INCUBATION TEMPERATURE AND POST-HATCH STRESS EFFECTS ON IMMUNE PARAMETERS, IMMUNE SYSTEM DEVELOPMENT, AND PERFORMANCE IN COMMERCIAL BROILERS

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ABSTRACT

Broiler performance is dependent on immunocompetency and the ability to respond to environmental challenges. Incubation temperature, post-hatch transportation, and vaccination may impose stress upon the embryo and post-hatch chick and impact immune system development and lifetime performance of the bird. The objective of the first study was to evaluate incubation temperature and post-hatch transportation environment on response parameters indicative of early immunity in the neonatal chick. Cobb 500 eggs (n=5200) were incubated with combinations of eggshell temperatures common to commercial multi-stage incubators during early and late incubation: low (**L**): 36.7°C, standard (**S**): 37.5°C, and high (**H**): 39.0°C. After hatch, chicks were transported under one of two conditions: control (**C**: 34°C) or distressed (**D**: 40°C), yielding 8 experimental treatments: LH-C, LS-C, SH-C, SS-C, LH-D, LS-D, SH-D, and SS-D. The objective of the second study was to examine the effects of incubation temperature profiles on response to vaccination in Cobb 500 broilers (n=2000). Temperature treatments were the same as the first study, and embryos were administered vaccinations for Marek's disease virus (**MDV**) at embryonic day (**ED**) 18, Newcastle disease virus (**NDV**) at hatch, the combination of MDV+NDV, or no vaccine (control). There were 16 resulting experimental groups: LH-Control, LH-MDV, LH-NDV, LH-MDV+NDV, LS-Control, LS-MDV, LS-NDV, LS-MDV+NDV, SH-Control, SH-MDV, SH-NDV, SH-MDV+NDV, SS-Control, SS-MDV, SS-NDV, and SS-MDV+NDV. Two and three way interactions ($P<0.05$) were observed for the parameters evaluated and are presented for both studies. These studies

suggest an influence of incubation temperature and post-hatch stressors on chick development and early immune response parameters.

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LIST OF ACRONYMS

BW- Body weight

d- Day

C- Control

D- Distressed

DOH- Day of hatch

ED- Embryonic day

EST- Eggshell temperature

Gal-2- Gallinacin-2

GAPDH- Glyceraldehyde-3-phosphate dehydrogenase

h- hour

H- High

HSP- Heat shock protein

iNOS- Inducible nitric oxide synthase

L- Low

MDV- Marek's disease virus

mRNA- Messenger ribonucleic acid

Muc-2- Mucin-2

NDV- Newcastle disease virus

S- Standard

T_3- Triiodothyronine

T_4- Thyroxine

Wk- Week

YFBW- Yolk-free body weight

CHAPTER I

INTRODUCTION

Genetic selection for high-yield broilers has resulted in an increased growth rate during embryonic development. Rapid growth causes an increase in metabolic heat production and limits the availability of oxygen, among other critical resources, that are necessary for optimal organ and tissue development in the embryo. The consequences of accelerated embryonic growth are further exacerbated by the environment within the incubator. Incubators have not been updated to compensate for the changing demands of the embryo as egg capacity has increased, and air cooling systems with adequate ventilation are not employed to dissipate the resultant elevated heat levels. Therefore, it is not uncommon for temperatures within setters and hatchers to deviate significantly from optimum environmental conditions, thus having a direct impact on development and physiological processes in the growing embryo and in the subsequent neonatal chick. Decreased hatchability, increased hatch window, and poor chick quality typically result in the hatchery and are followed by low body weight, decreased yolk sac utilization, and reduced organ weights in the chick during the initial post-hatch period.

The stress imposed upon the embryo during incubation may impact the bird's response to additional stressors during the post-hatch period. Incubation stress coupled with exposure to post-hatch stressors may culminate in suppressed function of the innate immune response and detrimental effects on small intestinal development and nutrient utilization, which equate to poor performance and productivity during the grow-out period. It is extremely critical to maintain optimum incubator conditions because embryonic development comprises approximately 1/3 of the broiler's life. If the required environmental conditions are not met, it is possible that the bird may never recover from these stressors, thus resulting in significant production losses.

To date, few studies have examined the effects of incubation temperature profiles and their direct effects on development of high-yield broiler embryos. Additionally, the impact of stress resulting from post-hatch transportation and vaccination in the previously stressed embryo on post-hatch development has not been investigated. The purpose of the following studies was to elucidate the bird's response to environmental stressors so as to tailor incubation and post-hatch transport management practices to better address bird demands and maximize productivity.

CHAPTER II

LITERATURE REVIEW

Incubation Management

Improvements in growth of modern broiler strains over the past 50 years have dramatically impacted embryological development and, consequently, hatchery management. However, this change in the embryo over time has not resulted in concomitant modifications and improvements in commercial incubators. The most common change in incubators has simply been an increase in automated control of the incubator environment (Deeming, 2002). The two incubation systems utilized in commercial hatcheries are single- or multi-stage. In single-stage incubation, all eggs are at the same developmental stage, whereas in multi-stage incubation, embryos representative of multiple developmental stages are incubated together. In multi-stage incubation, embryos that are more developmentally advanced produce more metabolic heat than eggs at a less mature stage of development and, therefore, may be used to help incubate the younger embryos (Hamidu *et al.,* 2007). However, it has been suggested that genetic strains of broiler breeders with different growth potentials result in embryos with varying metabolic rates (Tona *et al.,* 2004), which could result in inconsistent embryonic heat production, heat requirements, and incubation environment. In both single- and multi-stage programs, eggs are set in the incubator, also referred to as the setter, and remain there for 18 d. After 18 d of incubation, eggs are transferred to the hatcher and once the chicks have hatched, they are removed for sorting, processing, and distribution to the farm.

Higher environmental temperatures near the last few egg racks, or those racks containing embryos more developmentally advanced, have been noted in multi-stage incubators (Hamidu *et al.,* 2007). This situation creates complications in terms of establishing and maintaining an

appropriate environmental temperature to meet the needs of embryos developing at different rates. Broiler embryos require a 21 d incubation period, and the birds may remain in live growout on the farm for as little as 34 d and as many as 52 d, with an average of 42 d. Therefore, embryonic development currently represents 30 to 40% of the broiler's lifespan (Ricks *et al.,* 2003). Consequently, incubation plays a critical role in efficient growth and development of the embryo that will later impact post-hatch performance and commercial production management.

Temperature. Temperature may be considered the most crucial factor in incubation management. In order to achieve maximum hatchability (French, 1997) and chick quality, it is necessary to select an incubation temperature that will facilitate optimal growth and development. The most favorable incubation temperatures typically range between 37°C and 38°C, yet hatchability may be achieved between 35°C and 40.5°C (Wilson, 1991). Maintaining a temperature within this range may be challenging based on whether single- or multi-stage incubation management is utilized.

The hatchery's primary priority is to obtain a high hatch rate of viable chicks with a narrow spread of hatch (Decuypere and Bruggeman, 2007) consisting of quality, marketable chicks. Good quality chicks must be clean, dry, alert, and free from deformities with cleanly sealed navels and normal leg conformation (Decuypere and Bruggeman, 2007). Several scoring systems have been developed to evaluate chick quality at hatch, and chicks with the highest scores, or those chicks of optimal quality, showed highest relative growth up to 7 d post-hatch (Tona *et al.,* 2003). Evaluation of chick quality is therefore an effective indicator of broiler performance early in life and potential lifetime bird performance. However, chick quality may be greatly influenced by factors such as egg storage duration, breeder flock age, breeder

nutrition, and especially, environmental conditions within the incubator (Decuypere and Bruggeman, 2007).

It is evident that high incubation temperatures are a result of the differences in heat production by the embryo relative to heat transfer within the machine (Meijerhof, 2002), which is impacted by the ventilation system. Expelled embryonic heat will typically accumulate in the setters and hatchers. With every 1°C increase in incubation temperature, embryonic heat production was changed by 4.9% (Lourens *et al.,* 2006), and with advancing embryonic age, heat production was increased as reflected by allantoic fluid temperature (Janke *et al.,* 2002). It is suggested that increasing heat production may be attributed to embryonic growth processes and the subsequent increase in body mass incurring high metabolic activity (Janke *et al.,* 2002).

Ventilation is a critical component of hatchery management and is an important contributor to the balance of an optimal incubation environment. Incubators require an air conditioning system that distributes either warm or cool air that is circulated through the eggs and then returned back to the conditioning system (French, 1997). Air flow uniformity is dependent on the ability of the air to pass through egg trays, and as space between egg trays increases, the air speed required is decreased (French, 1997). Maintenance of a uniform and consistent incubation temperature may be challenging due to uneven air flow within the machine. However, adequate ventilation is necessary for the control of oxygen, carbon dioxide, and water vapor levels in the incubator environment. Oxygen, carbon dioxide, and water vapor are diffused between the egg and incubator environment during the process of embryonic respiration (Christensen, 2009). As the embryo ages and body mass increases, oxygen requirements increase as well. The conductance properties of the eggshell serve as the limiting factor for oxygen diffusion to the embryo. When oxygen availability is reduced, the embryo enters a

hypoxic state and is required to utilize other sources to obtain energy. During the first several days of incubation, oxygen consumption exponentially increases at a slow rate, which is followed by an almost linear increase, a plateau phase, and finally a secondary increase at the termination of incubation (Janke *et al.,* 2002). As alterations in temperature occur, oxygen consumption by the embryo is further modified, thus impacting embryonic heat production.

Variability Between Eggshell Temperature and Incubation Temperature. Appropriate environmental temperature surrounding the egg is extremely important in terms of facilitating normal embryonic development. Embryos are poikilothermic and are reliant on the surrounding environment to establish body temperature. The evaluation of the temperature experienced by the embryo is difficult to determine as it would require destructive measures that would deleteriously impact embryonic growth and development. Therefore, the use of eggshell temperature (**EST**) as a reflection of embryonic temperature is commonly accepted. Infrared fever thermometers are commonly used to determine EST (Hulet *et al.,* 2007). The temperature that the embryo experiences may be dependent on several factors, which include incubator temperature, the transfer of heat between the incubator and the embryo, and the production of metabolic heat by the developing embryo (French, 1997). Although an average EST of 37.8°C was observed in single stage incubators, the EST ranged anywhere between 5°C above or below average depending on stage of incubation and egg position within the incubator (Lourens, 2001).

One of the primary aims of commercial incubation management is to maintain an equal air temperature across all eggs at every location within the incubator. Air velocity may be used as a tool to achieve this uniform temperature; however, most incubators are unable to distribute an equal air velocity across all eggs. This may result in heat transfer differences between eggs, and consequently, varying embryonic temperatures (Meijerhof, 2002). Airflow uniformity

within an incubator is dependent on the ability of air to pass across the pores of the eggshell to eliminate heat (Hulet *et al.,* 2007). The number and size of pores, egg size, and eggshell thickness are determinants of heat dissipation between the embryo and incubator environment (Hulet *et al.,* 2007). Lourens (2001) reported that eggs located in a trolley in front of the ventilator experienced EST that were 0.3°C higher than the corner trolley at embryonic day (**ED**) 1. Significant EST differences were seen especially after ED10. At ED18, corner trolley eggs experienced EST of 40.5°C, which is 2.7°C higher than the optimum incubation temperature of 37.8°C. These substantial deviations in EST may very well be attributed to air flow across the eggs in differing locations within the machine.

When the environmental temperature in the incubator is reported at 37.3°C, the internal egg temperature may reach 38.2°C at ED14 (Leksrisompong *et al.,* 2007). In order for an internal egg temperature of 37.9°C to be maintained at ED19, the incubator temperature had to be reduced by 1.6°C to a set point of 36.3°C. These discrepancies between EST and incubator temperature support findings that embryo development and hatchability are more affected by actual embryo temperature than the surrounding environmental temperature (Lourens *et al.,* 2005). In multi-stage incubators, it is not uncommon for low EST to be reported at the start of incubation (Joseph *et al.,* 2006). Embryonic heat production is insignificant at the beginning of incubation; therefore, egg temperature is less than incubation temperature, because embryonic heat loss due to evaporative cooling exceeds heat produced by the embryo (French, 1997). High EST have been reported during the latter part of incubation in both single- and multi-stage incubators (Joseph *et al.,* 2006). During the third week of incubation, it is possible for environmental temperatures to reach as high as 40°C when airflow between trays is less than 0.1 m/s (Lourens, 2001). These high temperatures are not uncommon as embryonic heat production

significantly exceeds heat loss due to evaporative cooling and thus results in an egg temperature that is greater than incubator temperature (French, 1997).

Embryonic Growth and Development

Embryonic development during incubation may be divided into two distinct periods: differentiation and growth. The entire incubation period takes 21 d from the time of placement in the incubator until the chick hatches. The differentiation period involves the development of embryonic tissues, such that 90% of the organs are present by ED12 (Deeming, 2002). The differentiation stage also involves the development of extra-embryonic tissues that support overall developmental processes in the organism. The embryo will increase in size, and tissues will mature during the growth phase to prepare the chick for hatching (Deeming, 2002).

The avian embryo develops from a round ball of cells that forms the zygote. Once zygote formation is complete, initial embryonic development begins in the oviduct during the process of egg formation. By the time the egg has been laid, the blastoderm has reached the gastrula stage and consists of approximately 60,000 cells (Deeming, 2002). Only about 500 of these cells will contribute to the embryo itself as the remainder of the cells will be allocated to formation of extra-embryonic structures (Stevens, 1996). Development beyond this point will not proceed until the onset of continuous incubation. Vertebrate embryonic development typically begins with the anterior end, with the head developing before the tail. In avian species, the head begins development within 24 h of incubation, and brain and heart development commences by 30 h with the presence of distinguishable eyes by 48 h (Deeming, 2002). The alimentary tract and respiratory system begin development by ED3, and a fully formed bird is recognizable by ED12.

Low Temperatures During Early Incubation. Suboptimal incubation temperatures can greatly impact the commencement of physiological processes in the developing embryo.

Temperatures that are lower than the recommended optimum incubation temperature of 37°C to 38°C have been reported to delay development and, consequently, increase incubation time. However, the overall development of the chick was comparable to those incubated at optimum incubation temperatures (Suarez *et al.,* 1996; Black and Burggren, 2004) as the relative timing of internal and external pipping, two critical developmental events, were not affected by suboptimal incubation temperatures (Black and Burggren, 2004). Chicks incubated under low temperature conditions early in development required a higher machine temperature (or lower difference between machine temperature and EST) during the second and third weeks of incubation (Lourens *et al.,* 2005). Low EST may therefore lower the metabolic rate and overall heat production of the embryo. As a direct result, hatchability is typically decreased under conditions in which the EST is below standard within the setter (Joseph *et al.,* 2006).

A number of studies have reported the impact of low temperatures early in incubation on chick development. Embryo length and yolk-free body weight were reduced at ED7, 14, 18, and 21.5 in embryos incubated at a low incubation temperature of 36.7°C (Lourens *et al.,* 2005). Chick length was also reduced at hatch when eggs were exposed to 36.7°C during the first 10 d of incubation (Joseph *et al.,* 2006). At 3 and 6 wk post-hatch, chicks that were incubated at low setter EST had lower body weights and decreased body weight gain compared to chicks that had been exposed to standard temperatures (Joseph *et al.,* 2006). Additionally, low setter EST caused decreased carcass weight, increased abdominal fat pad yield, reduced fillet weight yield, and decreased tender weights compared to standard EST conditions. Joseph *et al.* (2006) further attributed decreased post-hatch growth to delayed development of the embryo during incubation. Turkey poults respond in a similar manner to broilers with decreased body and pectoral weight and number of semitendinosus muscle fibers at 16 d post-hatch after exposure to 35.5°C from

ED5 to ED8 (Maltby *et al.,* 2004). In contrast, earlier studies have shown that temperatures below 37°C during the first 10 days of incubation resulted in increased body weight, gastrointestinal tract weight, and liver weight at 2 and 3 wk post-hatch, which is suggestive of increased heat production due to compensatory growth. This enhanced growth rate may impact the growing embryo not only during incubation, but up to 3 wks post-hatch (Geers *et al.,* 1983).

High Temperatures During Late Incubation. Significant metabolic heat production by the embryo begins around ED4, and by ED9, embryonic temperature may be significantly higher than the surrounding environmental temperature (Hulet *et al.,* 2007). Therefore, dissipation of heat by the embryo is a critical factor influencing overall embryonic development, hatchability, chick quality, and subsequent post-hatch bird performance. The detrimental effect of high incubation temperature on hatchability often increases with an increase in temperature and exposure time (Wilson, 1991). Additionally, the effects of high incubation temperatures are very much dependent on the developmental stage of the embryo (French, 2000). Subcellular, cellular, tissue, or organ changes during critical phases of embryological development can impact growth, reproduction, and performance as well as thermoregulation and the subsequent acquisition of thermotolerance (Decuypere, 1984).

Incubation temperatures higher than the standard and their effects on organ and body weights in male broiler chicks have been demonstrated (Leksrisompong *et al.,* 2007). Heart and body weights were significantly smaller, and liver weights were significantly higher at hatch when chicks were exposed to 40.0°C from ED19 to 20 compared to 38.2°C. When egg temperatures of 38.4°C or 40.3°C were maintained from ED19 to 20, heart, gizzard, proventriculus, small intestine, and overall body weight were significantly smaller, and yolk sac weight was higher at hatch in chicks from the high incubation temperature. These findings are in

agreement with other studies that have reported decreased yolk-free body weight and decreased heart size and heart weight in overheated embryos compared to controls (Wineland *et al.,* 2000ab). Turkey embryos exposed to temperatures exceeding 37°C also expressed a decrease in heart weight and energy metabolism capabilities in cardiac tissue (Christensen *et al.,* 2004a). Hulet *et al.* (2006) reported that eggs exposed to temperatures between 37.8°C and 38.0°C during the last 5 or 7 d of incubation had a two percent greater hatch compared to eggs incubated at a temperature of 39.7°C and six percent greater than heat stressed embryos at 40.3°C. Heat stress in the hatcher also induced the incidence of dead embryos late in incubation, malpositions, red beaks, and enlarged yolk sacs (Hulet *et al.,* 2006). High incubation temperatures negatively impact chick quality by inducing poor coloring of the chicks, excessive blood in the eggshell, blood on the down and feathers, red hocks, unhealed navels, ectopic viscera, weakness, and a generally unthrifty appearance (Leksrisompong *et al.,* 2007).

The effects of high temperature on embryonic development reported by Lourens *et al.* (2005) suggested that high EST (38.9°C) during the third week of incubation caused a significant decrease in embryo length and yolk-free body weight at 21.5 d of incubation as compared to a constant EST of 37.8°C. Embryos heated to 39.6°C for 6 h daily from ED10 to18 had lower body weight, higher heart weight, and shorter tibia length at ED18 and longer shank length and decreased lung weight at ED21 relative to control embryos incubated at 37.8°C (Yalcin and Siegel, 2003). Thermal manipulation of embryos at 38.5°C for 3 h during ED16, ED17, and ED18 resulted in decreased body temperature and reduced plasma T_3 and T_4 levels at hatch compared to controls at 37.8°C (Yahav *et al.,* 2004). The reduction in plasma T_4 concentration suggests a decline in thyroid activity in thermal stressed embryos, while the T_3 decrease indicates reduced deiodination activity (Yahav *et al.,* 2004). The decreased body temperature together

with lower thyroid hormone concentrations in heat stressed eggs suggests a reduction in metabolic rate and may later impact the ability of these birds to respond to additional heat stressors during the post-hatch period. Body weights of broilers at 44 d of age were lower after incubation temperatures of 39.7°C compared to 38.6°C and 37.5°C (Gladys *et al.,* 2000). Additionally, feed conversion from 1 to 21 d post-hatch in the 39.7°C group was significantly higher (less efficient) compared to the other two incubation temperatures (Gladys *et al.,* 2000).

Elevated incubation temperatures not only impact broilers but non-optimal incubation temperature effects have been demonstrated in turkey embryos as well. Incubation temperatures exceeding 37.0°C at ED27 to 28 in turkey embryos impacted gut development as indicated by depressed embryonic jejunum weight (Christensen *et al.,* 2004b). Intestinal function was evaluated by measuring alkaline phosphatase (ALP), an enzyme that serves as an indicator of overall growth and maturation activities in the body. Total ALP activity was higher in poults exposed to 39.0°C than those exposed to 36.0°C, 37.0°C, or 38.0°C, which suggests a relationship between elevated incubation temperature stress and intestinal metabolism. Increased mortality in turkey embryos has been reported when poults are exposed to high incubation temperatures between ED15 to 20 and ED24 to 28. Additionally, these high temperatures may induce the incidence of excess albumin, ruptured yolk sacs, edematous heads, eye cataracts, and swollen down plumules (French, 1994). Hatchability in turkey poults was depressed when embryos were exposed to 38.5°C for different time periods throughout incubation relative to hatchability of the control temperature of 37.5°C (French, 2000).

