 35 QTL were identified across all traits and measurement phases.

Eight QTL were identified for BT phenotypes (Fig. 1a, 1b, 1c and 1d), i.e. (i) four for BT20 with two on GGA1 (GGA for Gallus gallus chromosome), one on GGA14, and one on GGA15; (ii) two for BT28 with one on GGA15 and one on GGA26; and (iii) two for BT28-20 (change in BT measured at pre-heat treatment and after one week of heat treatment) with one on GGA14 and one on GGA27. No QTL was found for BT22-20 (change in BT measured at pre-heat treatment and after one day of heat treatment). QTL co-localizations were identified on GGA14 for BT20 and BT28-20 and on GGA15 for BT20 and BT28.

Twenty-one QTL for BW phenotypes were identified, i.e. (i) seven for BW21 with one each on GGA1, 2, 4, 6, 7, 8, and 14; (ii) nine for BW28 with two on GGA1, two on GGA5, and one each on GGA2, 3, 4, 6, and 26; and (iii) five for BW28-21 (change in BW measured at pre-heat treatment and after one week of heat treatment) with one each on GGA1, 4, 6, 10, and 21 (Fig. 1e, 1f and 1g). QTL for all BW phenotypes co-localized on
GGA6 in a region containing four adjacent 1-Mb windows. QTL also co-localized on GGA1 for BW21 and BW28 and on GGA4 for BW28 and BW28-21.

Three QTL were identified for digestibility with one each on GGA19, 20 and 21 (Fig. 1h). QTL co-localized for digestibility and BW28-21 on GGA21.

For breast yield, an economically important trait, three QTL were identified with one each on GGA1, 15, and 22 (Fig. 1i). The QTL on GGA15 included five adjacent 1-Mb windows and cumulatively accounted for 24.5% of the genetic variation. The most significant single 1-Mb window in this region accounted for 15.4% of the genetic variation. QTL co-localization was not identified between breast yield and any of the other traits measured in the current study.

**Candidate genes**

For each trait, positional candidate genes were identified within a 200 kb region i.e. 100 kb upstream and 100 kb downstream of the SNP with the highest effect within the 1-Mb window that explained the highest percentage of genetic variation. Fifty annotated genes were identified (see Table 3). For BW measurements, five, four, and one positional candidate genes for BW21, BW28, and BW28-21, respectively, were found among the 18, 24, and six genes that were located within the corresponding 1-Mb windows. For BT measurements, seven, seven and 11 positional candidate genes for BT20, BT28, and BT28-20, respectively, were found among the 48, 38, and 29 genes that were located within the corresponding 1-Mb windows. For dry matter digestibility and breast yield, six and four positional candidate genes, respectively, were identified among the 19 and 10 genes that were located within the corresponding 1-Mb windows.
Discussion

The aims of this study were to identify and estimate the effect of QTL, and to identify positional candidate genes, for BT, BW, dry matter digestibility, and breast yield using a novel AIL under heat stress and a 600K SNP panel for genotyping.

Population used

Previous generations of this AIL were used for several QTL mapping studies and allowed the identification of many QTL including 257 QTL for growth and body composition (Abasht and Lamont 2007; Deeb and Lamont 2003; Li et al. 2003; Zhou et al. 2006a, 2006b), 93 for skeletal integrity (Zhou et al. 2007), 51 for metabolic traits (Zhou et al. 2007), and 12 for response to Salmonella enteritidis challenge (Kaiser and Lamont 2002; Kaiser et al. 2002; Liu and Lamont 2003). Therefore, collectively, a wide range of traits has been associated with a large number of QTL in previous generations of this AIL. The continued erosion of LD in this population over subsequent generations, combined with the availability of more dense SNP panels, creates a unique opportunity to more finely map the location of QTL that are in LD with a causal mutation.

Phenotypes and heritabilities

Phenotypic measurements for BT and BW consisted of repeated measures on individual birds. This allowed us to use both absolute measures and the differences between measures carried out before (pre-heat) and during heat treatments. Since, measurements of dry matter digestibility and breast yield required euthanization, they were only performed after seven days of heat treatment, on day 28.

A previous study reported a significant correlation between BT and survival in chickens during heat stress (Chen et al. 2013), which suggested that selection for BT during
heat stress has potential to reduce mortality. In our study, heritabilities were low (0.03 to 0.11) for the changes in BT and higher (0.10 to 0.11) for absolute BT than previously estimated for a broiler line i.e. 0.05 (El-Gendy and Washburn 1995). Heritabilities for changes in BT from pre-heat to acute and chronic heat conditions were both low i.e. 0.03, which could be due to the low precision and large variation of the measurements. This indicates that it will be challenging to genetically select for resistance to BT change during heat stress. More precise methods of BT measurement (e.g., infrared thermography) should be explored.

Heritabilities for BW were low to moderate (0.15 to 0.35), which agree with the heritabilities of 0.4 to 0.6 previously reported for BW in broiler lines (Chambers 1990). Heritability for breast yield was moderate i.e. 0.15 and agreed with previously reported estimates (Le Bihan-Duval et al. 1998).

In this study, dry matter digestibility was measured using a titanium oxide marker to calculate dry matter in the feed and ileal contents. We estimated a heritability of 0.33 for digestibility, which is similar to that (0.33 to 0.47) reported for broilers that were fed a wheat-based diet (Mignon-Grasteau et al. 2004). Many previous studies on feed conversion ratio in chickens reported moderate heritabilities (Aggrey et al. 2010; Thomas et al. 1958 Van Kaam et al. 1999). Feed intake and conversion ratios, which are associated with digestibility, are arguably the most costly impacts of heat stress. Because the heritability estimated for digestibility during heat stress is moderate, there may be potential for improvement of this trait via selection.
Generally, heritabilities estimated for most traits in our study are lower than those previously reported, which is likely due to lower genetic variation within the population studied.

**Genome-wide association study**

To date, seven QTL for BT have been reported on GGA2, 3, 4, 5, 6 and 11 (Demeure et al. 2013; Nadaf et al. 2009a; Pinard-Van Der Laan et al. 2009), but none overlapped with those detected here. This absence of QTL overlap may be due to differences in experimental protocols since, in these previous studies (Demeure et al. 2013; Nadaf et al. 2009a; Pinard-Van Der Laan et al. 2009), the traits that were measured were response to disease challenge and resting BT between lines selected for growth or fat accretion. In addition, these QTL may be population-specific.

The two QTL which co-localized for BT20 and BT28-20 and for BT20 and BT28 were near QTL for hematocrit on GGA14 (Navarro et al. 2005) and GGA15 (Pinard-Van Der Laan et al. 2009), respectively. One mechanism to regulate BT during periods of heat is to increase blood flow towards the body surface (Wolfenson et al. 1981), and it has also been well documented that panting behaviour occurs under high temperatures (Sandercock et al. 2001); both mechanisms result in changes in the blood system. Moreover, the co-localized QTL for BT on GGA15 was located near a QTL for corticosterone that was measured in response to manual restraint (Buitenhuis et al. 2003). This co-localization suggests that it may be a good candidate for further investigation of the pleiotropic response to stress.

Heat stress specific QTL were identified for BT28 and BT28-20 on GGA26 and 27, respectively. Both regions present considerable overlap with previously reported QTL for growth (Ambo et al. 2009; Ankra-Badu et al. 2010; Lu et al. 2012; Nadaf et al. 2009b;
Podisi et al. 2013; Zhou et al. 2006b, 2006a). The large overlap between BT and QTL for growth is not surprising given the highly negative correlation between BT and growth during heat stress in chickens (Cooper and Washburn 1998), although we attempted to account for this relationship by fitting BW as a co-variate in the GWAS analysis.

The co-localized QTL for all BW measurements on GGA6 was near a previously reported QTL for growth in many different chicken populations, including a broiler x layer cross (Ambo et al. 2009; Atzmon et al. 2007; Podisi et al. 2013; Sewalem et al. 2002;), White Plymouth Rock, New Hampshire and White Leghorn chickens (Nassar et al. 2012), a commercial broiler line (McElroy et al. 2006), high and low growth broiler lines (Nadaf et al. 2009a), white leghorn x red jungle fowl (Le Rouzic et al. 2008), and the F2 broiler x Fayoumi generation used in the current study (Zhou et al. 2006a). The QTL that we detected on GGA6 is confirmed by previously reported QTL in this region in a wide range of chicken populations, which suggests a conserved QTL, and supports our results. This region explained a relatively large percentage of the genetic variation for BW21 (3.0 %), BW28 (8.1 %), and BW28-21 (4.6 %), which confirms the importance of this QTL.

The co-localized QTL for BW21 and BW28 on GGA1 were also localized near QTL for growth that were previously reported in a large range of chicken populations including crosses between Silkie fowl and Cornish broiler (Lu et al. 2012), White Recessive Rock and Xinghua chicken (Xie et al. 2012), broiler and layer (Podisi et al. 2013), and Shamo and White Plymouth Rock (Uemoto et al. 2009). As observed for the co-localized QTL on GGA6, this region on GGA1 is probably highly conserved given the variety of populations for which QTL have been reported near this region.
Several QTL for average daily gain are located near the region where QTL for BW28 and BW28-21 co-localize on GGA4, in two chicken populations including a Silky fowl x White Plymouth rock cross (Gu et al. 2011), and a broiler x layer cross (Podisi et al. 2013). Again, this overlap between the QTL detected in our study and previously reported QTL supports our results.

Although it is interesting to discuss overlapping QTL for measurements performed at different phases, it is also relevant to examine the QTL that were identified specifically for traits measured during heat stress i.e. BW28 on GGA26 and BW28-21 on GGA10 and 21. Indeed, the QTL for BW28 on GGA26 was located within the 2-Mb QTL region for BT28 and as discussed above, many QTL for growth have been identified in this region. Similarly, QTL for growth have also been reported in the QTL regions on GGA10 and GGA21 that we detected here.

Although feed represents a large proportion of poultry production costs (51.8 % in 2001 and 68.7 % in 2008) and these costs continue to increase due to the increased demands for grain in other industries such as ethanol for fuel (Donohue and Cunningham 2009), very few QTL related to feed use have been identified (37 of the 4795 QTL listed in www.animalgenome.org). In a recent study that evaluated layer hens for feed intake and feed use and performed a GWAS using the 600K array, eight QTL were identified (Yuan et al. 2015), but none co-localized with the QTL reported here. The three QTL identified for digestibility were all located near previously reported QTL for growth-related traits. Very recently, Mignon-Grasteau et al. (Mignon-Grasteau et al. 2015) identified a QTL for dry excreta weight on GGA19 within the same interval as that reported here for digestibility. The fact that the QTL regions for these two feed-use traits overlap provides evidence that this
region on GGA19 is indeed a true QTL and should be further investigated given the economic importance of these traits to the poultry industry.

A strong QTL for breast yield was identified on GGA1. This region contains a large number of QTL related to abdominal fat and growth traits that were detected across diverse chicken populations (www.animalgenome.org). Surprisingly, no QTL for breast muscle has been reported in this region even in studies on previous generations of the same AIL. Thus, we suggest that this QTL may be specific to breast muscle growth during heat stress conditions. Furthermore, the QTL for breast yield that we detected on GGA15 and 22 overlap with previously reported QTL for breast muscle on GGA15 (Nadaf et al. 2009a) and GGA22 (Lu et al. 2012). The region on GGA1 warrants further investigation as a QTL specific to heat stress.

**Candidate genes**

All positional candidate genes were identified for each trait within 200 kb of the most significant SNP. Cellular response to heat stress has been extensively reviewed and involves a range of biological mechanisms, i.e. inhibition of DNA synthesis, transcription, and translation, cell cycle arrest, denaturation of proteins, enhanced degradation of proteins by ubiquitin and lysosomal pathways, disruption of the integrity of the cytoskeleton (Fuquay 1981), and increased apoptosis (Matsuki et al. 2003). In addition, heat stress induces metabolic changes and increased intracellular ion concentrations. Previously, Coble et al. (Coble et al. 2014) observed that, in broiler chickens, heat stress induced transcriptional changes and Morimoto (Morimoto 1998) reported an increased expression of heat shock protein genes in response to heat stress. Heat shock proteins form an evolutionarily conserved family across all multicellular organisms (Waters et al. 1996).
Genes involved in the disruption of DNA synthesis, transcription, RNA processing, and translation were identified near the QTL for all traits analyzed in this study except for BW28 and BW28-21. We identified three genes that code for separate subunits of the mediator complex (MED 9, 15, and 31). The mediator complex is required for the regulation of eukaryotic RNA polymerase II transcripts (Malik and Roeder 2005). In yeast, the mediator complex interacts directly with heat shock proteins and serves as a bridge between heat stress and transcriptional regulation of heat shock related genes (Park et al. 2001).

Disruption of progression through the cell cycle and resulting apoptosis occur during cellular stress. We identified five genes that are involved in cell cycle progression that were located near some of the QTL detected in our study for all BT measurements and digestibility. Among the cell cycle checkpoints, two crucial checkpoints, between G1/S and G2/M transitions, are arrested in response to heat stress (Kühl and Rensing 2000). Apoptosis is induced during extreme stress conditions. In this study, we identified genes involved in apoptosis near QTL for both digestibility and breast yield.

Other categories of functions that were associated with the candidate genes detected in our study include glucose regulation, disruption of the cytoskeleton, free radical damage, and blood vessel development. The ACE gene, involved in blood vessel development, was located near the QTL for BT28-20, thus we hypothesize that it may play a role in reducing BT during periods of heat stress by enhancing blood flow to the body surface (Wolfenson et al. 1981).

Genes involved in membrane permeability and changes in cellular ion concentrations were located near the QTL for BT, BW, and digestibility. In chickens, Ait-Boulahsen et al. (Ait-Boulahsen et al. 1989) showed that Na+, K+, and Cl- plasma levels increase in response
to heat stress, which can have an effect on the endocrine system, for example as a secondary messenger, and subsequently on stress response. Two genes of this functional category were identified near the QTL for digestibility i.e. SLC13A5 that encodes a citrate transporter and PITPNM3 that encodes a calcium ion binding protein; both these genes are involved in ion movement, which is impacted by heat stress.

Genes related to the immune system were identified near the QTL for BT20 and BT28, and breast yield. It has been shown that, compared to animals not exposed to high ambient temperatures, laying hens that are exposed to cyclic heat stress have decreased T-cell and B-cell proliferations and decreased antibody titre to sheep red blood cells and an increased total white blood cell count (Mashaly et al. 2004), which supports the hypothesis that the immune function is disrupted during heat stress. We identified the MIF and DDT genes near the QTL for BT22 and BT28, respectively. These genes function as proinflammatory cytokines involved in the immune response (Calandra and Roger 2003). One of the hallmarks of inflammation is to increase BT. If inflammation can be suppressed in birds subjected to heat stress, this might decrease the negative impact of high ambient temperatures. The SOCS2 gene that encodes a suppressor of cytokine signaling was identified near the largest QTL for breast yield and may be a good candidate gene for future studies on the mechanisms that influence breast muscle yield in chickens.

Candidate genes with a role in cell signaling were identified near the QTL for BT and BW. Cell signaling increases during response to stress. One gene of particular interest in cell signaling is the MAP3K3 gene that was found near the QTL for BT28-20 since that the MAPK signaling pathway is known to be involved in the cellular response to stress (Cowan and Storey 2003).
Breast muscle yield is an extremely important trait because of its economic impact in the broiler industry. The 1-Mb window QTL with the largest effect that we identified here was for breast yield and explained 15.4% of the total genetic variation. The favourable allele of the SNP with the largest effect within the window that explained the largest proportion of genetic variation was fixed in the Fayoumi line but was also segregating in the broiler line. Thus, it was not possible to determine which line contributed the favourable allele. The best candidate gene in this region is SOCS2, which has a role in suppressing cytokine signalling. We hypothesize that the effect of this QTL on muscle accretion may be heat-specific because no other QTL for breast yield was identified in this region.

Conclusions

SNPs were identified for BT, BW, digestibility, and breast yield in a unique chicken AIL measured under heat stress. A major QTL for breast yield under heat stress explained more than 24% of the genetic variation. Exploiting this information for genomic selection to breed heat-tolerant chickens is feasible. The QTL regions that we identified contain many genes with functions that suggest a role in response to heat stress and, thus, these genes are both positional and functional candidates.

Competing Interests

The authors acknowledge no competing interests.

Authors' Contributions

AVG participated in sample collection, carried out all statistical analyses and drafted the manuscript. KJB participated in sample collection and contributed digestibility data for analysis. CMA and MEP participated in the design of the study, sample collection and acquisition of funding. MFR and CJS participated in the design of the study and acquisition
of funding. SJL participated in the design of the study, participated in sample collection, participated in acquisition of funding and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors thank the Iowa State University Poultry Research Center staff for animal care; the Lamont, Persia, Rothschild and Ashwell lab personnel for assistance; Jack Dekkers, Dorian Garrick, Rohan Fernando and their research groups for GWAS advice; Melanie Hess and Andrew Hess for their statistical and coding advice. This research was supported by USDA-NIFA-AFRI Climate Change Award #2011-67003-30228; the USDA National Institute of Food and Agriculture, Hatch project #5358; and AVG was supported by a USDA National Needs Fellowship, Award #2011-38420-20050.

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Figure 1 Genome-wide plot of percentage of genetic variance for traits measured during heat stress.
Traits were measured before heat treatment (day 20 or 21), during acute heat treatment (day 22) and chronic heat treatment (day 28), and the differentials between trait measurements due to one day of heat treatment (day 22-20) and to seven days of heat treatment (day 28-20 or day 28-21) were calculated. Only traits that reached significance in the GWAS (≥ 0.05 % of the genetic variation) are displayed. Plots for body temperature (BT) measured on days 20, 22, 28, and the differential 28-20 (A, B, C, and D); plots for body weight (BW) measured on days 21, 28, and the differential 28-21 (E, F, and G); plot for digestibility, measured from ileal content, on day 28 (H); plot for % of breast weight (I), calculated from % of total body weight, and measured on day 28. Results show the percentage of genetic variance that is explained by each non-overlapping 1-Mb window, labelled by the index number of the windows coloured and ordered by chromosome (1 to 27, and Z).

Tables

Table 1 Phenotypic means and heritabilities (h²)

<table>
<thead>
<tr>
<th>Trait</th>
<th>Mean ± SEM (range)</th>
<th>h²(SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT20</td>
<td>42.3 ± 0.01 (41.5-42.9)</td>
<td>.11 (0.06)</td>
</tr>
<tr>
<td>BT22</td>
<td>42.4 ± 0.02 (41.2-43.2)</td>
<td>.10 (0.06)</td>
</tr>
<tr>
<td>BT 22-20</td>
<td>0.1 ± 0.02 (-1.4-1.5)</td>
<td>.03 (0.04)</td>
</tr>
<tr>
<td>BT28</td>
<td>42.3 ± 0.01 (41.4-43.1)</td>
<td>.10 (0.06)</td>
</tr>
<tr>
<td>BT28-20</td>
<td>-0.02 ± 0.02 (-1.1-1.3)</td>
<td>.03 (0.04)</td>
</tr>
<tr>
<td>BW21</td>
<td>253.6 ± 1.58 (157.6-352.0)</td>
<td>.24 (0.17)</td>
</tr>
<tr>
<td>BW28</td>
<td>402.6 ± 2.58 (238.7-555.5)</td>
<td>.35 (0.11)</td>
</tr>
<tr>
<td>BW28-21</td>
<td>149.2 ± 1.57 (48.1-203.3)</td>
<td>.15 (0.11)</td>
</tr>
<tr>
<td>Digestibility</td>
<td>90.6 ± 0.98 (86.2-95.8)</td>
<td>.33 (0.14)</td>
</tr>
<tr>
<td>Percent breast weight</td>
<td>8.81 ± 0.03 (5.1-12.6)</td>
<td>.15 (0.08)</td>
</tr>
</tbody>
</table>

Body temperature (BT) measured on days 20, 22, 28, and the differentials 22-20 and 28-20; body weight (BW) measured on days 21, 28, and the differential 28-21; digestibility, measured from ileal content, on day 28; percent of breast weight, calculated from percent of total body weight, and measured on day 28.

Table 2 Identified windows that explain a significant percentage (≥ 0.5 %) of the genetic variance

<table>
<thead>
<tr>
<th>Windows explaining ≥ 0.5 % of genetic variance</th>
<th>SNP with highest model freq within window</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trait</td>
<td>Chr Pos (Mb)</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>BT20</td>
<td>1</td>
</tr>
<tr>
<td>BT20</td>
<td>1</td>
</tr>
<tr>
<td>BT20</td>
<td>14</td>
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<td>BT20</td>
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<td>BT22</td>
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<td>BT28</td>
<td>15</td>
</tr>
<tr>
<td>BT28</td>
<td>26</td>
</tr>
<tr>
<td>BT28-20</td>
<td>14</td>
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<tr>
<td>Table 2 continued</td>
<td></td>
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<tr>
<td>------------------</td>
<td></td>
</tr>
<tr>
<td>BT28-20</td>
<td>27 2 0.58</td>
</tr>
<tr>
<td>BW21</td>
<td>1 130 0.66</td>
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<td>2 46 2.37</td>
</tr>
<tr>
<td>BW21</td>
<td>2 47 0.65</td>
</tr>
<tr>
<td>BW21</td>
<td>4 29 0.75</td>
</tr>
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<td>BW21</td>
<td>6 17 0.58</td>
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<td>8 20 0.59</td>
</tr>
<tr>
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<td>14 2 0.5</td>
</tr>
<tr>
<td>BW28</td>
<td>1 129 0.69</td>
</tr>
<tr>
<td>BW28</td>
<td>1 130 0.5</td>
</tr>
<tr>
<td>BW28</td>
<td>1 175 0.51</td>
</tr>
<tr>
<td>BW28</td>
<td>2 46 0.57</td>
</tr>
<tr>
<td>BW28</td>
<td>3 7 0.56</td>
</tr>
<tr>
<td>BW28</td>
<td>4 35 0.8</td>
</tr>
<tr>
<td>BW28</td>
<td>4 36 1.05</td>
</tr>
<tr>
<td>BW28</td>
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</tr>
<tr>
<td>BW28</td>
<td>5 4 0.7</td>
</tr>
<tr>
<td>BW28</td>
<td>6 17 1.1</td>
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<tr>
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<td>6 18 1.67</td>
</tr>
<tr>
<td>BW28</td>
<td>6 19 2.48</td>
</tr>
<tr>
<td>BW28</td>
<td>6 20 2.11</td>
</tr>
<tr>
<td>BW28</td>
<td>6 21 0.77</td>
</tr>
<tr>
<td>BW28</td>
<td>26 3 0.54</td>
</tr>
<tr>
<td>BW28-21</td>
<td>1 2 1</td>
</tr>
<tr>
<td>BW28-21</td>
<td>4 35 1.2</td>
</tr>
<tr>
<td>BW28-21</td>
<td>4 36 1.36</td>
</tr>
<tr>
<td>BW28-21</td>
<td>6 17 0.62</td>
</tr>
<tr>
<td>BW28-21</td>
<td>6 18 0.86</td>
</tr>
<tr>
<td>BW28-21</td>
<td>6 19 1.31</td>
</tr>
<tr>
<td>BW28-21</td>
<td>6 20 1.29</td>
</tr>
<tr>
<td>BW28-21</td>
<td>6 21 0.55</td>
</tr>
<tr>
<td>BW28-21</td>
<td>10 4 0.56</td>
</tr>
<tr>
<td>BW28-21</td>
<td>21 1 0.53</td>
</tr>
<tr>
<td>BW28-21</td>
<td>21 5 0.64</td>
</tr>
<tr>
<td>Digestibility</td>
<td>19 9 0.63</td>
</tr>
<tr>
<td>Digestibility</td>
<td>20 13 0.62</td>
</tr>
<tr>
<td>Digestibility</td>
<td>21 5 0.53</td>
</tr>
<tr>
<td>% breast weight</td>
<td>1 42 0.58</td>
</tr>
</tbody>
</table>

*Body temperature (BT) measured on days 20, 22, 28, and the differentials 22-20 and 28-20; body weight (BW) measured on days 21, 28, and the differential 28-21; digestibility, measured from ileal content, on day 28; percent of breast weight, calculated from percent of total body weight, and measured on day 28.
Table 3 Positional candidate genes categorized by function for windows explaining the largest percentage of genetic variation