Incubation temperatures that are too low or too high have been shown to impact overall embryo development, post-hatch chick quality, organ function and development, and bird performance. The effects of non-optimal incubation temperatures on development of the

immune system and initiation of the immune response in neonatal chicks have not been evaluated. However, based on previous reports of these temperature effects on the previously mentioned parameters, it is possible that post-hatch chick immunocompetency is impacted by exposure to early low or late high incubation temperatures.

Gastrointestinal Tract

The most anterior portion of the embryonic gut is known as the fore-gut, which serves as the derivative for the esophagus, crop, proventriculus, gizzard, duodenum, liver, gall bladder and pancreas. It is the first portion of the gastrointestinal tract to form anterior to posterior from the primitive gut tube. Fore-gut formation is considered complete by late ED2 to early ED3 (Romanoff, 1960). The hind-gut forms next, from posterior to anterior, and gives rise to the cloaca, rectum, large intestine, and ceca. The mid-gut is the last region to form, and contributes to the components of the small intestine, with the exception of the duodenum, which is previously formed from the fore-gut.

The gastrointestinal tract undergoes dramatic post-hatch morphological changes. At hatch, the intestinal villi are very small, and crypts are not detectable (Bar-Shira and Friedman, 2005). Crypts become well-defined by 2 to 3 d post-hatch and continue to increase in number until a plateau stage 48 to 72 h after hatch (Bar-Shira and Friedman, 2005). The number of intestinal enterocytes increases dramatically due to the increase in villus length during the first several days after hatch (Geyra *et al.,* 2001). The initial post-hatch period is critical in terms of intestinal structure and function as the chick is transitioning from dependence on yolk as the primary nutrient source to carbohydrate and protein-rich feed (Noy and Sklan, 2001), and this dietary transition period fuels intestinal development. Utilization of the yolk occurs through two

routes, the first of which involves direct transfer through circulation, and the second in which the yolk is transferred through the yolk stalk into the small intestine (Noy and Sklan, 2002).

The post-hatch period is crucial not only from a dietary transition standpoint and subsequent establishment of intestinal microflora, but also in the maturation of the gut-associated lymphoid tissue (**GALT)**, which is imperative for the induction of the immune response to intestinal pathogens. The maturation of GALT occurs in two distinct phases. The first stage occurs during the first week post-hatch, and the second stage occurs during the second week post-hatch. These respective stages are characterized by increases in mRNA levels of the cytokines interleukin-2 (**IL-2**) and interferon-γ (**IFN-γ**) (Bar-Shira *et al.,* 2003). Exposure to environmental antigens and the transition to feed induces the onset of GALT maturation, which is initiated by an increase in lymphocytes and natural killer cells in the intraepithelial lymphocyte compartment in the newly hatched chick (Bar-Shira *et al.,* 2003). New T lymphocytes arrive at 3 d post-hatch and promote the maturation of the lamina propria. The GALT B lymphocyte population begins to increase around 4 d after hatch and continues to increase throughout the first 2 weeks of life. Bar-Shira *et al.* (2003) further suggested that cellular responses mature earlier and are required in order for a humoral response to occur. Therefore, a lack of antibody response of the chick suggests T lymphocyte immaturity. It is understood that the innate immune response reaches maturity around 5 d post-hatch, while the adaptive response doesn't mature until the second week of life. Therefore, maternal antibodies play a crucial role in providing initial immune protection until the immune system has developed and matured.

During the last three days of incubation, intestinal weight as a proportion of body weight increases from 1% at ED17 to nearly 3.5% at hatch (Uni *et al.,* 2003b). However, small intestinal development may be impacted by a variety of environmental factors, one of which is

exposure to thermal stress. Twenty-eight day old male broilers reared in climatic chambers for two wks and exposed to thermally stressing temperatures of 30.0°C had decreased small intestinal weight relative to birds that were maintained at a non-stressful rearing temperature of 20°C (Garriga *et al.,* 2006). Twenty-four h exposure to heat stress (30.0°C) in 44 d broilers caused decreased crypt depth in the ileum compared to control temperatures (Burkholder *et al.,* 2008). Uni *et al.* (2001) evaluated the effects of early post-hatch thermal stress and its influence on intestinal morphology and proliferation of intestinal epithelial cells. After 24 h exposure of 3 d old male broiler chicks to 36.0°C, the jejunal mucosa exhibited narrow villi with large spaces between them and decreased villus volume 24 h post treatment. Immediately after heat exposure, the percentage of proliferating cell nuclear antigen (PCNA)-positive crypt cells was reduced in heat stressed chicks. Stress induced imbalances in the gastrointestinal tract can impact normal microbial populations and intestinal integrity and thus reduce innate protective mechanisms and increase subsequent susceptibility to pathogenic infections (Burkholder *et al.,* 2008).

The production of inflammatory cytokines in the intestinal mucosa is predominantly mediated by intraepithelial lymphocytes and intestinal epithelial cells. Intestinal enterocyte secretion of interleukin (**IL**) -6, IL-8, or TNF-α occurs during an inflammatory response (Sikora and Grzesiuk, 2007). Many of the pro-inflammatory cytokines produced are mediated by the transcription activator, NF-κB, through the NF-κB pathway. Thus, the blockage of this pathway will inhibit the production of pro-inflammatory cytokines and will minimize inflammation. Anti-inflammatory cytokines, such as IL-10 and IL-4 or a heat shock response also may mediate steps in the NF-κB pathway but have the ability to repress pro-inflammatory cytokine production (Malago *et al.,* 2002).

Mucins and Goblet Cells. The mucus gel layer serves as the first line of defense to pathogens that invade the intestine. This layer is formed through the secretion of mucins, which are large glycoproteins that maintain the integrity of the protective intestinal barrier. These mucins hydrate and gel to form a shield to destructive pathogens. The mucus gel is also comprised of water, electrolytes, sloughed epithelial cells, and immunoglobulins (Specian and Oliver, 1991). Secretory mucins are secreted by one of two processes: baseline secretion or compound exocytosis (Deplancke and Gaskins, 2001). Newly synthesized mucin granules are slowly and continually released during baseline secretion to maintain the protective mucus blanket, whereas an acute release of centrally stored mucin granules takes place during compound exocytosis. The release of hormones, neuropeptides, cytokines, and lipids may induce compound exocytosis (Deplancke and Gaskins, 2001).

Goblet cells are responsible for producing mucins. Goblet cells originate from stem cells at the base of the crypt or from poorly differentiated cells in the lower crypt. Once propagated, these immature goblet cells begin producing and secreting mucin granules immediately (Specian and Oliver, 1991). Immature goblet cells that are found deep within the crypts of the small intestine produce neutral mucins that contain little sialic acid; however, as the goblet cells mature and migrate towards the tip of the villus, the sialic acid concentration increases (Specian and Oliver, 1991). Goblet cell migration from the crypt to the villus tip and the process of cell sloughing into the intestinal lumen takes approximately 2 to 3 d; goblet cells are therefore being consistently replaced (Geyra *et al.,* 2001).

In the chicken, goblet cell development takes place during the late embryonic to immediate post-hatch period (Smirnov *et al.,* 2006). Intestinal goblet cells observed in the embryo at ED17 contained only acidic mucins, yet at hatch and up to 7 d post-hatch, both neutral

and acidic goblet cells were observed (Uni *et al.,* 2003a). When male broiler chicks were delayed access to feed and water for 48 h after hatch, they experienced increased density of goblet cells in the duodenum at d 2 and in the jejunum at d 2 to 3 (Uni *et al.,* 2003a). Additionally, mRNA gene expression of mucin in 28 d old male broilers was significantly higher in the duodenum and jejunum in those birds fasted for 72 h relative to those with unlimited access to feed. Results indicate that fasting can cause a goblet cell mediated innate and protective response in the small intestine, but it may be further suggested that embryonic or other post-hatch stress could elicit similar modifications in mucin dynamics and intestinal integrity.

Avian Immune System

In vitro studies have shown that the fundamental mechanisms of the immune response are identical in avian species, mammals, and most other vertebrates (Jeurissen *et al.,* 1994). The primary lymphoid organs in chickens consist of the bursa of Fabricius and the thymus. Secondary lymphoid organs include the spleen, bone marrow, and mucosa-associated lymphoid tissues. Within each respective lymphoid organ, there exists specialized sections in which antigen presentation to T cells occurs, T and B cells interact, or immunoglobulins (**Ig**) are produced (Jeurissen *et al.,* 1994). Immunoglobulins, the proteins that constitute antibodies, are present on the surface of cells and serve as receptors for antigen recognition by B cells. When antigen stimulation occurs, B cells respond by differentiating into plasma cells that produce antibodies that are antigen-specific. Three main classes of antibodies are produced by avian species: IgM, IgA, and IgG, the latter of which may be referred to as IgY in avians. The primary antibody response is elicited by IgM, followed by subsequent IgA and IgY production. Immune system development begins early during embryogenesis. Bursal precursor cells may be detected by ED7, and cells expressing surface IgM, IgG, and IgA may be detected at ED10,

ED14, and ED16, respectively (Sharma, 1999). Cells expressing surface CD3 appear at ED9 and surface T-cell receptor (TCR) are evident by ED12 (Sharma, 1999).

Embryonic Origin of Blood Cells. The nucleated avian erythrocyte is the only blood cell that is formed during the first two days of incubation (Lillie, 1952). Leukocytes develop from the mesenchyme and do not appear until later in development. Granulocytes may be recognizable in the blood stream by ED3, but they do not differentiate into mature adult cells until the latter portion of embryonic development (Lillie, 1952). Heterophils appear by ED5 but are not numerous in number until ED7. Eosinophils may be observed as early as ED7, but are typically not distinguishable until the last few days of incubation, and basophils are noted by ED14 (Lillie, 1952).

Primordial lymphoid cells develop from the mesenchyme after the second day of incubation. At the beginning of the twelfth day of incubation, the spleen begins to produce primitive lymphoid cells which will differentiate into lymphocytes and erythrocytes (Lillie, 1952). The embryonic mesenchyme is active in lymphoid cell formation; however, towards the end of the incubation period, hematopoietic activity is reserved for the spleen, thymus, bone marrow, bursa of Fabricius, and lymphatic tissue of the gastrointestinal tract (Lillie, 1952).

Bursa of Fabricius. The bursa of Fabricius appears around d 3 to 4 of embryonic development in chickens. It contains the following layers from exterior to interior: thin serosa, muscularis, mucosa, and epithelium of cylindrical or cuboidal cells. The thickest portion, the mucosa, establishes a structural foundation for the bursal follicles, which are embedded in the mucosa.

The immunological role of the bursa of Fabricius in antibody production was first documented by Glick and colleagues (Glick *et al.,* 1956). It serves as the primary lymphoid

organ for B cell maturation and the generation of antibody diversity. It is dorsal to the cloaca and composed of many longitudinal folds, called plicae, which consist of cuboidal epithelium. The bursal follicle, the functional unit of the bursa, originates from epithelial buds that appear at ED12 (Glick, 1983). The follicles arise from the epithelial cells in the lamina propria and are colonized by lymphoid cells. The follicles may be divided into both an inner medulla and an outer cortex separated by a basal membrane and epithelial cells. The medulla possesses epithelial cells, secretory cells, macrophages, plasma cells, lymphoblasts, and lymphocytes. The cortex region, lacking secretory cells, becomes much more apparent post-hatch (Glick, 1993). The cells of the medulla are separated from the bursal lumen by follicle-associated epithelium (FAE), which is derived in the embryonic bursa from mucosal surface epithelium. There are an estimated 8,000 to 12,000 FAE areas, or follicles per bursa (Glick, 1988). These FAE areas play a critical role in cloacal sampling, which suggests that environmental antigens may be taken up by the bursal lumen and FAE. This mechanism has been reported in chicken embryos beginning at ED15 (Sorvari *et al.,* 1977).

According to Toivanen and colleagues (1986), pre-bursal stem cells enter the bursa between ED8 to 14. By ED12, Ig genes undergo rearrangement and beginning on ED12, bursal stem cells with surface IgM appear. Antigen-independent production of the antibody repertoire occurs between ED12 to 21. The bursa experiences its most rapid growth between 2 and 3 wks of age and plateaus between 3 and 8 wks, followed by involution and regression (Glick, 1983). From the time of hatching until the involution of the bursa, the antibody repertoire is expanded and selected based on antigen-dependent processing of environmental antigens.

The lamina propria contains large blood vessels between the bursal follicles. The blood vessels vascularize the follicles, whereas lymph vessels do not enter the follicles but originate at

the follicle exterior (Ekino *et al.,* 1979). Approximately 5% of lymphocytes per day (Lassila, 1989) migrate from the cortex of the follicle to the lymph vessels (Ekino *et al.,* 1979). The medulla and cortex regions of the follicles consist mainly of B lymphocytes, while CD3 positive T lymphocytes may be found in the lamina propria between follicles, under epithelium, and intraepithelially (Jeurissen *et al.,* 1994). Additionally, mononuclear phagocytes may be recognized in the medulla and outer cortex as well as in the lamina propria, and dendritic cells may be detected in the medulla (Jeurissen *et al.,* 1994).

Spleen. During embryonic development, the spleen plays a critical role in erythropoiesis and granulocytopoiesis. It appears around ED5 in the developing embryo. During a brief period after hatch, the spleen is the primary organ to receive early post-bursal stem cells from the bursa (Toivanen, 1986). The spleen serves as a secondary lymphoid organ in the chicken and is surrounded by collagen and reticulum fibers. It is comprised of both red and white pulp, which contains both scattered lymphoid and nonlymphoid cells and densely packed lymphoid and nonlymphoid cells, respectively. Peri-arteriolar lymphoid sheaths (**PALS**) are found surrounding veins and venules and are made up of T cells. During a humoral response, germinal centers form in the PALS regions and consist primarily of B lymphocytes. Additional B cell areas exist in the peri-ellipsoid lymphocyte sheaths (**PELS**), as do macrophages, and plasma cells. It has been reported that B cells originating from PELS in the spleen may selectively bind antigen and assist in the initiation of the humoral immune response (Jeurissen, 1993). After intravenous injection of an antigen, antigens localize in the ellipsoids regardless of the type of antigen (Jeurissen *et al.,* 1994).

Thymus. The thymus is composed of two rows of seven lobes alongside the jugular veins on both sides of the neck. Each thymic lobe consists of a lobule, which contains both a

medulla and cortex. The thymus consists primarily of T lymphocytes, and its primary role is to promote the proliferation and maturation of the T lymphocytes. Within the cortex, T lymphocytes are found with CD3, CD4, and CD8 markers (Jeurissen *et al.,* 1994). Most cells in the medulla are CD3 positive. About 5 to 20% of the B lymphocyte population is found exclusively in the medulla, depending on the age of the bird (Jeurissen *et al.,* 1994). Germinal centers filled with B cells often form within the medulla, and the entrance of environmental antigens is restricted to the medulla due to the presence of the blood-thymus barrier in the cortex (Jeurissen *et al.,* 1994).

The lymphoid organs work in concert to maintain a functional and efficient system prepared to combat any adverse physiological changes and to identify and eliminate invading pathogens. However, if the onset of development of these organs is either delayed or accelerated due to environmental stressors, the competency of the lymphoid tissues and the capability of the immune system to elicit a response may be impacted.

Vaccination

Vaccination of flocks is a critical aspect of commercial poultry production to assist in preventing and minimizing spread of disease. In order for commercial producers to remain profitable, a vaccination program and strict biosecurity practices should be followed to maximize flock health. Birds that are exposed to infectious agents may experience subclinical infections, clinical disease, or death that may be characterized by immunosuppression and poor performance (Sharma, 1999). Several viral agents are known to cause recurring infections in commercial flocks, such as Newcastle disease virus (**NDV**) and Marek's disease virus (**MDV**). It is standard to protect flocks from these pathogens through vaccination programs. The poultry industry utilizes both active and passive immunization methods for disease control, although active

immunization through live vaccine administration is standard (Sharma, 1999). Post-hatch vaccine delivery mechanisms include aerosol, spray, eye drop, injection, or administration through drinking water (Sharma, 1999). In ovo injection systems serve as an effective vaccination method for embryos in the hatchery.

In ovo Vaccination. The commercial poultry industry currently employs labor-saving automated technology for in ovo vaccination of birds with minimized chick handling and improved hatchery management. In ovo vaccination involves injection of vaccine through the eggshell at ED18 when eggs are being transferred from the setter to the hatcher. At this stage of incubation, the embryo is well developed, fills most of the space within the egg with the exception of the air cell, and is surrounded by the amnion, which is enclosed in the allantoic membranes (Ricks *et al.,* 2003). The in ovo systems work by lowering an injection head onto the top of the egg that creates a small hole to allow a needle to descend into the egg to administer the vaccination (Johnston *et al.,* 1997). An advantage to in ovo vaccination is that protection from vaccination requires several days to develop, and by vaccinating the embryos, the time between vaccine administration and environmental exposure is increased (Johnston *et al.,* 1997). Additionally, egg injection systems reduce labor costs, ensure precise vaccination delivery, and reduce bird stress due to minimized handling (Ricks *et al.,* 2003). In ovo vaccination has replaced post-hatch injection of broiler chickens for MDV.

Marek's Disease Virus. Marek's disease is a destructive alphaherpes virus, and infection occurs by inhalation of dust contaminated with the virus from the feather follicle epithelium of infected birds. Although MDV pathogenesis is not fully understood, it is believed that macrophages from the lungs transport the virus to the bursa, thymus, and spleen, and it targets

the lymphocyte populations (Nair, 2005). Typically, MDV has atrophic effects on the bursa and thymus (Nair, 2005) and has an apoptotic effect on both B and T cells (Schat and Xing, 2000)

The first phase of MDV infection involves the destruction of B lymphocytes by apoptosis. Replication of the virus will then switch to activated T cells, and a latent infection will be established in these cells. This phase occurs around 7 d post-infection, and the infected T cells will carry the virus through the bloodstream to visceral organs, peripheral nerves, and feather follicle epithelium, the site of MDV replication (Baigent *et al.,* 2006). Cells occupying the visceral organs and nerves that are carrying the latent virus typically proliferate to form lymphoid tumors, inducing morbidity and mortality and therefore minimizing productivity and performance in infected birds (Baigent *et al.,* 2006). The last phase involves a reactivation of the virus which may cause a secondary infection cycle followed by further immunosuppression (Xing and Schat, 2000).

The activation of cytokines in response to MDV occurs during the first 4 to 5 d following infection and especially involves TNF-α and iNOS (Schat and Xing, 2000). Nitric oxide (**NO**) is a free radical generated by NO synthase (**NOS**) that plays a role as a chemical messenger in many physiological processes. Its inducible form, iNOS, may be expressed by macrophages as a result of cytokine production and the presence of bacterial toxins (Xing and Schat, 2000). Interestingly, NO has been found to inhibit the replication of MDV in vitro and in vivo (Xing and Schat, 2000). This finding provides plausible explanation for the importance of macrophages in MDV pathogenesis. The mechanism by which NO suppresses viral replication remains elusive; however, it is possible that NO has an antiviral effect on the host cells in which viral replication occurs (Xing and Schat, 2000).

Newcastle Disease Virus. Newcastle disease is caused by Newcastle disease virus (**NDV**) or avian paramyxovirus 1. Of all poultry species, chickens are the most susceptible to NDV, while geese and ducks are the least likely to be affected (Wakamatsu *et al.,* 2006). In gallinaceous birds, viral shedding is usually 1 to 2 wks in duration. Newcastle disease virus isolates may be categorized into three main pathotypes: lentogenic isolates which are mildly virulent and do not typically cause disease in adult birds, mesogenic isolates that cause respiratory disease, and velogenic isolates that are characterized by high mortality (Seal *et al.,* 2000). Exotic Newcastle disease (END) is a term used to describe NDV infection in birds exposed to a velogenic isolate and may induce nearly 100% mortality in unvaccinated flocks (Wakamatsu *et al.,* 2006). Clinical signs include sneezing, coughing, gasping, drooping wings, muscle tremors, paralysis, or sudden death (Wakamatsu *et al.,* 2006). Wakamatsu and colleagues (2006) isolated END from infected chickens, inoculated 4 wk old, specific pathogen-free White Leghorn chickens and reported enlarged and mottled spleens 2 and 3 d post-inoculation. These results correspond with those reported by Sijtsma *et al.* (1991), in which birds infected with NDV exhibited a significant increase in relative splenic weight, possibly induced by the formation of germinal centers. The most effective means for controlling NDV is vaccination using either live or inactivated vaccine strains. Nonvirulent vaccine strains used for vaccination protect the birds from NDV by eliciting an antibody response locally, systemically, or both (Al-Garib *et al.,* 2003). The humoral immune response to vaccination may be detected after 6 to 10 d in the blood and also locally (Al-Garib *et al.,* 2003). The Hitchner B1 strain of NDV is a commonly administered vaccination strain, and the oculotopical administration of this vaccine can enhance IgG, IgM, and IgA production in serum and tears (Russell and Ezeifeka, 1995). These results suggested that day old chicks respond well to live virus vaccination and

that IgM is the most actively involved in clearance of NDV infection. Vaccination of flocks for NDV helps eliminate the clinical signs that are induced by the virus; however, depending on the vaccination strain used, viral shedding and replication may not be fully eliminated (Russell and Koch, 1993).

Vaccination is a standard practice in commercial hatchery processing. However, chick processing at the hatchery involves many other procedures that may be inducers of stress, and these stress effects have not been a focus of research. Vaccination at hatch requires chick handling, as do other processing procedures such as sexing and sorting. Each of these procedures alone may impose stress upon the bird, but collectively, may impose an even greater stress upon the newly hatched chick. Stress experienced at the hatchery may continue to affect the chicks at time of placement at the farm and into the early brooding period.

Stress

Stress may be defined as a disruption of an organism's physiological homeostasis or well-being. Response to physical stressors is dependent on both the neural and endocrine systems. The neurogenic system (**NS**), consisting of the central nervous system and adrenal medulla, works in concert with the hypothalamus-pituitary-adrenal axis (**HPA**) to elicit a stress response. Response by the NS is rapid and is primarily mediated by the catecholamines, epinephrine and norepinephrine, which are critical players in the "fight or flight" response. These catecholamines ultimately induce the production of cyclic adenosine monophosphate (**cAMP**) which is crucial in energy reactions, but they also increase antibody formation, suggesting the impacts of stress on immune response efficiency. Activation of the HPA axis is typically induced in response to chronic stress. It begins with hypothalamic stimulation and induces an increase in adrenocorticotropic hormone (**ACTH**) from the anterior pituitary, which

subsequently causes increased production of adrenal cortical steroids, such as corticosterone. The effects of corticosteroid production include decreases in bursa, thymus, and spleen tissue, a reduction in circulating lymphocytes, and an increase in heterophils (Siegel, 1985).

Transportation Stress. Immediately before, during, and following transport, birds may be exposed to various stressors that include catching, handling, loading, motion, acceleration, impact, environmental temperature fluctuations, and withheld feed and water (Mitchell *et al.,* 1992). Most studies involving transportation stress have been conducted at slaughter age rather than post-hatch transport from the hatchery to the farm. Studies have suggested that stress experienced during transportation may increase disease susceptibility by suppressing the immune response due to production of corticosteroids through the hypothalamo-adrenocortical system and catecholamines from the sympatho-adrenal system (Warriss, 2004). The transportation stressor that is possibly of the greatest concern is heat stress due to its association with high mortality, decreased productivity, performance, and meat quality (Mitchell and Kettlewell, 1998). Mitchell and Kettlewell (1998) evaluated the effects of thermal stress during transport. Broilers that were 42 d of age were transported in crates for a 3 h time period with in-crate temperatures set between 22.0°C and 30.0°C and heat load being modified by adjusting water vapor density within the crate. Results showed that birds confined to crates during transport experienced hyperthermia as indicated by rectal temperatures. Creatine kinase (**CK**), an enzyme critical for the maintenance of energy levels, has shown elevated levels in stressed birds (Mitchell and Sandercock, 1995), which was in agreement with the elevated plasma CK levels observed in this study. Corticosterone was also elevated as reflected by heterophil to lymphocyte (**H:L**) ratios. Increased respiratory hypocapnia and alkalosis were reported in birds incurring higher heat loads during transport. In order to overcome thermal stress during

transportation and maintain birds in their thermoneutral zone, it has been suggested to ensure adequate air movement around the birds through the use of fans that would consistently circulate air (Webster *et al.,* 1993).

When stress effects prior to and following transportation were evaluated during October and July in vehicles with closed or open curtain sides, respectively, H:L ratios were nearly doubled, plasma CK levels were elevated, and eosinophil counts were reduced by almost 50% following transport (Mitchell *et al.,* 1992). Vibration and mechanical stress may also induce a stress response in transported broilers (Carlisle *et al.,* 1998). Broilers that were 41 d old were placed on hydraulic platforms that delivered varying levels of vibration selected to simulate the vibrations experienced during transport. Plasma CK levels were influenced by both vibration treatment and time, with peak plasma CK levels reported 8 h following treatment. Plasma glucose concentrations tended to fall in all treatment groups at 24 h post-vibration exposure. Plasma corticosterone levels increased during treatment, decreased to sub-basal levels at 8 h, and returned to basal levels by 24 h post-treatment. These changes suggest a physiological stress response to transport vibrations that may induce muscle damage and activation of the pituitary-adrenocortical axis. It appears that a variety of factors, such as temperature, time, and vibration, induce stress during transport of slaughter-age birds. These same factors may also cause stress to chicks during transportation from the hatchery to the growout farm.

Blood System Response to Stress. The different cells of the blood system possess distinct characteristics that enable them to carry out their respective physiological functions. In avian species, erythrocytes and thrombocytes are nucleated cells that function to transport oxygen and carbon dioxide and to play a role similar to platelets, respectively. Leukocytes may be subdivided into granulocytes and agranulocytes. Granulocytes consist of eosinophils,

basophils, and heterophils. Eosinophils play a defensive role against parasites such as worms or protozoa. Basophils function as mediators in the early inflammatory response, and heterophils serve to defend the host from bacterial pathogens (Maxwell, 1993). Agranulocytes include B and T lymphocytes, which assist in pathogen recognition and destruction associated with the immune response. Natural killer cells play a role in the control of bacteria, viruses, and parasites and present a cytolytic response against tumor cells (Multhoff, 2002).

According to Yahav and colleagues (1997), animals may be faced with acute cold or heat stress, seasonal temperature changes, and diurnal temperature cycle changes. Changes in the blood may be observed in response to these stressors, but it remains unclear as to whether these changes are attributed to the acclimation to environmental temperature or to the temperature stress itself. Yahav *et al.* (1997) reported that during wk 5 to 8 post-hatch, male broilers exposed to a constant ambient temperature of 10.0°C had significantly higher blood volume values represented as percent body weight than those exposed to 20.0°C. Hematocrit and hemoglobin concentrations decreased with increased ambient temperature (10.0°C, 20.0°C, or 30.0°C).

The H:L ratio is a good indicator of stress in chickens. A continuous dose of ACTH caused an increase in H:L ratios 4 and 7 d following the onset of ACTH administration (Puvadolpirod and Thaxton, 2000). Heterophil:lymphocyte ratios have been shown to increase during crating (Zulkifli *et al.*,2009) and after exposure to environmental heat stress, and increase further after exposure to multiple stressors (Macfarlane and Curtis, 1989). Birds exposed to heat stress lasting 3 h at 36 and 37 d of age caused increased H:L ratios and number of basophils, the latter of which is indicative of basophilia, a response to severe heat stress (Altan *et al.,* 2003). Environmental stressors appear to induce changes in cell dynamics in the blood system as a protective mechanism. Heat stress, especially, may result in elevated cell counts, making

evaluation of these parameters a valuable indicator of homeostasis disruption and initiation of a defensive response.

Immune Response to Stress. A variety of environmental or physiological stressors may induce a lymphoid tissue response, and to demonstrate this, exogenous administration of ACTH may be utilized to mediate the adrenal gland response. Continuous administration of ACTH via mini-osmotic pumps caused decreased relative bursa, thymus, and spleen weights 4 and 7 d following pump-implantation compared to control (Puvadolpirod and Thaxton, 2000). Oral corticosterone administration retarded spleen weights in 21 d old broilers (Post *et al.,* 2003). Heat stress in laying hens decreased antibody production to sheep red blood cells (**SRBC**) 1 and 4 wks following heat exposure (Mashaly *et al.,* 2004). These findings are in agreement with previous studies that have reported significantly lower antibody titers in heat stressed broilers (Khajavi *et al.,* 2003). Additionally, in vitro analysis of effects of hyperthermic conditions on T cell proliferation suggested that helper T cells are targeted during heat stress and increase in number in response to these hyperthermic conditions (Jampel *et al.,* 1983).

Heat Shock Proteins

Heat shock proteins (**HSP**) are molecular chaperones that may increase in expression under stress conditions. They are organized into superfamilies based on molecular weight (kDa), including HSP 110, HSP 90, HSP 70, HSP 60, HSP 47, and a group of smaller HSPs. These proteins are intracellular in nature under resting conditions, and they may be found in cytoplasm, mitochondria, endoplasmic reticulum, or cellular nuclei depending on the family to which they belong (Chen *et al.,* 2007). They function as molecular chaperones to prevent misfolding, denaturation, and aggregation of proteins. HSPs can be either inducible or constitutive. Inducible HSPs are not produced prior to stress exposure, whereas constitutive HSPs involve

proteins that are synthesized prior to stress and experience augmented synthesis under stress conditions.

Heat Shock Proteins and Immunity. Heat shock proteins are activators of the innate immune response and may function as their own adjuvant in inducing the immune response (Wallin *et al.,* 2002). They have the capacity to stimulate the production of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-1, IL-6, and IL-12, and the release of nitric oxide by monocytes, macrophages, and dendritic cells (Tsan and Gao, 2004). During the immune response, antigen presenting cells (**APC**), such as dendritic cells, present recognized antigen, which in turn induces signals for T cell activation. However, several alternative methods for adaptive immune response activation exist. Matzinger (1998) proposed that the adaptive response may be activated by endogenous substances that have been released from damaged or stressed tissues. This "danger theory" therefore suggests that HSPs alert the immune system of tissue damage through their upregulation in times of stress.

Heat shock proteins may have either cytoprotective or apoptotic cellular effects. These contrasting effects are dependent on the onset of HSP expression relative to the induction of the inflammatory response. This is termed the "heat shock paradox" as explained by DeMeester *et al.* (2001). The paradox suggests that if the heat shock response and HSP expression occurs prior to an inflammatory response, the subsequent effect on cells is cytoprotective. Conversely, if cells are "primed" by inflammation and then incur a heat shock response, cell death may occur. Another approach to the paradox is to consider that cytoprotection occurs when severe stress is followed by mild stress, whereas an apoptotic effect would be observed if the events occurred in the opposite order (Sikora and Grzesiuk, 2007). The heat shock paradox is not fully understood

but suggests a plausible explanation for differential cellular and tissue responses with regard to HSPs.

The cellular stress response will depend on the state of the cell and whether it is in a basal or activated state (Cobb *et al.,* 1996). Cellular responses to mild and severe heat stress will vary as mild heat stress will typically induce an adaptation in growth conditions, whereas severe heat stress will invoke cell death or morbidity (Park *et al.,* 2005). Regardless of whether the stressor is mild or severe, cell membrane integrity may be impacted, which thus causes activation of different signal transduction pathways, such as the Ras signal pathway or mitogen-activated protein kinases (MAPK). These pathways elicit the onset of the heat shock response (Park *et al.,* 2005).

When cells undergo necrotic cell death, HSPs are released into the extracellular space and release signals to elicit dendritic cell maturation and activate the NF-κB pro-inflammatory pathway (Basu *et al.,*2000). These events may therefore suggest the role of extracellular HSPs in innate immunity (Chen *et al.,* 2007). In contrast, as suggested by DeMeester *et al.* (2001), intracellular HSPs may play an anti-inflammatory role by inhibiting the NF-κB pathway. However, the inhibition of this pathway is dependent on NF-κB pathway suppression prior to cellular stress. These contrasting outcomes contribute to the ambiguity of the heat shock paradox, as the mechanisms by which HSP may be cytotoxic or cytoprotective by either promoting or inhibiting the pro-inflammatory pathway have not been fully elucidated.

Heat Shock Proteins and Broiler Heat Stress. The presence of HSPs during broiler embryological development is crucial for protein synthesis, newly synthesized protein binding, and the prevention of protein misfolding while offering a protective effect against stressful incubation conditions (Leandro *et al.,* 2004). Studies evaluating tissue responses to heat and/or

cold stress have reported that the different HSP expression levels may be attributed to varying levels of heat shock factors (Nakai and Morimoto, 1993). Heat shock factors activate a heat shock element that is an upstream promoter sequence in the heat shock gene. Under conditions of increased internal temperature, expression of hsp70 and induction of genes related to hsp70 synthesis may occur (Leandro *et al.,* 2004). Tissues that are essential for normal bodily function might be impacted by thermal stress more rapidly than less critical tissues (Flanagan *et al.,* 1995). When broiler chick embryos were exposed to heat stress (40.0°C), expression of hsp70 protein levels was significantly higher in cardiac tissue at ED13 and ED19 and in lung tissue at ED19 compared to control incubation temperatures of 37.8°C and to expression levels in liver, brain, and breast muscle (Leandro *et al.,* 2004). The heart is extremely active during incubation, and its viability is variable with temperature (Romanoff, 1960). Additionally, the tissue differences seen between the heart and lungs may be attributed to the fact that the heart develops earlier during incubation, while the lungs become active during the hatching process, specifically with the onset of internal pipping during the latter period of incubation (Leandro *et al.,* 2004). Increased expression of hsp70 appears to occur after exposure to elevated environmental temperatures, or thermal stress. Studies have evaluated hsp70 expression levels during embryonic development, but also during the post-hatch period in heat stressed broilers. Although no studies have evaluated hsp70 expression in the neonatal chick following incubation, it would be expected that the response would be similar to what has been previously reported.

Summary

It is evident that non-optimal embryonic incubation temperatures serve as stressors that can have a direct effect on organ development, acquisition of immunocompetency, and performance in broilers. Additionally, the exposure of embryos to non-optimal temperatures

may cause them to experience an increased sensitivity to subsequent stressors during the post-hatch period. Elevated incubation temperatures are a direct result of increased selection for growth rate in modern broiler strains, which correspondingly accelerates rate of growth and metabolic heat production of the embryo. Undesirably high temperatures are prevalent for late stages of embryonic development in multi-stage incubation systems due to differing embryo ages and different levels of metabolic heat production. Single-stage systems allow for better manipulation of incubation environment as all embryos require the same environmental conditions due to their comparable developmental stages; however, multi-stage programs yield lower operating costs as older embryos are used to heat the younger embryos. Regardless of the incubation system utilized, it is necessary to remember that many of the machines have not been modified to satisfy the changed demands of the modern bird. Therefore, there is much interest in the impact of non-optimal environmental conditions in the incubator on post-hatch chick development and performance.

The objectives of these experiments were to evaluate the effects of non-optimal incubation temperature profiles and post-hatch transportation and vaccination stressors. These objectives were tested by exposing embryos to temperature profiles selected to simulate multi-stage incubation environments followed by post-hatch exposure to transportation stress or vaccination. The effects from these parameters of interest were evaluated through expression of genes reflective of innate immune activity, peripheral blood cell profiles, antibody titers to vaccination, intestinal morphology, immune organ development, and performance.

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CHAPTER III

Incubation Temperature and Post-Hatch Transportation Stress Effects on Indicators of Immune System Development and Early Immune Response of Commercial Broilers

ABSTRACT Increased heat production by high yield broiler embryos, a result of genetic selection for rapid growth, often results in elevated temperatures during the latter phase of incubation and induces embryonic heat stress. Many commercial incubators have not been updated to compensate for the changing demands of the embryo as capacity for eggs has increased, and air cooling systems with adequate ventilation are not employed to dissipate the accumulated excess heat. Chick processing at the hatchery, followed by transport to the farm, may further impose stress upon the bird. The combined exposure of non-optimal incubation temperature and post-hatch transportation stress may impact immune system development and the immune response, which could further influence the bird's ability to respond to additional post-hatch stressors and affect bird performance. In this study, Cobb 500 eggs (n=5200) were incubated with the following shell temperatures during early and late incubation: low (**L**: 36.7°C), standard (**S**: 37.5°C), and high (**H**: 39°C). Eggs were incubated at S from embryonic day (**ED**) 8 to 14, and combinations of L or S during early (ED 0 to 7) incubation and S or H during late (ED 15 to 21) incubation, yielding four incubation treatment groups: LH, LS, SH, and SS. Chicks were separated into two transport groups at hatch: control (**C**: 34°C) and distressed (**D**: 40°C), for 8 total treatments: LH-C, LH-D, LS-C, LS-D, SH-C, SH-D, SS-C, and SS-D. Samples from the small intestine and immune organs were collected at day of hatch (**DOH**) both prior to and after transportation, d 4, and d 6 for evaluation of intestinal goblet cells and mRNA gene expression of heat shock protein70, Gallinacin-2, and Mucin-2 through relative quantative real-time PCR. Immune organ weights were collected at DOH (pre - and post-transportation)

and d 2, d 4, and d 6 for evaluation of relative organ weight. Birds were immunized with sheep red blood cells (**SRBC**) at d 14 post-hatch, and antibody titers to SRBC were evaluated at 6 d and 14 d post-SRBC administration. Transportation decreased bursa (P=0.0480) and increased spleen (P=0.0167) weights as a percentage of body weight (**BW**). An interaction (P=0.0141) of transportation and temperature influenced antibody production to SRBC 6 d after immunization. There was an interaction (P=0.0004) of transportation, age, and intestinal segment and an interaction (P=0.0004) of temperature and age for goblet cell number. Differences in mRNA gene expression of Gal-2 and Muc-2 in the small intestine presented an interaction (P=0.0033 and P=0.0056, respectively) of temperature and age, while intestinal gene expression of hsp70 showed an interaction (P=0.0147) of temperature, transportation, and age. Expression of hsp70 in the immune organs exhibited an interaction (P=0.056) of temperature and age. Collectively, these results provide insight into an influence of incubation and transportation stressors on development of the immune system and on the early immune response of the chick.
Key words: incubation, transportation, immune, broiler

INTRODUCTION

Selection for high-yield broiler lines over the past five decades has resulted in increased growth rate and improvement in feed conversion. This genetic progress, as reflected by improved performance parameters during the post-hatch period, directly impacts development and growth of the embryo through a substantial increase in embryonic heat production. Currently, the embryonic incubation period represents 30 to 40% of the broiler's lifespan (Ricks *et al.,* 2003); therefore, it is critical to provide an incubation environment that promotes efficient development so as to maximize post-hatch growth and performance.

Internal egg temperature, or the temperature directly experienced by the embryo, impacts embryonic development and hatchability more than the air temperature within the incubator (Joseph *et al.,* 2006). Therefore, eggshell temperature (**EST**) is accepted as a common representative of embryo temperature that is measurable without destroying the embryo or the eggshell. Under commercial incubation conditions, the optimum incubation temperature ranges between 37°C and 38°C (Hulet *et al.,* 2007). However, even if the average EST is 37.8°C, the age of the embryo and location of eggs within the incubator may cause EST discrepancies of up to 5°C (Lourens, 2001). In multi-stage incubators, it is not uncommon for low EST to be reported during early incubation as the amount of heat produced by the embryo is much less than the amount of heat lost due to evaporative cooling (French, 1997). Metabolic heat production by the embryo begins around embryonic day (**ED**) 4 and is substantial by ED 9 (Hulet *et al.,* 2007). Therefore, during the latter period of incubation, heat produced exceeds heat lost by the embryo, resulting in EST higher than the optimum (French, 1997). These dynamics in incubation temperature result from the difference in embryonic heat production and transfer of heat to the embryo by the machine itself (Meijerhof, 2002), factors directly related to the ventilation system within the incubator. The automation of incubators has increased with time (Deeming, 2002), but the majority of machines have not been modified to reflect the changing demands of the high-yield embryo, and as a result, heat often accumulates within the machine. With every 1°C increase in incubation temperature, the amount of heat produced by the embryo increases by 4.9% (Lourens *et al.,* 2006). The effects of elevated incubation temperatures on yolk-free body weight, chick length, hatchability, organ weights, and post-hatch performance have been reported (Lourens *et al.,* 2005; Hulet *et al.,* 2007; Leksrisompong *et al.,* 2007). Additionally,

high incubation temperature exposure has resulted in unhealed navels, ectopic viscera, red hocks, and overall weakness of the birds (Leksrisompong *et al.,* 2007).