<table>
<thead>
<tr>
<th>Function</th>
<th>Trait</th>
<th>Gene name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disruption of DNA synthesis, transcription, RNA processing, and translation</td>
<td>BW21</td>
<td>HHEX</td>
<td>Gallus gallus hematopoietically expressed homeobox (HHEX), mRNA. [Source:RefSeq mRNA;Acc:NM_205252]</td>
</tr>
<tr>
<td>BT20</td>
<td>MED9</td>
<td>Gallus gallus mediator complex subunit 9 (MED9), mRNA. [Source:RefSeq mRNA;Acc:NM_001277637]</td>
<td></td>
</tr>
<tr>
<td>BT22</td>
<td>MED15</td>
<td>mediator complex subunit 15 [Source:HGNC Symbol;Acc:HGNC:14248]</td>
<td></td>
</tr>
<tr>
<td>BT28-20</td>
<td>RNF113A</td>
<td>Gallus gallus ring finger protein 113A (RNF113A), mRNA. [Source:RefSeq mRNA;Acc:NM_001004396]</td>
<td></td>
</tr>
<tr>
<td>BT28-20</td>
<td>DDX42</td>
<td>Gallus gallus DEAD (Asp-Glu-Ala-Asp) box polypeptide 42 (DDX42), mRNA. [Source:RefSeq mRNA;Acc:NM_001030926]</td>
<td></td>
</tr>
<tr>
<td>Digestibility</td>
<td>MED31</td>
<td>mediator complex subunit 31 [Source:HGNC Symbol;Acc:HGNC:24260]</td>
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</tr>
<tr>
<td>Breast yield</td>
<td>MRPL42</td>
<td>mitochondrial ribosomal protein L42 [Source:HGNC Symbol;Acc:HGNC:14493]</td>
<td></td>
</tr>
<tr>
<td>Disruption of progression through the cell cycle</td>
<td>BT22 &amp; BT28</td>
<td>TBX6</td>
<td>Gallus gallus T-box 6 (TBX6), mRNA. [Source:RefSeq mRNA;Acc:NM_0010030367]</td>
</tr>
<tr>
<td>BT22</td>
<td>KLHL22</td>
<td>kelch-like family member 22 [Source:HGNC Symbol;Acc:HGNC:25888]</td>
<td></td>
</tr>
<tr>
<td>BT28-20</td>
<td>LIMD2</td>
<td>Gallus gallus LIM domain containing 2 (LIMD2), mRNA. [Source:RefSeq mRNA;Acc:NM_001006330]</td>
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<tr>
<td>BT28-20</td>
<td>STRADA</td>
<td>Gallus gallus STE20-related kinase adaptor alpha (STRADA), mRNA. [Source:RefSeq mRNA;Acc:NM_001012844]</td>
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<tr>
<td>Digestibility</td>
<td>KIAA0753</td>
<td>Gallus gallus membrane-associated ring finger (C3HC4) 5 (MARCH5), mRNA. [Source:RefSeq mRNA;Acc:NM_001097537]</td>
<td></td>
</tr>
<tr>
<td>Increase protein degradation by ubiquitination</td>
<td>BW21</td>
<td>MARCH5</td>
<td>Gallus gallus membrane-associated ring finger (C3HC4) 5 (MARCH5), mRNA. [Source:RefSeq mRNA;Acc:NM_0010030367]</td>
</tr>
<tr>
<td>BW28</td>
<td>PCGF5</td>
<td>Gallus gallus polycomb group ring finger 5 (PCGF5), mRNA. [Source:RefSeq mRNA;Acc:NM_0010277361]</td>
<td></td>
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<tr>
<td>BW28</td>
<td>HECTD2</td>
<td>HECT domain containing E3 ubiquitin protein ligase 2 [Source:HGNC Symbol;Acc:HGNC:26736]</td>
<td></td>
</tr>
<tr>
<td>BT20</td>
<td>USP22</td>
<td>Ubiquitin carboxyl-terminal hydrolase [Source:UniProtKB/TrEMBL;Acc:F1NG36]</td>
<td></td>
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<tr>
<td>Membrane permeability and ions</td>
<td>BW28-21</td>
<td>GRID2</td>
<td>glutamate receptor, ionotropic, delta 2 [Source:HGNC Symbol;Acc:HGNC:4576]</td>
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<tr>
<td>BT20</td>
<td>PEMT</td>
<td>Gallus gallus phosphatidylethanolamine N-methyltransferase (PEMT), nuclear gene encoding mitochondrial protein, mRNA. [Source:RefSeq mRNA;Acc:NM_001006164]</td>
<td></td>
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<tr>
<td>BT28-20</td>
<td>CYB561</td>
<td>cytochrome b561 [Source:HGNC Symbol;Acc:HGNC:2571]</td>
<td></td>
</tr>
<tr>
<td>BT28-20</td>
<td>KCNH6</td>
<td>potassium voltage-gated channel, subfamily H (eag-related), member 6 [Source:HGNC Symbol;Acc:HGNC:18862]</td>
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<tr>
<td>BT28-20</td>
<td>CCDC47</td>
<td>coiled-coil domain containing 47 [Source:HGNC Symbol;Acc:HGNC:24856]</td>
<td></td>
</tr>
<tr>
<td>BT28-20</td>
<td>MYL4</td>
<td>myosin, light chain 4, alkali; atrial, embryonic [Source:HGNC Symbol;Acc:HGNC:7558]</td>
<td></td>
</tr>
<tr>
<td>Digestibility</td>
<td>SLC13A5</td>
<td>solute carrier family 13 (sodium-dependent citrate transporter), member 5 [Source:HGNC Symbol;Acc:HGNC:23089]</td>
<td></td>
</tr>
<tr>
<td>Digestibility</td>
<td>PITPNM3</td>
<td>PITPNM family member 3 [Source:HGNC Symbol;Acc:HGNC:21043]</td>
<td></td>
</tr>
<tr>
<td>Immune system activation</td>
<td>BT20</td>
<td>TNFRSF13B</td>
<td>Gallus gallus tumor necrosis factor receptor superfamily, member 13B (TNFRSF13B), mRNA. [Source:RefSeq mRNA;Acc:NM_001097537]</td>
</tr>
<tr>
<td>BT22</td>
<td>DDT</td>
<td>Gallus gallus D-opochromine tautomerase (DDT), mRNA. [Source:RefSeq mRNA;Acc:NM_001030667]</td>
<td></td>
</tr>
<tr>
<td>BT22</td>
<td>CABIN1</td>
<td>calcineurin binding protein 1 [Source:HGNC Symbol;Acc:HGNC:24187]</td>
<td></td>
</tr>
<tr>
<td>All characterized genes within a 200-kb region, i.e. 100 kb upstream and 100 kb downstream of the SNP which was most frequently included in the MCMC iterations (post-burn-in), and is in the window explaining the largest amount of genetic variation for each trait. Body temperature (BT) measured on days 20, 22, 28, and the differential 28-20; body weight (BW) measured on days 21, 28, and the differential 28-21; digestibility, measured from ileal content, on day 28; percent of breast weight, calculated from percent of total body weight, and measured on day 28.</td>
<td></td>
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</tbody>
</table>
CHAPTER 3
QUANTITATIVE TRAIT LOCI IDENTIFIED FOR BLOOD CHEMISTRY COMPONENTS OF AN ADVANCED INTERCROSS LINE OF CHICKENS UNDER HEAT STRESS

Modified from a paper published in BMC Genomics on April 14, 2016:

Angelica Van Goor, Chris M. Ashwell, Mike E. Persia, Max F. Rothschild, Carl J. Schmidt and Susan J. Lamont

Author’s contribution
AVG participated in sample collection, isolated DNA for genotyping, carried out all statistical analyses, and drafted the manuscript.

Abstract

Background

Heat stress in poultry results in considerable economic losses and is a concern for both animal health and welfare. Physiological changes occur during periods of heat stress, including changes in blood chemistry components. A highly advanced intercross line, created from a broiler (heat susceptible) by Fayoumi (heat resistant) cross, was exposed to daily heat cycles for seven days starting at 22 days of age. Blood components measured pre-heat treatment and on the seventh day of heat treatment included pH, pCO₂, pO₂, base excess, HCO₃, TCO₂, K, Na, ionized Ca, hematocrit, hemoglobin, sO₂, and glucose. A genome-wide association study (GWAS) for these traits and their calculated changes was conducted to identify quantitative trait loci (QTL) using a 600K SNP panel.
Results

There were significant increases in pH, base excess, HCO$_3^-$, TCO$_2^-$, ionized Ca, hematocrit, hemoglobin, and sO$_2$, and significant decreases in pCO$_2$ and glucose after 7 days of heat treatment. Heritabilities ranged from 0.01-0.21 for pre-heat measurements, 0.01-0.23 for measurements taken during heat, and 0.00-0.10 for the calculated change due to heat treatment. All blood components were highly correlated within measurement days, but not correlated between measurement days. The GWAS revealed 61 QTL for all traits, located on GGA (Gallus gallus chromosome) 1, 3, 6, 9, 10, 12-14, 17, 18, 21-28, and Z. A functional analysis of the genes in these QTL regions identified the Angiopoietin pathway as significant. The QTL that co-localized for three or more traits were on GGA10, 22, 26, 28, and Z and revealed candidate genes for birds’ response to heat stress.

Conclusions

The results of this study contribute to our knowledge of levels and heritabilities of several blood components of chickens under thermoneutral and heat stress conditions. Most components responded to heat treatment. Mapped QTL may serve as markers for genomic selection to enhance heat tolerance in poultry. The Angiopoietin pathway is likely involved in the response to heat stress in chickens. Several candidate genes were identified, giving additional insight into potential mechanisms of physiologic response to high ambient temperatures.

Background

Climate change has increased the frequency of severe heat waves and the global temperature is projected to become increasingly warmer (USDA 2012). Heat stress in poultry negatively impacts animal production and welfare resulting in economic losses estimated to
be between $125-165 million for the U.S. broiler poultry industry (St-Pierre 2003). During a severe heat wave in Iowa, over 1.5 million layer hens died (NOAA 1995).

To reduce core body temperature during periods of heat stress, blood flow to internal organs decreases and blood flow to the combs and other surface tissues increases in chickens (Wolfenson et al. 1981). During periods of heat stress, blood volume and oxygen carrying capacity are altered (Yahav et al. 1997) and dehydration, caused by increased respiration, can increase hematocrit (Borges et al. 2004). Energy availability, as determined by plasma glucose level, is increased in chickens exposed to heat stress (Garriga et al. 2006).

During high ambient temperatures, chickens reduce feed intake by as much as 17%, which reduces growth (Austic 1985). However, metabolic and endocrine changes during heat stress also contribute to reduction in growth in broilers, as demonstrated by a pair-feeding study (Lin et al. 2004).

A major change in blood components is caused by heat-induced increased respiration, which results in respiratory alkalosis, a disturbance in the acid base balance characterized by an increase in blood pH accompanied by a decrease in pCO₂. Respiratory alkalosis occurs in broilers during heat stress and is associated with reduced growth rate (Teeter et al. 1985). Metabolic alkalosis is an additional measure of disturbances in acid base balance and is defined by a decrease in the fixed acid concentrations and an increase in fixed base concentrations within the extracellular fluid (Galla 2000).

Electrolyte balance is essential for acid-base balance, maintenance of cellular homeostasis, synthesis of tissue protein, and electrical potential of cell membranes, enzymatic reactions, and maintaining osmotic pressure (Borges 2007). Altering electrolyte
amounts in feed partially ameliorates the negative impacts of heat stress in broiler chickens (Borges et al. 2003).

The goal of the current study was to identify the physiological changes and genomic regions associated with response to heat stress in chickens as characterized by the blood chemistry components, including pH, pCO₂, pO₂, base excess (BE), HCO₃, TCO₂, K, Na, ionized Ca (iCa), hematocrit (Hct), hemoglobin (Hb), sO₂, and glucose (Glu). In a commercial egg laying population, developmental measures have been established with hopes of using measures of blood chemistry components for selection (Schaal et al. 2015). To date, few studies have identified QTL for blood components in chicken (Jacobsson et al. 2005; Nadaf et al. 2009; Park et al. 2006; Zhou et al. 2007). We used a 600K SNP panel to identify QTL regions associated with levels of blood components of chickens under thermoneutral and heat stress conditions, and changes induced by heat.

**Results**

**Blood component measurements and heritabilities**

Phenotypic means and heritabilities are given in Table 1 for blood components measured pre-heat (day 20 of age), after 7 days of heat treatment (day 28 of age), and the calculated change due to heat treatment (day 28-20). After 7 days of heat treatment, pH, BE, HCO₃, TCO₂, iCa, Hct, Hb, and sO₂ significantly increased while pCO₂ and glucose significantly decreased. There were no significant changes in pO₂, K, and Na due to heat treatment.

Heritabilities ranged from 0.01-0.21 for pre-heat measurements, 0.01-0.23 for measurements taken during heat, and 0.00-0.10 differential due to heat treatment.
**Trait correlations**

Correlations between blood components at each measurement phase are given in Figure 1 as a heat map. Almost all blood components were positively correlated with all other variables measured on the same day. Very few significant correlations, however, occurred between variables measured on different days.

**Genotyping**

Of the 480 genotyped birds, 458 Advanced Intercross Line (AIL) and all 12 parental line birds passed the whole animal DishQC criterion. Of the 580,961 SNPs on the array, filtering based on SNP call rate ≥ 95% removed a small proportion (59,789 SNPs), whereas filtering based on MAF removed a much larger proportion (311,055 SNPs), yielding 210,117 SNPs for subsequent analyses.

**GWAS**

The results from the GWAS for each trait are depicted in Figure 2. A wide range of genetic variation (0.5-9.8%) was explained by each significant window and detailed information is found in Table 2. Adjacent windows that were significant for a single trait are discussed below as a single QTL region.

Six QTL for pH phenotypes were identified: three for pH20 with two on GGA18 and one on GGA28, one for pH28 on GGA12, and two for pH28-20 with one each on GGA6 and GGA10.

Nine QTL for pCO₂ measurements were identified: one for pCO₂20 on GGA28, four for pCO₂28 located on GGA1, 9, 10, and 27, and four for pCO₂28-20 (change due to heat treatment) on GGA3, 10, 23, and 28. No QTL were identified for pO₂20 or for pO₂28-20. One QTL was identified for pO₂28 on GGA13.
A total of five QTL were identified for BE traits: two for BE20 on GGA18, three for BE28 trait with one each on GGA1, 21, and 27, and none for BE28-20. Nine QTL were identified for TCO_2 traits: none for TCO_220; eight for TCO_228 one each on GGA6 and GGA26, and six on GGAZ, and one for TCO_228-20 on GGA10. No QTL were identified for HCO_320 or HCO_328-20, while seven were revealed for HCO_328 with one on GGA6 and six on GGAZ.

Five QTL for K traits were identified: four for K20 with two on GGA10, one on GGA12, and one on GGA26, none for K28 and one for K28-20 located on GGA12. No QTL were identified for the Na phenotypes. A single QTL was identified for ionized Ca phenotypes: for Ca28 on GGA26.

We identified five QTL for Hct measurements: none for Hct20 or Hct28-20, and five for Hct28 located one each on GGA1, 10, 14, 22, and two on GGA28. Seven QTL were identified for Hb: none for Hb20, six for Hb28 located one each on GGA1, 10, 14, 22, and two on 28, and one for Hb28-20 on GGA22. There were three QTL for sO_2 phenotypes: none for sO_220, two for sO_228 located on GGA24 and GGA25, and one for sO_228-20 on GGA17. Four QTL were identified for Glu: one for Glu20 on GGA10, and three for Glu28 with one on GGA22 and two on GGAZ.

**Pathway analysis**

The pathway analysis of all annotated genes within significant QTL regions across all measured traits, and separately for genes in the regions of QTL co-localization, and the top 20 significant (P ≤ 0.05) canonical pathways for each group are listed in Table 3. Of the 999 genes identified within all significant QTL regions, 682 genes were annotated within IPA and used for the pathway analysis. Two canonical pathways of interest for all identified QTL
include the AMPK signalling and Angiopoietin signalling pathways. Of the 226 genes in regions of QTL co-localization, 185 were annotated within IPA and used for pathway analysis. A pathway of particular interest that was revealed was the Cardiac Hypertrophy signalling pathway.

**Candidate genes**

We explored regions of QTL co-localization in detail to identify candidate genes that may give insight into the complex biological mechanisms that control blood component response to heat stress. Candidate genes were identified using Ensemble Biomart within the 1 Mb windows that were significant for 3 or more traits (Supplementary Table 1).

**Discussion**

The aim of this study was to identify and estimate the effect of QTL, and to perform a functional analysis using positional candidate genes, for blood components (pH, pCO₂, pO₂, base excess, HCO₃, TCO₂, K, Na, ionized Ca, Hct, Hb, sO₂, and Glu) using a novel AIL of chickens under heat stress and a 600K SNP panel for genotyping. The blood components measured were within the accepted range reported for chicken (Martin et al. 2010). Blood chemistry components are grouped into functional categories (i.e., respiratory alkalosis, metabolic alkalosis, blood volume and oxygen carrying capacity, electrolytes, and glucose) for discussion.

**Population studied**

Previous generations of this AIL were used for several QTL mapping studies and allowed the identification of many QTL including 257 for growth and body composition (Abasht and Lamont 2007; Deeb and Lamont 2003; Li et al. 2003; Zhou et al. 2006a, 2006b), 93 for skeletal integrity (Zhou et al. 2007), 51 for metabolic traits (Zhou et al. 2007), 12 for
response to *Salmonella enteritidis* challenge (Kaiser and Lamont 2002; Kaiser et al. 2002; Liu and Lamont 2003), and 35 for response to heat stress (Van Goor et al. 2015). Therefore, collectively, a wide range of traits have been associated with a large number of QTL in previous generations of this AIL. The continued erosion of Linkage Disequilibrium (LD) in this population over subsequent generations, combined with the availability of larger SNP panels, creates a unique opportunity to more finely map the location of QTL that are in LD with a causal mutation.

**Respiratory alkalosis**

**Phenotypic measurements**

During periods of intense heat, chickens increase the depth and frequency of respiration to decrease core body temperature (Randall and Hiestand 1939). Broilers that are heat stressed increase panting and display signs of respiratory alkalosis (Teeter et al. 1985), which is caused by an increase in the amount of CO₂ expelled from the lungs, and a consequent increase in pH within the blood, and an increase in pO₂ within the blood. We investigated blood pH, pCO₂, and pO₂ to characterize respiratory alkalosis induced by heat stress.

Occurrence of respiratory alkalosis was clearly demonstrated in the current study by a significant increase in blood pH and significant decrease in pCO₂ due to heat treatment, in agreement with previous studies. Heat stress for two hours at 32°C in broilers at 35 days of age significantly increases blood pH and decreases pCO₂ (Sandercock et al. 2001) and, in another study using broilers, heat stress at 32°C for 2 weeks at 28 days of age in birds that were panting (Teeter et al. 1985). We found pO₂ increased in response to heat treatment,
although not significantly. In a study using 35 day old broilers, blood pO$_2$ significantly increased after cyclical heat stress for 10 days at 35°C (Deyhim and Teeter 1991).

**Heritabilities**

Only one other published study has estimated heritabilities of blood components in chickens under thermal stress (Closter et al. 2009). The current study, therefore, adds substantially to the body of information on response of birds to thermal stress by estimating heritabilities of blood component levels and changes under heat stress and thermoneutral conditions. In broiler chickens at 22 days of age reared under cold stress conditions, heritabilities for blood pH, pCO$_2$, and pO$_2$ were estimated at 0.15, 0.15, and 0.03, respectively (Closter et al. 2009), in agreement with the current study’s estimates for thermoneutral and heat conditions. Our estimates for the changes in these blood components due to heat treatment was much lower, suggesting that the ability to select for the response to heat stress may be difficult.

**GWAS**

To our knowledge, QTL for blood pH, pCO$_2$, and pO$_2$ in chickens have not been previously reported. Identification of QTL for blood pH on different chromosomes across measurement phases, indicates that genetic control of these traits exists and is partly dependent on the environment. Co-localized QTL for pCO$_2$20 and pCO$_2$28-20 on GGA28, and for pCO$_2$28 and pCO$_2$28-20 on GGA10, suggest that the same genetic regions contribute to control of pCO$_2$ level independent of environmental temperature. The presence of co-localized QTL between measurement phases was not expected, based on the lack of phenotypic correlations (r=0.00).
Metabolic alkalosis

Phenotypic measurements

Metabolic alkalosis occurs when there is a disturbance in the fixed acids and bases in the extracellular fluid (Galla 2000). Imbalance of dietary Na, K, or Ca can result in metabolic alkalosis (Dagher 2008), which is characterized by an increase in blood pH, HCO$_3^-$, and base excess, and can be induced in growing layers by high levels of calcium in feed (Guoa et al.). Base excess is considered a comprehensive measure of the metabolic components of bases, which reflects the nonrespiratory contribution to changes in acid-base disturbances (Chin et al. 2012). Base excess can be altered by changing the cation:anion ratio in the diet of broiler chickens and is associated with body weight and bone density (Halley et al. 1987). In the current study, base excess significantly increased after heat treatment, which is consistent with the hypothesis that chickens experience metabolic alkalosis under heat stress.

HCO$_3^-$ is the most abundant buffer in the blood, is primarily regulated by the kidneys, and is a metabolic component of acid-base balance (Chin et al. 2012). We observed a significant increase in HCO$_3^-$ due to heat treatment. These results contrasted with a previous study using broilers at 28 days of age in which blood HCO$_3^-$ significantly decreased in panting birds under acute heat stress (Teeter et al. 1985), and another study using male broilers that reported a decrease in HCO$_3^-$ after a heat stress at 32°C for 10 hours (Borges et al. 2003). TCO$_2$ also increased in response to heat treatment. It was unexpected to observe a decrease in base excess, consistent with metabolic alkalosis, while HCO$_3$ and TCO$_2$ increased, because the traits are highly positively correlated within all treatment phases ($r \geq 0.95$).
**Heritabilities**

We estimated heritability of base excess between 0.00-0.10, of HCO₃ between 0.03-0.23, and of TCO₂ between 0.01-0.13. In broiler chickens at 22 days of age reared under cold stress conditions, blood HCO₃ and TCO₂ heritability were both estimated at 0.19 (Closter et al. 2009).

**GWAS**

We are the first to report QTL in chickens for blood base excess, HCO₃, and TCO₂, which are related to metabolic alkalosis. QTL for base excess are located on separate chromosomes for all measurement phases, indicating a strong genetics by environmental (G x E) temperature interaction. The phenotypic correlations for base excess between measurement phases were both very low (r=0.03). The QTL for base excess on GGA18 overlap with pH measured at thermoneutrality and were highly correlated (r=0.78). Surprisingly, QTL for HCO₃ were only identified during heat treatment and were on GGA6 and GGAZ. Ten of the eleven QTL for TCO₂ measured during heat co-localized with QTL for HCO₃ and these co-localized regions were located on GGA6, 26, and Z.

**Electrolytes**

**Phenotypic measurements**

Blood K and Na levels numerically increased and iCa statistically increased in response to heat treatment. This is in disagreement with previous reports of decreasing levels of both K and Na in response to heat stress, likely due to increased water intake which results in decreased concentrations of electrolytes within the blood (Ait-Boulahtsen et al. 1989, Borges et al. 2004; Borges et al. 2003).
Heritabilities

Heritability of K and Na blood levels in humans has been estimated to be very low, 0.03 and 0.04, respectively (Meyer et al. 2010), in agreement with our low heritability estimates during heat and for the calculated differential. In contrast, our estimates for heritability under thermoneutral conditions for K and Na were higher, 0.20 and 0.08, respectively. Estimated heritability was 0.02 for ionized Ca measured during heat stress, lower than the 0.19 of mice in thermoneutral conditions (Tordoff et al. 2007). The estimated heritability was low, for both thermoneutral (0.04) and the differential due to heat (0.01), indicating the genetic component for ionized Ca is dependent upon environmental conditions at the time of measurement. The low heritabilities of these traits during heat and for the calculated differential due to heat treatment suggest it may be difficult to select for these traits.

GWAS

This research is the first to describe QTL for the electrolyte-balance traits of blood K, Na, and ionized Ca in the chicken. In swine, QTL have been identified for these traits (Reineret al. 2009). QTL for blood K were located on GGA10, 12, and 26. QTL were identified for K across the thermoneutral and differential due to heat measurement phases, indicating genetic control of this component in this region on GGA12 despite environmental temperature. The correlation between thermoneutral and the differential was moderate (r=0.10). No significant QTL for Na were identified in the current study and a single QTL for ionized Ca was located on GGA26 for the measurement taken during heat.
**Blood volume and oxygen saturation**

**Phenotypic measurements**

Changes in blood volume and oxygen carrying capacity occur in chickens during periods of heat stress (Yahav et al. 1997). Both hematocrit and hemoglobin significantly increased due to heat treatment, which may be the result of dehydration. This result contrasts with a previous study using male broilers in which both decreased after an acute heat stress at 32°C for 10 hours (Borges et al. 2004). Blood sO₂ is a measure of oxyhemoglobin in relation to total hemoglobin that is able to bind oxygen (Chin et al. 2012), significantly increased during heat treatment.

**Heritability**

The heritability of Hct was estimated as very low at 0.01 and 0.02 for pre-heat and the differential, respectively, while during heat was moderately heritable at 0.21. Heritability has been estimated for hematocrit at 0.39 in domestic fowl (Washburn 1967). The increase in heritability when measured during heat stress indicates that this trait may be useful for selection. Heritability estimates of sO₂ were very low (0.01-0.03), which is in general agreement with a previously reported value of 0.07 in cold-stressed broiler chickens at 22 days of age (Closter et al. 2009).

**GWAS**

Seven QTL for haematocrit have been identified in chickens (www.animalgenome.org). In a broiler by layer F2 intercross, QTL for hematocrit were located on GGA1, 2, 6, and 14 (Boschiero et al. 2013); in a Fayoumi by Leghorn F2 intercross on GGA1 and GGA15 (Pinard-Van Der Laan et al. 2009), and in a broiler by layer cross on GGA1 (Navarro et al. 2005). Our current work confirmed previously identified QTL
for Hct28 on GGA1 and GGA14. Novel QTL for Hct were on GGA10, 22, and 28. Most of the QTL identified in the current study for Hb co-localized with those identified for Hct, with the addition of a relatively large QTL for Hb28-20 on GGA22, explaining 1.7% of the genetic variation. The co-localization of QTL among Hct and Hb is expected because they have very high positive phenotypic correlations across all measurement phases (r ≥ 0.99). We identified novel QTL for sO$_2$ on GGA17, 24, and 25, none of which overlapped between measurement phases, indicating separate genetic control of this trait dependent upon environmental temperature. A previous study using a commercial broiler line identified one on GGA16 (Ewald et al. 2007). Thus, QTL for sO$_2$ appear to be population specific.

**Glucose**

**Phenotypic measurement**

Glucose is the body’s primary source of energy, and blood Glu significantly decreased due to heat treatment in the current study. In contrast, male broilers had a significant increase in Glu after heat stress at 32°C for 10 hours (Borges et al. 2004), and in broiler chicks of 5 weeks of age at 35-40°C (Khan et al. 2002). In chicken lines divergently selected for blood glucose concentration, the low glucose line was less efficient at food utilization compared to the high glucose line (Leclercq et al. 1987), indicating the decrease in glucose we see during heat stress may contribute to inefficiency in food utilization.