Post-hatch transportation induces a multitude of stressors upon the bird, one of which is the fluctuation of environmental temperature (Mitchell *et al.,*1992). Transportation-induced stress may result in a suppressed immune response due to corticosteroid and catecholamine production (Warris, 2004). Heat stress during transport to processing may induce hyperthermia as indicated by rectal temperature (Mitchell and Kettlewell, 1998) and has been shown to result in high mortality, decreased performance, and reduction in meat quality (Mitchell and Kettlewell, 1998). The transport stressors that impact the bird prior to slaughter may also influence the chick during transportation from the hatchery to the growout facility.

To date, studies evaluating development and response of the immune system due to inadequate incubation temperatures or stressors associated with post-hatch transportation from the hatchery have not been reported. Burkholder *et al.* (2008) showed that imbalances in the gastrointestinal tract due to stress exposure could result in disruptions in intestinal integrity, thus impacting innate immune defenses and influencing protective mechanisms in the gut. Several studies have investigated the effects of heat stress on immune response parameters (Donker *et al.,* 1990; Khajavi *et al.,* 2003; Mashaly *et al.,* 2004), but these experiments focused exclusively on heat stress during the post-hatch period, not during embryonic development. The objective of this study was to evaluate the effects of different incubation temperature profiles reflective of those seen in commercial multi-stage incubators coupled with post-hatch transportation stress on development and response parameters indicative of early immunity in the neonatal chick. To fulfill some of the experimental objectives of this study, mRNA gene expression of several indicators of innate immunity were evaluated. Mucins are critical components of the protective

intestinal barrier and are secreted by intestinal goblet cells. Gene expression of the secretory mucin, **Muc-2**, is reflective of goblet cell activity and innate intestinal defenses. The antimicrobial peptide, **Gal-2** has been observed in heterophils (Sugiarto and Yu, 2004) and thus could play a role in innate immunity. Finally, a heat shock protein, **hsp70**, has shown increased expression with high environmental temperatures in broilers (Leandro *et al.,* 2004) and may therefore serve as a useful indicator for thermal stress. Changes in mRNA expression of these selected genes may provide insight into the response of the immune system to environmental stress.

MATERIALS AND METHODS

Animal Welfare

This experiment was approved and conducted under the guidelines set forth by the Institutional Animal Care and Use Committee at Virginia Tech.

Incubation Temperature

Cobb 500 eggs (n=5200) were obtained from a 31-week-old breeder flock. Eggs were incubated[1] at a North Carolina State University hatchery facility. To simulate a commercial multi-stage incubation environment, four temperature profiles were established to represent the range of temperatures experienced by the growing embryo during incubation. Temperatures were established as low (**L**: 36.7°C) or high (**H**: 39.0°C) relative to the industry standard temperature (**S**: 37.5°C). From embryonic day (**ED**) 0 to ED7, half of the eggs were incubated under L conditions and half were incubated under S conditions. All eggs were incubated at S from ED8 until ED14. From ED15 to ED21, half of the eggs were maintained at S and the other half were incubated at H. These temperature combinations yielded four total incubation

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[1] Natureform I40 Incubators, Jacksonville, FL

treatments: eggs incubated at L during the early phase and H during the late phase (**LH**), eggs incubated at L during the early phase and S during the late phase (**LS**), eggs incubated at S during the early phase and H during the late phase (**SH**), and eggs incubated at S during both the early and late phases (**SS**). Incubation temperature was regulated by thermistors connected to microprocessors with a temperature sensitivity of ± 0.05°C. Humidity was controlled with a comparable system by humidity sensors. Digital thermometers[2] were placed in each incubator tray to monitor temperature. Incubation temperature, humidity levels, and EST were evaluated and logged daily. At DOH, all chicks were sexed at the hatchery facility.

Transportation

Chicks were placed in hatchery chick trays and divided into two groups for a four-hour transport period to Virginia Tech. Two wooden transport boxes were built to regulate and control the environment during transportation. Each box consisted of an observational window, two air inlets, and a fan for ventilation control. Air entered the transport boxes via the air inlets, and the fan pulled the air across the box and across the stacked chick trays to ensure adequate ventilation. The transport boxes were placed in two cargo vans, and an equal number of chicks from each incubation treatment were placed in each. Ventilation was manually regulated in the control (**C**: 34°C) transport group to ensure that all chicks remained in their thermoneutral zone. The environment in the distressed transport group (**D**: 40°C) was manipulated to increase the respiratory rate of the chicks. The D group had increased panting and vocalization due to the increased environmental temperature, decreased ventilation, and reduction in oxygen availability. Chick box temperatures were recorded by an electronic data logger throughout transport. The combination of the four incubation temperature treatments with the two transportation treatments

[2] Cox, Lexington, NC

yielded 8 total treatment groups consisting of: LH-C, LH-D, LS-C, LS-D, SH-C, SH-D, SS-C, and SS-D.

Diets

Upon arrival at Virginia Tech, 23 males and 23 females (n=46 chicks) from each treatment were randomized and placed in floor pens (0.76ft^2/bird; n=8 replicates/treatment) with fresh pine shavings. Birds were reared under lighting conditions recommended for Cobb broilers and had *ad libitum* access to water and a corn-soybean based diet formulated to meet or exceed Cobb 500 nutritional requirements. Three dietary phases were utilized and consisted of a starter diet from d 0 to d 14, a grower diet from d 14 to d 28, and a finisher diet from d 28 until the termination of the study at d 42. Starter and grower diets contained monensin (Coban[3]) and bacitracin methylene disalicylate[4]. The finisher diet was medicated with Coban and virginiamicin (Stafac[5]).

Tissue Collection for RNA Isolation

Intestinal and immune tissue samples were collected at DOH (pre- and post-transportation), d 4, and d 6 post-hatch from 3 female birds/treatment for evaluation of hsp70, Muc-2, and Gal-2 mRNA gene expression. Each sampling bird was weighed and euthanized by cervical dislocation. Intestinal tissue samples were collected immediately from the duodenum (ascending loop), jejunum (from pancreatic duct to Meckel's diverticulum), and ileum (from Meckel's diverticulum to ileocecal junction). Each segment was rinsed in PBS, minced using razor blades, and homogenized. Homogenized tissue was weighed, and 20 to 30mg of sample was allocated to two microcentrifuge collection tubes to create duplicate samples. Samples were then snap frozen in liquid nitrogen and stored at -80°C until further analysis. Bursa, thymus, and

[3] Elanco Animal Health, Greenfield, IN
[4] Alpharma, Animal Health Division, Fort Lee, NJ
[5] Phibro Animal Health, Ridgefield, NJ

spleen samples were also collected, snap frozen in liquid nitrogen, and stored at -80°C until further analysis. The frozen immune organ samples were cut and weighed (20 to 30mg) over dry ice at the time of RNA isolation. Total RNA was isolated from both the intestinal and immune tissues using the RNeasy Miniprep Kit[6] using the animal tissue isolation protocol provided by the manufacturer. A nanodrop spectrophotometer[7] was utilized to evaluate total RNA concentration and purity by examining 260/280 and 260/230 ratios. RNA quality was determined by examination of 18s and 28s bands after gel electrophoresis using ethidium bromide stain.

Primer Design

Primer Express software (Version 3)[8] was used to design and synthesize forward and reverse primer pairs. Primer pair sequences utilized are presented in Table 3.1.

Reverse Transcription and Real-Time PCR

Total RNA was diluted to 0.2 µg/µL in RNAse-free water. The High Capacity cDNA Archive Kit[8] was utilized to perform reverse transcription and convert total RNA to cDNA. Following reverse transcription, cDNA was diluted to 1:30 and stored at -20°C until real-time PCR was performed. Relative quantification real-time PCR was conducted using an ABI PRISM 7300 Real-Time PCR System[8]. Samples were evaluated in duplicate in 96 well plates. Each well contained a reaction comprised of 2µl cDNA, 12.5 µl SYBR green PCR master mix[8], 0.5 µl forward primer (5µM), 0.5 µl reverse primer (5µM), and 9.5 µl RNAse-free water. The following machine settings were used to perform real-time PCR: 50°C for 2 min (1 cycle), 95°C for 10 min (1 cycle), 95°C for 15s and 60°C for 1 min (40 cycles). A dissociation curve was evaluated to ensure amplification of one product after completion of each run. Settings for the

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[6] Qiagen, Darlington Lab, Valencia, CA
[7] Thermo Scientific, NanoDrop™ 1000, Wilmington, DE
[8] Applied Biosystems, Foster City, CA

dissociation curve were as follows: 95°C for 15s, 60°C for 30 min, and 95°C for 15 min. The Ct values (for duplicate samples) for the target and endogenous reference gene, Glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**), were averaged, and the difference between the target and GAPDH averages were calculated to obtain the ΔCt value. The ΔΔCt values were obtained by taking the average ΔCt values for the designated intestinal tissue calibrator (duodenum SS-C) and each individual immune organ calibrator (bursa SS-C, thymus SS-C, and spleen SS-C). Fold changes in gene expression were then obtained by calculating $2^{-\Delta\Delta Ct}$ and are the data presented. Statistical significance was determined through evaluation of ΔCt values only.

Intestinal Morphology

Six birds per treatment were randomly selected for evaluation of intestinal morphology at DOH (pre- and post-transportation), d 4, and d 6. Tissue samples (3cm) were collected from the duodenum (mid-section of the ascendant loop), jejunum (mid-point from the pancreatic duct to Meckel's diverticulum), and the ileum (mid-point from Meckel's diverticulum to the ileocecal junction). Intestinal segments were flushed with cold PBS and fixed in 10% neutral buffered formalin. Each tissue was cut into 5 (10mm) sections and placed in a tissue cassette. Tissues were processed, embedded in paraffin, cut into 5µm sections, and mounted onto slides. Slides were stained using routine procedures for Alcian Blue (AB) periodic acid-Schiff reagent (PAS) staining (Luna, 1968; Carson and Pickett, 1983) and evaluated using a light microscope. Goblet cells along the perimeter of 4 villi were counted in each of 3 of the 5 tissue cross sections for each intestinal section. For each villus from which goblet cell number was evaluated, total villus area was calculated using SigmaScan Pro 5 software[9]. Villus area was measured from the

[9] Olympus America, Inc., Melville, NY

opening of the crypt to the tip of the villus. The total goblet cell count per villus area was log transformed prior to analysis (n=12 measurements/bird, 6 birds/treatment).

Immune Organ Development

Bursa and spleen samples were collected to evaluate immune organ development. At DOH (pre- and post-transportation), d 2, d 4, and d 6, sampling birds (n=20 at DOH; n=16 d 2 to d 6) were weighed and euthanized by cervical dislocation, and immune organs were collected and weighed. Immune organ weights are reported as a percentage of total body weight.

Antibody Response

To evaluate antibody response, subsets of each treatment (n=40/treatment) were transferred to grower batteries and divided into two groups (n=20/treatment): non-immunized control and sheep red blood cell (**SRBC**) immunized. Administration of SRBC (0.1mL of 0.5% SRBC suspension administered via the brachial vein) occurred at d 14. Blood samples were collected from the brachial vein at 6 d and 14 d following immunization. Antibody titers to SRBC were determined by the HI method described by Wegmann and Smithies (1966).

Statistical Analysis

Data were evaluated as a completely randomized experimental design arranged as a 4 x 2 factorial (4 incubation temperature treatments and 2 transportation treatments). Analysis of variance was performed using the GLM procedure of SAS version 9.1[10]. Values are reported as least squares (LS) means ± SEM. Data reported as a percentage were transformed prior to analysis using arc-sine (square root of percent), and SEM values were pooled. Immune organ development was analyzed with a statistical model comprised of the main effects of incubation temperature, post-hatch transportation, and age and all two and three way interactions for each

[10] SAS Institute, Inc., Cary, NC

immune organ. The statistical model for intestinal morphology included main effects of incubation temperature, post-hatch transportation, intestinal segment, and age, and all two, three, and four way interactions. The model for antibody titers consisted of the main effects of incubation temperature, post-hatch transportation, SRBC treatment, and all two and three way interactions. Main effects from incubation temperature, post-hatch transportation, age, tissue (or segment), and all two, three, and four way interactions comprised the model for the evaluation of gene expression. Gene expression of the immune organs is reflective of the bursa, thymus, and spleen analyzed collectively. Differences between treatments were adjusted using Tukey's test. Significance was established at $P \leq 0.05$. Pre-transportation DOH data were analyzed independently from all post-transportation data.

RESULTS

Immune Organ Development

The main effects of temperature and transportation resulted in significant differences in bursa weight as a percentage of BW at DOH pre-transport and post-transport, respectively (Table 3.2). Birds incubated under LH or LS conditions had significantly reduced bursa weights (P=0.0142) compared to the SS incubation group, while SH had similar weights to all incubation treatments. There were no differences in spleen weights prior to transport. Bursa weight as a percentage of BW (Table 3.2) of chicks transported under distressed conditions was decreased as compared to control transport conditions (P=0.0480). A two-way interaction (P=0.0327) of post-hatch transportation and age impacted spleen weight as a percentage of BW as spleen weight was increased in birds transported under distressed conditions compared to the control at all days except d 2 where spleen weight was slightly higher in control transported chicks (Figure 3.1).

Greater differences in spleen weight between control and distressed transport was observed at d 4 and d 6.

Antibody Response to SRBC

At 6 d post-immunization, there was a two-way interaction (P=0.0141) of temperature and transportation on antibody titer to SRBC (Figure 3.2). Birds that were incubated under early low and late high conditions (LH) had increased antibody titers in transportation stressed birds as compared to control transport, while the opposite was seen in birds incubated under SS conditions. In LS and SH incubated birds, there was little difference in antibody titers between control and distressed transport. Transportation impacted antibody production differently in non-immunized control and SRBC immunized birds as indicated by the two way interaction (P=0.0177) of transportation and SRBC treatment 14 d post-administration of SRBC (Figure 3.3). Birds transported under control conditions had similar antibody titers in non-immunized control and SRBC immunized groups. However, in birds that were stressed during transport, the SRBC immunized group had higher antibody titers than did the non-immunized controls. There were no main effects of temperature or transportation on antibody production to SRBC at either 6 d or 14 d post-immunization.

Goblet Cell Number

Exposure to transportation stress resulted in differential goblet cell responses in intestinal segments over time as reflected by the three-way interaction (P=0.0004) of transport, age, and segment (Figure 3.4). Under control transportation conditions, there was a decrease in goblet cell number from DOH to d 4 in all three intestinal segments, but the decrease was greater in the duodenum and jejunum. From d 4 to d 6, the ileum exhibited an increase in goblet cell number in contrast to the duodenum and jejunum, which both continued to decrease to d 6. In each

intestinal segment, transporting the birds under distressed conditions resulted in a decrease in goblet cell number from DOH to d 4, which was either maintained or increased slightly to d 6. Incubation temperature impacted intestinal goblet cell number with increased age as indicated by the two-way interaction (P=0.0004) of temperature and age (Figure 3.5). Birds incubated under early S or late H conditions exhibited a dramatic decrease in goblet cell number from DOH to d 4, which was maintained to d 6 in the SH and SS groups, but increased to d 6 in the LH group. In contrast, LS incubation conditions resulted in the fewest number of goblet cells at DOH, which decreased slightly to d 4, exhibiting the most goblet cells relative to the other treatment groups. The LS group then showed a dramatic decrease in goblet cells from d 4 to d 6. There was no main effect of incubation temperature on goblet cell number at DOH pre-transport.

mRNA Gene Expression

For each gene evaluated, there existed tissue, age, or a combination of tissue and age differences. However, these data will not be presented as the focus of this research was on the differences resulting from treatment effects.

Gal-2. Relative mRNA gene expression of Gal-2 in the small intestine was influenced by the interaction (P=0.0033) of incubation temperature and age (Figure 3.6). Birds incubated under LS conditions exhibited nearly a 2-fold higher level of Gal-2 gene expression compared to the other respective incubation treatments at DOH, and this expression decreased rapidly to d 4. However, all treatments showed a decrease in expression from DOH to d 4 and maintained a low level of expression to d 6. There were no differences in Gal-2 intestinal expression pre-transport. Additionally, no pre- or post-transportation differences in Gal-2 expression existed in immune tissue.

hsp70. A three way interaction (P=0.0147) of incubation temperature, transportation, and age resulted in varying levels of hsp70 gene expression in the small intestine with the most differential responses occurring at DOH and d 4 (Figure 3.7). At hatch, chicks from the LS incubation temperature group transported under control conditions exhibited a 2- to 2.5-fold higher level of hsp70 expression relative to the other treatments, which showed comparable expression levels relative to each other. However, this LS-C group experienced a dramatic decrease in gene expression from DOH to d 4. In contrast to the decreased expression of LS-C, the groups most stressed by incubation temperature and transportation, LS-D and SH-D, had increased expression. Other groups had relatively smaller changes in expression or remained similar to DOH expression levels. Between d 4 and d 6, all birds transported under distressed conditions exhibited a decrease in expression, with the exception of SS which showed an increase similar to the LS control transport group. Other treatments resulted in a slight decrease to no change in expression from d 4 to d 6. Incubation temperature and transportation resulted in a two way interaction (P=0.0317) in the intestine (Figure 3.8). Birds incubated under early L conditions and then exposed to transportation stress exhibited a reduction in hsp70 expression, while early S incubation temperatures coupled with transport stress resulted in an increase in expression. No differences were seen in intestinal expression of hsp70 prior to transportation at DOH.

Immune organ expression of hsp70 was altered by interactions of incubation temperature and age, as well as by the main effects of incubation temperature and transportation. There was a main effect of temperature (P=0.0224, Figure 3.9) at DOH prior to transportation. Birds incubated under LS and SH conditions exhibited higher hsp70 expression compared to LH and SS incubated birds.

The incubation temperature and age interaction (P=0.056, Figure 3.10) indicated a 2.5-fold higher level of hsp70 expression in immune tissue of LS birds compared to all other incubation treatments, which were at similar expression levels at hatch. Between DOH and d 4, however, LS birds experienced a decrease in expression, while expression by other treatments remained relatively constant. All groups had increased hsp70 expression from d 4 to d 6, and those groups with late H incubation, LH and SH, increased more as compared to the late S incubated birds. By d 6, birds incubated under late H conditions exhibited the highest level of gene expression compared to late S. Transportation distress resulted in a higher (P=0.057) level of hsp70 expression compared to control transport conditions (Figure 3.11).

Muc-2. The interaction (P=0.0056) of incubation temperature and age indicated the highest expression of Muc-2 in the small intestine under LH incubation conditions relative to other incubation treatments at DOH (Figure 3.12). All groups exhibited a decrease in expression from DOH to d 4, with the most dramatic decrease in the LH birds to reach expression levels comparable to other treatments by d 4. Expression level was maintained from d 4 to d 6 in all treatment groups except SH, which exhibited a slight increase in expression. Muc-2 expression at DOH pre-transportation was not significantly different between treatments.

DISCUSSION

Once fertilization of the ovum occurs and embryogenesis is initiated, embryonic development is heavily influenced by the environment (Wilson, 1991). Results from this study suggest that exposing embryos to temperatures above or below the optimum during specific periods of incubation (early L or late H) or to combinations of these temperatures potentially impact the onset of certain developmental or growth processes in the embryo, which ultimately influences post-hatch development in the young chick. Transportation stress, although

previously evaluated predominantly at slaughter age, may result in modifications of the immune response due to the production of corticosteroids as mediated by the hypothalamo-adrenocortical system (Warris, 2004).

In this study, the spleen and bursa responded differently to post-hatch transportation treatment as measured by organ weight. The spleen exhibited an increase in weight after transportation stress. A study conducted by Heitmeyer (1988) reported increased spleen mass in female mallards during molting. This increased organ size was attributed to a seasonal increase in blood volume, suggesting a greater amount of blood needing to be processed by the spleen. The stress imposed upon the chicks during transportation in this study induced hypoxia due to reduced ventilation and increased temperature. Due to this reduction in oxygen availability, it is possible that total blood volume was increased to attempt increased distribution of oxygen to tissues in response to this hypoxic stress. Additionally, the cardiovascular system may assist in mediating thermoregulatory processes by modulating dissipation of heat (Yahav *et al.,* 1997), which may be achieved through increased blood plasma volume as seen in chickens exposed to hyperthermic conditions (Whittow, 1964). The splenic response in this study is seen up to the last measurement at d 6, suggesting a potential long-term impact of transportation stress on organ function.

In contrast, the bursa responded to transportation stress with decreased organ weight. In a study evaluating stress effects due to restraint in rats, elevated plasma corticosterone was accompanied by decreased lymphocyte numbers, and interestingly, the number of B lymphocytes incurred a greater decrease compared to T lymphocytes (Dhabhar *et al.,* 1995). Peripheral blood leukocyte numbers were inversely related to plasma corticosterone levels, suggesting a role of the endocrine system in turnover or redistribution of immune cells (Dhabhar *et al.,* 1994).