**Heritability**

The current study estimated heritabilities for glucose ranging between 0.02-0.19. In a study using chickens divergently selected for blood glucose concentration, heritability was estimated at 0.25 (Leclercq et al. 1987).
We identified QTL for Glu20 and Glu28 on GGA10, 22, and Z, while QTL were mapped to GGA2, 7, and Z in the F2 generation of the same chicken population under thermoneutral conditions (Zhou et al. 2007). The two studies may have detected the same QTL on chromosome Z and, due to the breakdown of LD over the generations, the current study may have mapped the QTL more accurately. In an F2 intercross between fat and lean broilers, QTL were identified for blood glucose on GGA3 and GGA18 (Demeure et al. 2013), and for fasting plasma glucose on GGA5, 6, 13, and 26 (Nadaf et al. 2009). A study using an F2 of broilers divergently selected for growth, identified QTL for plasma glucose on GGA20 and GGA27 (Park et al. 2006). Thus, QTL location for blood glucose level appears to be heat and/or population-specific.

Pathway analysis

Considering all measured traits, we identified a total of 32 unique QTL. All annotated genes within the QTL regions were used for pathway analysis using IPA and many significantly associated canonical pathways were identified including AMPK signalling and Angiopoietin signalling were identified. The top 20 pathways are found in Table 3. AMPK is a master metabolic regulator involved in metabolism (Mihaylova and Shaw 2011) and, thus, may be a pathway which warrants further investigation for involvement in production traits during heat stress. During high ambient temperatures chickens redirect blood flow to the body surface to decrease body temperature (Yahav et al. 1997), and the angiopoietin signalling pathway functions in blood vessel development which may help alleviate temperature stress.
The co-localized regions resulted in many significant canonical pathways and the top 20 pathways are found Table 3. Of particular interest is the Cardiac Hypertrophy signalling pathway \((P=4.35E^{-02})\). QTL for hemoglobin and hematocrit represent 3 (7 total) regions of co-localization and there is a positive linear relationship between hematocrit and heart weight in chickens under heat stress (Yahav et al. 1997); therefore, this pathway likely contributes to the response to heat stress in chickens.

**Candidate genes for co-localized QTL**

The QTL regions that co-localized for three or more traits were further investigated for positional, functional candidate genes to give further insight into the biological mechanisms involved in the response of blood components to heat stress. The identified genes are located in Supplementary Table 1.

There are 51 genes in the region on GGA10 between 3-6 Mb that contained QTL for Glu20, pCO\(_2\)28, and TCO\(_2\)28-20. With 2 of these 3 traits associated with CO\(_2\) concentration, \(CA12\) (carbonic anhydrase) is a likely candidate gene involved in the CO\(_2\) response to heat stress. Carbonic anhydrases catalyse the reaction of CO\(_2\) and H\(_2\)O to form HCO\(_3\) and H+, and thus may stabilize blood acid base balance during heat stress. Another strong functional candidate in this region is \(HSP40\), a member of the heat shock protein family that functions as a molecular chaperone to prevent cellular damage during heat stress (Glover and Lindquist 1998). A candidate gene in this region for glucose level is \(GCNT3\), a glucosamine acetyl transferase which is associated with glucose metabolism in humans (Dostrovsky et al. 2011). Fourteen genes were identified on chromosome 10 between 16-17 Mb, where QTLs co-localized for pH differential, Hct during heat, Hb during heat, and K20. Many QTL in chicken have been identified in this region including those related to growth (Ambo et al.)
2009; Nassar et al. 2015; Zhou et al. 2006a; Zhou et al. 2010), abdominal fat (Campos et al. 2009 Demeure et al. 2013; Zhou et al. 2006b;), and the stress-associated trait of fear response (Buitenhuis et al. 2004). A strong candidate gene is ALDH6 (aldehyde dehydrogenase) which functions to convert aldehydes to carboxylic acids. This gene may function to maintain blood acid base balance during heat stress. Another gene in this region is IGF1 (insulin like growth factor 1), which has many roles and is a biomarker for growth (Renehan et al. 2004).

Four genes were identified on chromosome 22 between 3-4 Mbs, where QTL were co-localized for Hct during heat, Hb during heat, Hb differential due to heat treatment, and glucose during heat treatment. To our knowledge, no QTL have been reported in this region. Because all traits were measured during heat treatment or as the differential, we propose these to be heat specific QTL. Candidate genes TGFA (pretransforming growth factor) and ADRA1A (adrenergic receptor) both regulate cell growth. It is known that metabolic changes occur during periods of heat in chickens that contribute to reduction in growth, independent upon feed intake (Lin et al. 2004).

There are 48 genes in the 1 Mb region on GGA26 between 3-4 Mbs, where QTL co-localized for TCO28, K20, and iCa28. Notably, a QTL for tibia bone mineral density identified in a commercial broiler and layer cross is located within this region (Schreiweis et al. 2006). This co-localization suggests that this locus might be involved in both blood calcium and bone density, and therefore, may be an ideal candidate for further investigation to understand the physiological response to heat stress on bone mineral density.

There are 86 genes in the 2 Mb region on GGA28 between 3-5 Mb where QTLs co-localize for pH pre-heat, Hb during heat, Hct during heat, pCO2 pre-heat, and pCO2 differential. A QTL for heart weight, relating to susceptibility of pulmonary hypertension
(Rabie et al. 2005) co-localizes with those identified here. Many of these genes are related to membrane transport of solutes and DNA transcription. The solute carriers SLC39A3, SLC25A42 and SLC35E1 were identified, as well as CHERP and CIB3, involved in calcium homeostasis. Transcription-related genes include SUGPI, which is involved in RNA splicing; RFXANK, a DNA-binding protein; NR2C2AP, a nuclear receptor protein; DDX49, an RNA helicase; ELL, an RNA polymerase II elongation factor; and SIN3B a transcriptional regulator.

On GGAZ, 2 genes were identified between 5-7 Mbs, where QTL co-localize for glucose during heat treatment, HCO$_3$ during heat treatment, and TCO$_2$ during heat treatment. The only reported QTL near this region is for antibody response to KLH antigen (Sun et al. 2013). Heat stress is known to reduce antibody titre in chickens (Mashaly et al. 2004), and this locus may be involved in the complex interaction of heat and antibody titre. Although, antibody levels were not measured in the current study. During periods of heat stress, DNA transcription, RNA translation, and cellular proliferation are altered (Kültz 2005) and we observed several genes in this region related to these particular responses including: KIAA1328, involved in chromosomal integrity during mitosis; and TPGS2, involved in tubulin formation.

On GGAZ, 21 genes were identified between 69-71 Mbs, where QTL co-localize for glucose during heat treatment, HCO$_3$ during heat treatment, and TCO$_2$ during heat treatment. The one QTL that is near this region was identified in a previous generation of the same AIL as the current study, and is for bone mineral density (Zhou et al. 2007). A recent study found that heat stress in broilers results in decreased bone mineral density (Hosseini-Vashan et al. 2015). In humans, low serum bicarbonate levels are associated with decreased bone
mineral density (Chen et al. 2015). Although this relationship has yet to be elucidated in the chicken, further studies should investigate the association between blood chemistry variables and bone mineral density. The genes identified in the current study that are primarily involved in DNA transcription include XPA, which is a DNA repair protein, FOXE3 which is part of the forkhead box, and SNORA66 which is small nuclear RNA. Additionally, microRNAs gga-mir-2954, gga-mir-2131, and gga-mir-1583 were identified in this region. An additional gene of interest identified was DNAJA1, which is part of the heat shock family of proteins.

**QTL for blood components reveal orthologous genes between chicken and swine**

QTL for blood pCO₂ in the current study were located on GGA1, 3, 9, 10, 23, 27, and 28. In swine, QTL for blood pCO₂ are on chromosomes 6, 7, 8, 9, and X (Reiner et al. 2009). We identified a region of synteny between chicken GGA1, 110-111 Mb, and pig chromosome X, 43-44 Mb (Figure 3 A), which contains a pCO₂ QTL and several orthologous genes including FUNDC1, EFHC2, NDP, and MAOA. Another region of synteny exists between chicken chromosome 10, 1-4 Mb, and pig chromosome 7, 53-65 Mb (Figure 3 B/C), which contains several orthologous genes including, but not limited to, UBE2Q2, DNAJ, GRAMD2, ADPGK, NEO1, CLK3, SCAMP5, CSK, and MPI. This region contains the carboxylic anhydrase gene (CA12) in chicken, which is involved in calcium metabolism, but this gene maps on pig chromosome 1, a chromosome on which no QTL have been reported for blood chemistry measurements. The region on GGA10, 1-4 Mb, contains QTL for glucose pre-heat, pCO₂ during heat, pCO₂ differential due to heat, and TCO₂ differential due to heat. The syntenic region in swine contains co-localized QTL for pCO₂, HCO₃, TCO₂, and base excess (Reiner et al. 2009).
A QTL for blood K level mapped to syntenic regions in chicken chromosome 10, 16-17 Mb, in our line and swine chromosome 1, 63-226 Mb (Figure 3 D) in a previous study (Reiner et al. 2009). An orthologous gene of interest in this region is *IGF-1*.

Conclusions

The results of this study contribute to the currently sparse knowledge of levels and heritabilities of several blood components under thermal neutral and heat stress conditions in chickens. Most blood components changed in response to heat treatment. Mapped QTL may serve as markers for genomic selection to enhance heat tolerance in poultry and several candidate genes were identified which may give additional insight into mechanisms of physiologic response to high ambient temperatures.

Methods

Ethics statement

Animal experiments were approved by the Institutional Animal Care and Use Committee of Iowa State University: Log #4-11-7128-G.

Chicken lines

We used the F18 and F19 generations of an AIL between chicken lines divergent for thermotolerance created by crossing a single broiler sire to six highly inbred Fayoumi dams (Deeb and Lamont 2002). Birds were reared in floor pens with wood shavings bedding and had *ad libitum* access to water and feed that met all NRC requirements (NRC 1994).

Heat stress experimental design

A total of 631 birds from four hatches (two hatches in each of the two generations) were used for independent heat stress experiments (four replicates). At 17 days of age, birds were transferred to environmentally controlled chambers and acclimated for five days.
Multiple chambers, each containing 6 pens, were used per replicate. Ten to 12 birds were placed in each pen. From day 22 to 28 of age, the chambers heated to 35°C for 7 hours per day and remained at 25°C at all other times.

**Blood variable measurements**

Blood was collected from the wing vein on day 20 (pre-heat) and day 28 (during heat) using a heparinized syringe and needle, and analysed immediately using an iSTAT Portable Clinical Analyser (Chin et al. 2012). The iSTAT CG8+ cartridge was utilized to measure thirteen blood variables including: pH, pCO₂, pO₂, base excess, HCO₃, TCO₂, K, Na, ionized Ca, hematocrit, hemoglobin, sO₂, and glucose.

**DNA isolation and genotyping**

Blood was collected from the wing vein by using an EDTA-coated syringe and needle, and stored at -20°C. DNA was extracted using a salting out method. Briefly, whole blood was incubated with lysis buffer containing proteinase K. Proteins were precipitated out using 5M NaCl while the supernatant remained. The supernatant was combined with 70% ethanol to precipitate out DNA. The DNA isolated from 468 AIL, 6 broiler, and 6 Fayoumi chickens was genotyped on the Affymetrix 600K chicken SNP axiom array (Kranis et al. 2013) by GeneSeek Inc., Lincoln, NE. SNP chromosomal locations were based on the Gallus_gallus_4.0 assembly through Ensembl.

**Statistical analyses**

Calculations of means and standard errors, fixed effects and covariates for the GWAS were calculated based on ANOVA (analysis of variance), and significant terms were fit as fixed effects with a P value ≤ 0.05 using JMP statistical software (SAS Institute 2000).
Heritabilities were estimated with an animal model using ASReml software (Gilmour et al. 2009).

Parameters for inclusion of SNP genotypes included SNP call rate ≥ 95% and minor allele frequency ≥ 5%. Genotyping console (Affymetrix) software was used to create genotyping calls and quality control based on whole animal DishQC score ≥ 0.7. The SNPolisher (Affymetrix) R package was used for quality control of individual SNP in all animals with passing DishQC scores.

The GWAS of phenotypic traits with SNP genotypes was done using GenSel software (Fernando and Garrick 2008). Bayes B, which fits all SNPs simultaneously as random effects, was used for the analysis. The mixed model used for the GWAS:

\[ y = Xb + \sum_{j} z_{j} \alpha_{j} \delta_{j} + \varepsilon. \]

Where \( y \) = vector form of phenotypes, \( X \) = incidence matrix to account for fixed effects on phenotypes, \( b \) = vector of fixed effects, \( z_{j} \) = vector of genotypes for SNP \( j \) based on the number of B alleles (-10, 0, +10, or the average of the genotypes at SNP \( j \)), \( \alpha_{j} \) = allele substitution effect for SNP \( j \), \( \delta_{j} \) = whether SNP \( j \) was included in the Markov chain Monte Carlo (MCMC) chain, and \( \varepsilon \) is the error associated with the analysis.

The genomic markers were split into 1001 non-overlapping 1 Mb windows across the genome. A total of 41,000 MCMC iterations were run for each analysis and the first 1000 iterations were discarded (burn in). The \( \delta_{j} \) was set so that \( \pi = 0.9978 \) to avoid fitting more SNPs than number of animals in a given iteration. In a true infinitesimal model, each window is expected to explain 0.1% (100%/1001) of the genetic variation; therefore, a 1 Mb window was considered significant if it explained ≥ 0.5% of the total genetic variation, corresponding to 5 times more observed than expected.
Pathway analysis

To further investigate QTL regions, we conducted a pathway analysis using Ingenuity Pathway Analysis (IPA) software. All annotated genes within significant (explaining ≥ 0.05% of the genetic variation) 1 Mb windows for any measured trait were identified using Ensemble biomart. This gene list was used as input into IPA and a core analysis was completed using default parameters to identify significant (P ≤ 0.05) canonical pathways and the top 20 significant pathways were reported. Additionally, a gene list was created using the regions of QTL co-localization (3 or more traits) and analysed as described for all QTL regions.

Candidate genes

Candidate genes were identified for regions of QTL co-localization (3 or more traits). All genes within the region were identified using ENSEMBL biomart (Cunningham et al. 2015).

Syntenic regions between chicken and swine

To identify syntenic regions for reported QTL for the same blood chemistry component measurements between chicken and pig, the Comparative Genomics option was used in Ensembl (Cunningham et al. 2015).

Availability of Data and Materials

The dataset supporting the conclusions of this article is available in the Animal QTLdb (animalgenome.org) repository and can be found at http://www.animalgenome.org/cgi-bin/QTLdb/GG/pubtails?PUBMED_ID=ISU0082. The phenotypic dataset supporting the conclusions of this article is included within the article as an additional file (Supplementary Table 2).
Abbreviations

AIL: advanced intercross line
GWAS: genome wide association study
QTL: quantitative trait loci

Competing Interests

The authors have no competing interests.

Authors' Contributions

AVG participated in sample collection, carried out all statistical analyses and drafted the manuscript. CMA and MEP participated in the design of the study, sample collection and acquisition of funding. MFR and CJS participated in the design of the study and acquisition of funding. SJL participated in the design of the study, participated in sample collection, participated in acquisition of funding and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors thank the Iowa State University Poultry Research Center staff for animal care; the Lamont, Persia, Rothschild and Ashwell lab personnel for assistance; Jack Dekkers, Dorian Garrick, Rohan Fernando and their research groups for GWAS advice; Melanie Hess and Andrew Hess for statistical and coding advice. This research was supported by USDA-NIFA-AFRI Climate Change Award #2011-67003-30228; the USDA National Institute of Food and Agriculture, Hatch project #5358; and AVG was partly supported by a USDA National Needs Fellowship, Award #2011-38420-20050.
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**Figures**
Figure 1 Heat map of phenotypic correlations between blood chemistry components. Heat map showing phenotypic correlations between blood chemistry components measured on day 20 (pre-heat), day 28 (during heat), and day 28-20 which is the difference due to heat treatment. Traits are clustered together based on function. The colors represent the correlation coefficient ($r^2$) with red indicating a positive correlation and blue indicating a negative correlation.
Figure 2 Genome-wide plot of percentage of genetic variance for traits measured during heat stress.

Traits were measured before heat treatment (day 20) and during heat treatment (day 28), and the differentials were also calculated. The traits that reached significance in the GWAS (≥ 0.05 % of the genetic variation) are displayed. Results show the percentage of genetic variance that is explained by each non-overlapping 1-Mb window, labeled by the index number of the windows, and are colored and ordered by chromosome (1 to 28, and Z). Plots display: pH on days 20 and 28, and the differential 28-20 (A, B, and C); partial CO₂ (pCO₂) on days 20, 28, and the differential 28-20 (D, E, and F); partial O₂ (pO₂) on day 28, (G); base excess on day 28 and day 28, (H and I); bicarbonate (HCO₃⁻) on day 28 (J); total CO₂ (TCO₂) on day 28 and the differential 28-20 (K and L); potassium (K) on days 20 and the differential 28-20 (M and N); ionized calcium (iCa) on day 28 (O); hematocrit (Hct) on day 28 (P); hemoglobin (Hb) on day 28 and the differential 28-20 (Q and R); saturated oxygen (SO₂) on day 28 and the differential (S and T); glucose on days 20 and 28 (U and V).
Figure 3 Syntenic regions between chicken and swine.

Syntenic regions between chicken and pig containing QTL for blood component traits. A. QTL for pCO\textsubscript{2} in both chicken and pig. Chicken QTL on GGA1 at 110-111 Mb in chicken syntenic with pig on chromosome X, 43-44 Mb. B/C. GGA10 1-2 Mb in chicken and pig chromosome 7 53-60 Mb D. GGA10 16-17 Mb and swine chromosome 1, 63-226 Mb.

Tables

Table 1 Phenotypic means and heritabilities (h\textsuperscript{2})

<table>
<thead>
<tr>
<th>Trait</th>
<th>Day 20</th>
<th>Day 28</th>
<th>Day 28-20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>h\textsuperscript{2} (SE)</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>pH</td>
<td>7.50 ± 0.0\textsuperscript{a}</td>
<td>.17 (0.08)</td>
<td>7.53 ± 0.003\textsuperscript{b}</td>
</tr>
<tr>
<td>pCO\textsubscript{2}, mmHg</td>
<td>31.9 ± 0.1\textsuperscript{a}</td>
<td>.21 (0.06)</td>
<td>31.1 ± 0.2\textsuperscript{b}</td>
</tr>
<tr>
<td>pO\textsubscript{2}, mmHg</td>
<td>43.3 ± 0.3\textsuperscript{a}</td>
<td>.06 (0.04)</td>
<td>43.9 ± 0.2\textsuperscript{a}</td>
</tr>
<tr>
<td>BE, mM</td>
<td>1.8 ± 0.1\textsuperscript{a}</td>
<td>.10 (0.05)</td>
<td>3.3 ± 0.2\textsuperscript{b}</td>
</tr>
<tr>
<td>HCO\textsubscript{3}, mM</td>
<td>25.0 ± 0.1\textsuperscript{a}</td>
<td>.05 (0.04)</td>
<td>26.0 ± 0.1\textsuperscript{b}</td>
</tr>
<tr>
<td>TCO\textsubscript{2}, mM</td>
<td>25.9 ± 0.1\textsuperscript{a}</td>
<td>.02 (0.03)</td>
<td>26.9 ± 0.1\textsuperscript{b}</td>
</tr>
<tr>
<td>K, mM</td>
<td>4.8 ± 0.0\textsuperscript{a}</td>
<td>.20 (0.01)</td>
<td>4.9 ± 0.0\textsuperscript{a}</td>
</tr>
<tr>
<td>Na, mM</td>
<td>137.0 ± 0.2\textsuperscript{a}</td>
<td>.08 (0.6)</td>
<td>137.2 ± 0.3\textsuperscript{a}</td>
</tr>
<tr>
<td>iCa, mM</td>
<td>1.25 ± 0.0\textsuperscript{a}</td>
<td>.04 (0.01)</td>
<td>1.28 ± 0.01\textsuperscript{b}</td>
</tr>
<tr>
<td>Hct, % PCV</td>
<td>22.5 ± 0.2\textsuperscript{a}</td>
<td>.01 (0.03)</td>
<td>23.2 ± 0.1\textsuperscript{b}</td>
</tr>
<tr>
<td>Hb, g/dL</td>
<td>7.7 ± 0.1\textsuperscript{a}</td>
<td>.07 (0.05)</td>
<td>7.9 ± 0.0\textsuperscript{b}</td>
</tr>
</tbody>
</table>
Blood chemistry components were measured pre-heat (day 20), on the seventh day of heat treatment (day 28), and the calculated differential due to heat (day 28-20). Different superscript letters within row represent significant differences (P≤0.05).

### Table 2 Windows explaining a significant percentage (≥ 0.5) of genetic variance

<table>
<thead>
<tr>
<th>Traita</th>
<th>Chr (Mb)</th>
<th>% of genetic variance explained</th>
<th>Nb of SNPs</th>
<th>Freq of iterations with (P &gt; 0)b</th>
<th>SNP namec</th>
<th>SNP pos (bp)d</th>
<th>Model freqe</th>
<th>Allele freqf</th>
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</thead>
<tbody>
<tr>
<td>pH20</td>
<td>18 3</td>
<td>1.29</td>
<td>401 0.94</td>
<td>AX-75894740 3342614</td>
<td>0.0111</td>
<td>0.652</td>
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</tr>
<tr>
<td>pH20</td>
<td>28 4</td>
<td>1.01</td>
<td>328 0.85</td>
<td>AX-76384843 4097788</td>
<td>0.0090</td>
<td>0.294</td>
<td></td>
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</tr>
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<td>pH20</td>
<td>28 3</td>
<td>0.64</td>
<td>437 0.92</td>
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<td>0.0092</td>
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<td>28 10</td>
<td>0.50</td>
<td>372 0.89</td>
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<td>0.0066</td>
<td>0.298</td>
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<tr>
<td>pH20</td>
<td>28 3</td>
<td>0.75</td>
<td>437 0.93</td>
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<td>0.0048</td>
<td>0.711</td>
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<td></td>
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<td>pH220</td>
<td>28 4</td>
<td>0.49</td>
<td>328 0.89</td>
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<td>0.0239</td>
<td>0.706</td>
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<td>0.59</td>
<td>462 0.94</td>
<td>AX-75706074 19358758</td>
<td>0.0070</td>
<td>0.416</td>
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<td>pH220</td>
<td>11 110</td>
<td>0.54</td>
<td>194 0.38</td>
<td>AX-80866127 110487208</td>
<td>0.0098</td>
<td>0.510</td>
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<tr>
<td>pH220</td>
<td>27 2</td>
<td>0.53</td>
<td>650 0.96</td>
<td>AX-76356017 2038872</td>
<td>0.0065</td>
<td>0.653</td>
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<td>pH220</td>
<td>10 3</td>
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<td>447 0.91</td>
<td>AX-76507032 3037730</td>
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<td>pH220</td>
<td>28 4</td>
<td>0.61</td>
<td>328 0.83</td>
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<td>0.0076</td>
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<tr>
<td>pH220</td>
<td>23 2</td>
<td>0.57</td>
<td>388 0.86</td>
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aBlood chemistry components were measured pre-heat (day 20), on the seventh day of heat treatment (day 28), and the calculated differential due to heat (day 28-20).
bFrequency in which the window was included in the MCMC iterations (post-burn-in).
cSNP within the specified window which was most frequently included in the MCMC iterations (post-burn-in), and is therefore predicted to have the greatest effect on the phenotype.
dPosition of SNPs in base pairs on Gallus-gallus (version 4.0) chromosome.
eFrequency in which the SNP was included in the MCMC iterations (post-burn-in) model.
fAllele frequency of the SNP in the genotyped population (N = 458).
Table 3. Top 20 canonical pathways for QTL identified for all traits, and for co-localized QTL.