Unfortunately, plasma corticosterone levels were not evaluated in this study; however, Mitchell and Kettlewell (1998) reported elevated corticosterone production as reflected by H:L ratios in response to transportation. Additionally, 35 d old broilers administered various levels of dietary corticosterone showed bursa involution with increased corticosterone administration (Malheiros *et al.,* 2003). Exogenous administration of adrenocorticotropin (ACTH) to 6 wk old broilers resulted in a reduction in relative weight of the bursa (Puvadolpirod and Thaxton, 2000). It can therefore be suggested that exposure to transport stress modulates corticosteroid secretion, which could have a negative impact on the immune system and subsequent bursal activity. A strong immune response is also expensive in terms of energy expenditure and may disrupt normal physiological activity (Wakelin, 1992), so it is possible that the priority of the bird following exposure to transport stress is to first attempt heat dissipation and distribution of oxygen to hypoxic tissues mediated by the spleen as previously suggested.

The reduction in bursa weight due to early L incubation temperatures may correspond to the fact that developing embryos are poikilothermic (Black and Burggren, 2004) and are therefore highly sensitive to temperatures to which they are exposed during incubation. These low incubation temperatures delay embryonic development resulting in longer incubation periods (Suarez *et al.,* 1996; Black and Burggren, 2004). This overall delay in development may directly impact the onset of bursa development beginning around ED3 to ED4, which is within the time period that embryos were exposed to suboptimal incubation temperatures in this study.

The humoral response during heat stress in broilers has shown a reduction in antibody titers to SRBC both 1 and 4 wk following heat exposure (Mashaly *et al.,* 2004) and a reduction in Newcastle disease antibody titers 4 and 7 d after initiation of heat stress (Zulkifli *et al.,* 2000). An increase in cytokine production due to stress (Ogle *et al.,* 1997) has been shown to result in

increased production of corticotropin releasing factor by the hypothalamus (Sapolsky *et al.,* 1987), which ultimately causes increased corticosteroid production by the adrenal glands. The end result is a reduction in mediators of the immune response (Sapolsky *et al.,* 2000). In this study, antibody titers to SRBC at 20 d was lower in birds incubated under SS conditions and transported under distressed conditions relative to control transport. It is possible that because these birds were not stressed during incubation, once they were exposed to transportation stress, the stress response was elicited and reduced the immune capacity of the birds. In contrast, birds exposed to either early L or late H incubation temperatures had already been exposed to a "conditioning" environmental stress, so when exposed to transportation stress, the detrimental effects on immune system function were minimized. Embryos that were heat stressed from ED16 to ED18 and then thermally challenged at 3 d post-hatch exhibited a reduction in corticosterone and decreased body temperature compared to control birds (Yahav *et al.,* 2004). This response may have implications in terms of epigenetic adaptations to temperature which has been suggested to influence the reaction of embryos or hatchlings to conditions to which they are later exposed (Tzschentke, 2007).

Mucus production plays a critical role in mucosal protection. Production of mucin is mediated by goblet cells, and cytokines modulate mucin production and proliferation of goblet cells (Blanchard *et al.,* 2004). Goblet cell density increases slightly in the duodenum with increasing development, but density increased more rapidly in both the jejunum and ileum during late incubation and post-hatch (Uni *et al.,* 2003). Interestingly, this response was seen in chicks transported under distressed conditions as opposed to control conditions, but it did not begin until 4 d post-hatch. Currently, no studies exist that have evaluated the effects of broiler chick post-hatch transportation on intestinal morphology. Control transported chicks had a decrease in

goblet cell number with increasing age, suggesting possibly that transportation itself, even with the effects of environmental stressors eliminated, has an impact on intestinal development, which is especially critical in the newly hatched chick. Alternatively, between d 4 and d 6 post-hatch, distressed transport resulted in an increase or a maintained number of goblet cells in all three intestinal sections. This suggests that stress possibly altered cytokine induction necessary to increase mucin production and promote intestinal development. However, it appears as if transportation serves as a stressor, and when non-optimal transportation is incurred, the birds are somewhat more responsive.

The LH and LS groups were most affected in terms of goblet cell number relative to the other incubation temperature treatments, specifically between d 4 and d 6. Previous research from our laboratory has shown that between d 4 and d 6, villus height to crypt depth ratios in the jejunum were decreased in the LS group and increased in the LH group, which is the same trend that was seen in goblet cell number in all intestinal segments in this study. Taken together, these responses could be a result of cellular turnover in the intestine as goblet cells are short-lived (Uni *et al.,* 2003) and undergo a migratory process from the crypt to the tip of the villus along with epithelial cells (Geyra *et al.,* 2001). It is possible then that cellular turnover in the LS and LH groups is accelerated and delayed, respectively. Gene expression of Muc-2 corresponded with decreased goblet cell number in the LS group and, as expected, showed a gradual decline in expression between d 4 and d 6. Expression of Muc-2 was maintained between d 4 and d 6 in the LH group, which does not directly relate to the increased number of goblet cells observed, but suggests the influence of other factors in promoting the production of intestinal mucin. Overall, these observations indicate an influence of non-optimal incubation temperatures during early and late embryonic developmental periods on early intestinal development and gut

integrity. These results have further implications in terms of establishment of intestinal microflora in the newly hatched chick, as mucin promotes proliferation of specific microflora due to its high carbohydrate content (Deplanke and Gaskins, 2001) and could therefore influence subsequent susceptibility to enteric disease.

The avian β-defensin, Gal-2, has been reported in heterophils (Sugiarto and Yu, 2004), which is supportive of its potential indirect role in the innate defense system. The gradual decrease in Gal-2 expression during the first week post-hatch observed in this study corresponds to results previously reported (Bar-Shira and Friedman, 2006). Increased heterophil counts are indicative of stress in chickens (Siegel, 1985), which may provide explanation as to why the LH and LS temperature groups exhibited the highest level of Gal-2 expression at DOH. Additionally, during the first 24 h immediately after hatch, there is a significant outpouring of heterophils from the spleen (Lucas and Jamroz, 1961), which could further contribute to Gal-2 expression and also provide further explanation for increased splenic activity as induced by stress as previously discussed. Therefore, it is possible that an elevated number of heterophils could directly impact Gal-2 gene expression and serve as a further indicator of exposure to stress. However, hematological evaluation would need to be conducted to further validate this theory.

Gene expression of hsp70 in both the immune organs and intestinal tissue was highest at hatch in the LS incubated birds. Expression of hsp70 has been shown to increase under conditions of elevated internal temperature in broilers (Leandro *et al.,* 2004), but interestingly, the LS birds were never exposed to elevated incubation temperatures. A study by Geers *et al.* (1983) reported that low incubation temperature resulted in compensatory growth due to increased metabolic heat production. Therefore, it is possible that the LS birds produced an overall greater amount of metabolic heat at hatch to compensate for early suboptimal

temperature exposure, thus resulting in higher hsp70 expression. Late H temperatures resulted in higher hsp70 expression by d 6, suggesting a delayed onset of expression due to high temperature exposure.

The immune organs and intestinal tissue exhibited different responses to transportation treatment in terms of hsp70 expression. The heat shock paradox proposed by De Meester *et al.* (2001) offers a plausible explanation for differential cellular and tissue responses with regard to HSPs. The paradox suggests that if inflammation occurs prior to heat shock, cellular death occurs, whereas if inflammation occurs after heat shock, the effect is cytoprotective. Evaluation of hsp70 expression in the bursa, thymus, and spleen, collectively (data not shown), showed that a late H incubation temperature plus transport stress resulted in elevated hsp70 expression, which could have occurred because these birds were essentially heat stressed twice, both during incubation and transportation. Therefore, resources were allocated towards protection of the immune organs in response to heat exposure. This response may be due to the fact that these organs are exclusively responsible for lymphocyte production and proliferation, and if the functionality of the organs were compromised, the bird would be unlikely to elicit an efficient response to any immunogen. In the intestinal tissue, early L incubation temperatures followed by transport stress resulted in decreased gene expression. It is possible that the increased metabolic heat production due to low temperature exposure may be more physiologically taxing than heat stress imposed exogenously. The response of the bird may therefore be to first protect from this physiological stress, and the effects of heat stress become a secondary focus. When followed by heat shock, which alone has protective effects that one would think could complement the inflammatory response, more damage may be elicited, thus resulting in decreased hsp70 expression.

It appears that the effects of transportation may have been more dramatic than those effects related to incubation temperature, specifically in terms of hsp70 expression. Both transportation and incubation temperature serve as stressors, however the type of stress and the subsequent responses to the stress that they impose could be very different. Non-optimal incubation temperatures impose obvious heat or cold stress, as well as hypoxic and oxidative stressors. Transportation inflicts those same stressors, but additionally, could also result in stress due to the handling, motion, acceleration, and impact of transport (Mitchell *et al.,* 1992). These other transportation stressors that are not experienced by the bird during incubation may be the reason that transportation appears to more dramatically affect hsp70 gene expression. Additional impacts of post-hatch chick transportation beyond thermal stress should be evaluated to further identify the effects of these additional stressors.

These data suggest the influence of incubation temperature and transportation stress, alone or in combination, on immune and intestinal development and the corresponding functions of these respective organ systems. In general, it appears as if these stressors at times may have detrimental additive effects when applied in combination, but it was also observed that exposure to one stressor may prime the bird for exposure to subsequent stressors. The innate defense mechanisms in the stressed chicks seem to be delayed, but the adaptive response at a later post-hatch age remains relatively unaffected despite previous stress exposure. These results warrant further research to better elucidate the mechanisms responsible for inducing these responses.

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Gene	GenBank ID	Description	Sequence: forward/reverse
GAPDH	NM_204305	Glyceraldehyde-3-phosphate dehydrogenase	GCCGTCCTCTCTGGCAAAG/ TGTAAACCATGTAGTTCA
hsp70	NM_001006685	Heat shock protein 70	ACTGCTCTCATCAAGCGTAACAC/ GTCTGAGTAGGTGGTGAAGGTCTG
Gal-2	NM_204992	Gallinacin 2	GTCCCAGCCATCTAATCAAAGTC/ AGGCCATTTGCAGCAGGAA
Muc-2	XM_421035	Mucin 2	CGTACACTGGTGGAAAATATTCTGA/ CAACATCCTTTGTTAGCTCAATAGCT

Table 3.1 Forward and reverse primers used for relative quantification real-time PCR. Primers were designed by Primer Express software (Applied Biosystems, Foster City, CA).

Incubation Temperature	**Bursa % BW DOH pre-transport**
LH	0.095 ± 0.0011^{b}
LS	0.092 ± 0.0011^{b}
SH	0.102 ± 0.0011^{ab}
SS	0.123 ± 0.0011^{a}
P	0.0142

Transportation	**Bursa % BW DOH to d 6**
Control	0.140 ± 0.009^{a}
Distressed	0.134 ± 0.009^{b}
P	0.0480

Table 3.2 Effect of incubation temperature and transportation on bursa weight (% of BW) of Cobb 500 broiler chicks. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Transportation treatments were designated as Control (C: 34°C) or Distressed (D: 40°C). Data are presented as LS means ± SEM. There was a main effect (P= 0.0480) of transportation from DOH to d 6 (n=68/treatment) and a main effect (P=0.0142) of temperature at DOH prior to transportation (n=20/treatment).

[a,b] Data within a column lacking a common superscript differ significantly

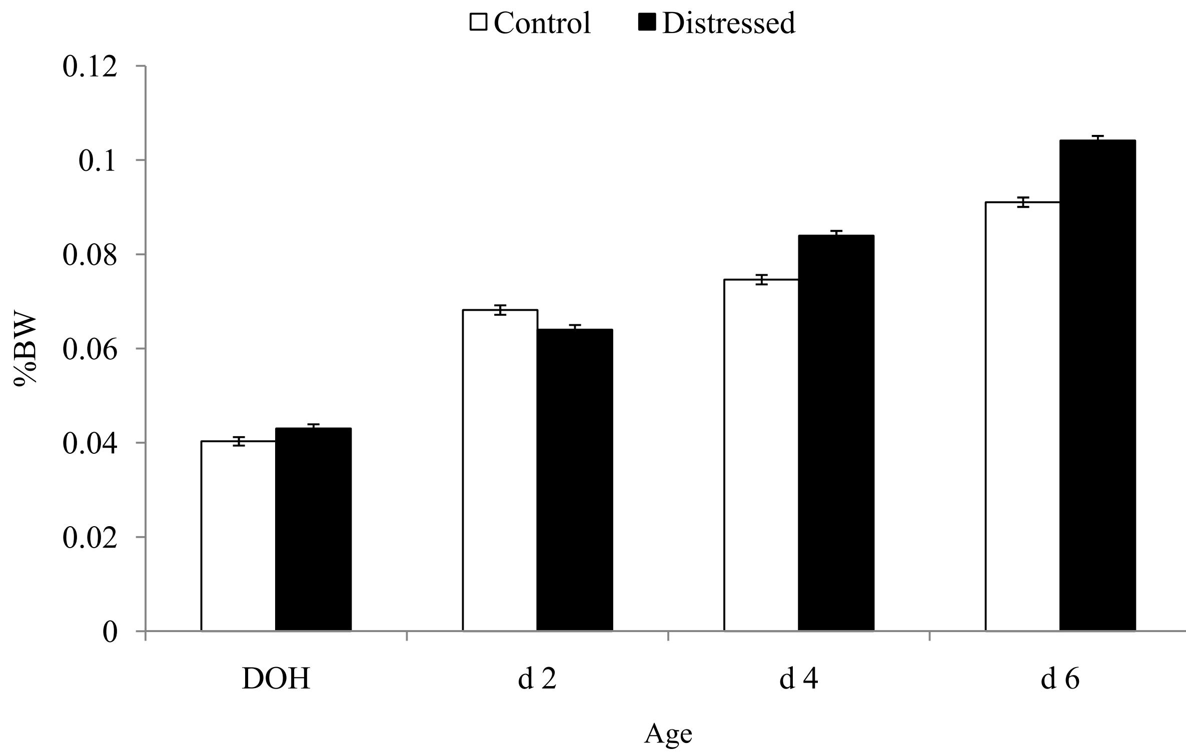

Figure 3.1 Effect of post-hatch transportation and age on spleen weight (% of BW) of Cobb 500 broiler chicks. Transportation treatments were designated as Control (C: 34°C) or Distressed (D: 40°C). Data are represented as LS means ± SEM (n=20/treatment at DOH; n=16/treatment at all other ages). There was a two-way interaction (P=0.0327) of transportation and age.

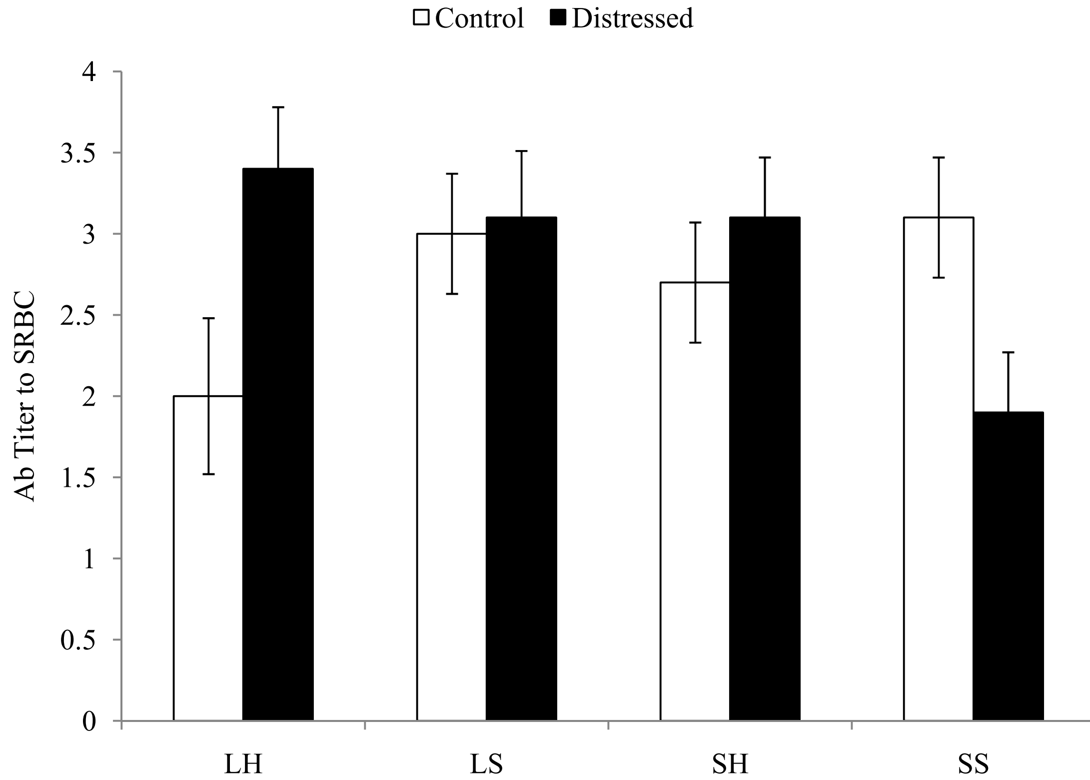

Figure 3.2 Effect of incubation temperature and transportation on antibody response of 20 d old Cobb 500 broilers 6 d following sheep red blood cell (SRBC) administration at d 14. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Transportation treatments were designated as Control (C: 34°C) or Distressed (D: 40°C). Data are presented as LS means ± SEM (n=40/treatment). There was a two-way interaction (P=0.0141) of incubation temperature and transportation.

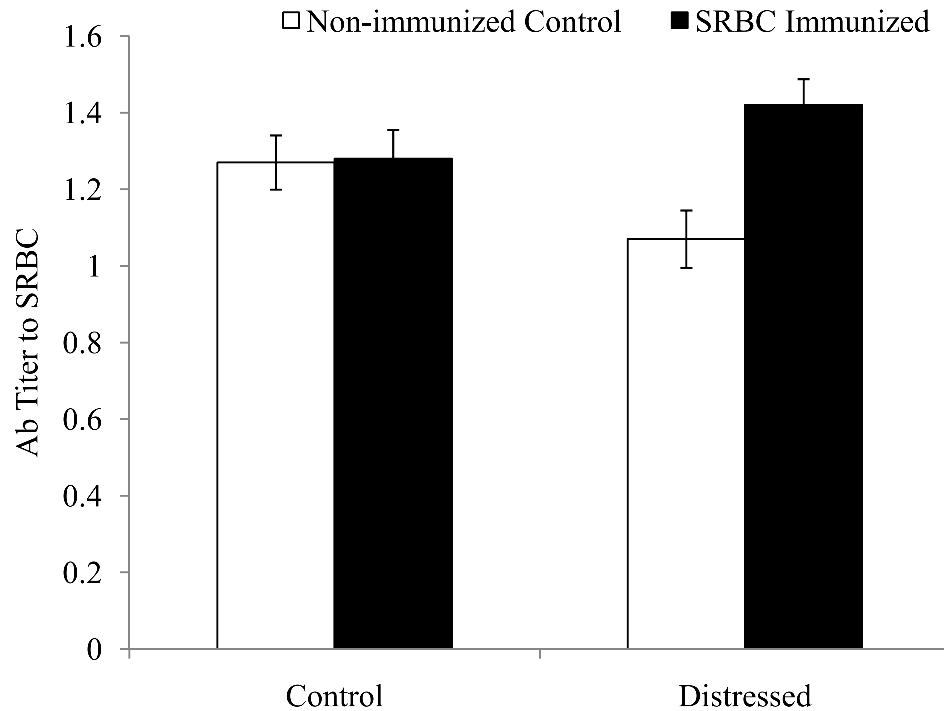

Figure 3.3 Effect of SRBC administration and transportation on antibody response of 28 d old Cobb 500 broilers 14 d following sheep red blood cell (SRBC) administration at d 14. SRBC treatments were designated as non-immunized control or SRBC immunized. Transportation treatments were designated as Control (C: 34°C) or Distressed (D: 40°C). Data are presented as LS means ± SEM (n=80/treatment). There was a two-way interaction (P=0.0177) of SRBC administration and transportation.

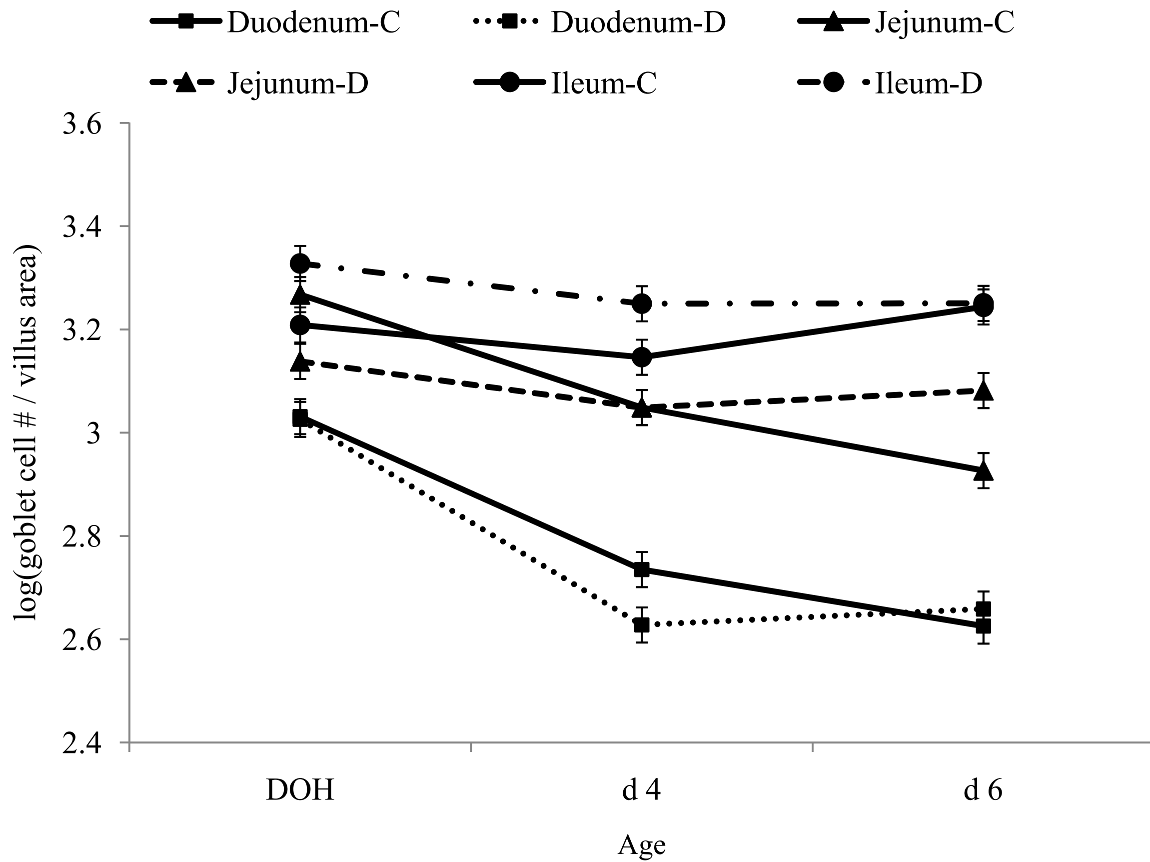

Figure 3.4 Effect of transportation, age, and intestinal segment on goblet cell number in Cobb 500 broilers. Transportation treatments were designated as Control (C: 34°C) or Distressed (D: 40°C). Intestinal tissue samples were taken from the duodenum, jejunum, and ileum. Data are presented as LS means ± SEM (n=24/treatment). There was a three-way interaction (P=0.0004) of transportation, age, and intestinal segment.

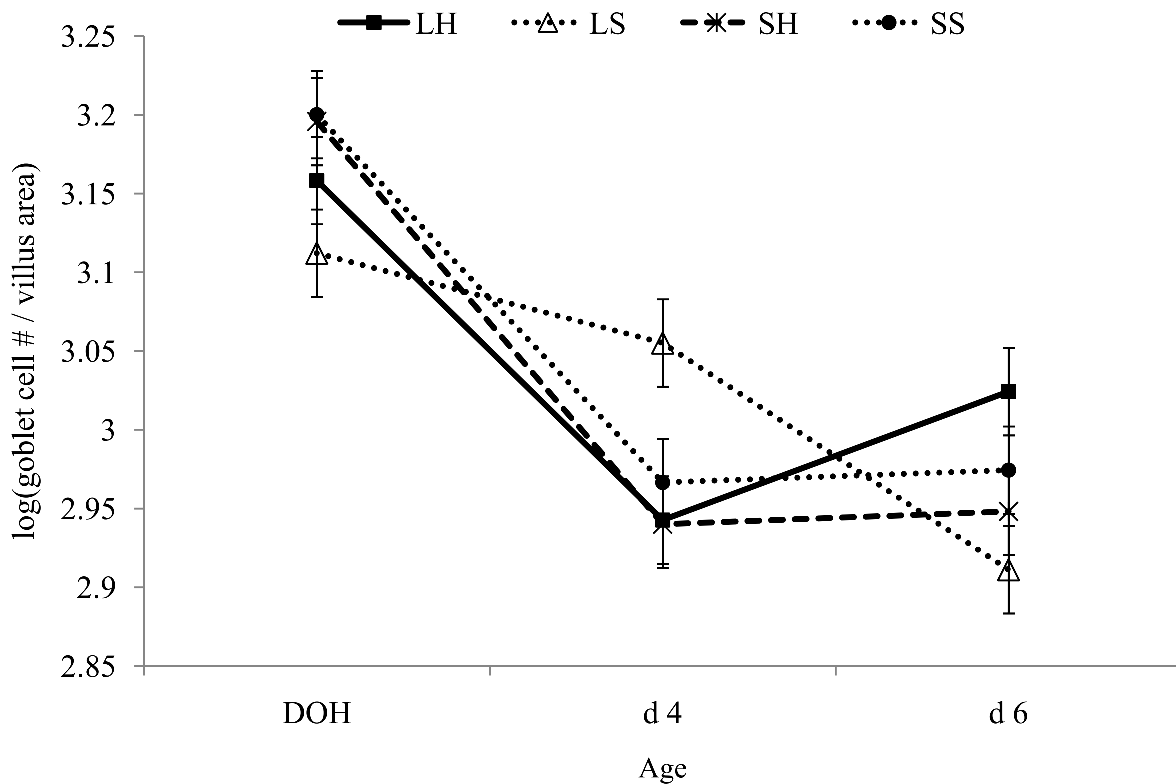

Figure 3.5 Effect of incubation temperature and age on intestinal goblet cell number in Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Data are presented as LS means ± SEM (n=36/treatment). There was a two-way interaction (P=0.0004) of incubation temperature and age.

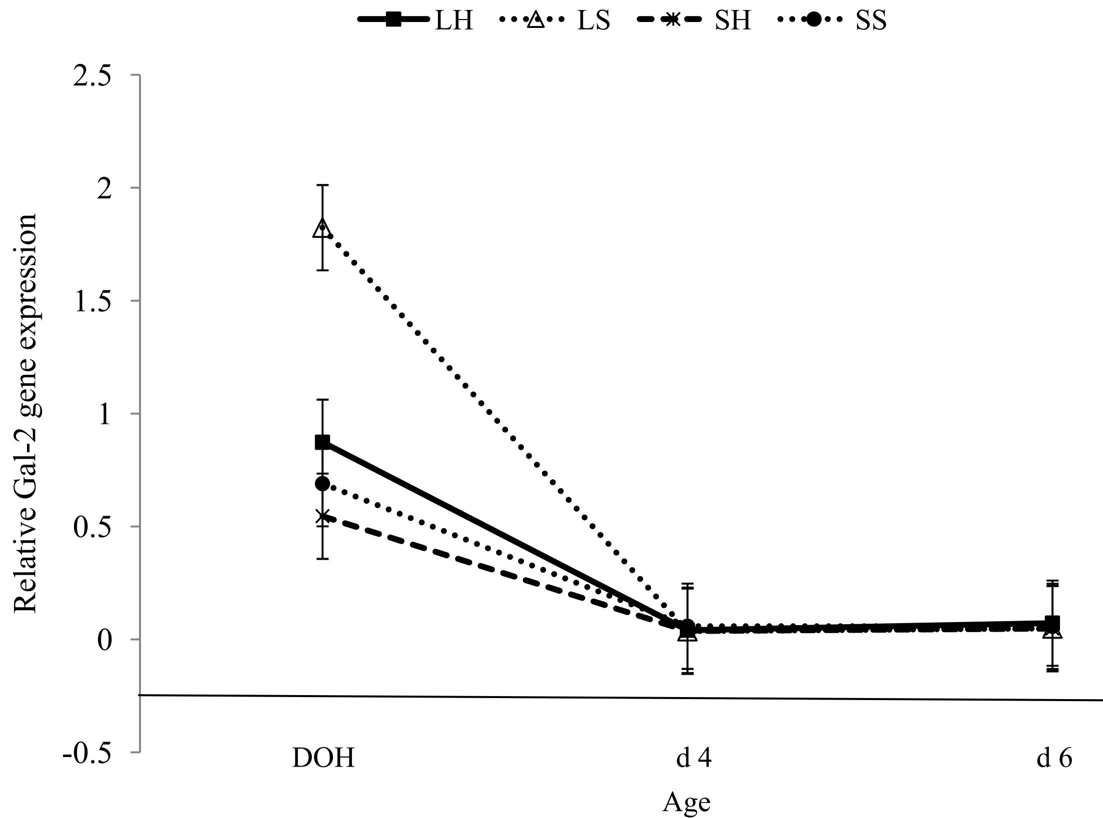

Figure 3.6 Effect of incubation temperature and age on relative mRNA expression of Gal-2 in the small intestine of Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Relative gene expression ($2^{-\Delta\Delta Ct}$) for Gal-2 was calculated using the ΔΔCt method with the average ΔCt value for duodenum in the SS-C group as the calibrator. Data are presented as LS means ± SEM (n=18/treatment). There was a two-interaction (P=0.0033) of temperature and age.

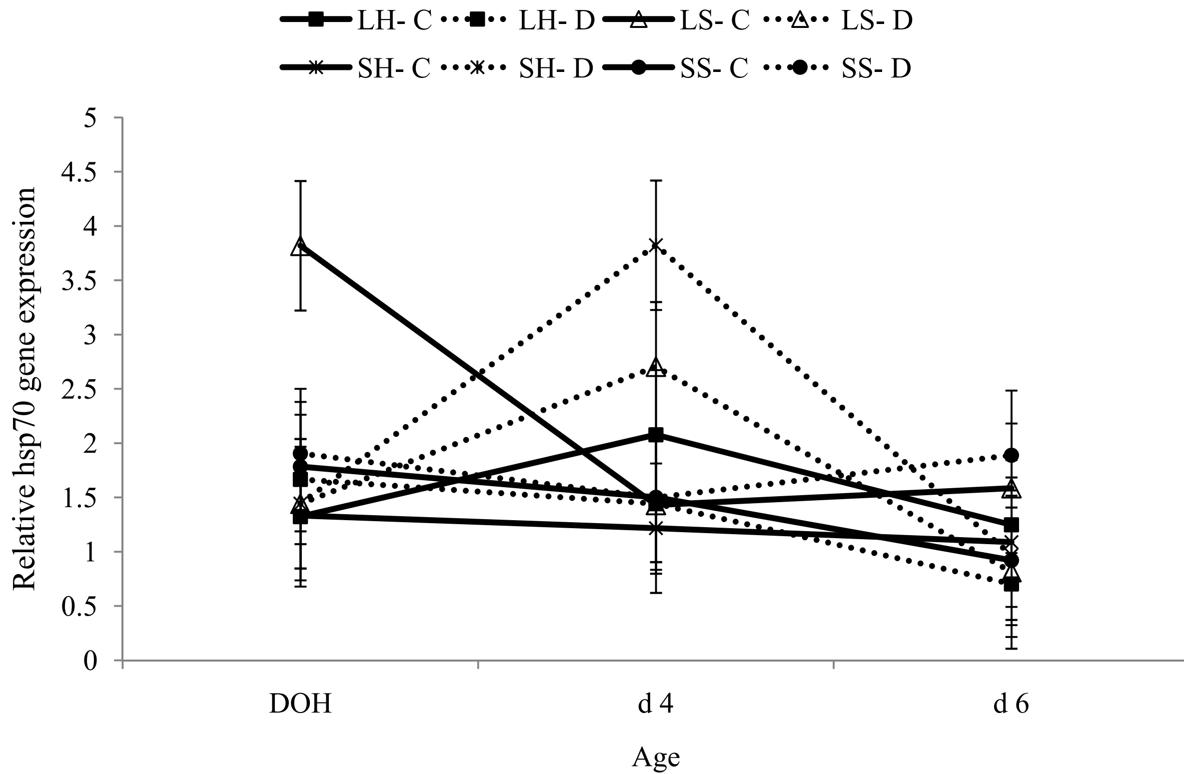

Figure 3.7 Effect of incubation temperature, transportation, and age on relative mRNA expression of hsp70 in the small intestine of Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Relative gene expression ($2^{-\Delta\Delta Ct}$) for hsp70 was calculated using the ΔΔCt method with the average ΔCt value for duodenum in the SS-C group as the calibrator. Data are presented as LS means ± SEM (n=9/treatment). There was a three-way interaction (P=0.0147) of temperature, transportation, and age.

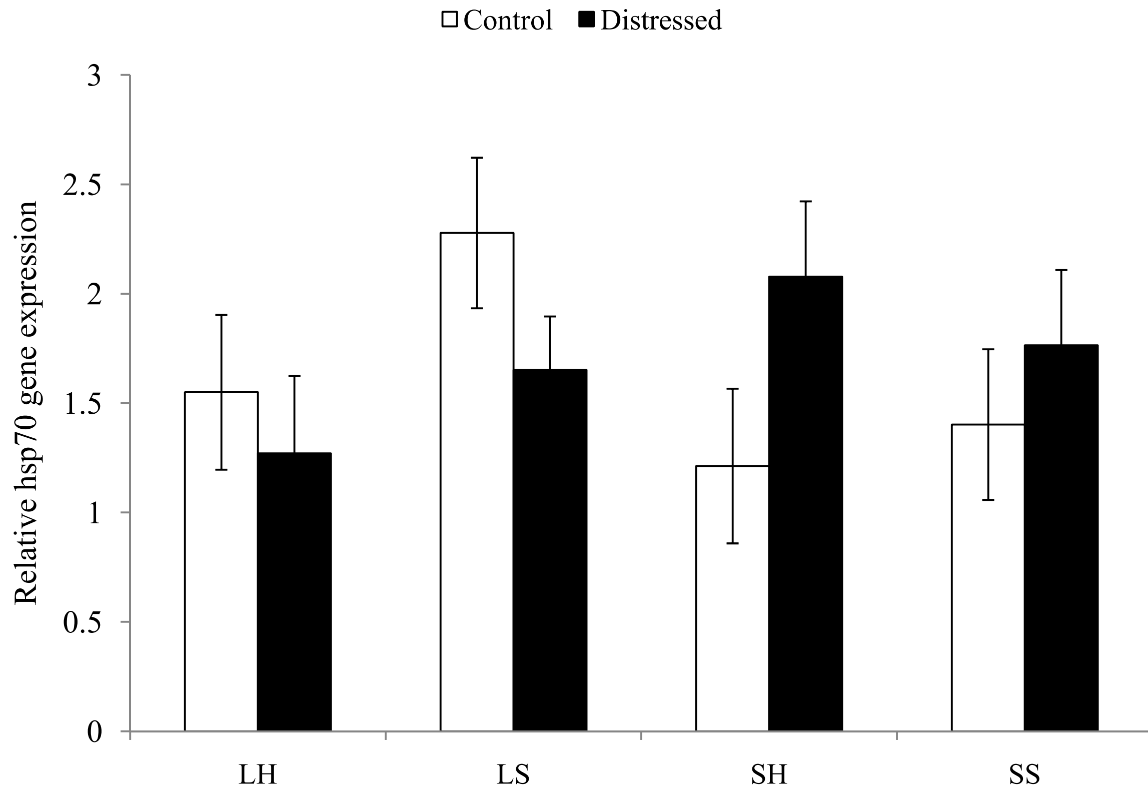

Figure 3.8 Effect of incubation temperature and transportation on relative mRNA expression of hsp70 in the small intestine of Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Relative gene expression ($2^{-\Delta\Delta Ct}$) for hsp70 was calculated using the ΔΔCt method with the average ΔCt value for duodenum in the SS-C group as the calibrator. Data are presented as LS means ± SEM (n=27/treatment). There was a two-way interaction (P=0.0317) of temperature and transportation.

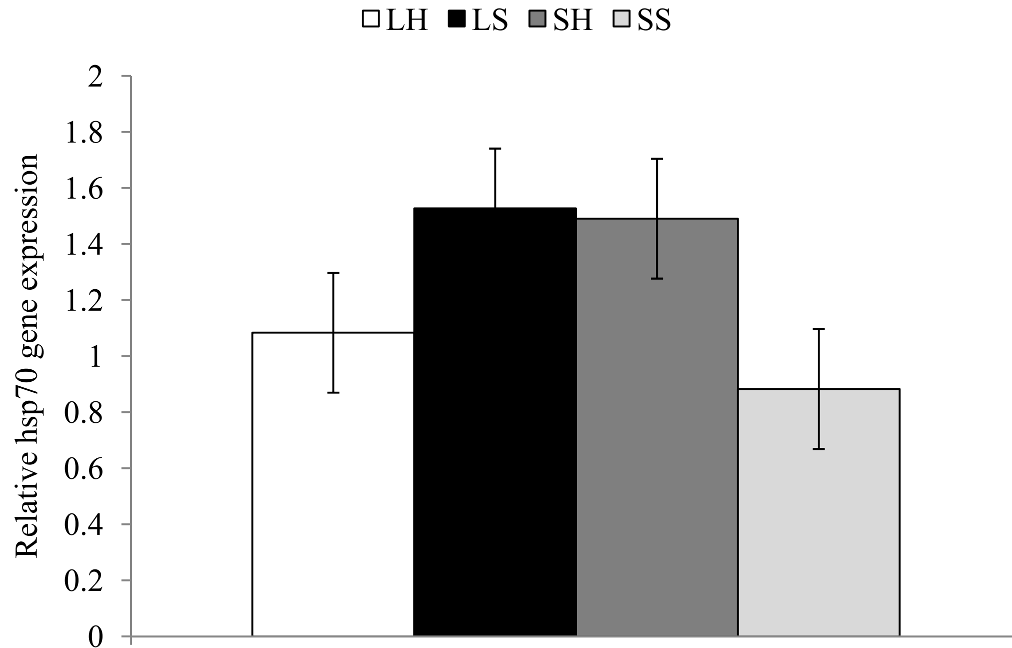

Figure 3.9 Effect of incubation temperature on relative mRNA expression of hsp70 in the immune organs (bursa, thymus, and spleen, collectively) at DOH prior to transportation in Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Relative gene expression ($2^{-\Delta\Delta Ct}$) for hsp70 was calculated using the ΔΔCt method with the average ΔCt values for bursa, thymus, and spleen in the SS-Control group, respectively, as the calibrator(s). Data are presented as LS means ± SEM (n=16/treatment). There was a main effect (P=0.0224) of temperature.

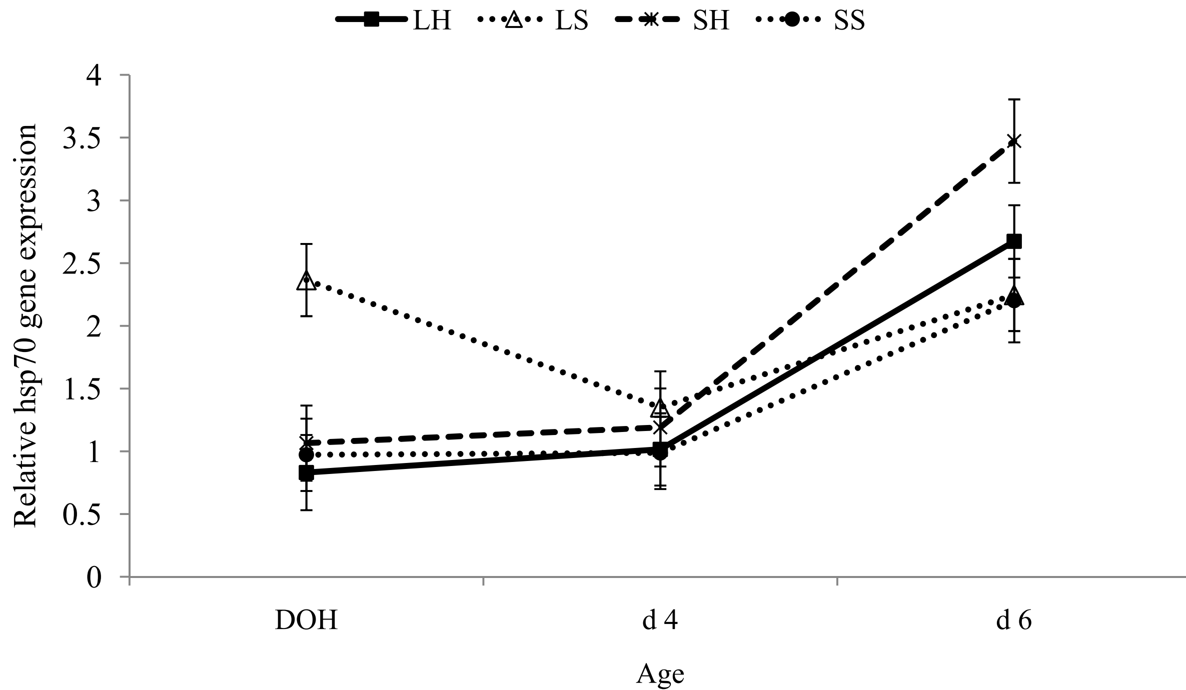

Figure 3.10 Effect of incubation temperature and age on relative mRNA expression of hsp70 in the immune organs (bursa, thymus, and spleen, collectively) of Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Relative gene expression ($2^{-\Delta\Delta Ct}$) for hsp70 was calculated using the ΔΔCt method with the average ΔCt values for bursa, thymus, and spleen in the SS-C group, respectively, as the calibrator(s). Data are presented as LS means ± SEM (n=18/treatment). There was a two-way interaction (P=0.056) of temperature and age.