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<td>INPP5E,IPMK,SEC16A,PMPCA</td>
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<td>AMPK Signaling</td>
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<td>CHRNA5,MTOR,STRADA,AK8,INSR,CHRNA3,PPM1J,CHRNB4,PIK3R2,ADRA2A,TSC1,FOXO1,ADRA1A</td>
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<td>NRAS,PIK3R2,BIRC5,CASP9,IKBAP,FOXO1</td>
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<td>CALR,CHRNA5,MYL4,CHRNB4,CAMK4,CHRNA3,CAMK1G,MEF2D,TPM1,RAP1A,MEF2A</td>
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<td>MTOR,MYL4,CAMK4,RHOC,IGF1R,NRAS,PIK3R2,RHOT1,A DRA2A,MEF2D,MAP3K3,CACNA1D,MEF2A,ADRA1A</td>
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<td>ABL1,TGFA,NRAS,PIK3R2,CASP9,RXRA</td>
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<tr>
<td>Nur77 Signaling in T Lymphocytes</td>
<td>1.26E-03</td>
<td>7/57</td>
<td>MAP2K5,SIN3B,CASP9,RXRA,CAMK4,MEF2D,MAP3K3</td>
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<tr>
<td>Putrescine Degradation III</td>
<td>2.84E-03</td>
<td>4/21</td>
<td>ALDH1A1,ALDH1A3,MAOB,ALDH4A1</td>
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</table>
Table 3 continued

<table>
<thead>
<tr>
<th>Pathway</th>
<th>P-value</th>
<th>Ratio:</th>
<th>Genes in pathway that were identified in current study</th>
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<tbody>
<tr>
<td>Superpathway of D-myo-inositol (1,4,5)-trisphosphate Metabolism</td>
<td>4.71E-03</td>
<td>4/24</td>
<td>INPP5E,IPMK,SEC16A,PMPCA</td>
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<tr>
<td>Thyroid Cancer Signaling</td>
<td>9.69E-04</td>
<td>6/40</td>
<td>NRAS,RET,RXRA,NTRK1,TCF7L2,NGF</td>
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<tr>
<td>Tryptophan Degradation X (Mammalian, via Tryptamine)</td>
<td>4.02E-03</td>
<td>4/23</td>
<td>ALDH1A1,ALDH1A3,MAOB,ALDH4A1</td>
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<tr>
<td><strong>Pathways identified for co-localized QTL</strong></td>
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<tr>
<td>2-oxobutanoate Degradation I</td>
<td>4.22E-02</td>
<td>1/5</td>
<td>MCEE</td>
</tr>
<tr>
<td>AMPK Signaling</td>
<td>4.42E-03</td>
<td>6/178</td>
<td>CHRNA5,PPM1J,CHRNB4,INSR,CHRNA3,ADRA1A</td>
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<td>Calcium Signaling</td>
<td>1.55E-04</td>
<td>8/178</td>
<td>CALR,CHRNA5,CHRNB4,CHRNA3,CMK1G,TPM1,RAP1A,MEF2A</td>
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<tr>
<td>Cardiac Hypertrophy Signaling</td>
<td>4.35E-02</td>
<td>5/223</td>
<td>IGF1R,NRAS,RHOC,MEF2A,ADRA1A</td>
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<tr>
<td>CDK5 Signaling</td>
<td>4.94E-02</td>
<td>3/105</td>
<td>NRAS,PPM1J,NGF</td>
</tr>
<tr>
<td>Cholecystokinin/Gastrin-mediated Signaling</td>
<td>4.95E-02</td>
<td>3/245</td>
<td>NRAS,RHOC,MEF2A</td>
</tr>
<tr>
<td>CTLA4 Signaling in Cytotoxic T Lymphocytes</td>
<td>4.01E-02</td>
<td>3/88</td>
<td>PPM1J,PTPN22,AP1M1</td>
</tr>
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<td>ERK5 Signaling</td>
<td>1.69E-02</td>
<td>3/63</td>
<td>NRAS,NGF,MEF2A</td>
</tr>
<tr>
<td>Germ Cell-Sertoli Cell Junction Signaling</td>
<td>4.93E-02</td>
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<td>NRAS,TJP1,RHOC,RAB8B</td>
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<td>Glioblastoma Multiforme Signaling</td>
<td>3.73E-02</td>
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<td>WNT2B,IGF1R,NRAS,RHOC</td>
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<tr>
<td>Glioma Signaling</td>
<td>1.01E-02</td>
<td>4/98</td>
<td>TGFA,IGF1R,NRAS,CAMK1G</td>
</tr>
<tr>
<td>Integrin Signaling</td>
<td>3.33E-02</td>
<td>5/207</td>
<td>NRAS,TSPAN2,RHOC,TLN2,RAP1A</td>
</tr>
<tr>
<td>Methylmalonyl Pathway</td>
<td>3.39E-02</td>
<td>1/4</td>
<td>MCEE</td>
</tr>
<tr>
<td>mTOR Signaling</td>
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<td>5/187</td>
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<tr>
<td>NF-κB Signaling</td>
<td>1.65E-02</td>
<td>5/172</td>
<td>TGFA,IGF1R,NRAS,INSR,NGF</td>
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<td>PTEN Signaling</td>
<td>1.89E-02</td>
<td>4/118</td>
<td>IGF1R,NRAS,INSR,MAGI3</td>
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<tr>
<td>Renal Cell Carcinoma Signaling</td>
<td>2.32E-02</td>
<td>3/71</td>
<td>TGFA,NRAS,RAP1A</td>
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<tr>
<td>STAT3 Pathway</td>
<td>2.49E-02</td>
<td>3/73</td>
<td>IGF1R,NRAS,INSR</td>
</tr>
</tbody>
</table>
All characterized genes within a significant QTL regions were used as input for Ingenuity Pathway Analysis (IPA) software. The Top 20 significant (P ≤ 0.05) pathways are listed. The results are displayed for pathways identified when using all QTL regions (61 total QTL) which resulted in 682 (999 total) annotated genes used for pathway analysis. The bottom section of the table displays for pathways identified when using only the co-localized QTL regions (7 total co-localized QTL regions) which resulted in 185 (226 total) annotated genes used for pathway analysis. The pathways are the top canonical pathways identified by IPA and are listed in alphabetical order. The ratio refers to the number of genes that were identified in the current study compared to the total number of genes that are in the pathway according to IPA.

<table>
<thead>
<tr>
<th>TCA Cycle II (Eukaryotic)</th>
<th>1.65E-02 2/23</th>
<th>IDH3A,ACO1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid Cancer Signaling</td>
<td>4.62E-02 2/40</td>
<td>NRAS,NGF</td>
</tr>
</tbody>
</table>
CHAPTER 4
UNIQUE GENETIC RESPONSES REVEALED IN RNA-SEQ OF THE SPLEEN OF CHICKENS STIMULATED WITH LIPOPOLYSACCHARIDE AND HEAT

Modified from a paper in preparation for submission to PLOSOne

Angelica Van Goor, Chris M. Ashwell, Mike E. Persia, Max F. Rothschild, Carl J. Schmidt and Susan J. Lamont

Author’s contribution
AVG participated in sample collection, isolated RNA, created cDNA libraries, validated RNA-seq results with Fluidigm technology, carried out all statistical analyses, and drafted the manuscript.

Abstract
Climate change and disease have large negative impacts on poultry production, but little is known about the interactions of responses to these stressors in chickens. Fayoumi (heat and disease resistant) and broiler (heat and disease susceptible) chicken lines were stimulated at 22 days of age, using a 2x2x2 factorial design including: breed (Fayoumi or broiler), inflammatory stimulus [lipopolysaccharide (LPS) or saline], and temperature (35°C or 25°C). Transcriptional changes in spleens, at 7 hours post temperature stimulus and 3.5 hours post inflammatory stimulus, were analyzed using RNA-sequencing on the Illumina HiSeq 2500. Thirty-two individual cDNA libraries were sequenced (four per treatment) and an average of 22 million reads were generated per library, and an average of 16 million reads were mapped to the chicken genome.

Stimulation with LPS induced more detectable differentially expressed genes (DEG, log2 fold change ≥ 2 and FDR ≤ 0.05) in the broiler (N=283) than the Fayoumi (N=85), whereas heat treatment resulted in fewer detectable DEG in broiler (N=22) compared to
Fayoumi (N=107). The double stimulus of LPS+heat induced the largest numbers of changes in gene expression, for which broiler had 567 DEG and Fayoumi had 1471 DEG of which 399 were shared between breeds. More DEG may mean an increased responsiveness to treatment. Further analysis of DEG revealed pathways impacted by these stressors such as Remodelling of Epithelial Adherens Junctions due to heat stress, Granulocyte Adhesion and Diapedesis due to LPS, and Hepatic Fibrosis/Hepatic Stellate Cell Activation due to LPS+heat. The genes and pathways identified provide deeper understanding of the response to the applied stressors and may serve as biomarkers for genetic selection for heat and disease tolerant chickens.

**Introduction**

Climate change will increase the degree and frequency of severe weather patterns, and the global average temperature is expected to become increasingly warmer (USDA 2012), which will have large negative impacts on poultry production (St-Pierre et al. 2003). Heat stress generally decreases immunocompetence in chickens, characterized by decreased relative weights of immune tissues (Niu et al. 2009; Quinteiro-Filho et al. 2010; Smith 2003), decreased antibody production (Donker et al. 1990; Mashaly et al. 2004), increased incidence of bacterial colonization of the spleen (Quinteiro-Filho et al. 2012), higher susceptibility to infections (Quinteiro-Filho et al. 2012), and decreased macrophage activity (Quinteiro-Filho et al. 2010). St-Pierre estimated $158 million dollars in production losses annually in poultry due to heat stress in the U.S. alone (St-Pierre et al. 2003), and disease is estimated to cause 20% of production losses in the poultry industry (Biggs 1982), and is a concern for animal welfare and human health.
Several studies have identified decreases in adaptive immunity in chickens during heat stress conditions. A 5-week period of heat stress in layers decreased total white blood cell count, antibody production, and lymphocytes activity (Mashaly et al. 2004). Another study using layers under heat stress identified a reduction in the relative weight of liver (Felver-Gant et al. 2012). Examination of the gut epithelial cells in laying hens during heat stress identified a decrease in the number of immune cells (Deng et al. 2012). Broilers that are heat stressed have lower weights of immune organs including the bursa of Fabricius, thymus, and spleen (Niu et al. 2009; Quinteiro-Filho et al. 2010; Smith 2003). Lower relative weight in lymphoid organs is undesirable, and has been associated with decreased immune response to Newcastle Disease in chickens (Sijtsma et al. 1991). Total circulating antibody and specificity are decreased in broilers under heat stress (Smith 2003). When broiler chicken lines selected for high and low antibody titre to SRBC were subjected to heat stress, the highly responsive line had decreased antibody production to SRBC under heat stress conditions compared to thermoneutral conditions (Donker et al. 1990). In addition to adaptive immune cell reaction to heat stress, broilers have increased susceptibility to mild enteritis, characterized by increased concentrations of white blood cells in lamina propria of the jejunum (Quinteiro-Filho et al. 2012). On the contrary to immunosuppressive effect, short bouts (1-2 hours) of heat stress have been shown to increase antibody production to SRBC in broilers (Heller et al. 1979). The few studies that have characterized innate immune response in chickens under heat have reported decreases in activity. Macrophages have lower basal and bacterial induced oxidative burst activity during heat stress (Quinteiro-Filho et al. 2010). Broilers heat stressed and challenged with *Salmonella Enteritidis* have increased bacterial invasion of the spleen, and the authors speculate this may be due to gut barrier dysfunction.
during heat stress (Quinteiro-Filho et al. 2012). Broilers under heat stress conditions increase intestinal permeability (Quinteiro-Filho et al. 2012; Quinteiro-Filho et al. 2010; Star et al. 2009), and layers have altered gut morphology of microvilli (Deng et al. 2012). Increase intestinal permeability causes a rapid increase in bacteria within the blood, which can lead to endotoxic shock, sepsis, and death, largely due to a pro-inflammatory cytokine storm (Maes 2008). The major contributing factor to the cytokine storm caused by disruption of the gut barrier is thought to be lipopolysaccharide (LPS) (Mass et al. 2008). LPS is an essential component of gram negative bacteria and a major contributor to the fatality of heat stroke in humans (Graber et al. 1971). The endotoxemia caused by an increased amount of circulating LPS during periods of heat stress could contribute to increased mortality and decreased production. The double stimulation of LPS and heat stress could increase body temperatures beyond the thermal comfort zone, resulting in increased mortality. However, the type of stressor and the time of exposure determines the immune response (Flier et al. 1998). To our knowledge, the spleen in chicken has never been tested for response to heat stress and immune stimulation in vivo. In the current study, the spleen transcriptome was sequenced to investigate the effect of LPS, heat stress, and LPS+heat.

**Materials and Methods**

**Ethics statement**

All animal experiments were approved by the Institutional Animal Care and Use Committee at Iowa State University: Log #4-11-7128-G.

**Experimental design and tissue sampling**

Chicks were produced from the same breeders (16 sires and 30 dams) in two hatches (replicates). The Fayoumi (heat and disease resistant) and the broiler (heat and disease...
susceptible) breeds were assessed for response to heat stress and LPS stimulation using a full factorial design including the factors; breed, thermal treatment, and inflammatory stimulus. A total of 48 chickens (24 per breed) were used for RNAseq. At 17 days of age, birds were transferred to environmentally-controlled chambers and acclimated for five days. Floor pens had wood shavings bedding and birds had *ad libitum* access to water and corn-soy feed that met all NRC requirements for the duration of the study (NRC 1994). There were four environmental chambers, each containing four pens, per replicate. One to two chickens were sampled per pen per replicate to have equal representation from pens.

At 22 days of age, two of the chambers were heated to 35°C (heat stress, HS), while the other two remained at 25°C (thermoneutral, TN). After 3.5 hours of thermal treatment, half of the birds were subcutaneously injected with LPS (LPS) (*Salmonella enterica* serotype *typhimurium*) in the amount 100 μg/kg of body weight, or phosphate buffered saline (PBS). This design resulted in 8 treatment groups: 1. Fayoumi, TN, PBS (F_TN_PBS); 2. Fayoumi, HS, PBS (F_HS_PBS); 3. Fayoumi, TN, LPS (F_TN_LPS); 4. Fayoumi, HS, LPS (F_HS_LPS); 5. Broiler, TN, PBS (B_TN_PBS); 6. B, HS, PBS (B_HS_PBS); 7. Broiler, TN, LPS (B_HS_LPS) 8. Broiler, HS, LPS (B_HS_LPS). After a total of 7 hours of heat stress (3.5 hours post LPS stimulation) birds were euthanized and the spleens were harvested, placed in RNAlater, and stored at -20°C until further use.

**Body temperature and blood chemistry component measurements**

Cloacal body temperature was measured by inserting a digital thermometer approximately 2.5 cm into the cloaca on days 20 (pre-treatment) and 22 of age (at the end of treatment). The precision of the digital thermometer was 0.1°C. Blood was collected from the wing vein on day 20 (pre-heat) and day 22 (at the end of treatment after body temperature
was measured) using a heparinized syringe and needle, and analysed immediately using an iSTAT Portable Clinical Analyser (Chin et al. 2012). The iSTAT CG8+ cartridge was utilized to measure thirteen blood variables including: pH, pCO$_2$, pO$_2$, base excess, HCO$_3$, TCO$_2$, K, Na, ionized Ca, hematocrit, hemoglobin, sO$_2$, and glucose. The blood was measured on a total of 244 birds and analysed with a Students’ T test with correction for multiple testing.

**Library generation and sequencing**

The total RNA was isolated from 4 chicken spleens per treatment group (32 birds total) with the RNAqueous kit (Ambion) using all manufacturers recommendations as previously described (Cheeseman et al. 2007), and treated with DNA-free kit (Ambion). The RNA quality was quantified using the Agilent 2000 Bioanalyzer. Only samples with an RNA Integrity Number (RIN) greater than 9 were used for cDNA library construction using the TruSeq RNA Library preparation kit v2 (Illumina) that preferentially amplifies PolyA mRNA. The cDNA libraries created from the spleen of 32 chickens were run on the HiSeq 2500 machine (Illumina) with the option of 100 bp single end reads at the Iowa State University DNA Facility. Four lanes were used for RNA-sequencing with 8 multiplexed samples per lane (one sample per each treatment).

**Detection of differential gene expression**

Quality control of RNA-seq reads was conducted using FASTQC and FASTX Clipper (version 0.0.13; http://hannonlab.cshl.edu/fastx_toolkit/) with the following options; phred score of 30, minimum base pair length of 30, and adapter sequences were removed.

Reads were mapped to the *Gallus gallus* genome version 4.0 (4.78 GTF Ensembl) using Tophat (version 2.0.9) (Kim et al. 2013) using default parameters. Counting of mapped
reads to a gene was done using HTSeq (version 3.0). Table 1 includes the average (N=4/treatment group) number of generated reads before and after quality filtering, number of mapped and the percentage of transcriptome coverage. There are annotated 15,508 coding genes in *Gallus gallus* 4.0 genome. To calculate transcriptome coverage we used the average number of annotated genes that were expressed (3 of 4 samples per treatment group must have at least 1 read count for each gene) in our dataset and divided by the number of annotated coding genes. The resulting number is within range of predicted coverage based on sequencing depth (Wang et al. 2011). Differential gene expression was detected using edgeR (version 1.00), with Benjamini Hochberg method used for multiple testing correction, and maximum FDR ≤0.05. A pairwise comparison was used to detect DEG within breed contrasting the most naive group, i.e. TN_PBS. The contrasts contained 4 individuals per treatment group and were as follows; F_TN_PBS vs F_HS_PBS, F_TN_PBS vs F_TN_LPS, F_TN_PBS vs F_HS_LPS, B_TN_PBS vs B_HS_PBS, B_TN_PBS vs B_TN_LPS, and B_TN_PBS vs B_HS_LPS.

**Fluidigm expression verification of RNA-seq data**

We used Fluidigm gene expression technology to confirm the mRNA expression detected by RNA-seq in the current study. As described in the Library Generation and Sequencing section, total RNA was isolated and treated with a DNA-free kit. Gene expression analysis was performed using a microfluidic Reverse Transcription quantitative PCR (RT-qPCR) (Fluidigm Corporation, San Francisco, CA, USA). All procedures were conducted according to manufacturer’s recommendations, unless otherwise noted. Briefly, 50 ng of the total RNA was reversely transcribed using the Fluidigm Reverse Transcription Master Mix (Fluidigm Corporation, San Francisco, CA, USA). cDNA was pre-amplified
with PreAmp Master Mix (Fluidigm Corporation, San Francisco, CA, USA), using 12 cycles of pre-amplification. Exonuclease I (New England Biolabs, UK) treatment was applied to remove unincorporated primers. Pre-amplified and purified cDNA samples were diluted 10x in TE buffer and stored at -20°C until further analyses. RT-qPCR analysis was completed for 22 target genes and 2 reference genes, listed in Table 2. These genes were selected to assay because they represented a range of fold change expression values from extremely changed (LFC ≥ ±30) to not changed due to treatment (LFC = 0). The FlexSix Integrated Fluid Circuits (IFCs) (Fluidigm Corporation, San Francisco, CA, USA) was used to assay mRNA expression. Sample assay included 1.35 µl of pre-amplified and Exo I treated cDNA, 1.5 µl of the SsoFast™ EvaGreen® Supermix with Low ROX™ (2x) (Bio-Rad) and 0.15 µl of the FlexSix Delta Gene Sample Reagent (Fluidigm Corporation, San Francisco, CA, USA). Primer assays were prepared as 20 µl stock by mixing 1 µl of each primer (100 µM) with 10 µl of the 2x Assay Loading Reagent and adjusted to 20 µl with DNA suspension buffer (low EDTA TE buffer). The samples, assays and the loading reagents were then loaded onto IFCs microfluidic channels using the RX loading station (Fluidigm Corporation, San Francisco, CA, USA). RT-qPCR was performed on the Biomark™ HD (Fluidigm Corporation, San Francisco, CA, USA) using the fast program that consisted of an incubation step at 95°C for 60 s followed by 30 cycles: 96°C for 5 s and 60°C for 20 s. Fluorescence emission was recorded after each cycling step. Upon RT-qPCR completion, melting curves were generated by increasing temperature from 60 to 95°C, followed by continued fluorescence acquisition.

RT-qPCR data were analyzed as follows: raw qPCR data were analyzed and checked for quality using Real-Time PCR Analysis Software (Fluidigm Corporation, San Francisco, CA, USA). Main effects were estimated using least square means method implemented in
JMP Pro 10.0.2 software (SAS Institute, Cary, NC, USA). Chicken line (Fayoumi or broiler), thermal treatment (TN or HS), and immune stimulus (PBS and LPS) as well as the interaction between line and treatments were fitted in the model. Analyses were performed separately for each line, gene, and treatment using dCt values (Ct target – Ct reference). To determine the relative gene expression, ddCt method was used (Livak and Schmittgen 2001). Delta Ct values were obtained by normalizing the Ct values of the target genes with the geometrical mean of the two reference genes (H6PD and RPL4). Fold induction of the gene expression was estimated as $2^{-\Delta \text{ddCt}}$. Untreated (control) samples were used as calibrators.

**Table 1 Forward and reverse primer sequences used with Fluidigm to validate RNA-sequencing results.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H6PD</td>
<td>ATGTACCGGGTGGACCACTA</td>
<td>AACTGACGTTCTGATCTGGAAA</td>
</tr>
<tr>
<td>RPL4</td>
<td>TTCTGCCTTGCCAGCATCA</td>
<td>AGGAAATCTGGGATCTCCCA</td>
</tr>
<tr>
<td>IL-10</td>
<td>CATGCTGCTGGGCTGTTA</td>
<td>CGTCTCCTTGATCTGGTTGATG</td>
</tr>
<tr>
<td>IL-6</td>
<td>GCTGCGGGCTTGA</td>
<td>GGTAGGCTGAAAGGCGAACAG</td>
</tr>
<tr>
<td>IL-1beta</td>
<td>TCTCTCGTGGAGTGTCAC</td>
<td>GCCATCTGCCAGTTGCA</td>
</tr>
<tr>
<td>iNOS</td>
<td>GACCCAGCCTGTTGTGAGATA</td>
<td>AGCAGCTGTAGTGATGATCCA</td>
</tr>
<tr>
<td>IFN-gamma</td>
<td>AACCTTCTGATGGGCGTTAAA</td>
<td>GCTTTGCCTGGATCTTCA</td>
</tr>
<tr>
<td>CCL4</td>
<td>CTCATCAGAGGACTCCACCA</td>
<td>GCTTGACGCTCTGCAGGTA</td>
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<tr>
<td>ANGPTL4</td>
<td>TGTGACATGACTGCAGAAGG</td>
<td>CAGCCAGAAGTCACCAGTAAA</td>
</tr>
<tr>
<td>CD40</td>
<td>AGCCTGTGGATGCTGTTGAA</td>
<td>CTCACAGGTTGTGCGAGCA</td>
</tr>
<tr>
<td>IL18</td>
<td>CTGGGCAGCTTGGAGATGTA</td>
<td>CTGAATGCAACAGGCGATCC</td>
</tr>
<tr>
<td>HSPA2</td>
<td>CCACCATTCCACCAAAACCA</td>
<td>ATACACCTGGACGGACGACAC</td>
</tr>
<tr>
<td>HS4F</td>
<td>TTCTGTTGGCCAGCTCCAA</td>
<td>ACAACCCACTGTCACAGCATCA</td>
</tr>
<tr>
<td>TLR4</td>
<td>CCTGCTGTGGCATGGA</td>
<td>TGTTCTGCTGTGCATCTGAA</td>
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<td>S100A1</td>
<td>AGCTGAGAGAGAGAGAGCTG</td>
<td>GCTCTGCTGAGATCTTCTCC</td>
</tr>
<tr>
<td>MAPK9</td>
<td>TTACAGAGCGCCAGGTGTA</td>
<td>TCTCCCATGATGCAAACAC</td>
</tr>
</tbody>
</table>

**Data Deposition**

The data discussed in this publication will be deposited in the Gene Expression Omnibus at NCBI (Edgar et al. 2002).

**Results**
Body temperature and blood chemistry component response

The body temperature and blood chemistry results are presented in Figure 1. Body temperature significantly (P ≤ 0.05) increased in both breeds in response to the single treatments of heat or LPS, and the increases were of the same magnitude for each treatment. The double stimulus of heat+LPS resulted in a significant increase; i.e., it was statistically (P ≤ 0.05) higher than the single treatments. Blood chemistry components are presented as percent change from pre-heat values. Between-breed comparisons reveal relatively few (10 with P≤0.05 statistically significant differences, possibly because of the large variability. The double stimulus of heat+LPS resulted in the most differences between breeds.

Alignment and mapping to the chicken genome

The RNA-seq generated from 32 individual cDNA libraries (4 per treatment group) as reported in Table 2 were mapped to *Gallus gallus* 4.0. Across all treatment groups, the average number of reads generated per cDNA library was 21,502,88; post filtering resulted in 17,645,401; the percentage of reads that mapped to the chicken genome was 89%; and the percentage of the spleen transcriptome that was detected as expressed was 82%.

Table 2
Average number of RNA-sequencing reads generated within each treatment group (N=4) before and after FASTQC filtering, and number of mapped reads, and percentage of transcriptome coverage.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Reads generated</th>
<th>Reads post-filtering</th>
<th>Mapped reads (%)</th>
<th>Transcriptome coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>F_TN_PBS</strong></td>
<td>24,200,942</td>
<td>19,841,278</td>
<td>17,480,166 (88.1%)</td>
<td>12,987 (83.7%)</td>
</tr>
<tr>
<td><strong>F_HS_PBS</strong></td>
<td>20,203,988</td>
<td>16,721,600</td>
<td>14,915,667 (89.2%)</td>
<td>12,885 (83.1%)</td>
</tr>
<tr>
<td><strong>F_TN_LPS</strong></td>
<td>28,607,883</td>
<td>23,464,251</td>
<td>21,023,969 (89.6%)</td>
<td>12,534 (80.8%)</td>
</tr>
<tr>
<td><strong>F_HS_LPS</strong></td>
<td>22,828,821</td>
<td>18,843,821</td>
<td>20,568,587 (90.1%)</td>
<td>12,717 (82.0%)</td>
</tr>
<tr>
<td><strong>B_TN_PBS</strong></td>
<td>17,551,738</td>
<td>14,404,416</td>
<td>12,863,143 (89.3%)</td>
<td>12,692 (81.8%)</td>
</tr>
<tr>
<td><strong>B_HS_PBS</strong></td>
<td>18,184,950</td>
<td>14,859,218</td>
<td>13,239,563 (89.1%)</td>
<td>12,568 (81.0%)</td>
</tr>
<tr>
<td><strong>B_TN_LPS</strong></td>
<td>19,724,297</td>
<td>16,050,493</td>
<td>14,236,787 (88.7%)</td>
<td>12,675 (81.7%)</td>
</tr>
<tr>
<td><strong>B_HS_LPS</strong></td>
<td>20,720,682</td>
<td>16,978,131</td>
<td>15,059,602 (88.7%)</td>
<td>12,632 (81.5%)</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>21,502,888</td>
<td>17,645,401</td>
<td>16,173,436 (89.1%)</td>
<td>12,711 (82.0%)</td>
</tr>
</tbody>
</table>

Differentially expressed genes
Differentially expressed genes (DEG) within the spleen were detected using EdgeR software with a LFC ≥ ±2 and FDR ≤ 0.05 and are depicted in Figure 2 as a heat map that indicates total number of DEG as well as the direction of fold change. Broilers responded to LPS (B_TN_PBS vs B_TN_LPS) by 283 DEG, with only 25 (less than 10%) downregulated. Fayoumis responded to LPS (F_TN_PBS vs F_TN_LPS) by 85 DEGs, with more than 80% upregulated. Broilers responded to thermal treatment (B_TN_PBS vs B_HS_PBS) by only 22 DEG, with 15 upregulated and 7 downregulated. Fayoumis responded to thermal treatment (F_TN_PBS vs F_HS_PBS) with almost 5 times more (107) DEG compared to broilers, with nearly two-thirds (79) upregulated and one-third (28) downregulated. Broilers responded strongest to LPS+heat (B_TN_PBS vs B_HS_LPS) treatment by 567 DEG, with the majority (328) upregulated and 239 downregulated. The contrast which resulted in the most DEG was in Fayoumis that responded to treatment with LPS+heat (F_TN_PBS vs F_HS_LPS) by 1471 DEGs, and nearly twice as many were downregulated (972) than upregulated (499). The shared DEGs within breed between treatment groups, and the shared DEGs between breeds within treatment groups are depicted as Venn Diagrams in Figure 3. Within-breed comparison for DEG due to treatment reveal that there are few genes in common between LPS and heat (Fayoumi 0 and broiler 7). Also, the Fayoumi has 6 times (31 DEG) more genes that are shared between all three treatments than the broiler (5 DEG). Comparing between lines within treatments, LPS treatment resulted in sharing of 72% (61 out of 85 possible) of DEG. A different trend was observed for shared genes responsive to heat treatment with only 32% (7 out of 22 possible). The largest number of shared genes between breeds is for the LPS+heat treatment, 399 DEG (out of a possible 568), corresponding to 70%. ).
Validation of RNA-sequencing data using Fluidigm technology

Validation of the RNA-sequencing data was completed using Fluidigm gene expression technology. A total of 32 RNA samples (4 per treatment) were assayed in triplicate on a FlexSix IFC where we tested mRNA expression of 14 test genes and 2 housekeeping genes. The comparison of fold changes calculated from Fluidigm and RNA-sequencing are depicted in Figure 4. The correlation between the two technologies was \( R^2=0.84 \).

GO enrichment analysis

The DEG from each of the contrasts (6 total) were used for GO enrichment analysis (www.geneontology.org) for biological process enrichment and the top ten enrichments are shown in Figure 5.

Ingenuity pathway analysis

All DEG from each contrast were used for input into Ingenuity Pathway Analysis (IPA) software. The results of top canonical pathways are shown in Table 3 for each of the contrast groups. The top predicted upstream regulators are shown in Table 3. All genes with FDR \( \leq 0.05 \) were included in the pathway analysis. The resulting number of genes input and annotated within IPA for each treatment group are: F_HS_PBS had 79 annotated genes of 116 total, F_TN_LPS had 66 annotated genes of 85 total, F_HS_LPS 2925 annotated genes of 3707 total, Br_HS_PBS had 18 annotated genes of 25 total, Br_TN_LPS had 264 annotated genes of 346 total, and Br_HS_LPS had 798 annotated genes of 971 total.

Table 3

<table>
<thead>
<tr>
<th>Breed</th>
<th>Number of Genes Input and Annotated in IPA</th>
<th>Total Genes</th>
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</thead>
<tbody>
<tr>
<td>F_HS_PBS</td>
<td>79</td>
<td>116</td>
</tr>
<tr>
<td>F_TN_LPS</td>
<td>66</td>
<td>85</td>
</tr>
<tr>
<td>F_HS_LPS</td>
<td>2925</td>
<td>3707</td>
</tr>
<tr>
<td>Br_HS_PBS</td>
<td>18</td>
<td>25</td>
</tr>
<tr>
<td>Br_TN_LPS</td>
<td>264</td>
<td>346</td>
</tr>
<tr>
<td>Br_HS_LPS</td>
<td>798</td>
<td>971</td>
</tr>
</tbody>
</table>

Ingenuity pathway analysis results for differentially expressed genes due to treatment within breed. The top 5 significant (\( P \leq 0.05 \)) canonical pathways, upstream regulators, and diseases and disorders are listed for each contrast.
<table>
<thead>
<tr>
<th>Canonical pathways</th>
<th>F_TN_PBS vs. F_HS_PBS</th>
<th>Br_TN_PBS vs. Br_HS_PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute Phase Response Signaling</td>
<td>2.02E-07</td>
<td>Lipid Antigen Presentation by CD1</td>
</tr>
<tr>
<td>Extrinsic Prothrombin Activation Pathways</td>
<td>2.60E-05</td>
<td>Remodeling of Epithelial Adherens Junctions</td>
</tr>
<tr>
<td>Intrinsic Prothrombin Activation Pathway</td>
<td>1.64E-04</td>
<td>Macropinocytosis Signaling</td>
</tr>
<tr>
<td>Retinoate Biosynthesis I</td>
<td>2.42E-04</td>
<td>Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes</td>
</tr>
<tr>
<td>Coagulation System</td>
<td>2.89E-04</td>
<td>Paxillin Signaling</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Upstream Regulators</th>
<th>F_TN_PBS vs. F_TN_LPS</th>
<th>Br_TN_PBS vs. Br_TN_LPS</th>
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<tr>
<td>TNF</td>
<td>2.81E-13</td>
<td>CYTH2</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>2.37E-12</td>
<td>IPCEF1</td>
</tr>
<tr>
<td>IKBK</td>
<td>3.09E-12</td>
<td>SUV39H2</td>
</tr>
<tr>
<td>IL1B</td>
<td>5.63E-12</td>
<td>LMCD1</td>
</tr>
<tr>
<td>GH1</td>
<td>1.31E-10</td>
<td>CYTH3</td>
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<table>
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<th>Diseases and Disorders</th>
<th>F_TN_PBS vs. F_HS_LPS</th>
<th>Br_TN_PBS vs. Br_HS_LPS</th>
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<tbody>
<tr>
<td>Cardiovascular Disease</td>
<td>3.68E-03</td>
<td>Connective Tissue Disorders</td>
</tr>
<tr>
<td>Developmental Disorder</td>
<td>3.68E-03</td>
<td>Inflammatory Response</td>
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<tr>
<td>Hematological Disease</td>
<td>3.68E-03</td>
<td>Skeletal and Muscular Disorders</td>
</tr>
<tr>
<td>Hereditary Disorder</td>
<td>3.68E-03</td>
<td>Infectious Diseases</td>
</tr>
<tr>
<td>Immunological Disease</td>
<td>3.68E-03</td>
<td>Cancer</td>
</tr>
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</table>

<table>
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<th>Upstream Regulators</th>
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<th>Br_TN_PBS vs. Br_TN_LPS</th>
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</thead>
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<tr>
<td>TNF</td>
<td>4.39E-22</td>
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</tr>
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<td>Lipopolysaccharide</td>
<td>1.48E-15</td>
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<td>TLR3</td>
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<td>IL1B</td>
<td>1.55E-12</td>
<td>NFKB (complex)</td>
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<td>Prostaglandin E2</td>
<td>1.56E-12</td>
<td>IFNG</td>
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<th>Br_TN_PBS vs. Br_HS_LPS</th>
</tr>
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<tbody>
<tr>
<td>Organismal Injury and Abnormalities</td>
<td>3.15E-03</td>
<td>Inflammatory Response</td>
</tr>
<tr>
<td>Inflammatory Response</td>
<td>3.15E-03</td>
<td>Organismal Injury and Abnormalities</td>
</tr>
<tr>
<td>Cancer</td>
<td>2.91E-03</td>
<td>Cancer</td>
</tr>
<tr>
<td>Gastrointestinal Disease</td>
<td>3.15E-03</td>
<td>Inflammatory Disease</td>
</tr>
<tr>
<td>Metabolic Disease</td>
<td>3.15E-03</td>
<td>Infectious Diseases</td>
</tr>
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</table>

Table 3 continued

<table>
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<tr>
<th>Canonical Pathways</th>
<th>F_TN_PBS vs. F_HS_LPS</th>
<th>Br_TN_PBS vs. Br_HS_LPS</th>
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</thead>
<tbody>
<tr>
<td>Hepatic Fibrosis/Hepatic Stellate Cell Activation</td>
<td>2.58E-10</td>
<td>Hepatic Fibrosis/Hepatic Stellate Cell Activation</td>
</tr>
</tbody>
</table>
For the first time, to our knowledge, we describe the birds’ phenotypic responses and the changes in the spleen transcriptome in response to thermal treatment (heat stress) and immune stimulation (LPS) alone, and in combination (LPS+heat), in unique lines of chickens. We hypothesized that the lines would differ in their response to these treatments, and that the response of the heat-tolerant and disease-resistant Fayoumis would give insights into the genetic mechanisms of favorable response to these stressors. The Fayoumis were imported to the U.S. from Egypt more than 60 years ago due to their disease resistant nature, and since have been highly inbred >99.9% (Deeb and Lamont 2002). Because the Fayoumi breed originated in a region that has a high-temperature climate, this breed has undergone natural selection for tolerance to heat. In contrast, the broilers were commercially selected for muscle mass accretion in temperate climates and were not deliberately inbred (Deeb and Lamont 2002). Because of the commercial selection for rapid and efficient growth, we
hypothesize the broilers will be less tolerant to heat than the Fayoumis. Studies of an advanced intercross between the Fayoumi and broiler lines determined that body temperature, body weight, breast yield, digestibility (Van Goor et al. 2015), and blood components (Van Goor et al. 2016) measured during heat stress were heritable and QTL were identified, which further illustrates the heat-response divergence of these two lines. In addition to divergence for heat tolerance, these lines have been characterized for differences in response to challenge with Salmonella Enteritidis, which show the Fayoumi to be more resistant than the broiler to (Abasht et al. 2009; Cheeseman et al. 2007). Therefore, the Fayoumi and broiler serve as a comparative model for heat and disease differences.

**Phenotypic responses**

To characterize the phenotypic response to LPS, heat, and LPS+heat, we measured body temperature and 13 different blood chemistry components. Body temperature increased to the same amount with LPS or heat treatment alone in both breeds. When birds were treated with LPS+heat, the body temperature increased statistically more i.e. beyond the individual treatments. The mean normal body temperature of chickens is 41°C and the highest productivity is within the thermoneutral temperature zone, which has been estimated between 20-26°C for adult broilers and layers (Deaton et al. 1978;; Meltzer 1983; Meltzer et al. 1982). The upper critical temperature is defined as the ambient temperature in which the animal continually increases body temperature until mortality results, and this is estimated between 36-37°C for broilers (Van Der Hel et al. 1991). Our experiment used an acute heat stress of 7 hours at 35°C. LPS is a well characterized inflammatory stimulus but the effect on body temperature varies considerably depending on breed, age, and exposure time (Cheng et al. 2004; De Boever et al. 2008; Jones et al. 1981; Leshchinsky and Klasing 2001; Jones et al.
Generally, these reports are consistent that a period of hypothermia precedes hyperthermia. Here we report that at 3.5 hours post injection a significant increase in body temperature is observed, with Fayoumis increasing body temperature higher than broilers. Little is known about the effect of heat stress on immune response. In mice, heat treatment alone doesn’t affect serum levels of TNF-α, IL-1, IL-1B, and acute phase proteins, whereas LPS+heat results in higher concentrations of these inflammatory cytokines compared to LPS alone (Ostberg et al. 2000). However, this result does not necessarily support an improved or reduced immune response, only that more inflammatory molecules are present. If inflammation is not strictly controlled, it may have detrimental results on the host. Additionally, because we observed a synergistic increase in body temperature when the double stimulus (LPS+heat) was done, this could be detrimental to the host. Blood chemistry components were measured that relate to acid-base balance, electrolytes, and glucose. During periods of heat stress, chickens increase the depth and frequency of breaths (Zhou and Yamamoto 1997), which results in alkalosis. Indeed, in the current study we identified changes related to respiratory alkalosis including increased pH, increased pCO₂, and increases in TCO₂. When birds were treated with LPS, the changes in blood components appears to be opposite compared to heat treatment. It may be that the chickens do not attempt to decrease body temperature by respiration in response to an inflammatory stimulus, because inflammation and fever in response to an immune challenge is an efficient method to kill the infectious agent. The changes in blood chemistry components related to acid base balance (pH, BE, HCO₃, and TCO₂) were more similar in response to LPS and to LPS+heat, than to heat. We hypothesize that, under heat stress, the birds may not be attempting to decrease body temperature when faced with an immune stimulant. This is
reflected in the blood chemistry components of the LPS+heat treatment resembling LPS treatment more than heat treatment. The most significant differences in blood components between breeds was due to treatment with LPS+heat. These blood components may be good biomarkers for combined heat tolerance and disease resistance because we observed significant differences between lines characterized for divergence of heat and disease response. Although, further investigation is required.

**Spleen transcriptomic responses**

**Heat stress**

Heat stress resulted in a larger number (N=107) of DEGs in Fayoumis compared to broilers (N=22) in the spleen, and most DEGs in each breed were upregulated. During periods of heat stress, intestinal permeability increases in chickens. Broilers under heat stress conditions increase intestinal permeability (Quinteiro-Filho et al. 2012; Quinteiro-Filho et al. 2010; Star et al. 2009), and layers have altered gut morphology of microvilli (Deng et al. 2012). The data presented here may indicate that broilers may be more susceptible to disruptions in tight junctions of the intestinal mucosa (“leaky gut”). In particular, we observe changes in pathways such as “Remodelling of Epithelial Adherens Junctions” and “Paxillin Signalling”. Interestingly, LPS is identified as an upstream regulator due to heat treatment in Fayoumis. This may be due to leaky gut during heat treatment, which in turn activates the proinflammatory immune response. We observed Acute Phase Response (APR) Signaling as the top canonical pathway in Fayoumi. The APR is known as the extreme specific change in plasma proteins due to systemic inflammation (Kushner 1982), and functions to restore homeostasis by non-specific immune mechanisms (Murata et al.2004). Typically, acute phase proteins are activated during infection, but we observed an increase during heat stress likely because of their pleiotropic effects. In humans, for example, acute phase proteins
increase angiogenesis (Epstein et al. 1999), which could help reduce body temperature. Clotting could reduce permeability of the intestine during heat stress and thus minimize the occurrence of leaky gut syndrome. A DEG of interest in the Fayoumi in response to heat stress was SOCS2, a suppressor of cytokine signalling. The SOCS2 gene was previously reported as a candidate gene for a very large QTL for breast muscle yield during heat stress in an intercross line between Fayoumi and broiler (Van Goor et al. 2015). Thus, this gene may represent a bridge among traits of heat tolerance, growth and immune response.

**LPS**

The broiler responded to LPS treatment with many more (N=283) DEG than the Fayoumi (N=85). The unresponsiveness in the spleen to LPS in the Fayoumi may be due to the effects of anti-inflammatory gene expression. An example of this in the current study is the upregulation of IL10R, which in turn promotes IL10 signalling resulting in immunosuppression by inhibition of transcription of proinflammatory cytokines (Murray 2005). A pathway that was strongly (P=2.27E-04) activated in the Fayoumi in response to LPS treatment was “Glucocorticoid Receptor Signalling”. Glucocorticoids are a class of corticosteroids that are highly conserved across all vertebrates (Yao et al. 2008), and are involved in reducing inflammation (Barnes 1998). In a previous study on chickens, LPS increased serum levels of corticosterone (Nakamura et al. 1998), which is a major glucocorticoid in chickens and the plasma concentration used as a measure of stress (Post et al. 2003). Heat stress is known to increase corticosterone concentrations in broilers during heat stress (Nathan et al. 1976), although we did not observe it as a top canonical pathway in either breed due to heat treatment. The IL-1R and IL-1R2 were both upregulated in the current study in response to LPS, and both receptors are involved in the anti-inflammatory
response (Colotta et al. 1993). Another top canonical pathway identified in Fayoumis in response to LPS was Granulocyte Adhesion and Diapedesis. During pathogen invasion, immune cells travel to the site of infection by expressing adhesion proteins and moving from circulation to the infected tissues. An extremely important type of granulocyte in chicken are referred to as heterophils. One study demonstrated that chickens with a reduced (3 to 9 fold) heterophil number have reduced ability to control Salmonella enteritidis disease pathogenesis (Kogut et al. 1994). The ability to activate heterophils during inflammation is desirable. The broiler responded strongly to LPS treatment by inflammatory response. A top pathway in both breeds is Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis, which is a disease characterized by uncontrolled inflammation, and we observed upregulation of genes such as IL-1beta, IL-6, and IL-22 which are pro-inflammatory cytokines. The IL-17 pathway was activated in response to LPS in the broiler, and this pathway is known to be activated by NFkB which was upregulated in the current study (Hwang et al. 2004). The broilers may be increasing inflammation during challenge with LPS which results in uncontrolled inflammation.

**LPS+heat**

During stimulation with LPS+heat, several shared pathways were identified between broiler and Fayoumi, such as “Hepatic Fibrosis/Hepatic Stellate Cell Activation” and “Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis”. The latter pathway was also identified in both breeds with LPS as the only stimulus, illustrating the important role of inflammatory response pathways to LPS under both thermoneutral and hot temperatures. The pathway related to hepatic function was not identified in either single stimulus. During periods of heat stress in chickens, the liver is sensitive to oxidative damage...
(Lin et al. 2006), and the liver is also a known site for bacterial pathogenesis (Nakamura et al. 1985). It may be that the double stressor of heat and LPS is resulting in liver damage due to oxidative damage, which can be detected in spleen transcriptome changes. After LPS+heat in the Fayoumi, the Aryl Hydrocarbon Receptor Signalling pathway was effected, which is important for immunological responses and inhibiting inflammation (Kimura et al. 2009). This is thought to occur through upregulation of IL-22 (Alam et al. 2010). Most of the Fayoumi’s DEG in response to LPS+heat were downregulated. The broiler showed the effected pathway “LPS/IL-1 Mediated Inhibition of RXR Function” which is thought to lead to impaired metabolism (Chartoumpekis et al. 2013), potentially a mechanism to reduce body heat generation.

Because no information is available about the spleen responding to environmental stressors, we have little context to interpret our results. We only investigated the spleen transcriptome response at one very acute treatment time (7h heat and 3.5h post LPS). The complete host response to heat and LPS cannot be fully understood by looking at one time point. It would, therefore be beneficial to study an even earlier time point such as 2 hour of heat and LPS, and chronic exposure to the stressors such as after 1 week. The broiler and Fayoumi have different developmental rates. The large differences in response to thermal treatment and immune stimulation could be attributed partly attributed to developmental differences. Another limitation of this study is that we chose to investigate only PolyA mRNA. Other types of RNA are of great importance to host response.

Conclusions
This is the first report of the spleen transcriptomic response in chickens to heat stress, LPS, and the double stimulation in unique genetic lines of chickens. Heat and LPS treatment increased body temperature to the same amount, and the double stimulation synergistically increased body temperature. However, blood chemistry components revealed different physiological responses to heat and LPS and, furthermore, heat+LPS resulted in a similar blood chemistry response to LPS alone. Many significant genes and pathways were identified and may serve as molecular markers to breed for more disease and/or heat resistant chickens. Of particular interest are the suggested roles of acute phase proteins, granulocytes and macrophages in modulating the response of birds to stressors.

Acknowledgements

The authors thank the Iowa State University Poultry Research Center staff for animal care; the Lamont, Persia, Rothschild and Ashwell lab personnel for assistance; Jack Dekkers, Dorian Garrick, Rohan Fernando and their research groups for GWAS advice. This research was supported by USDA-NIFA-AFRI Climate Change Award #2011-67003-30228; the USDA National Institute of Food and Agriculture, Hatch project #5358; and AVG was supported by a USDA National Needs Fellowship, Award #2011-38420-20050.

Competing Interests

The authors declare that they have no competing interests.

Authors’ Contributions

AVG participated in sample collection, isolated RNA, created cDNA libraries, carried out all statistical analyses and drafted the manuscript. CMA and MEP participated in the design of the study, sample collection and acquisition of funding. MFR and CJS participated in the design of the study and acquisition of funding. SJL participated in the design of the
study, participated in sample collection, participated in acquisition of funding and helped to draft the manuscript. All authors read and approved the final manuscript.

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**Figures**
A. Body temperature response to treatment

B. Blood chemistry component response to heat

C. Blood chemistry component response to LPS

D. Blood chemistry component response to LPS+heat
Figure 1
Phenotypic responses to treatments breeds are depicted by different colors with broiler in red and Fayoumi in black. A. Body temperature response to treatments. B. Blood chemistry component changes in response to heat, C. LPS, and D. heat+LPS. Measured in Fayoumi and broiler breeds at 22 days of age after 7 hours of heat treatment at 35°C, and 3.5 hours post LPS treatment with an iSTAT machine. The * indicates a significant (P ≤0.05) difference between genetic lines within a given component.

Figure 2
Number of DEG due to treatment in spleen

![Bar chart showing number of DEG for different treatments and genetic lines, with an iSTAT machine used for measurements.](chart)

Figure 2
A heat map showing the number of differentially expressed genes and the direction of log fold change (LFC) of expression values. Genes are presented with FDR ≤ 0.05 and LFC ≥ ±2.

**Figure 3**

Venn diagrams for overlapping differentially expressed genes. Green is treatment with LPS, red is heat, and blue is LPS+heat. Genes were considered differentially expressed with LFC ≥ 2 and FDR ≤ 0.05.
A  Broiler response to LPS

Unigene Numbers

1. defense response to protozoan
2. response to protozoan
3. lymphocyte chemotaxis
4. monocyte chemoattractant
5. positive regulation of chemokine production
6. neutrophil migration
7. granulocyte chemotaxis
8. regulation of chemokine production

B  Broiler response to LPS+heat

Unigene Numbers

1. lymphocyte chemotaxis
2. lymphocyte migration
3. neutrophil migration
4. granulocyte chemotaxis
5. granulocyte migration
6. positive regulation of leukocyte migration
7. positive regulation of leukocyte migration
8. leukocyte chemotaxis
C  Fayoumi response to LPS

Unigene Numbers

- Response to interleukin-1
- Cytokine-mediated signaling pathway
- Cellular response to cytokine stimulus
- Response to cytokine
- Positive regulation of immune system process
- Immune system process

D  Fayoumi response to heat

Unigene Numbers

- Chemokine-mediated signaling pathway
- Response to interleukin-1
- Cytokine-mediated signaling pathway
- Response to cytokine
- Defense response
Figure 4. Gene ontology enrichment analyses showing top significantly associated GO terms based on differentially expressed genes.

Figure 5. Validation of RNA-sequencing with Fluidigm technology.
CHAPTER 5
DISTINCT FUNCTIONAL RESPONSES TO STRESSORS OF BONE MARROW DERIVED DENDRITIC CELLS FROM DIVERSE INBRED CHICKEN LINES

Modified from a paper submitted to Developmental and Comparative Immunology on March 7, 2016. Under revision.

Angelica Van Goor*, Anna Slawinska*, Carl J. Schmidt and Susan J. Lamont

*These authors contributed equally to this study

Author’s contribution
AVG and AS both participated in experimental design, carried out all cell culture experiments, completed RNA isolation and mRNA expression using Fluidigm technology, carried out all statistical analyses, and drafted the manuscript.

Abstract

Differences in responses of chicken bone marrow derived dendritic cells (BMDC) to in vitro treatment with lipopolysaccharide (LPS), heat, and LPS+heat were identified. The Fayoumi is more disease resistant and heat tolerant than the Leghorn line. Nitric Oxide (NO) production, phagocytic ability, MHC II surface expression and mRNA expression were measured. NO was significantly induced in BMDC from both lines in response to LPS and LPS+heat stimulation; Fayoumi produced significantly more NO with LPS treatment. Fayoumi had significantly higher phagocytic ability and MHC II surface expression. Gene expression for the heat-related genes BAG3, HSP25, HSPA2, and HSPH1 was strongly induced with heat and few differences existed between lines. Expression for the immune-related genes CCL4, CCL5, CD40, GM-CSF, IFN-γ, IL10, IL-12β, IL-1β, IL-6, IL-8, and iNOS was highly induced in response to LPS and different between lines. This research contributes to the sparse knowledge of genetic differences in chicken innate immune responses.
Keywords: dendritic cells, Fayoumi, Leghorn, MHC-II, nitric oxide, gene expression

Introduction

Dendritic cells (DC) are the key types of antigen presenting cells (APC). Immature DC are present on all surfaces of peripheral tissues of the body, such as intestinal mucosa or skin epithelia. The DC’s major function is to monitor presence of antigens, which are then captured and processed. DC primed with antigen migrate to secondary lymphatic organs, where they present antigens to naïve T cells, which activates them towards Th2/Th2 polarization (Banchereau and Steinman, 1998). As such, DC are considered to bridge innate and adaptive immune responses. In chickens, several classes of DC have been identified [reviewed by (Nagy et al. 2016; Wu and Kaiser 2011)]; intestinal (mucosal) DC (Del Cacho et al. 2012), interdigitating DC (Del Cacho et al. 2009), follicular DC (Del Cacho et al. 2008) and epidermal DC (Igyarto et al. 2006). For research purposes, DC can be also generated in vitro, by differentiating bone marrow cells into DC lineage with GM-CSF and IL-4 cytokines (Wu et al. 2010b). In this manner, a population of bone marrow derived dendritic cells (BMDC) can be used to demonstrate their unique properties in response to environmental and inflammatory stressors.

BMDC can be primed in vitro with lipopolysaccharide (LPS), which triggers changes in their phenotype from immature to mature DC by modulating expression of surface molecules. It has been reported that the maturing BMDC under fever-like conditions release different levels of the cytokines than those that respond to an inflammatory stressor only (Tournier et al. 2003b). Thermal stress, which is usually associated with inflammation (local hyperthermia) or fever (systemic hyperthermia), delivers additional inflammatory cues to the immune system [reviewed by (Evans et al. 2015)]. Temperature elevated above physiological
level is sensed as a danger signal, which enhances DC function including antigen uptake, activation-associated migration, antigen presentation, T cell activation and cytokine release [reviewed by (Ostberg and Repasky 2006a)].

Chickens farmed in commercial settings face many inflammatory and environmental stressors including infectious diseases, intensive vaccination programs and management procedures. The latter are often influenced by the seasonal changes, such as waves of elevated ambient temperatures in the summer. Heat stress in farmed chickens leads to an immediate decrease in performance parameters and reproduction (Mashaly et al. 2004; Sandercock et al. 2001). Heat stress negatively influences intestinal microbiota composition and gut barrier function. As a consequence, it causes increased permeability of the junctional complexes in the intestines, known as “leaky gut” (Lambert 2009). Intra-abdominal influx of intestinal microbiota and gut-derived endotoxins leads to systemic endotoxemia, i.e. presence of endotoxins in the blood, which activates potent inflammatory responses and is a major cause of death during heat stroke (Leon and Helwig 2010). The intestinal DC population, present in the lamina propria of the gut-associated lymphoid tissue (GALT), samples, processes and presents antigens that were transferred across intestinal epithelium. In this way, intestinal DC create the first line of defense in heat stress-related endotoxemia (Fujita et al. 2006).