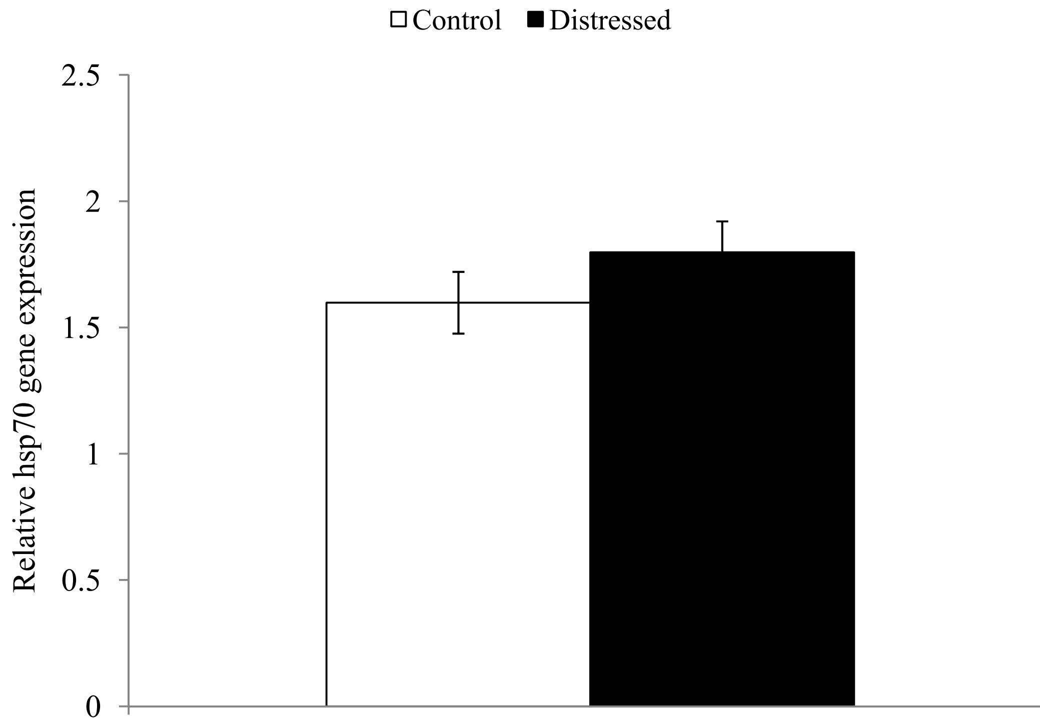

Figure 3.11 Effect of transportation on relative mRNA expression of hsp70 in the immune organs (bursa, thymus, and spleen, collectively) of Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Relative gene expression ($2^{-\Delta\Delta Ct}$) for hsp70 was calculated using the ΔΔCt method with the average ΔCt values for bursa, thymus, and spleen in the SS-C group, respectively, as the calibrator(s). Data are presented as LS means ± SEM (n=108/treatment). There was a main effect (P=0.057) of transportation.

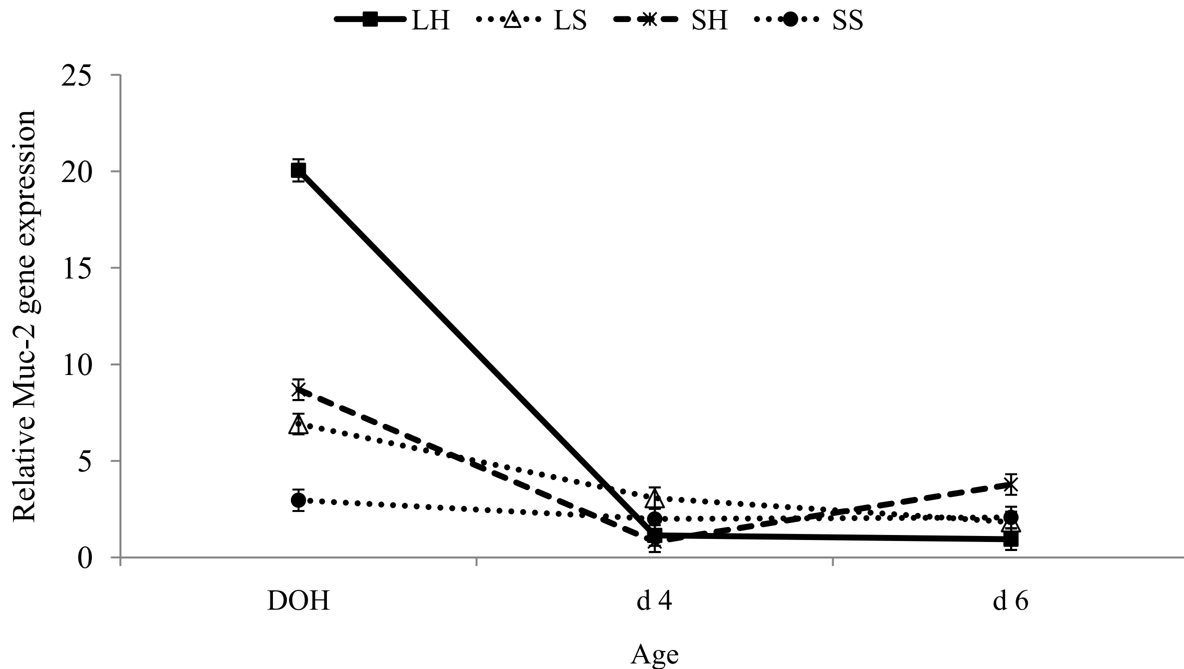

Figure 3.12 Effect of incubation temperature and age on relative mRNA expression of Muc-2 in the small intestine of Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Relative gene expression ($2^{-\Delta\Delta Ct}$) for Muc-2 was calculated using the ΔΔCt method with the average ΔCt value for duodenum in the SS-C group as the calibrator. Data are presented as LS means ± SEM (n=18/treatment). There was a two-way interaction (P=0.0056) of temperature and age.

CHAPTER IV

Incubation Temperature and Vaccination Effects on Immune Organ Parameters and Performance in Commercial Broilers

ABSTRACT Elevated temperatures during the second half of incubation of commercial broilers have become common in both single- and multi-stage incubators due to increased metabolic heat production by the embryo. This increased heat production is a direct result of genetic selection for high-yield broilers and lack of ventilation modifications in commercial incubators to assist in the dispersion of heat that accumulates within the machines. Consequently, this thermal stress may impact growth of the embryo and may further affect development of the bird during the post-hatch period. Vaccinations at the hatchery may induce stress upon the bird as well, and if the chicks have been previously exposed to temperature stress during incubation, the ability of the already stressed bird to respond to vaccination could be compromised, which may impact a multitude of physiological factors and ultimately affect bird performance. In this study, Cobb 500 eggs (n=2000) were incubated with the following shell temperatures during early and late incubation: low (**L**: 36.7°C), standard (**S**: 37.5°C), and high (**H**: 39°C). Eggs were incubated at S from embryonic day (**ED**) 8 to 14, and combinations of L or S during early (ED 0 to 7) incubation, and S or H during late (ED 15 to 21) incubation, yielding four incubation treatment groups: LH, LS, SH, and SS. Embryos were administered Marek's disease virus vaccine (**MDV**; n=125/incubation treatment) at ED 18, Newcastle disease virus vaccine (**NDV**; n=125/incubation treatment) at hatch, the combination of MDV+NDV (n=125/incubation treatment), or no vaccine (Control; n=500/incubation treatment). This led to a total of 16 treatment groups: LH-Control, LH-MDV, LH-NDV, LH-MDV+NDV, LS-Control, LS-MDV, LS-NDV, LS-MDV+NDV, SH-Control, SH-MDV, SH-NDV, SH-MDV+NDV, SS-Control, SS-MDV, SS-NDV, and SS-

MDV+NDV. Body weight (**BW**) and yolk sac weight were evaluated at day of hatch (**DOH**), d 2, d 4, d 10, and d 14 for determination of yolk-free body weight (**YFBW**). Immune organ weights were collected at DOH, d 2, d 4, d 10, and d 14 for evaluation of bursa and spleen development. Bursa and thymus samples were collected at DOH, d 4, d 10, and d 14 for evaluation of mRNA gene expression of hsp70 and iNOS through relative quantative real-time PCR. Whole blood was collected at d 5, d 15, and d 21 for determination of heterophil:lymphocyte (**H:L**) ratios, and blood serum was evaluated for antibody production to NDV using ELISA at d 15 and d 21. Incubation temperature impacted YFBW from d 2 to d 4 (P=0.0001). Yolk sac weight as a % of BW was affected by incubation temperature and age (P=0.0011) from d 2 to d 4 and incubation temperature alone at DOH (P<0.0001). There were no differences in bursa weight as % BW, but vaccination impacted spleen weight as % BW from d 2 to d 14 (P<0.0001). There was an interaction (P=0.0289) of incubation temperature, vaccination, and age and an interaction (P=0.006) of incubation temperature and vaccination on H:L ratios. Antibody titers in NDV vaccinated birds were significantly (P=0.0005 and P<0.0001) higher than in non-vaccinated birds at d 15 and d 21, respectively. Incubation temperature, vaccination, and tissue (P=0.0409) at DOH, and incubation temperature, tissue, and age (P=0.0177) from d 4 to d 14 impacted immune organ gene expression of hsp70. Gene expression of iNOS was influenced by the interaction of vaccination and tissue (P=0.0250) from d 4 to d 14. These results provide information that better explains effects of incubation temperature and vaccination stressors on immune system development and early immune response in broiler chicks.

Key words: incubation, vaccination, broiler, immune

INTRODUCTION

The optimum temperature within the incubator is considered that temperature required to maximize hatchability (Wilson, 1991). The desired temperature in commercial hatcheries ranges between 37°C and 38°C (Hulet *et al.,* 2007); however, several factors influence the temperature experienced by the embryo. Eggshell temperature (**EST**) is considered the most reliable reflection of embryonic temperature without compromising the structure of the eggshell and destroying the embryo. Elevated EST is not uncommon during the latter portion of the incubation period in both single- and multi-stage incubators (Joseph *et al.,* 2006). However, in single-stage incubation, all embryos are at the same developmental phase, which allows for better temperature manipulation and the ability to target the thermal requirements of the embryo at different stages (Lourens *et al.,* 2006). In both single- and multi-stage incubation, the commonly elevated EST are reflective of not only the genetic selection for high-yield broilers and the resulting increased metabolic heat production of these embryos during development, but also the lack of modifications within the incubator to meet the needs of the rapidly growing embryo. High incubation temperatures are typically a result of the differences in heat production by the embryo relative to heat transfer within the machine (Meijerhof, 2002). Expelled embryonic heat will often accumulate in the machine, thus inducing heat stress upon the developing embryo. Ventilation is a critical component of hatchery management, and maintenance of an appropriate and consistent incubation temperature may be challenging if airflow in the machine is not uniform. Metabolic heat production by the embryo begins around embryonic day (**ED**) 4, and by ED 9, the temperature of the embryo exceeds the air temperature within the incubator (Hulet *et al.,* 2007). Therefore, dissipation of heat from the embryo is critical and influences hatchability (Hulet *et al.,* 2007).

Organ development and yolk sac utilization have been impacted after exposure to high incubation temperatures (Wineland *et al.,* 2000ab; Leksrisompong *et al.,* 2007). High EST during the third week of incubation has resulted in a reduction in yolk-free body weight (**YFBW**) at hatch (Lourens *et al.,* 2005). Embryos that have been overheated during incubation have shown a reduction in hatchability and chick quality (French, 2000). Additionally, exposure of embryos to elevated incubation temperatures resulted in decreased body weight at d 44 (Gladys *et al.,* 2000). These data are suggestive of the detrimental impact of non-optimal incubation temperatures not only at hatch but also on overall lifetime bird performance, as temperature during the last several days of incubation has been shown to greatly impact post-hatch performance (Hulet *et al.,* 2007).

Exposure to infectious agents may induce subclinical infection, clinical disease, or death (Sharma, 1999). Newcastle disease virus (**NDV**) and Marek's disease virus (**MDV**) are two common viral agents that may result in recurring infections in commercial poultry flocks. Marek's disease virus has been shown to have atrophic effects on the bursa and thymus (Nair, 2005) and an apoptotic effect on B and T lymphocyte populations (Schat and Xing, 2000). Newcastle disease virus has resulted in enlargement of the spleen (Sijtsma *et al.,* 1991; Wakamatsu *et al.,* 2006). Although many studies have evaluated the pathogeneses of these viral diseases and their impacts on performance and overall production, no studies have looked at bird response to vaccination after having been previously exposed to temperature stressors during incubation.

The effects of heat stress on immune parameters have been previously evaluated, with decreased NDV antibody titers in female broilers 4 and 7 d following initiation of heat stress (Zulkifli *et al.,* 2000). To date, however, antibody production to vaccination and immune system

development have not been evaluated in combination with other embryonic environmental stressors, especially incubation temperature stress. The objective of this study was to evaluate the effects of incubation temperature profiles common to commercial multi-stage incubators on response to vaccination, immune system development, early immune response parameters, yolk sac utilization, and performance to 3 wk post-hatch. To fulfill several of the objectives of this study, mRNA gene expression of several indicators of immune activity were evaluated, including heat shock protein 70 (**hsp70**) and the inducible form of nitric oxide synthase (**iNOS**). Heat shock protein 70 is a highly conserved, stress-inducible HSP considered the most thermally sensitive relative to other HSPs. The inducible form of NOS may be expressed as a result of increased cytokine production (Xing and Schat, 2000). Changes in mRNA expression of these genes could provide valuable information regarding the response of the immune system to environmental stressors.

MATERIALS AND METHODS

Animal Welfare

This experiment was approved and conducted under the guidelines set forth by the Institutional Animal Care and Use Committee at Virginia Tech.

Incubation Temperature

Cobb 500 eggs (n=2000) were obtained from a 31-week-old breeder flock and incubated at a North Carolina State University hatchery facility[11]. Four temperature profiles were established to simulate the conditions experienced by the embryo in commercial multi-stage incubators. Incubation temperatures were designated as low (**L**: 36.7°C) or high (**H**: 39.0°C) relative to the industry standard temperature (**S**: 37.5°C). From embryonic day (**ED**) 0 to ED7,

[11] Natureform I40 Incubators, Jacksonville, FL

half of the eggs were incubated under L conditions and half were incubated under S conditions. All eggs were incubated at S from ED8 until ED14. From ED15 to ED21, half of the eggs remained at S and the other half were incubated at H. Four incubation treatments were derived from these temperature combinations: eggs incubated at the low temperature during the early phase and the high temperature during the late phase (**LH**), eggs incubated at the low temperature during the early phase and the standard temperature during the late phase (**LS**), eggs incubated at the standard temperature during the early phase and the high temperature during the late phase (**SH**), and eggs incubation at the standard temperature during both the early and late phases (**SS**). Incubation temperature was regulated by thermistors connected to microprocessors with a temperature sensitivity of ± 0.05°C. Humidity was controlled with a comparable system by humidity sensors. Digital thermometers[12] were placed in each incubator to monitor temperature. Incubation temperature, humidity levels, and EST were evaluated and logged daily.

Vaccination

There were four vaccination treatment groups (n=125/incubation temperature treatment) including non-vaccinated control, MDV vaccinated, NDV vaccinated, and MDV in combination with NDV (**MDV+NDV**). Vaccination for MDV was administered in ovo at day 18 of incubation (n=250 birds/incubation temperature treatment). At DOH, chicks (n=125 previously non-vaccinated birds/incubation temperature treatment and 125 MDV vaccinated birds/incubation temperature treatment) were vaccinated subcutaneously with 0.1mL killed NDV[13]. The combination of incubation temperature treatments and vaccination resulted in a total of 16 treatments (Table 4.1): LH-Control, LH-MDV, LH-NDV, LH-MDV+NDV, LS-Control, LS-MDV, LS-NDV, LS-

[12] Cox, Lexington, NC
[13] Lohmann Animal Health, Winslow, ME

MDV+NDV, SH-Control, SH-MDV, SH-NDV, SH-MDV+NDV, SS-Control, SS-MDV, SS-NDV, and SS-MDV+NDV.

Transportation

Birds were placed in hatchery chick trays according to treatment (n=67 or 68 birds/box). The four vaccination treatments per incubation temperature treatment were stacked together, four trays high, in a cargo van. Stacks for each incubation treatment were placed at the front, middle, and back of the van to ensure representation of each treatment at all locations within the vehicle. Temperature and ventilation were manually controlled to maintain the chicks within their thermoneutral zone throughout the four hour transport time from the hatchery facility to the research facility at Virginia Tech.

Rearing and Diets

Upon arrival at Virginia Tech, chicks were randomized within treatment and assigned to one of six Petersime battery brooders (n=9 replicate pens/treatment with 12 chicks/pen at DOH). The birds were housed in an environmentally controlled room under constant fluorescent lighting with wall fans and gas heaters. Birds had *ad libitum* access to water and a corn-soybean based diet formulated to meet or exceed Cobb 500 nutritional requirements. A starter diet was administered from DOH until the termination of the study at d 21.

Performance Parameters

All chicks were weighed individually prior to transportation at DOH (n=2000). At d 2, d 4, d 10, and d 14 all sampling chicks were weighed (n=15/treatment) for evaluation of live body weight. At DOH, d 2, and d 4, chicks (n=15/treatment) were randomly selected for evaluation of yolk sac weight and YFBW. Birds were weighed,

euthanized by cervical dislocation, and yolk sacs were collected and weighed to calculate YFBW. Yolk sac weights are presented as a percentage (%) of body weight (**BW**), and BW is presented as both YFBW and total BW.

Tissue Collection

Bursa and spleen samples were collected prior to transportation at DOH and at d 2, d 4, d 10, and d 14 for evaluation of immune organ weights (n=10/treatment). Each bird was weighed and euthanized by cervical dislocation, and organs were collected. Bursa and spleen weights are presented as a % of total BW. Bursa and thymus samples were collected (n=4 birds/treatment) at DOH (pre-transportation), d 4, d 10, and d 14 for examination of hsp70 and iNOS mRNA gene expression. Birds were euthanized by cervical dislocation, and immune organs were immediately collected, placed in 1.5mL microcentrifuge tubes, and temporarily stored in a cooler containing dry ice. Immediately following organ collection from all birds, samples were transferred to -80°C until further analysis. Samples were prepared for RNA isolation by cutting 20 to 30mg of frozen tissue over dry ice followed by homogenization with a rotor tissue homogenizer. Total RNA was isolated from the homogenized immune tissue with the RNeasy Miniprep Kit[14] using the animal tissue isolation protocol provided by the manufacturer. A nanodrop spectrophotometer[15] was utilized to evaluate total RNA concentration and purity by examining 260/280 and 260/230 ratios. RNA quality was determined by examination of 18s and 28s bands after gel electrophoresis using ethidium bromide stain.

[14] Qiagen, Darlington Lab, Valencia, CA
[15] Thermo Scientific, NanoDrop™ 1000, Wilmington, DE

Primer Design

Primer Express software (Version 3)[16] was used to design and synthesize forward and reverse primer pairs. Primer pair sequences are presented in Table 4.2.