Chicken breeds with distinct genetic background, such as Leghorn and Fayoumi, may represent different levels of adaptation to pathogenic and environmental stressors. Leghorn is a major commercial egg laying breed, whereas Fayoumi represents a wild-type strain of chicken, originated in Egypt. Multiple studies have indicated that Fayoumi is a hardier genetic line than Leghorn, with a higher level of resistance to infections with Marek’s disease.
(Lakshmanan et al., 1996), coccidiosis (Pinard-Van Der Laan et al. 1998), Newcastle disease (S. Lamont, personal communication), avian influenza (Wang et al. 2014), and Salmonella (Redmond et al. 2011) compared to Leghorns. Both genetic lines used in this study, Leghorn (Ghs-6 line) and Fayoumi (M-5.1 line), are highly inbred (99.9%) (Zhou and Lamont 1999), and they have been used as an excellent discovery platform in numerous studies on avian immunogenetics and disease resistance (Coble et al. 2011; Kim et al. 2008; Redmond et al. 2009). We hypothesize, therefore, that the Fayoumi genetic line will be also superior to the Leghorn line in mounting a stronger innate immunity in response to complex environmental stimuli.

In this study, we address the questions of whether, and to what extent, genetic background influences inflammatory responses in chicken BMDC induced by heat and LPS. To our knowledge, this is a first report to provide functional comparisons between cellular and molecular responses to LPS, heat, and LPS+heat in chicken BMDC derived from distinct genetic lines. As such, this research contributes to the sparse knowledge generated by using in vitro assays to explore the complex interaction between genetic background, immune stimulation, and environmental stress on innate immune cells in vitro. The genes identified in the results of this study may serve as biomarkers to breed more disease and/or heat tolerant birds.

Materials and Methods

Animals

All animal maintenance and handling was approved by the Institutional Animal Care and Use Committee at Iowa State University: Log #4-03-5425-G. The highly inbred (99.9%) chicken lines Fayoumi (M-5.1) and Leghorn (Ghs-6) (Deeb and Lamont 2002) were used in
the current study. Adults were maintained in single bird cages and were artificially inseminated for production of fertile eggs that were incubated to produce embryos.

**Generation of bone marrow derived dendritic cells (BMDC)**

BM was collected from day 18 embryos of chicken inbred lines Fayoumi and Leghorn. BM was isolated and cultured as previously described (Wu et al. 2010b). Under sterile conditions, embryos were decapitated then femurs and tibias were removed using sterile instruments and placed into cold PBS. The ends of the bones were cut and BM was flushed through the bones using a syringe and needle with PBS, and the BM was pooled within chicken line. Debris was removed from BM using a 70 μm cell sieve. Cells were centrifuged for 10 minutes at 450 x G. The supernatant was discarded; then cell number and viability was determined using a hemocytometer with the trypan blue exclusion assay. Cells were frozen in 30% FBS and 10% DMSO in RPMI 1640 at a concentration of 1x10^7 viable cells/ml and stored at -70°C.

Cells were seeded at 1x10^6 cells/ml in 6-well tissue culture plates in pre-warmed RPMI-1640 (Sigma Aldrich, St. Louis, MO, USA), supplemented with 10% chicken serum (Sigma Aldrich, St. Louis, MO, USA), 1% L-glutamine (Gibco, Carlsbad, CA, USA), 1 U/ml penicillin and 1 μg/ml streptomycin (Gibco, Carlsbad, CA, USA) (complete medium), and supplemented with 10 ng/ml recombinant GM-CSF (KingFisher Biotech, St. Paul, MN, USA) and 10 ng/ml IL-4 (KingFisher Biotech, St. Paul, MN, USA), for 6 days at 41.5°C and 5% CO2. On day 3 of culture, three quarters of the medium was removed from each well, to remove non-adherent cells, and replaced with fresh complete medium containing recombinant cytokines. On day 5 of culture, cells were harvested using 0.25% trypsin-EDTA for 10 min (Gibco, Carlsbad, CA, USA) followed by trypsin deactivation with complete
medium, centrifuged for 10 minutes at 450 x G, and reseeded into 24-well tissue culture plates at a final concentration of 1x10^6 cells/ml with complete medium containing no recombinant cytokines. Cells re-adhered to tissue culture plates for 24h before experimental treatments were started, which were conducted on day 6 of culture.

**Treatment with LPS, heat, and LPS+heat**

A full factorial design, containing the factors of genetic line (Fayoumi and Leghorn), and treatment (control, LPS, heat, and LPS+heat) was used for all assays. The time post treatment that samples were assessed was dependent upon the assay. NO production was assayed at 24h, phagocytosis ability and MHC II surface expression was assayed at 4h, and cells for mRNA expression were harvested at 2h, 4h, and 8h post stimulus. On day 6 of culture, the medium was removed from wells and replaced with complete medium containing 200 ng/ml LPS (Sigma Aldrich, St. Louis, MO, USA), or medium alone as a control. Cells were immediately placed in an incubator at 45°C with 5% CO₂ (heat), or at 41.5°C with 5% CO₂ as a thermoneutral control (TN), for 2h. Therefore, the treatment groups include medium alone in TN conditions (control), LPS treatment in TN conditions (LPS), medium alone in heat conditions (heat), and LPS treatment in combination with heat conditions (LPS+heat).

Cells were harvested immediately (2h post treatment), or placed in an incubator at 41.5°C with 5% CO₂ for a temperature-recovery time. Supernatant (NO production assay) or cells (flow cytometry assays and RNA isolation) were collected for downstream analyses at different time points post-treatment (details presented below). Cells were harvested using 0.25% trypsin-EDTA for 10 min (Gibco, Carlsbad, CA, USA) followed by trypsin deactivation with complete medium. The cells were centrifuged then for 5 min at 200 x G and resuspended in the FACS buffer (PBS supplemented with 0.5% BSA and 0.05% NaN₃).
or cell lysis buffer from RNAqueous® Total RNA Isolation Kit (Ambion, Carlsbad, CA, USA) for flow cytometry or RNA isolation, respectively. Three technical replicates (wells) were assayed per genetic line and treatment and the experiment was replicated on three independent experimental days.

Nitric oxide production assay

BMDC were incubated for 24h post treatment (LPS, heat, and LPS+heat) at 41.5°C and 5% CO₂, then NO production in the cell supernatant was determined using the Griess reagent kit (Molecular Probes, Carlsbad, CA, USA). Supernatant was mixed with Griess reagents and incubated for 30 min at room temperature in dark conditions, and then measured at 540 nm on a spectrophotometer. The absorbance values were compared to the sodium nitrite standard curve to determine nitrite concentrations (μM).

Phagocytosis assay and MHC II surface expression

BMDC were incubated for 4h post treatment (LPS, heat, and LPS+heat) at 41.5°C and 5% CO₂, then assessed for phagocytic ability using 1 μm carboxylate-modified crimson beads (Molecular Probes, Carlsbad, CA, USA). Beads were suspended in pre-warmed complete medium, with or without 200 ng/ml LPS, and then BMDC were given 1 ml/well resulting in a final concentration of 20 beads/cell, then cells were harvested for flow cytometry at 4h post treatment. An ice control was used to inhibit phagocytosis. Briefly, cells were cooled on ice for 30 min, then given 20 beads/cell in pre-cooled complete medium, and then harvested at 4h post treatment. For both the treatment group and ice control, cells were harvested then washed twice in pre-cooled PBS and centrifuged at 450 x G for 5 min at 4°C. Cells were placed into 96 well round bottom plates and stained with MHC II-PE antibody (Southern Biotech, Birmingham, AL, USA) for 30 min, washed 3 times by 400 x G for 5
minutes centrifugation at 4°C, and resuspended in FACS buffer. Propidium Iodide (PI) was added to each sample and used as a viability marker in which only live cells were gated and utilized for downstream analyses. Cells were assessed for phagocytic ability using the FACSCanto machine (BD Biosciences, Palo Alto, CA, USA) at the Flow Cytometry Facility at Iowa State University.

**mRNA expression**

mRNA expression in BMDC stimulated with LPS, heat, and LPS+heat was analyzed after 2h, 4h and 8h post treatment. Total RNA was isolated using RNAqueous® Total RNA Isolation Kit (Ambion, Carlsbad, CA, USA). Gene expression analysis was performed using a microfluidic Reverse Transcription quantitative PCR (RT-qPCR) (Fluidigm Corporation, San Francisco, CA, USA). All procedures were conducted according to manufacturer’s recommendations, unless otherwise noted. Briefly, 50 ng of the total RNA was reverse transcribed using the Fluidigm Reverse Transcription Master Mix (Fluidigm Corporation, San Francisco, CA, USA). cDNA was pre-amplified with PreAmp Master Mix (Fluidigm Corporation, San Francisco, CA, USA), using 14 cycles of pre-amplification. Exonuclease I (New England Biolabs, UK) treatment was applied to remove unincorporated primers. Pre-amplified and purified cDNA samples were diluted 10x in TE buffer and stored at -20°C until further analyses. RT-qPCR analysis was done for 22 target genes and 2 reference genes, listed in Table 1. A 192x24 Integrated Fluid Circuits (IFCs) (Fluidigm Corporation, San Francisco, CA, USA) was used to perform RT-qPCR reactions. Sample assay included 1.35 µl of pre-amplified and Exo I treated cDNA, 1.5 µl of the SsoFast™ EvaGreen® Supermix with Low ROX™ (2x) (Bio-Rad) and 0.15 µl of the 192.24 Delta Gene Sample Reagent (Fluidigm Corporation, San Francisco, CA, USA). Primer assays were prepared as 20 µl
stock by mixing 1µl of each primer (100 µM) with 10 µl of the 2x Assay Loading Reagent and adjusted to 20 µl with DNA suspension buffer (low EDTA TE buffer). The samples, assays and the loading reagents were then loaded onto IFCs microfluidic channels using the RX loading station (Fluidigm Corporation, San Francisco, CA, USA). RT-qPCR was performed on the Biomark™ HD (Fluidigm Corporation, San Francisco, CA, USA) using fast program that consisted of incubation step at 95°C for 60 s followed by 30 cycles: 96°C for 5s and 60°C for 20s. Fluorescence emission was recorded after each cycling step. Upon RT-qPCR completion, melting curves were generated by increasing temperature from 60 to 95°C, followed by continued fluorescence acquisition.

**Statistical analyses**

Mean values of NO production, phagocytic ability, and MHC II surface expression were compared between the Fayoumi and Leghorn BMDC for all treatment groups using Students’ t test \( (P < 0.05) \) with correction for multiple comparisons, implemented in JMP Pro 10.0.2 software (SAS Institute, Cary, NC, USA). RT-qPCR data were analyzed as follows: raw qPCR data were analyzed and checked for quality using Real-Time PCR Analysis Software (Fluidigm Corporation, San Francisco, CA, USA). Main effects of the stimulation of the BMDC were estimated using least square means method implemented in JMP Pro 10.0.2 software (SAS Institute, Cary, NC, USA). Chicken line (Fayoumi and Leghorn), treatment (control, LPS, heat, and LPS+ heat) and time post treatment (2h, 4h, and 8h) as well as the interaction between line and treatment were fitted in the ANOVA model. Analyses were performed using an ANOVA fitting line, gene, and treatment using dCt values (Ct target – Ct reference). To determine the relative gene expression, the ddCt method was used (Livak and Schmittgen, 2001). Delta Ct values were obtained by normalizing the Ct
values of the target genes with the geometric mean of the two reference genes (H6PD and RPL4). Fold induction of the gene expression was estimated as $2^{-\Delta \Delta C_t}$. Untreated (control) samples were used as calibrators.

**Results**

**BMDC culture**

The average viability of BM cells after isolation was 90% and the number of viable cells obtained from each embryo averaged $1.5 \times 10^7$. Cultured BMDC displayed typical morphology similar to previous reports (Rajput et al. 2013; Wu et al. 2010b). Pictures of the growth and treatment morphology are found in Figure 1. Generally, the morphological characteristics of the two chicken lines were very similar to each other during both growth and treatment.

**Nitric oxide assay**

The results of NO assay are found in Figure 2. In thermoneutral temperature with medium alone (control), both chicken lines had detectable levels of NO production but the levels did not differ statistically. LPS treatment significantly ($P \leq 0.05$) increased NO production in both lines compared to controls, and Fayoumi produced significantly ($P \leq 0.05$) more NO compared to Leghorn. Heat treatment alone had no significant effect on NO production in either chicken line compared to control. Treatment with LPS+heat statistically ($P \leq 0.05$) increased NO production in both lines compared to control, with no difference between lines was detected.

**Flow cytometry, phagocytic ability and MHC II staining**

The general gating strategy for cells is found in Figure 3a. Cells were stained with PI as a viability marker and the average percent live cells was 78%. Gating for live cells and
identified as BMDC were utilized for downstream analyses. The gating strategy used to
determine phagocytic ability (percentage of bead+ cells) and MHC II surface expression
(percentage of MHC II+ cells) is found in Figure 3b. The phagocytic ability of cells is
quantified in Figure 3c. The percentage of bead+ cells in the ice control was not different
between lines (data not shown). Generally, treatment had little effect on phagocytic ability of
BMDC. For the treatment groups of control, LPS, and heat, a larger percentage of Fayoumi
cells were bead+ compared to Leghorns. This relationship is reversed in the LPS+heat
treatment group.

The surface expression of MHC II is quantified in Figure 3d. Treatment had no effect
on the surface expression of MHC II in the Leghorn line, but in the Fayoumi line, LPS
increased the surface expression of MHC II. In all treatment groups, Fayoumi had
statistically (P ≤ 0.05) higher surface expression of MHC II.

**mRNA expression in BMDC**

Table 2 presents significance of the effects in the gene expression study. The main
effects that were tested included breed (Leghorn vs. Fayoumi), treatment (control, LPS, heat,
and LPS+heat) and time point post-stimulation (2h vs. 4h vs. 8h). Treatment had a highly
significant effect on all the genes analyzed (P <0.0001). Breed had a significant effect on all
genes except *HSPH1, IFN-γ, IL-10, IL-12β* and *IL-6* (P < 0.05). Time had a significant effect
on all genes, except *IL-12β* (P < 0.05). The interaction of breed and treatment had a
significant impact on mRNA expression of *CASP9, CCL4, GM-CSF, HSP25, HSPH1, IL-15, IL-8, MHCII-β, TLR4* and *UBB* (P < 0.05) and a highly significant effect on *iNOS* (P <
0.0001). The interaction of breed and time had no significant effects on any gene tested and
was, therefore, removed from the model.
Gene regulation in response to LPS

Relative expression of the analyzed target genes is presented in Figures 4-6. Immune related genes are found in Figure 4. In BMDC from both chicken lines the genes with the highest level of expression in response to LPS treatment alone were *IL-10*, *IL-6*, *CCL4*, *IL-1β* and *iNOS* (*P* < 0.05). Their expression peaked at the 2h time point (reaching a fold change between approximately 300 and 2000), with exception of *iNOS* which had the highest mRNA fold change at 4h time point. The expression of *IL-10*, *IL-6*, *IL-1β* and *iNOS* was significantly higher in BMDC of Fayoumi than Leghorn (*P* < 0.05). The second set of genes that were also significantly up-regulated upon LPS treatment, but with lower fold change values (approximately between 10-70), included *CCL5*, *CD40*, *GM-CSF*, *IFN-γ*, *IL-12β* and *IL-8*. In general, induction of those genes (except *CCL5*) was also higher in BMDC from Fayoumi than Leghorn. Genes slightly up-regulated in response to LPS included *TGFβ2* (across all time points) as well as stress response genes (*BAG3*, *HSP25*, *HSPH1* and *HSPA2*) at 4h time point. LPS induced a clear down-regulation of some genes involved in immune (*IL-15*, *TLR4*) and stress response (*CASP9*, *HSPA2* and *UBB*). *MHC-IIβ* and *IL-4* mRNA expression in response to LPS was regulated inversely in Leghorns and Fayoumis (*P* < 0.05); both genes were up-regulated in Leghorns but down-regulated in Fayoumis.

Gene regulation in response to heat

Heat regulated genes are presented in Figure 5. Heat treatment induced mRNA expression of the stress response genes, including anti-apoptotic *BAG3* and heat stress response genes (*HSP25*, *HSPA2* and *HSPH1*). The highest expression of those genes was detected directly after heat treatment (2h); however, *HSP25* continued to be highly up-regulated also at later time points. The panel of immune response genes was not strongly
induced by heat alone. Their expression slightly fluctuated, but typically did not exceed a fold change between 0.5 and 2.

**Gene regulation in response to LPS+heat**

The synergistic effects of LPS combined with heat were demonstrated by a down-regulation of the immune-related genes (CD40, GM-CSF, IFN-γ, IL-12β, IL-15, IL-4, MHCIIβ and TLR4) and the stress response genes UBB and CASP9 (*P* < 0.05). This inhibition of mRNA synthesis was more evident at later time points, peaking at 8h (fold induction between 0.23 and 0.01). Down-regulation of the immune-related genes was in most cases stronger in BMDC derived from Fayoumis than Leghorns (*P*<0.05). Expression of the heat response genes (HSPH25, HSPA2 and HSPH1) maintained a similar profile to heat treatment alone. However, genes that were highly up-regulated in response to LPS alone (CCL4, CCL5 and IL-10) were still induced by the double stressor, but on much lower level.

**Differences in mRNA expression in BMDC derived from Fayoumi and Leghorn**

The relative gene expression of the inflammatory mediators upon LPS treatment was, in most cases, similar in Fayoumis and Leghorns. Overall, up-regulation of the Heat Shock Protein (HSP) genes upon heat treatment was similar between BMDC derived from both chicken lines. The major differences in response to LPS stimulation were determined for iNOS expression, that was higher in Fayoumi (4h – *P*=0.57, 8h - *P* <0.05), which corresponds with the increased NO production at 24h. Further, LPS induced four times higher mRNA expression of anti-inflammatory IL-10 in Fayoumi than Leghorn (*P* <0.05). In Leghorn, LPS induced higher mRNA abundance of CCL5, GM-CSF and IL-4 (*P* <0.05).

The main differences attributed to the genetic component occurred in response to combined treatment with LPS+heat. Stress response genes, such as HSPH1 and BAG3 were
up-regulated in Leghorns ($P < 0.05$). The inflammatory mediators $IL-1\beta$, $iNOS$, $IL-8$, $CCL4$ and $CCL5$ and markers of maturation $CD40$ and $TGF\beta2$ were higher in Leghorn stimulated with LPS+heat, especially at 8h time point ($P < 0.05$). At the same time, there was a very consistent and strong mRNA down-regulation in Fayoumi stimulated with LPS+heat in respect to maturation signatures ($MHCII-\beta$, $CD40$, $IL-15$), immune-related genes ($TLR4$ and $IL-4$) and stress response genes ($CASP9$ and $UBB$) ($P < 0.05$) in comparison to Leghorn.

**Discussion**

In the current study, we characterized the response of BMDC derived from unique and distinct inbred chicken lines to an inflammatory stimulus (LPS), an environmental stressor (heat), and the combination of both (LPS+heat). We chose a variety of *in vitro* assays (NO production, phagocytic ability, MHC II surface expression, and mRNA expression) to characterize the impact of genetics on the innate immune response of chicken BMDC. This research contributes to the sparse knowledge of *in vitro* response of primary chicken immune cells. The results of this research suggest it may be possible to identify resistant phenotypes *in vitro*. Because BMDC in chicken have been previously characterized as antigen presenting cells (Wu et al., 2010b), we chose to focus on the responses of these cells to stimulations, and to quantify the differences between unique breeds of chickens. We anticipated BMDC from the Fayoumi line to have a more primed and responsive innate immune system compared to Leghorn, including higher phagocytic ability, more NO production, higher surface expression of MHC II, and differences in RNA-expression. The treatments in the current study were chosen to represent important abiotic (high temperature) and biotic (LPS, a bacterial component immune stimulant) stressors frequently encountered in poultry production, and to better understand the interaction of the combined stressors.
Nitric oxide assay

There are two categories of NO: calcium-dependent constitutive NO that is produced by endothelial and neural cells, and calcium-independent inducible NO (iNOS) produced by mononuclear phagocytes (Gross et al. 1995; MacMicking et al. 1997a). The production of iNOS is an important host defense against invading bacteria (Hibbs et al. 1988). Production of NO inhibits microbial proliferation and when NO production is interrupted, microbial burden increases (MacMicking et al. 1997b; Stenger et al. 1996). In humans, increased NO production results in better clinical outcomes during malaria infection (Anstey et al. 1996), supporting the hypothesis that higher production of NO is indicative of a more effective innate immune system. In chickens, there is a strong genetic component in the amount of NO production and this is associated with enhanced immunocompetence. For instance, NO production is higher in macrophages derived from chickens selected for high antibody response (Guimarães et al. 2011). Also, embryo fibroblasts from chicken lines genetically resistant to Marek’s disease, generate higher NO levels upon LPS stimulation compared to a susceptible line (Xing and Schat 2000b).

In the current study, BMDC derived from Fayoumi birds produced significantly more NO compared to Leghorns in response to LPS stimulation. This difference in NO production was a modest amount and the relevance in vivo remains to be elucidated. However, previous studies have observed similar results as those reported here. Lines divergently selected for response to phytohemagglutinin-P show differences in resistance to E. coli attributed to Th1 activity levels (Sundaresan et al. 2005), and isolated PBMCs stimulated with Salmonella in the high selection line produced more NO, but this was not significant at 24 hours post stimulation (Singh et al. 2012). Additionally, studies of chickens differing in susceptibility to
Marek’s disease herpes virus show that the resistant birds produce more NO compared to susceptible birds, 8.8±2.0 and 2.3 ±1.6 µM, respectively (Djeraba et al. 2002), but this large difference occurs after stimulation \textit{in vivo} and a subsequent stimulation with IFN-gamma \textit{in vitro}. When evaluating the genetic differences without \textit{in vivo} stimulation and only \textit{in vitro} stimulation with IFN-gamma, the lines show modest differences (4.9±1.9 and 6.2 ±1.7 µM). Another study using lines different for Marek’s response found subtle differences in NO production in the spleen when stimulated with LPS; the resistant line had 3.3 µM and the susceptible line had 1.3µM NO production (Xing and Schat 2000a). Therefore, the degree of NO difference is dependent up the type of stimulation and time post stimulation. We propose that the measurement of NO production \textit{in vitro} using BMDC may be used to predict disease resistance in chickens. Although, this approach would have to be validated to assure that \textit{in vitro} responses indeed correspond to \textit{in vivo} responses.

Inducible differences in NO were identified between chicken lines in response to LPS stimulation, but not in response to heat or the combination of LPS+heat. In humans, heat stroke patients have higher levels of plasma NO levels (Alzeer et al. 1999). The increase in NO \textit{in vivo} could be attributed to “leaky gut” syndrome which occurs during periods of heat stress, and is caused by disruption of tight junctions in gut, allowing intestinal bacteria or LPS to enter into the blood stream (Dokladny et al. 2006). The current study produced no evidence that heat alone increases production of NO \textit{in vitro}. However, the double stimulus (LPS+heat) resulted in decreased NO production in both chicken lines compared to LPS alone. In conclusion, the NO production assay results suggest that one mechanism of the greater disease resistance of Fayoumis compared to Leghorns may partly be attributed to the function of cells of the innate immune system.
Phagocytic ability

Assessing phagocytic ability is one way to determine the efficiency of the host at removing pathogens during an infection. DC endocytose through both non-specific mechanisms, such as micropinocytosis, and specific mechanisms such as receptor-mediated endocytosis and phagocytosis (Trombetta and Mellman 2005). Upon maturation, APC alter chemokine and cytokine expression patterns and also undergo various morphological and functional changes, including changes in phagocytic ability (Guermonprez et al. 2002). Phagocytic ability is higher in macrophages from chickens selected for high antibody response (Guimarães et al. 2011), indicating genetic control of this function and also that chickens with a “primed” immune system phagocytize more. However, the primary role of specialized APC such as DC is not only to engulf, but also to process the antigens for presentation and subsequent activation of the adaptive immune system. Therefore, a critical function of APC is that upon maturation, phagocytic ability decreases so the cells can move to sites of T cells for antigen presentation (Sallusto et al. 1995).

Phagocytic ability was determined using fluorescently labeled beads and quantified using flow cytometry. The BMDC from Fayoumis had significantly higher percent bead+ cells compared to Leghorns under control conditions, LPS, and heat. Few differences in phagocytic ability were identified due to treatment. In conclusion, BMDC from Fayoumis more efficiently phagocytosed, compared to Leghorns. This result may indicate that Fayoumis have more “primed” innate immune cells, which results in better clearance of pathogens and, therefore, greater disease resistance compared to Leghorns, suggesting that phagocytic ability in vitro is a good predictor of disease resistance in chickens (Sun et al. 2008).
MHC II surface expression

Surface expression of MHC II is limited to a small number of cells including thymic epithelial cells, B cells, macrophages, and DC (Pieters 2000). Expression of MHC II is an intrinsic characteristic of APC, which allows them to present foreign antigens to T cells and subsequently activate the adaptive immune response (Steinman 1991). Maturation of APC by an immune stimulus, such as LPS, or pathogen challenge results in a redistribution of MHC II proteins from the intracellular compartments to the cell surface, resulting in an increase in MHC II surface expression (Cella et al. 1997; Pierre et al. 1997; Turley et al. 2000). However, few studies to date have identified the effect of high temperature on APC activity and maturation (Ostberg and Repasky 2006b). The cellular origin and thermal stress conditions are determining factors in whether an increase in MHC II surface expression (Ostberg et al. 2003), or no change (Tournier et al. 2003a), occurs.

BMDC from the Fayoumi responded to treatment with LPS and LPS+heat by increasing surface expression of MHC II, which was the expectation based on previous reports (discussed above). No difference in MHC II surface expression in BMDC from Leghorn was identified among any treatment groups, which was an unexpected outcome. BMDC from Leghorn had significantly lower surface expression compared to Fayoumi.

Because MHC II surface expression was only assayed at one time point (4h post stimulation), the Leghorn may simply be slower at moving MHC II to the cellular surface upon stimulation compared to Fayoumis. Another explanation for the observed difference in MHC II surface expression is that the genetic lines may have different avidity for the monoclonal antibody utilized to quantify the expression. We included MHC IIbeta in the mRNA expression portion of the experiment, and both lines significantly differed in their expression
at all time points and treatments. However, the mRNA expression and surface expression studies were contradictory. The Fayoumi displayed significant downregulation in all treatments, except heat stress at 4hr, whereas the direction of change due to treatment in the Leghorn was time and treatment dependent. Both lines showed extreme downregulation of MHC II mRNA expression when treated with LPS+heat.

In conclusion, BMDC from the Fayoumi had inherently higher levels of MHC II surface expression, in control and all treatment groups, compared to the Leghorn. The MHC II surface expression of BMDC from Fayoumi were responsive to treatments that included an inflammatory stimulus, but not to heat alone, while MHC II surface expression from BMDC from Leghorn did not respond to any treatment at the times assayed. The ability to rapidly change MHC II surface expression upon stimulation may be a marker for disease resistance.

**mRNA expression in BMDC treated with LPS, heat, and LPS+heat**

**Up-regulation of the inflammatory mediators with LPS**

Endotoxemia activates immature APC and rapidly modulates their gene expression towards secretion of the inflammatory signals. Inflammatory APC migrate further to spleen, where they regulate the adaptive immunity through recruitment of the microbe-specific T cells (Sallusto and Lanzavecchia 2000). In our study, LPS treatment induced high levels of pro-inflammatory cytokines \((IL-1β, IL-6)\) and chemokines \((CCL4, CCL5 and IL-8)\) as well as inducible nitric oxide synthase \((iNOS)\). Expression of these inflammatory mediators belongs to the core function of mature APC (Foti et al. 2006). Chemotaxis initiated by inflammatory chemokines aims to attract other cells with chemokine receptors, such as monocytes/macrophages, T and B cells, NK and immature DC to the inflammation site. DC are also involved in the migratory pattern that is an intrinsic feature of APC (Dieu-Nosjean et
al. 1999). Furthermore, LPS-activated BMDC expressed a high level of the anti-inflammatory cytokine, \textit{IL-10}, which alleviates the potentially harmful effects of prolonged inflammation by immunosuppressive activity via negative signaling of MyD88-dependent signaling (Chang et al. 2009). LPS also induced expression of \textit{iNOS}, which plays an important role in early antimicrobial defense (Serbina et al. 2003). Induction of \textit{iNOS} was in agreement with increased NO production in LPS-activated chicken BMDC in the current study, as discussed earlier. Even though inflammatory responses were up-regulated by LPS, the expression of the TLR4 receptor, which binds LPS, was down-regulated in our study. Such a phenomenon is in agreement with the literature and helps control the host inflammatory response by preventing excessive production of pro-inflammatory cytokines that lead to endotoxic shock (Liew et al. 2005). However, we did not investigate the surface expression of the TLR4 receptor and cannot conclude that this recycling mechanism was occurring in the current study. It would be useful to analyze the TLR4 surface expression in response to LPS, and to identify if the genetic lines differ in the ability to change the surface expression of the receptor upon stimulation. This study did assay downstream mRNA expression of the activation elicited by TLR4 activation such as IL-1beta, IL-6, iNOS, and INF-gamma, and the breeds exhibited similar expression patterns in these genes in response to stimulation.

\textbf{Th-1/Th-2 balance in BMDC treated with LPS}

An important function of APC, associated with their maturation stage, is polarization of the Th cell-mediated immune responses into Th-1 and Th-2 by secreting specific, immunoregulatory cytokines. Cross-regulatory cytokines \textit{IL-12} and \textit{IL-10} induce polarization of the T cells into Th-1 and Th-2, respectively (Corinti et al. 2001). \textit{IL-12} is one of the most
critical cytokines that activates naïve T cells recruitment into Th-1 cells, and therefore triggers cell-mediated immunity (Sartori et al. 1997). In contrast, autocrine *IL-10* expression was shown to inhibit *IL-12* (De Smedt et al. 1997) that is required for *IFN*γ production in BMDC (Fukao et al. 2001) and, as such, favors Th-2 immunity. In our study, *IL-10* mRNA abundance in LPS-treated BMDC was extremely high. However, there was also a significant up-regulation in expression of *IL-12β* and *IFN-γ*, which suggests the ability of the activated BMDC to induce Th-1 polarization even with the high levels of *IL-10*. However, the surface expression of *IL-12β* was not assayed in the current study. A commercially available antibody (KingFisher Biotech, Saint Paul, MN, US) is available for IL-12, but this is a polyclonal antibody that also recognizes IL-23 in chicken, thus limiting its usefulness.

Autocrine *IL-10* prevents spontaneous *in vitro* maturation of the APC and therefore plays an important regulatory role (Corinti et al. 2001). Mature APC can lose sensitivity to autocrine *IL-10* through reduction in the activity of the *IL-10* receptor on the cell surface (Corinti et al. 2001). Further, in this study, LPS treatment only minimally regulated *IL-4* mRNA expression in BMDC – a cytokine that drives Th-2 responses. BMDC do not produce high levels of *IL-4* in response to LPS in both mammalian (Yao et al. 2005) and avian (Wu et al. 2010a; Wu et al. 2010b) systems. Overall, the current results suggest that there was a bias towards Th-1 cell-mediated immune responses in chicken BMDC derived from both genetic lines in response to LPS.

Expression of markers of maturation in BMDC

Recognition of PAMPs by TLRs leads to increased expression of the markers of maturation in APC, such as CD40 or MHC II (Liang et al. 2013; Wu et al. 2010b). In this study, significant up-regulation of *CD40* mRNA upon LPS treatment indicated maturation of
BMDC. The surface receptor CD40 takes part in a cross-talk between APC and T and B cells
(Ma and Clark 2009). However, mRNA abundance of MHC-IIβ was practically unchanged in
LPS-stimulated BMDC, which is seemingly in disagreement with increased surface
expression of MHC II reported in our study. Inhibition of MHC-IIβ mRNA by LPS was
previously reported through the MyD88 pathway (Simmons et al. 2012). Regarding increased
surface expression of MHC II molecule, it had been demonstrated that immature APC
effectively and continuously express pathogen-associated peptide-MHC II complexes and
their rapid turnover is regulated by ubiquitination. APC activation with LPS decreases MHC
II ubiquitination, endocytosis and degradation and therefore increases retention of existing
MHC II molecules (Walseng et al. 2010).

**Heat treatment triggers molecular chaperones in BMDC**

Heat treatment greatly increased expression of HSP25 and HSPH1 and moderately
increased HSPA2 expression in BMDC in both chicken lines. These genes encode ATP-
dependent (HSPA2 and HSPH1) and ATP-independent (HSP25) chaperones that are
responsible for maintaining proper folding of the cytosolic proteins, unfolded during heat
stress. Temperature-inducible chaperones are responsible for achieving thermo-tolerance.
Induction of HSP by heat stress has been reported by other authors studying chicken using *in
vitro* (Slawinska et al., submitted), (Sun et al. 2015) and *in vivo* (Wang et al. 2013) models,
which indicates their conserved physiological role in heat response. In addition, HSP can
modulate the immune system by acting like a cytokine (Ferat-Osorio et al. 2014). APC
recognize chaperones that bind to their surface receptors and trigger release of inflammatory
cytokines and chemokines. Recently, it has been proposed to use chaperones as vaccine
adjuvants to deliver the peptide directly to APC MHC II molecules for T cell priming
Here, we report expression of three major HSP genes in chicken BMDC that could be potential targets for vaccine development.

**Heat treatment down-regulates LPS-induced immune genes**

Heat treatment combined with endotoxemia did not modify the high level of mRNA abundance of the heat response genes, i.e. HSP (HSP25, HSPH1 and HSPA2) and anti-apoptotic BAG3. In contrast, immune-response genes (IL-1β, IL-6, IL-8, IL-10, iNOS, CCL4 and CCL5) were expressed at lower level, compared to LPS treatment alone. The most distinct changes in the gene expression under combined LPS and heat treatment resulted in the significant down-regulation of immune- and maturation-related genes TLR4, IL-4 and GM-CSF, MHC-IIβ, CD40, IFN-γ, IL-12β and IL-15, as well as stress-response genes UBB and CASP9, especially at the later time point (8h). The question emerges, whether those changes result from the negative effect of heat treatment on cells or are they rather induced by the protective role of HSP against elevated inflammatory processes. High expression of the molecular chaperones combined with strong down-regulation of the apoptotic gene (CASP9) and ubiquitin B gene (UBB) involved in the protein degradation, suggests induction of the protective mechanisms in BMDC subjected to biotic (LPS) and abiotic (heat) stresses.

However, HSP are also known to modulate expression of inflammatory mediators (Ferat-Osorio et al. 2014). Ferlito and Maio (2005) reported induction of TNFα in heat stressed and LPS-treated promonocytic cells, but not in the differentiated macrophage-like cells (Ferlito and De Maio 2005), suggesting an impact of heat stress temperature, temperature-recovery period and differentiation stage of the cells on the immunomodulatory effect of heat treatment. In our earlier study (Slawinska et al., submitted), a chicken macrophage-like cell line (HD11) was stimulated with LPS, heat, and LPS+heat using
identical conditions to the current study. LPS+heat treatment increased expression of inflammatory cytokines in HD11 cells even more than LPS treatment alone. However, BMDC studied here are much more differentiated in comparison to HD11 cell line. As such, the BMDC may have developed distinct HSP mediated regulatory mechanisms to protect cells from endotoxic shock compared to the HD11 cells.

**Genetic background influences mRNA expression in BMDC under heat and LPS**

BMDC analyzed in this study were derived from two distinct chicken genetic lines (Leghorn and Fayoumi), characterized by different immune responses (regular vs. robust). The genetic background of the bone marrow donors was reflected in gene expression of BMDC treated with LPS+heat. Leghorn BMDC were characterized by overall higher inflammatory responses to LPS under heat conditions than Fayoumi BMDC. Intestinal inflammation is one of the major detrimental effects of heat treatment combined with *Salmonella* challenge in poultry (Quinteiro-Filho et al. 2012). It may lead to systemic inflammation, multi-organ failure and sepsis (Leon and Helwig 2010). In such cases, the ability of the organism to lessen the inflammatory responses may be a pro-survival strategy. The mechanism of this strategy is to reduce LPS signal transduction from the surface receptor TLR4 to the inside of the cell. In Fayoumi BMDC, expression of *TLR4* was significantly lower than the in Leghorn BMDC, followed by decreased expression of inflammatory cytokines and chemokines. The APC with their potent immunoregulatory role are a good target of the further evaluation of the genetic adaptation to heat.

**Conclusions**

APC serve as a bridge between the innate and adaptive arms of the immune system. For the first time, differences in response of chicken BMDC to treatment with an
inflammatory stimulus (LPS), an environmental stimulus (heat), and the combination of both (LPS+heat) were characterized using BM sourced from inbred Fayoumi (disease resistant) and Leghorn (disease susceptible) lines. A variety of in vitro assays characterized the innate response to these complex stimulations and included NO production, phagocytic ability, MHC II surface expression, and mRNA expression. Upon stimulation, BMDC from Fayoumis produced more NO, had higher phagocytic ability, and inherently had higher MHC II surface expression. Gene expression for the heat related genes BAG3, HSP25, HSPA2, and HSPH1 was strongly induced in response to heat treatment with few differences between lines, indicating conservation of this response. Gene expression for the immune related genes CCL4, CCL5, CD40, GM-CSF, IFN-γ, IL10, IL-12β, IL-1β, IL-6, IL-8, and iNOS were highly induced in response to LPS and differences between lines were both gene- and time-dependent. Genes that were strongly reduced due to all treatments include CASP9, IL-15, IL-4, MHC-IIβ, TGFβ2, TLR4, and UBB, and were largely different between lines. This research contributes to the sparse knowledge of genetic differences in innate immune response in chickens using in vitro methods. The results of this research may contribute to future strategies used to develop effective immunomodulators and vaccines, and to breed for more disease resistant and heat tolerant chickens.

Acknowledgements

This research was supported by USDA-NIFA-AFRI Climate Change Award #2011-67003-30228; the USDA National Institute of Food and Agriculture, Hatch project #5358; and AVG was supported by a USDA National Needs Fellowship, Award #2011-38420-20050. Polish-American Fulbright Commission is acknowledged for supporting a 2014/2015 Senior Advanced Research Award of AS at Iowa State University. The authors thank Dr.
Lonneke Vervelde, Dr. David Hume, and Dr. Pete Kaiser and their lab groups at the Roslin Institute, University of Edinburgh, for excellent advice and technical training; Michael Kaiser for advice on mRNA expression in chickens; Dr. Shawn Rigby, Flow Cytometry Facility at Iowa State University, for excellent advice on experimental design and also for performing the flow cytometry.

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### Tables

**Table 1.** Primer sequences used in the gene expression study

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**Table 1 continued**

| IFN-γ  | 396054    | AACCTTCCTGATGGCGTGAA | GCTTTGCCTGGGATTCTCAA | A   |


Table 2. Significant effects of treatment on mRNA expression of chicken bone marrow derived antigen presenting cells (BM-APC)

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Table 2 continued

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References: A. (Slawinska et al. 2015, submitted); B. (Avery et al., 2004b), C. (Kaiser et al., 2000), D. (Rothwell et al., 2004), E. (Kaiser et al., 2003), and F. designed in house. 2Reference genes.
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Twenty two genes were tested for mRNA expression levels. Chicken line (Fayoumi and Leghorn), treatment (control, LPS, heat, and LPS+ heat) and time post treatment (2h, 4h or 8h) as well as the interaction between line and treatment were fitted in the model. Analyses were performed separately for each line, gene, and treatment using dCt values (Ct target – Ct reference). To determine the relative gene expression, ddCt method was used. All treatments contained 3 technical replicates, and the experiments were replicated on 3 independent experimental days. Significance is considered if P ≤ 0.05.

**Figures**

Figure 1. Growth and treatment pictures of chicken bone marrow derived dendritic cells (BMDC). Cells differentiated from bone marrow of chicken day 18 embryos displayed typical growth and morphology (day 1, 3, and 6) with Fayoumi and Leghorn BMDC. On day 6 of culture, cells were stimulated with various treatments. Treatments of cells included thermal neutral (41.5°C) and medium alone (control), thermal neutral with 200 ng/mL lipopolysaccharide in the medium (LPS), heat for 2 hours at 45°C followed by temperature recovery and medium alone (heat), and 200 ng/mL LPS in combination with heat treatment for 2 hours at 45°C followed by temperature recovery (LPS+heat). Fluorescently modified beads were given in combination with treatments to assess phagocytic ability of cells. Pictures are representative examples of all experiments.
Figure 2: Nitric oxide production in chicken bone marrow derived dendritic cells (BMDC). Nitric oxide was measured using the supernatant of BMDC 24hr post treatment. Comparative analysis between BM-APC from Fayoumi (black bars) and Leghorn (grey bars). Treatments of cells included thermoneutral and medium alone (control), thermoneutral with 200 ng/mL LPS in the medium (LPS), heat for 2hr at 45°C followed by temperature recovery and medium alone (heat), and 200 ng/mL LPS in combination with heat treatment for 2hr at 45°C followed by temperature recovery (LPS+heat). Data are depicted as mean ± SEM; N=7-9 from 3 independent experimental days. Different letters indicate significance with multiple testing correction between all treatment groups P<0.05.
Figure 3: Flow cytometry gating strategy of chicken bone marrow derived dendritic cells (BMDC).

a) General gating strategy to identify BMDCs. Cells were stained with PI as a marker of viability and gating was done on live cells. Using the live cell population, BMDC were identified based on forward scatter and side scatter (auto fluorescence) characteristics, then used for downstream analyses. Flow cytometry plots are representative samples from all experiments.

b) Gating strategy to determine phagocytic ability (bead+), MHC-II surface expression (MHC-II+), and an illustration for samples which were both bead+ and MHC-II+. Flow cytometry plots are representative samples of all experiments.

c) Phagocytosis ability using chicken BMDC. Fluorescently labeled polystyrene beads were administered at 20 beads/cell to BMDC to compare phagocytic ability differences between treatment and genetic line. Treatments of cells included thermoneutral and medium alone (control), thermoneutral with 200 ng/mL LPS in the medium (LPS), heat for 2hr at 45°C followed by temperature recovery and medium alone (heat), and 200 ng/mL LPS in combination with heat treatment for 2hr at 45°C followed by temperature recovery (LPS+heat). Data are depicted as mean ± SEM; N=7 from 3 independent experiments. Different letters indicate significance with correction for multiple testing; P<0.05.

d) MHC-II surface expression using chicken BMDC. Cells were stained with MHC-II-PE antibody after treatments. Treatments of cells included thermal neutral and medium alone (control), thermal neutral with 200 ng/mL lipopolysaccharide in the medium (LPS), heat for 2 hours at 45°C followed by temperature recovery and medium alone (heat), and 200 ng/mL lipopolysaccharide in combination with heat treatment for 2hr at 45°C followed by temperature recovery (LPS+heat). Data are depicted as mean ± SEM; N=6-7 from 3 independent experiments. Different letters indicate significance with correction for multiple testing with all treatment groups; P<0.05.
Figure 4: Immune-related mRNA expression in chicken bone marrow derived dendritic cells (BMDC). Ten genes were tested for immune-related mRNA expression levels. Treatments of cells included: (1) LPS: 200 ng/mL LPS in the medium, thermoneutral (TN) conditions, (2) heat: heat for 2hr at 45°C followed by temperature recovery (heat), (3) LPS+heat: 200 ng/mL LPS in combination with heat treatment for 2hr at 45°C followed by temperature recovery. Untreated (control) samples (4) were kept in medium alone, in TN conditions during whole experiment. All samples were taken at 2h, 4h, and 8h post-
treatment. Treatments contained 3 technical replicates, and the experiments were replicated on 3 independent experimental days. To determine relative gene expression, ddCt method was used. Cycle threshold (Ct) of the target genes were normalized with the geometrical mean of two reference genes (\textit{H6PD} and \textit{RPL4}) (dCt = Ct target – Ct reference). Data are depicted as fold change (FC) of the gene expression. FC was estimated as $2^{-\text{ddCt}}$. Untreated (control) samples (not presented on graphs) were used to calibrate expression at FC = 1. Genes were considered up-regulated when FC > 1 and down-regulated with FC < 1. A Student’s T-test was used to make pairwise comparison of dCt values (within line, treatment, and hour post stimulus). Significance was considered if $P \leq 0.05$ and marked by *. Log2 scale was used on Y axis for improved visualization.

Figure 5: Stress-related mRNA expression in chicken bone marrow derived dendritic cells (BMDC). Six genes were tested for heat-related mRNA expression levels. Treatments of cells included: (1) LPS: 200 ng/mL LPS in the medium, thermoneutral (TN) conditions, (2) heat: heat for 2hr at 45°C followed by temperature recovery (heat), (3) LPS+heat: 200 ng/mL LPS in combination with heat treatment for 2hr at 45°C followed by temperature recovery. Untreated (control) samples (4) were kept in medium alone, in TN conditions during whole experiment. All samples were taken at 2h, 4h, and 8h post-treatment. Treatments contained 3 technical replicates, and the experiments were replicated on 3 independent experimental days. To determine relative gene expression, ddCt method was used. Cycle threshold (Ct) of the target genes were normalized with the geometrical mean of two reference genes (\textit{H6PD} and \textit{RPL4}) (dCt = Ct target – Ct reference). Data are depicted as fold change (FC) of the gene expression. FC was estimated as $2^{-\text{ddCt}}$. Untreated (control) samples (not presented on graphs) were used to calibrate expression at FC = 1. Genes were considered up-regulated when FC > 1 and down-regulated with FC < 1. A Student’s T-test was used to make pairwise comparison of dCt values (within line, treatment, and hour post stimulus). Significance was considered if $P \leq 0.05$ and marked by *. Log2 scale was used on Y axis for improved visualization.
and $RPL4$) dCt values ($Ct_{target} - Ct_{reference}$). Data are depicted as fold change (FC) of the gene expression. FC was estimated as $2^{\text{ddCt}}$. Untreated (control) samples (not presented on graphs) were used to calibrate expression at FC = 1. Genes were considered up-regulated when FC > 1 and down-regulated with FC < 1. A Student’s T-test was used to make pairwise comparison of dCt values (within line, treatment, and hour post stimulus). Significance was considered if $P \leq 0.05$ and marked by *.. Log2 scale was used on Y axis for improved visualization.

**Figure 6: Maturation-related mRNA expression in chicken bone marrow derived dendritic cells (BMDC).** Six genes were tested for heat-related mRNA expression levels. Treatments of cells included: (1) LPS: 200 ng/mL LPS in the medium, thermoneutral (TN) conditions, (2) heat: heat for 2hr at 45°C followed by temperature recovery (heat), (3) LPS+heat: 200 ng/mL LPS in combination with heat treatment for 2hr at 45°C followed by temperature recovery. Untreated (control) samples (4) were kept in medium alone, in TN conditions during whole experiment. All samples were taken at 2h, 4h, and 8h post-treatment. Treatments contained 3 technical replicates, and the experiments were replicated on 3 independent experimental days. To determine relative gene expression, ddCt method was used. Cycle threshold (Ct) of the target genes were normalized with the geometrical mean of two reference genes ($H6PD$ and $RPL4$) dCt values ($Ct_{target} - Ct_{reference}$). Data
are depicted as fold change (FC) of the gene expression. FC was estimated as $2^{-\Delta\text{Ct}}$. Untreated (control) samples (not presented on graphs) were used to calibrate expression at FC = 1. Genes were considered up-regulated when FC > 1 and down-regulated with FC < 1. A Student’s T-test was used to make pairwise comparison of dCt values (within line, treatment, and hour post stimulus). Significance was considered if $P \leq 0.05$ and marked by *. Log2 scale was used on Y axis for improved visualization.
CHAPTER 6
DISCUSSION, CONCLUSIONS, AND FUTURE RESEARCH

Introduction

As discussed in the literature review, heat stress in chickens results in economic losses due to decreased production, feed utilization, and decreased immune function. Many studies have characterized the phenotypic response to heat stress but none have investigated the genomics of heat stress and immune stimulation in chicken. To eliminate this gap in knowledge, we chose to investigate the response to heat stress and/or immune stimulation using unique genetic resources at the level of a population, a tissue, and a cell population. The divergent genetic lines used serve as discovery platforms for response to stressors. Chapters 2 and 3 utilized an AIL population, created from a cross between Fayoumi (heat tolerant) and broiler (heat susceptible), to identify the genomic regions associated with body temperature (BT), body weight (BW), percent breast yield, digestibility, and blood chemistry components in conditions of heat stress alone. Chapter 4 focused at the tissue level by contrasting Fayoumi and broiler lines for the spleen transcriptomic response to LPS, heat and LPS+heat. Chapter 5 contrasted cell populations from the Fayoumi and Leghorn (disease susceptible) lines, in which bone marrow derived dendritic cells (BMDC) were differentiated in vitro and assayed for nitric oxide production, phagocytic ability, MHC-IIbeta surface expression, and mRNA expression in response to LPS, heat and LPS+heat.

The research reported in this dissertation utilized state of the art technologies. Identification of QTL was possible by the use of the highly dense 600K chicken array. The array became available to the public in 2012, shortly after I began my graduate studies. The
global transcriptomic response of the spleen was completed using RNA-seq, a technology that was starting to be fully characterized in chickens in 2011 (Wang et al. 2011). It wasn’t reported until 2010 by Pete Kaiser’s research group that generation of BMDC in chicken was possible by cloning and addition of GM-CSF and IL-4 recombinant cytokines (Wu et al. 2010). The USDA National Needs Fellowship supported my training in BMDC culture techniques with Pete Kaiser and colleagues at the Roslin Institute for 6 months during my graduate career. The research techniques learned from Roslin were then implemented at Iowa State University.

The research reported in this dissertation is novel and highly advances the scientific knowledge in the area of heat stress and immune response in chickens. This is the first report of QTL identified in chicken for traits measured during heat stress. Others have identified traits for heat stress in cattle (Charoensook et al. 2012; Dikmen et al. 2013) and in Japanese quail (Minvielle et al. 2005) but never in chicken. This research was the first to report transcriptomic studies using RNA-seq on heat and immune stimulus, and the first to contrast genetic lines using the in vitro method of BMDC culture.

**QTL, Differentially Expressed Genes, and Pathways that Affect Response to Heat Stress and Immune Stimulation**

Chapters 2 and 3 presented identification of QTL using the same genotyped population (AIL) of chickens. The scope of the findings were too large to be well-described in a single manuscript. Here, we attempt to integrate the phenotypic and QTL information gained from the study on body temperature, body weight, breast yield, and digestibility with those identified for blood chemistry components. The benefit of integrating the results is that we can more fully understand the relationships between various types of traits measured using several different methods, an important aspect for future application of the findings for
genetic improvement of populations. The phenotypic correlations between all traits measured in Chapters 2 and 3 are illustrated as a heat map in Figure 1. The traits were measured pre-heat (day 20 or day 21), during the first day of heat (day 22), during the seventh day of heat (day 28), the difference due to acute heat (day 22-20), and the difference due to chronic heat (day 28-20). Note that blood chemistry components were not measured on day 22.

Phenotypic correlations are presented between all measured traits for the QTL mapping studies in Figure 1. The most striking correlations are observed for the blood chemistry components discussed in Chapter 3 with strong positive phenotypic correlations between the traits describing metabolic alkalosis (base excess, HCO₃, and TCO₂), and strong negative correlations between traits describing respiratory alkalosis (pH and pCO₂). When we focus on comparing correlations from traits described in Chapters 2 and 3, it is apparent that BT measures have on average low correlations (medians -0.024 to 0.025) with blood chemistry components. The strongest correlations between BT measures and blood chemistry components are the negative (average 0.22) correlations of BT with measures of acid-base balance including pH, base excess, TCO₂, and HCO₃. The correlations between BT and acid-base balance measures are very low (average 0.02) for measurements taken pre-heat (day 20) with the BT at any measurement during heat stress and the calculated differences due to heat treatment. A high phenotypic correlation between BT and acid-base during heat stress was expected because a major method of birds to reduce BT is to increase evaporative loss by increasing respiration, which results in a disturbance to the blood acid-base balance (Cooper and Washburn 1998). In summary, measuring any of the blood chemistry components before heat stress is not predictive of the BT the birds would have during heat stress. As discussed in Chapter 2, our study did not support the use of BT as a good measure of heat stress as we
measured it with a digital thermometer. This conclusion was reached due to the extreme variability of the measurement which resulted in no significant changes in BT due to heat stress, contradictory to the literature (see Chapter 2 discussion). We posit that a different technology, such as infrared imagery, might yield more repeatable measurements of body temperature and is worth exploring in the future in poultry heat-stress studies.

Feed represents a large proportion of poultry production costs (51.8% in 2001 and 68.7% in 2008) and these costs continue to increase due to the increased demands for grain in other industries such as ethanol for fuel (Donohue and Cunningham 2009). Climate change will certainly continue to impact feed prices, yet little is known about the mechanisms of heat stress on feed utilization in chicken. Moderate positive phenotypic correlations were observed for digestibility with BE28 (r=0.27) and with TCO₂₂₈ (r=0.28), indicating that the ability to maintain high levels of these molecules may be beneficial for digestibility during heat stress. A more desirable correlation would be one that could be measured pre-heat (day 20) and be predictive of the phenotypic response to heat stress. Examples of this are the correlations between digestibilities (measured after 7 days of heat stress) with pre-heat measurements of the ions Na, K, and iCa that have correlations of 0.15, 0.12, and 0.19, respectively. It is well established that the proper concentration of ions in feed affects digestibility, moreover, increasing Na, K, and Ca during periods of heat stress helps to ameliorate negative effects in chickens (Borges et al. 2003; Borges et al. 2004). Our datum suggest that chickens with higher initial concentrations of these electrolytes within the blood, digest feed more efficiently in heat stress conditions compared to animals with lower initial blood concentrations. The mechanistic relationship between these electrolytes and digestibility during heat stress has yet to be elucidated.
Co-localization of QTL could indicate genes with pleotropic effects such as a transcription factor location that influences the expression of multiple genes involved in various phenotypes. The co-localization of QTL for blood chemistry components was extensively discussed in Chapter 3. Here we discuss the regions of co-localization of QTL identified in both Chapter 2 and Chapter 3 to further understand the relationship between traits related to response to heat stress in chickens. Of the 96 total QTL identified in Chapters 2 and 3, there were only 4 regions of QTL co-localization, which are located on GGA10, 22, 26, and 27. On GGA10 at 4 Mb QTL for Glu20 and BW28-20 co-localized. The relationship between BW and Glu may reflect the ability to effectively use insulin, which can lead to higher blood glucose levels, although this relationship has not been studied in chickens under heat stress. An interesting candidate gene in this region is GCNT3, a glucosaminytransferase gene involved in mucin-type biosynthesis, which is important in intestinal homeostasis (Bergstrom and Xia 2013). Another region of co-localization was on GGA22 at 3 Mb for QTL for Glu28, Hb28, Hct28, and breast yield. Breast yield was extensively discussed in Chapter 2 because a large QTL explaining more than 15% in a single 1 Mb window on GGA1 was the largest QTL found across any of the studies. The QTL for breast yield on GGA22 explained a small amount of GV (1.11%). Only two previous studies have identified QTL in this region: one for age of sexual maturity (Xu et al. 2011) and another very recently reported for feed utilization (Reyer et al. 2015). On GGA26 at 3 Mb, QTL co-localized for TCO28, K20, iCa28, and BW28 and the QTL for all these traits explained a relatively small proportion of the GV between 0.50-0.54 %. This region is well represented in previously identified QTL (19 QTL reported in animalgenome.org, March 2016) including those for BW (Nadaf et al. 2009) and tibia breaking force and mineral density (Schreiweis et
al. 2006; Schreiweis et al. 2005). The K and iCa blood concentrations could be involved in tibia bone quality. There is a high incidence of leg problems particularly in the broiler industry because of rapid growth (Julian 1998). Bone health is a major threat to the poultry industry and has been estimated to account for $80-120 million dollars annually within the U.S. broiler industry (Sullivan 1994). In this region on GGA26 is CAMK1G, which is a calcium calmodulin gene that may be involved in the process of calcium regulation and growth. The final region of QTL co-localization was on GGA27 at 2 Mb for traits pCO₂₂₈, BE₂₈, and BT₂₈-₂₀. This region contains many previously reported QTL (48 QTL animalegenome.org) with 29 of them as QTL for BW. Some very interesting genes are located within this region including CRHR1, a corticotrophin releasing factor that has been shown to be highly upregulated in response to restraint stress in chickens (Goerlich et al. 2012). The ACE, an angiotensin converting enzyme, was also identified in this region and SNPs within this gene are related to exercise heat tolerance in humans (Heled et al. 2004). In summary, the regions of co-localization between the two QTL studies gives insight into genes within the regions that may have a pleiotropic effect during heat stress in chickens.

Another comparison that was not done in the single chapters was comparison of the agreement of mRNA expression between Chapters 4 and 5. There are extremely important differences in the experimental design of these studies although both contrast the unique genetic lines in response to treatment with LPS, heat stress, and the combination. Chapter 4 used an in vivo approach contrasting the Fayoumi and broiler, and assayed mRNA expression of the spleen using a global transcriptome approach of RNA-sequencing. Chapter 5 used an in vitro approach and contrasted Fayoumi and Leghorn lines and assayed expression of BM-DC using a targeted gene approach with Fluidigm technology. Many of the same genes were
DEG and will briefly be discussed here. Firstly, in both studies we see high increases in the pro-inflammatory cytokines IL-1beta, IL-6, and IL-8 in response to LPS and LPS+heat, but not when heat was used alone. Heat stress resulted in numeric (although not statistically significant) decreases in these cytokines, and the magnitude of fold change in the LPS+heat group is lower than that of LPS alone. HSPs are an important class of proteins that function as chaperones and maintenance of homeostasis during high temperatures. The BM-DC results were in agreement with the literature, where HSP25, HSPA2, HSP1, and BAG3 were all highly upregulated in response to heat stress. On the contrary, in the spleen we detected a mixture of increased and decreased expression levels of HSPs. In response to heat+LPS, HSP1 and BAG3 slightly (~LFC=2) increased in expression, whereas HSFA2 and HSF4 extremely (LFC~34) decreased in expression in all treatment groups in the spleen. It is well characterized that LPS is recognized by the host by TLR4 surface receptor. In Chapters 4 and 5 we see agreement in a strong decrease in expression of TLR4 in response to LPS alone and LPS+heat treatments. However, we did not investigate the surface expression of the TLR4 receptor. It would be useful to analyze the TLR4 surface expression in response to LPS, and to identify if the genetic lines differ in the ability to change the surface expression of the receptor upon stimulation. Additionally, both studies identified a decrease in MHC-IIbeta surface expression in these treatment groups. We did measure the MHCII surface expression using flow cytometry in the BMDC. We propose that a recycling mechanism is being used by these cell surface proteins. Although it is common to measure mRNA expression and make assumptions about protein expression, several studies have shown only a moderate correlation between the two measures. For example, the correlation between mRNA and protein expression (using a Pearson correlation coefficient) has been estimated at 0.59 for
mouse (Tian et al. 2004) and 0.76 for yeast (Futcher et al. 1999). One way that mRNA expression and protein expression may not agree with one another, as observed in Chapter 5 for the MHCII expression, is that the cell could increase recycling of the protein. Rather than changing expression at the mRNA level, regulation may occur at the protein level.

**General Conclusions for Stress Response Based on Various Types of Data**

For the GWAS studies in Chapters 2 and 3, we measured BT, BW, breast yield, digestibility, and blood chemistry components and found that most of these traits were lowly to moderately heritable under heat stress conditions. This suggests that it’s possible to breed for these traits in chickens to improve response to heat stress. However, because the AIL utilized here as a discovery platform is not representative of current commercial populations, a heat stress trial should be conducted using commercial birds to determine if the same genetic contributions exist. A total of 93 QTL were identified across the two studies and were located on GGA (*Gallus gallus* chromosome) 1-10, 12-15, 17-28, and Z. Almost every chromosome was represented as having QTL related to heat stress, although some of the QTL were identified for traits measured pre-heat treatment. The largest QTL we identified here was for percentage breast yield during heat stress on GGA1 that cumulatively explained 24% of the genetic variation. Because breast muscle yield is such an economically important trait, and this QTL was of large effect, a detailed analysis of this region should be a future research goal. However, the majority of the QTL identified had small effects (0.5-1.0% of the genetic variation), indicating that the traits reported here adhere to the infinitesimal model, where many loci act on the phenotypes.

In Chapter 4 the Fayoumi and broiler lines were contrasted for response to LPS, heat, and LPS+heat. These lines were used as a discovery platform to identify genes and pathways
in an immune tissue that are associated with resistance and susceptibility to stressors. The phenotypic data (see Chapter 4) revealed that the birds responded by a statistical increase to the double stimulation, as demonstrated by the increase in BT compared to either treatment alone. The acute response (7 hours post heat and 3.5 hours post LPS) revealed that the Fayoumi was relatively unresponsive to immune stimulation compared to the broiler based on the number of DEG, but the opposite was true under heat stress conditions. The double stimulation resulted in the largest number of DEG for both breeds, and most were downregulated. Therefore, we hypothesize that the combination of thermal stress and immune stimulation decrease immune pathways within the spleen. The Fayoumi may be better at reducing the inflammatory response by upregulating the anti-inflammatory IL-10 pathways during heat stress, which results in a better tolerance to heat stress. The study reported in Chapter 5 demonstrated that differences between genetic lines could be identified in vitro by generation of BMDC. This was observed for thermal and immune stressors. The Fayoumi displayed a more resistant phenotype than the Leghorn, although these differences were modest. Many of the genes chosen for mRNA expression were based on previous literature for experiments in vivo, and our results largely agree with those reported. We propose that this method to test responsiveness in vitro using BMDC could be used as preliminary evidence before testing genetic differences in vivo. The results of Chapter 5 indicate that the double stimulus of heat and LPS has a statistical increase effect, characterized by an increase in body temperature and number of DEG that was higher than for either stimulation alone. More research should be done on the innate immune response in the face of thermal stressors, to better develop animals that are adapted to heat, as the climate is rapidly changing.
Application of Research Findings

The human population continues to grow at an exponential rate. In 2016 it is estimated that the human population is 7.4 billion and it is predicted that by 2050 this will increase to 9.6 billion (Gerland et al. 2014). With increases in the population it is projected that food production will have to increase by 70-100% by 2050 (Tilman et al. 2011). With the urbanization and development of countries, the dietary preference for animal based proteins also increases (Regmi and Dyck 2001). According to a report by the FAO (FAO), the global consumption for meat poultry is projected to have the largest percentage increase in consumption compared to other animal meat products, rising to 181 million tons by 2050.

With increasing global average temperatures, and increase in demand for poultry products, one way to meet consumption trends is to increase production of poultry in the face of increasing temperatures. More emphasis should be placed on breeding for disease resistance and robustness rather than production in a clean and thermoneutral environment. Genetic selection began in the commercial poultry industry in the early 20th century and drastic improvements have resulted in broilers for growth and feed efficiency, and in layers for reproduction and feed efficiency. Feed costs represent 65-70% of production costs in livestock and thus the major breeding goal is to have the highest production traits with lowest feed intake (Herd et al. 2003; Luiting 1990). The age to reach market weight in the 1940s was 16 weeks and by 1990 broilers reached market weight by 6-7 weeks (Griffin and Goddard 1994). However, this rapid growth and improved feed efficiency has come at a cost to reproductive ability, cardiovascular ability, nervous system, and immune response. Production traits have been shown to be negatively correlated with the traits mentioned. Some even speculate that there are no limits to response to selection in pigs and poultry
(Fredeen 1984; Hunton 1984). Havenstein et al. (1994) compared a random bred control line from 1957 with that of a commercial broiler line from 1991 and when both populations were fed the 1991 diet, they estimated that 85-90% of the increased growth was due to increases in genetic merit of the birds, but the faster growing chickens had 3 times (9.1%) mortality compared to random bred chickens (3.3%). The mortality was associated with flip-overs, ascites, and leg problems. Problems associated with high production levels are continuously being revealed in the poultry industry, and breeders are trying to address them in their genetic improvement programs. But, for many of the traits, sparse information about their genetic basis is available. Some of the problems that have been increasing in incidence in the poultry industry include wooden breast syndrome (Sihvo et al. 2013) and white striping (Kuttappan et al. 2009) which have similar histological myopathies which is a consequence of rapid breast muscle growth and is thought to be caused by an excessive infiltration of connective tissues. Currently, some of the largest problems in broilers is sudden death syndrome, ascites, skeletal problems, and disease, while in the laying hen industry it is leg breakage, and disease (Julian 2005). It is anticipated that production losses will increase due to climate change and incidence of infectious disease (McMichael et al. 2007). In 2015, the U.S. experienced the largest animal disease disaster experienced in this age with a highly pathogenic avian influenza epidemic. According to the USDA, over 38 million birds were depopulated due to positive testing on the farm where they were housed. Interesting, only layers and turkeys were affected by the disease, whereas no deaths were recorded for broilers. Climate change is anticipated to expand the range and emergence of pathogens, as the range and migration patterns of insects and wild animals change.
According to the USDA 2015-2020 dietary recommendations, most Americans would benefit from increasing variety in their diet to include more fruits, vegetables, grains, and decreasing meat consumption (USDA2015). Clearly, breeding goals are shifting based on consumer demands for animal welfare. Regulations have been put in place in California’s Proposition 2 that requires a minimum space for each bird and other parts of the world are concerned with animal welfare such as the EU which is cage free. In an environment where the birds are exposed to the stressors of the environment, production will surely decrease.

When concern arises on feeding the world, livestock receive a small proportion of funding. Only 20% of all funding from NGOs is used for funding livestock research. Genetically modified organisms (GMOs) have been widely adopted in some parts of the world including the U.S., Argentina, Brazil, Canada, India, and China. The use of GMOs has revolutionized plant production in those countries. Some examples exist of animals with useful traits introduced using GMO technologies, for example, chickens resistant to the spread of avian influenza, pigs that reduce methane admissions, and fast growing salmon (FAO). The FDA recently (2015) approved the GM salmon, but with consumer kickback we have yet to see this item for purchase. I speculate we are a long way away from GMO in livestock which is available to the consumer. Therefore with the changing climate and increased threat of infectious livestock diseases, we should focus on improving robustness in the current livestock populations.

In the context of the research presented in this dissertation, the results are a starting place for future investigations into the genetic influence of heat and/or disease resistance.
Limitations of Research

As with all scientific work, that presented in this dissertation has limitations. An issue of great importance is the genetic lines of chickens that were tested for every research chapter. In Chapter 4 we contrast Fayoumi and broiler in their spleen transcriptome response, and in Chapter 5 we contrast Fayoumi and leghorn in the BMDC mRNA expression and functional response both contrasts in response to heat stress and LPS. We refer to the broiler as representative of the genetics within the commercial broiler industry, but the broiler sire was sourced from a commercial population over two decades ago (Deeb and Lamont 2002), and therefore may not be the best representation of the current commercial population. The massive increase in growth of the broiler chickens over the past 60 years is largely attributed to intense genetic selection for breast muscle accretion, whereas the genetic lines in the current study do not impose selection for any trait. The broilers maintained at the Iowa State University poultry farm are a closed population, and this has likely caused an increase in inbreeding within our broilers compared to the commercial sector. The Leghorn line maintained at ISU was previously commercially selected for egg production. Generally, egg laying birds are more disease resistant compared to commercial broilers (Leshchinsky and Klasing 2001). The Fayoumi breed was brought to the United States in the 1950s from the Fayoum region of Egypt. This breed was found to be highly resistant to Avian Leucosis, a disease that was rampant in the US at the time. The idea was that crossing of the Fayoumis with egg laying chickens would result in birds with good disease-resistance and egg-laying traits. However, this was largely unsuccessful because the Fayoumis and their offspring were not docile enough for commercial production. Dr. Arne Nordskog decided to maintain the Fayoumi line at ISU and begin an inbreeding program. Since the 1950s, the Fayoumi and
Leghorn lines have been inbred by half-sib mating, and it was estimated that these are greater than 99.9% inbred (Deeb and Lamont 2002). The Fayoumi, broiler and Leghorn have been well-studied because they allow contrast of the Fayoumi which is a “village chicken”, unselected, highly robust breed, the broiler, which is a commercially selected breed considered to be less robust due to intense selection, and the Leghorn, which was previously commercially selected for egg production.

Darvasi and Soller first described the use of an AIL for fine genetic mapping (Darvasi and Soller 1995). When a population is created from two inbred or divergent lines, and the progeny subsequently inter-mated for several generations, there is an increased probability of recombination between loci. This phenomenon allows accurate fine-mapping of QTL. In Chapters 2 and 3, the F18 and F19 generations of an AIL of chickens was utilized for GWAS for various traits measured in response to heat treatment. This intercross line originated from crossing a single male broiler sire with 6 highly inbred Fayoumi dams. Although this population has limited variability due to the initial mating, the broiler sire was characterized by the offsprings’ phenotypic means and variances of body composition phenotypes, which showed that it was representative of the entire broiler population (Deeb and Lamont 2002). We hypothesize that the highly inbred Fayoumi breed became fixed for alleles that in general had the highest frequency in the founder line. Thus, this AIL population is a powerful resource to identify QTL. Previous generations of this AIL were used for several QTL mapping studies and allowed the identification of many QTL including 257 QTL for growth and body composition (Abasht and Lamont 2007; Deeb and Lamont 2003; Li et al. 2003; Zhou et al. 2006a, 2006b), 93 for skeletal integrity (Zhou, Deeb, et al. 2007), 51 for metabolic traits (Zhou et al. 2007), and 12 for response to Salmonella enteritidis challenge.
(Kaiser and Lamont 2002; Kaiser et al. 2002; Liu and Lamont 2003). Therefore, collectively, a wide range of traits has been associated with a large number of QTL in previous generations of this AIL. The continued erosion of LD in this population over subsequent generations, combined with the availability of more dense SNP panels, creates a unique opportunity to more finely map the location of QTL that are in LD with a causal mutation. Although the AIL is a valuable resource population, the limitation is that these QTL could not be directly selected upon because they would have to validated within the commercial population to verify those QTL are present, and to estimate the effect on the traits of interest. Because we used a highly AIL, we propose that these QTL are indeed very close to causative genes/mutations that influence the phenotype, and will likely be found in commercial population. Additionally, we detected QTL that explained a relatively small percentage of the genetic variation (average = 0.6%). Selecting on markers that have low impact will take many generations to change the population mean.

In Chapter 4, whole transcriptome sequencing was discussed. Some of the major considerations when doing transcriptomic profiling include breed, age of the animal, tissue, experimental design (treatment and time), and what type of RNA to assay. We chose to contrast the Fayoumi and broiler breeds, but because the broiler is outbred more variation was observed in the transcriptome and decreased power to detect DEG. Another option would have been to contrast the highly inbred Leghorn line with Fayoumi. This would have increased our power, but there is less evidence of detrimental effects of heat stress on the Leghorn compared to the broiler. We chose to investigate the spleen transcriptome because of the critical function in innate and adaptive immunity, and the cross-talk between the immune system and general metabolism. Immune stimulation (LPS) was not the only stressor
birds were exposed to, as heat stress was also a factor. No previous work has quantified differences in RNA expression in an immune organ in response to heat stress. Most previous work including some from our lab and this animal study (Coble et al. 2014) has focused on the liver, which is the metabolic powerhouse. Because no information is available about the spleen responding to environmental stressors, we have little context to interpret our results. We only investigated the spleen transcriptome response at one very acute treatment time (7h heat and 3.5h post LPS). The complete host response to heat and LPS cannot be fully understood by looking at one time point. It would, therefore be beneficial to study an even earlier time point such as 2 hour of heat and LPS, and chronic exposure to the stressors such as after 1 week. The broiler and Fayoumi have different developmental rates. The large differences in response to thermal treatment and immune stimulation could be attributed partly attributed to developmental differences. Another limitation of this study is that we chose to investigate only PolyA mRNA. Other types of RNA are of great importance to host response. MicroRNA has been shown to be associated with response to heat stress, and long non-coding RNA is a rapidly expanding field. The limited choices in experimental design may result in missing some very important RNA players in the response to heat and/or LPS. The study reported in Chapter 5 has many of the same limitations as those in Chapter 4. Notably, the functional assays were all performed at a single time point. To gain a full understanding of chicken dendritic cells, a time course study should be completed in the future.

**Future Directions**

Chapters 2 and 3 reported a total of 96 QTL for production and physiological traits measured pre-heat, during heat, and the difference due to heat treatment. Many positional
candidate genes were identified and discussed in the context of response to heat stress. We hypothesized that the genes near QTL influenced the phenotypes, a common practice used to elucidate the biological mechanism of a response. Yet, much of a QTL’s effect on phenotype may be because of trans effects such as a transcription factor binding site. The 600K chicken genotyping panel was designed to represent the genome by distributing the SNPs uniformly according to map distance, to represent the micro chromosomes that have high rates of recombination (Kranis et al. 2013). Missing on the 600K chip are several regions of the micro chromosomes and chromosome 16, which contains the MHC region, because the chicken was so poorly annotated in these regions due to high recombination and high GC content, and 15% of all SNPs on the chip remain to be assigned to chromosomal locations. The GWAS should be rerun using version 5.0 of the *Gallus gallus* genome that was released in January 2016 (after the manuscripts on this dissertation’s GWAS studies had already been published or submitted to journals), which will likely improve assignment of SNPs and QTL effect estimates. Declining sequencing costs have made it feasible to do whole genome sequencing instead of SNP arrays. Whole genome sequencing would certainly improve mapping genomic regions that contribute to response to a stressor. The largest QTL identified in the GWAS studies was on chromosome 1 for breast muscle yield during heat stress which is a very important economic trait. The SOCS2 gene was near this QTL and true effect that this gene has on breast muscle yield should be studied. One way to test the function of SOCS2 is to use CRISPR/CAS9 technology to knock out function the gene.

Chapter 4 identified many genes and pathways influenced by heat stress and/or LPS treatment, contrasting genetic lines. We assayed only mRNA but other types of RNA should be sequenced as well. microRNA are small (<30 bp) transcribed sequences within the
genome that are not translated. They function in gene regulation by binding to complementary sequences on expressed transcripts and tag them for degradation. Recently microRNA has been shown to regulate response to heat stress in several plant species including Arabidopsis (Guan et al. 2013), wheat (Xin et al. 2010), Brassica rapa (Yu et al. 2011), and others that has been extensively reviewed (Sunkar 2012). One study identified microRNA expression signatures in chickens infected with Marek’s Disease (Tian et al. 2012). Another emerging class is the long non coding RNA (lncRNA) which are long (>200 bp) non-protein coding sequences. These lncRNA have many functions and have been shown to be involved in development and disease, chromatin remodeling, and post-transcriptional regulation of mRNA as discussed in an extensive review (Ulitsky and Bartel 2013). Chapter 4 assayed mRNA expression levels, which may not have perfect correlation with the amount of protein that is produced in response to treatment. The protein levels of genes should be investigated using western blot technology. I would recommend to test the protein expression of IL-10 because of the large impact on reducing the inflammatory response. Assaying this protein in blood samples of potential breeding animals, if the association with reduction of inflammation is confirmed, would allow the protein to be used as a biomarker in genetic selection because the animals would not be killed in the process of collecting the sample to assay.

Future research should investigate more tissues for the transcriptomic response to heat stress and/or LPS in chickens. The stress response tissues such as the hypothalamus and pituitary should be investigated. The anterior hypothalamus, in conjunction with the pituitary gland, regulate body temperature (Bligh 1966). Many studies have investigated that stress activates the hypothalamus is associated with decreases in immune response (Cao et al. 2013;
Dhabhar 2014; Eskilsson et al. 2014; Herman et al. 2013; Pijanowski et al. 2015). Microarray has been used to characterize the hypothalamus response to heat stress in broilers (Sun et al. 2015) where 1,239 differentially expressed genes were identified. This indicates that the transcriptome is responsive to heat treatment, but an immune stimulus, such as LPS, should be investigated as well to elucidate the interaction between thermal stressors and immune stressors. Another tissue that should be investigated in response to heat stress and immune stimulation is the liver, because of its major role in metabolism. The liver transcriptome has been investigated using RNA-seq in response to heat stress in broilers where relatively few (43) DEG (Coble et al. 2014), and in a chicken hepatocellular carcinoma cell line where 812 DEGs, were identified (Sun et al. 2015). Other immune related tissues should be assayed to investigate the responsiveness to heat and LPS to further elucidate the relationship between these stressors such as bursa, thymus, and PBMCs.

When investigating changes in gene expression, a crucial aspect is understanding the chromatin structure. Chromatin is organized within the cell by wrapping around histones. When chromatin is loosely wrapped around histones, the genes within the open region can be transcribed. Chromatin structure has never been investigated in chickens for either heat stress or immune response. To investigate the chromatin structure, a DNase-I hypersensitivity assay should be implemented. Another method to investigate the transcriptomic response is methylation status. Increased methylation is negatively correlated with transcription levels. A well accepted method to characterize the methylome is bisulfate sequencing that can be completed whole genome scale.

Chapter 5 discusses functional and mRNA expression level changes in response to heat stress and LPS in BM-DCs from Fayoumi and Leghorn chickens. Differences in
responses were identified between lines and among treatments, but these experiments should be conducted using ex vivo cells collected from experiments completed on animals. PBMCs are easily accessible and do not require euthanizing the animal for collection, and would be a good choice to contrast genetic lines and treatments.

In conclusion, this dissertation greatly increases the scientific knowledge on the genetic component of response to heat stress and immune stimulation in chickens. We utilized unique genetic lines as a discovery platform along with state of the art technologies. The results of this research are useful as a starting point for future scientific studies.

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**Figures**

![Figure 1 Heat map of phenotypic correlations between blood chemistry components](image-url)
and production traits from Chapters 2 and 3.
Heat map showing phenotypic correlations between blood chemistry components measured on day 20 (pre-heat), day 22 (acute heat), day 28 (chronic heat), and day 28-20 and day 22-20 which are the differences due to heat treatment. Traits are clustered together based on function. The colors represent the correlation coefficient ($r^2$) with red indicating a positive correlation and blue indicating a negative correlation.