Reverse Transcription and Real-Time PCR

Total RNA was diluted to 0.2 µg/µl in RNAse-free water. The High Capacity cDNA Archive Kit[16] was utilized to perform reverse transcription and convert total RNA to cDNA. Following reverse transcription, cDNA was diluted to 1:30 and stored at -20°C until real-time PCR was performed. Relative quantification real-time PCR was conducted using a 7500 Fast Real Time PCR System[16]. Samples were evaluated in triplicate in 96-well plates. Each well contained a reaction volume comprised of 1µl cDNA, 5µl Fast SYBR green master mix[16], 0.2 µl forward primer (5µM), 0.2 µl reverse primer (5µM), and 3.6 µl RNAse-free water. The following machine settings were utilized to perform real-time PCR: initial denaturation at 95°C for 20 sec, 40 cycles of 95°C for 3 sec, and 60°C for 15 sec. A dissociation curve was evaluated to ensure amplification of one product after completion of each run. Settings for the dissociation curve were as follows: 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, and 60°C for 15 sec. The Ct values (for triplicate samples) for the target and endogenous reference gene, glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**), were averaged, and the difference between the target and GAPDH averages were calculated to obtain the ΔCt value. The ΔΔCt values were obtained by taking the average ΔCt values for each individual immune organ calibrator (bursa SS-Control and thymus SS-Control). Fold changes in gene expression were then obtained by calculating $2^{-\Delta\Delta Ct}$, and are presented as such. Statistical significance was determined through evaluation of ΔCt values only.

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[16] Applied Biosystems, Foster City, CA

Heterophil/Lymphocyte Ratios

Blood samples (n=8 birds/treatment) were collected from the jugular or brachial vein at d 5, d 15, and d 21 for evaluation of heterophil to lymphocyte (**H:L**) ratios. Immediately following blood collection, a thin blood smear was prepared on a clean microscope slide and allowed to air dry. Smears were stained with a modified Wright-Giemsa stain using the protocol provided by the manufacturer[17]. Smears were evaluated by counting a total of 100 lymphocytes and heterophils per slide under 100X oil immersion. Heterophil:lymphocyte ratios were calculated by dividing the total number of heterophils by the total number of lymphocytes for each blood smear.

NDV ELISA

Antibody titers to NDV were evaluated at d 15 and d 21 (n=15 birds/treatment). Approximately 1 mL of blood was collected from the jugular or brachial vein, and the blood sample was placed in a polypropylene culture tube and sealed. Culture tubes were placed horizontally on trays to allow serum to separate, after which time tubes were placed upright and stored at 4°C overnight. Samples were centrifuged at 3000 RPM for 10 minutes, and serum was removed and aliquoted to 1.5mL microcentrifuge tubes. Serum was stored at -20°C until further analysis. Newcastle disease virus antibody detection was conducted using an NDV antibody test kit following the manufacturer protocol[18]. Samples were run in triplicate and evaluated using a plate reader[19] and corresponding analysis software[20].

[17] Sigma-Aldrich, St. Louis, MO
[18] ProFLOCK® PLUS NDV ELISA Kit, Synbiotics, San Diego, CA
[19] Thermo Multiskan Ascent®, Vantaa, Finland
[20] Ascent Software, Version 2.6, Vantaa, Finland

Statistical Analysis

Data were evaluated as a completely randomized experimental design arranged as a 4 x 4 factorial (4 incubation temperature treatments and 4 vaccination treatments). Analysis of variance was performed using the GLM procedure of SAS[21]. Values are reported as least squares (LS) means ± SEM. Data reported as a percentage were transformed prior to analysis using arc-sine (square root of percent). Immune organ development was analyzed with a statistical model comprised of the main effects of incubation temperature, vaccination, and age, and all two and three way interactions for each respective immune organ. Main effects from incubation temperature, vaccination, age, tissue, and all two, three, and four way interactions comprised the model for the evaluation of gene expression. Gene expression of immune organs is reflective of the bursa and thymus analyzed collectively. Heterophil:lymphocyte ratios were evaluated with a model including the main effects of incubation temperature, vaccination, and age, and all two and three way interactions. Antibody titers to NDV were analyzed with a model containing incubation temperature and vaccination for each day and the corresponding two way interactions. Differences between treatments were adjusted using Tukey's test. Significance was established at $P \leq 0.05$. All DOH data was analyzed independently of data from subsequent sampling days. At DOH, only birds from the control and MDV vaccinated treatments were sampled and evaluated, as NDV vaccine was administered at hatch and would not have presented any effects at that time. All subsequent sampling days evaluated all 16 treatments.

[21] SAS, version 9.1, SAS Institute Inc., Cary, NC

RESULTS

Performance Parameters

There were no differences in YFBW at hatch. However, from d 2 to d 4, YFBW was affected by incubation temperature (P=0.0001; Table 4.3) with both the SH and SS groups exhibiting heavier body weights compared to LH, which was heavier than LS. Body weight (**BW**) of all chicks at DOH was impacted by incubation temperature, with LS and LH birds exhibiting significantly higher BW compared to SS, which was higher than SH (P<0.0001; Figure 4.1). Body weight of sampling birds was unaffected by incubation temperature at DOH (data not shown), but differences were evident from d 2 to d 14 (Figure 4.2). Birds incubated under SH and SS conditions had heavier BW compared to the LS group, which was comparable to the intermediate group of LH (P=0.0003).

There was a main effect (P<0.0001) of incubation temperature at DOH on yolk sac weight as a % of BW (Figure 4.3). Birds incubated under LS, SH, or SS conditions had significantly lighter yolk sacs as a % of BW compared to LH. Yolk sac weight as a % of BW was affected by the two way interaction (P=0.0011) of incubation temperature and age (Figure 4.4) from d 2 to d 4. As expected, yolk sacs at d 2 were heavier than at d 4. Incubation treatments responded similarly between d 2 and d 4, but some groups appeared to absorb more yolk than others. Birds incubated under LH or LS conditions exhibited heavier yolk sacs as percent a % of BW compared to SS on both d 2 and d 4, and these groups appeared to have a slightly faster rate of yolk absorption from d 2 to d 4 as compared to SH. Chicks in the SS group had the smallest yolk sacs as a % of BW at both d 2 and d 4.

Immune Organ Development

Neither incubation temperature nor vaccination had an effect on bursa or spleen weight as % BW at DOH, and bursa weight was not affected from d 2 to d 14 (data not shown). There was

a main effect of vaccination (P<0.0001) on spleen weight as a % of BW from d 2 to d 14 (Figure 4.5). Birds vaccinated for MDV or the combination of MDV+NDV had heavier spleens compared to the control and NDV vaccine treatments.

H:L Ratios

There was a three-way interaction (P=0.0289) of incubation temperature, vaccination, and age on H:L ratios (Figure 4.6). At d 5, all treatments exhibited comparable H:L ratios with the exception of the birds incubated under SS conditions and vaccinated for NDV, which exhibited a slightly higher ratio relative to the other treatments. Between d 5 and d 15 and d 15 and d 21, multiple differential responses were observed. Birds from the LH-Control and from the LS-MDV exhibited a dramatic increase in H:L ratios relative to other treatments between d 5 and d 15. Between d 15 and d 21, LH-Control birds had a dramatic decrease in H:L ratio, while all other treatments either maintained ratios to d 21 or incurred a more slight increase or decrease. The SS-MDV and SS-NDV were the only groups with a decrease in H:L ratios from d 5 to d 15, and these groups responded similarly with a slight increase in H:L ratios from d 15 to d 21. Incubation temperature and vaccination (P=0.006; Figure A.1) as well as incubation temperature and age (P=0.0425; Figure A.2) effects on H:L ratios presented two-way interactions.

NDV ELISA

There was a main effect of vaccination on antibody production to NDV (Table 4.4) at both d 15 (P=0.0005) and d 21 (P<0.0001). At both days, there were no differences in titer values between birds vaccinated for NDV or for MDV+NDV. However, as expected, both NDV and MDV+NDV vaccinated birds had significantly higher titers than control non-vaccinated birds. No differences in titer values were observed between incubation temperature treatments.

mRNA Gene Expression

For each gene evaluated, there existed tissue, age, or a combination of tissue and age differences. However, these data will not be presented as the focus of this research was on the differences resulting from treatment effects.

hsp70. The expression of hsp70 at DOH was affected by incubation temperature, vaccination, and tissue (P=0.0409; Figure 4.7). In the bursa, hsp70 expression was not affected by incubation temperature in control (non-vaccinated) birds. In contrast, expression of hsp70 in the thymus of control birds was elevated in the LS and SH incubated birds as compared to LH or SS. In birds vaccinated for MDV, hsp70 expression was highest in the bursa of SH incubated chicks and in the thymus of LS incubated chicks. Expression of hsp70 in the bursa was lower in SS incubated and MDV vaccinated chicks, but this decrease was not seen in the thymus of these birds. There was also a three-way interaction (P=0.0177) of incubation temperature, tissue, and age (Figure 4.8). In general, at d 4 and d 14, hsp70 expression in the thymus was higher than expression in the bursa in all incubation temperature treatments. In the thymus, hsp70 expression was similar between LS and SH birds with little change in expression from d 4 to d 14. In contrast, in the thymus of LH incubated birds, hsp70 decreased from d 4 to d 10 and then increased from d 10 to d 14 to reach levels comparable to expression at d 4. The SS incubated birds had a continual increase in hsp70 expression in the thymus from d 4 to d 14. In the bursa, expression of hsp70 in LH and SH increased from d 4 to d 10 and decreased from d 10 to d 14. The LS and SS incubated birds had similar and relatively unchanging hsp70 expression in the bursa from d 4 to d 14. Vaccination for MDV+NDV resulted in a lower (P=0.0049) level of hsp70 expression compared to MDV alone from d 4 to d 14 (Figure 4.9). There were no hsp70

expression differences in the control and NDV vaccinated groups relative to the other vaccination treatments.

iNOS. Vaccination and tissue presented a two-way interaction (P=0.0250) that influenced iNOS gene expression (Figure 4.10). In general, thymic tissue showed lower iNOS expression compared to the bursa, and each tissue responded differently to vaccination. Vaccination for MDV resulted in relatively no change in iNOS expression in the bursa or thymus. Vaccination for NDV resulted in decreased expression relative to the control in the bursa; however, expression in NDV vaccinated birds was comparable to the control in the thymus. The combined vaccination for MDV+NDV resulted in increased iNOS expression relative to control in the bursa and no change in the thymus compared to control (non-vaccinated). There were no differences in iNOS gene expression at DOH and no differences resulting from incubation temperature.

DISCUSSION

Temperature may be considered the most critical aspect of incubation management. Metabolic heat production by today's high-yield embryos is much greater than the heat output of broiler lines of the past, and the corresponding result is overheating in the incubator that could influence embryo survivability and chick quality (Hamidu *et al.,* 2007). Incubation is one of the most challenging aspects of broiler management due to the discrepancies between the temperature of the embryo and the air temperature within the machine. These respective temperatures must be considered independently in order to create optimal environmental conditions, as the developing embryo is more influenced by its own internal temperature rather than the temperature of the surrounding air (Lourens *et al.,* 2005). With these key points in mind, it may be suggested that incubation temperatures deviating from the standard during early

or late phases of incubation may not only influence the embryo but could also affect development and ultimately performance of the bird during the post-hatch period. Chick processing at the hatchery, which includes vaccination, may impose stress upon the newly-hatched chick. The response to vaccinations administered at the hatchery after previous exposure to incubation temperature stress has not been previously reported; however, it has been suggested that exposure to acute thermal stress post-hatch resulted in reduced levels of circulating antibodies and suppressed cell mediated immunity (Thaxton and Siegel, 1970; 1972).

In this study, incubation temperature affected YFBW from d 2 to d 4, with LS birds exhibiting the lowest YFBW. These results are in agreement with previous research, which reported reduced YFBW at hatch in chicks exposed to low incubation temperatures (Lourens *et al.,* 2005). Additionally, low incubator temperatures resulted in reduced BW at 3 and 6 wk post-hatch compared to embryos incubated under optimum conditions (Joseph *et al.,* 2006), suggesting the deleterious impact of low temperatures not only in the neonatal chick, but up to market age. A study conducted in rainbow trout (*Onchrhynchus mykiss*) embryos reported that suboptimal environmental conditions resulted in delayed expression of myogenic regulatory factors (Xie *et al.,* 2001), which are critical to vertebrate myogenesis (Maltby *et al.,* 2004). Early low incubation temperatures in turkey embryos caused a reduction in the number of muscle fibers in the semitendinosus muscle (Maltby *et al.,* 2004). Number of myofibers is believed to be fixed at hatch, so it is possible that a reduction in the number of myofibers present may further impact growth potential during the post-hatch period (Joseph *et al.,* 2006), as environmental conditions early in development may greatly influence phenotype in poikilothermic organisms (Maltby *et al.,* 2004).

Chicks from the LH group exhibited lower YFBW from d 2 and d 4 compared to the SS incubation group. Previous studies have reported a reduction in YFBW after embryo exposure to elevated temperatures during the latter phase of incubation (Wineland *et al.,* 2000ab; Leksrisompong *et al.,* 2007). Chick embryos exposed to elevated incubation temperatures responded with accelerated growth (Wilson, 1991); however, accelerated development can negatively impact BW of the newly-hatched chick (Gladys *et al.,* 2000). An elevated incubation temperature applied after ED14 reduced relative small intestinal weight compared to embryos exposed to a standard incubation temperature (Leksrisompong *et al.,* 2007). This decrease in small intestinal weight could correspondingly translate to delayed intestinal maturation and delayed yolk sac absorption, which would directly impact the absorption and utilization of nutrients and ultimately influence productivity of the bird. Heavier absolute yolk sac weights as a % of BW were observed in the LH group at DOH and in the LH and LS groups at d 2. At d 4, the SS group had the smallest yolk sac as a percentage of BW compared to all other incubation treatments, suggesting better absorption of yolk contents in this group. Issacks *et al.* (1964) suggested that lipid turnover in the developing embryo increases dramatically after ED13. In addition, fatty acid oxidation serves as the primary source of energy and metabolic water in the embryo (Rahn and Paganelli, 1991). It is therefore possible that exposure to early L, and to a lesser extent late H, temperatures results in modification of lipid metabolism in the growing embryo. Slower uptake of the yolk has been attributed to slower embryonic growth (Peebles *et al.,* 2001). Delayed utilization of the yolk has further implications in terms of stimulation and development of the gastrointestinal tract, which could influence bird performance. The yolk is considered one of the main pathways by which maternal immunoglobulins are transferred from the hen to the chick (Gharaibeh *et al.,* 2008). The transmission of maternal antibodies protects

the young chick from pathogens as its immune system develops. This protective mechanism could be somewhat compromised if yolk uptake is delayed and could result in the young chick being more susceptible to pathogen invasion.

Embryos vaccinated with herpes virus of turkeys (HVT) have been evaluated for viral loads in various organs of embryos, and the highest viral loads were found in the spleen (Tan *et al.,* 2007). Birds were evaluated for several weeks following vaccination, and bursal atrophy was reported after wk 5. The spleens of challenged birds were enlarged, exhibited tumors, and presented increased spleen:BW ratios during wks 6 to 8 following initial vaccination at ED11 or ED17 (Tan *et al.,* 2007). In this study, birds that were vaccinated for MDV alone or the combination of MDV+NDV exhibited the highest spleen weights as a % of BW, suggesting the crucial role and function of the spleen in MDV pathogenesis. Marek's disease virus has been shown to have an apoptotic effect on both B and T cells (Schat and Xing, 2000), directly affecting function of the bursa and thymus. Therefore, increased splenic activity may be demanded to compensate for the minimized function of the primary lymphoid organs, thus explaining the spleen's increased weight. Birds vaccinated for NDV alone had spleen weights comparable to the control, but less than those of birds vaccinated for MDV or MDV+NDV. It is possible that the spleen is not as actively involved in NDV pathogenesis. Additionally, a killed NDV vaccine was administered in this study. Killed vaccine preparations may result in an immune response lasting for a shorter period of time and a weaker cell-mediated and mucosal immune response (Horzinek and Thiry, 2009). Vaccine adjuvants, which potentiate the immune response in inactive vaccines, vary in their modes of action and therefore differentially influence the activity and recruitment of lymphocytes in response to vaccination, thus possibly further influencing the activities of lymphoid tissues.

Heterophil:lymphocyte ratios are considered a reliable indicator of stress in chickens (Siegel, 1995). To date, these ratios have not been evaluated in response to vaccination coupled with embryonic incubation temperature profiles. Typically, a higher H:L ratio suggests a higher stress level. Immune cells are influenced by the release of glucocorticoids, such as corticosterone, in response to environmental stressors and pathogen exposure (Shini *et al.,* 2008). This modulation of corticosterone production and secretion causes a reduction in lymphocyte circulation and an increase in heterophils (Siegel, 1985). At d 5, chicks from the SS incubation temperature group that were vaccinated for NDV exhibited the highest H:L ratio. This may have occurred because these birds were not stressed during incubation and were then subjected to handling and vaccination stress at hatch. The administration of NDV vaccine at hatch was these birds' first experience with environmental stress, which subsequently resulted in elicitation of a stress response, which would presumably involve modifications in corticosterone production. Birds exposed to early L and late H incubation conditions without vaccination showed the highest H:L ratios at d 15. Environmental stress during both the early and late phases of incubation may result in a suppressed stress response initially following stress exposure, which could explain why the LH group had a moderate H:L ratio at d 5. However, as the immune system matures and the chicks recover from the initial depressed response, the stress exposure effects become evident. Interestingly, it does not appear as if these stress effects are sustained long-term, suggesting that incubation temperature and vaccination stressors may impact the bird early on, but they are able to elicit recovery as the immune system and other critical organ systems develop and mature.

As there were no effects of incubation temperature on NDV titers, it may be suggested that incubation temperature stress does not negatively influence the bird's ability to produce

antibodies to NDV and therefore does not compromise the immune system in response to this particular virus. Additionally, administering NDV vaccine in combination with MDV did not cause antibody titer reduction, which suggests a lack of suppression of the immune response.

In general, incubation under LS conditions resulted in the highest relative expression of hsp70 in the immune organs at hatch, particularly in the thymus. These results agree with earlier results from our laboratory (data not published) evaluating hsp70 gene expression in lymphoid tissue. One would expect hsp70 expression to be highest under thermal stress conditions, as it has been documented that hsp70 expression increases under elevated internal temperature conditions in broilers (Leandro *et al.,* 2004). However, as we have previously suggested, exposure to low incubation temperatures resulted in compensatory growth as a result of elevated metabolic heat production (Geers *et al.,* 1983). Therefore, LS birds were likely producing a greater amount of metabolic heat to compensate for the exposure to early L incubation temperatures. Consequently, hsp70 expression was higher in the LS treated birds. Embryos exposed to the LH treatment did not have as high a level of hsp70 expression as would be expected due to late H temperature exposure during incubation. This response could be due to the fact that the elevated temperature exposure during the latter portion of incubation did not require these chicks to compensate as much as chicks from the LS treatment and increase accumulation and expression of hsp70.

The Marek's disease virus oncoprotein, Meq, plays a role in oncogenicity as it has been reported that deletion of this oncoprotein prevents the accumulation of viral tumors (Zhao *et al.,* 2009). Confocal microscopic detection has shown that MDV infection resulted in accumulation of hsp70 and its co-localization with Meq, further suggesting the importance of the interaction of hsp70 and Meq in MDV oncogenesis (Zhao *et al.,* 2009). In this study, birds vaccinated for

MDV had the numerically highest level of hsp70 gene expression. Because of the oncogenic nature of MDV and susceptibility of MDV infected birds to virus-induced lymphomas (Zhao *et al.,* 2009), this elevated expression of hsp70 would be expected. The mechanism by which stress proteins are synthesized in response to paramyxoviruses remains unknown (Collins and Hightower, 1982). A study conducted by Collins and Hightower (1982) reported that the increase in stress proteins in NDV-infected cells reflected an increase in accumulation of stress mRNAs. Further, it was reported that NDV itself, in addition to thermal and chemical stress, may induce stress proteins in avian cells (Collins and Hightower, 1982). Gene expression of hsp70 in NDV vaccinated birds was comparable to the level of hsp70 expression in MDV vaccinated birds. Although the mechanism of pathogenesis for these two respective viruses are vastly different, it appears as if both viruses result in increased accumulation of hsp70.

The bursa plays a crucial role in MDV pathogenesis, as B cells in the bursa are responsible for harboring the cytolytic phase of viral replication. Birds infected with MDV have been shown to have higher gene expression of iNOS in the bursa compared to non-infected birds (Abdul-Kareem *et al.,* 2008); however, little difference was observed between the control and MDV vaccinated birds' expression of iNOS in the bursa. The lower relative iNOS expression in the bursa in NDV vaccinated birds may have resulted because these birds were administered the vaccine at hatch. Interestingly, iNOS may be expressed by macrophages as a result of cytokine production (Xing and Schat, 2000). The stress of bird handling and vaccination upon immediate hatch may have resulted in a suppressed response (cytokine downregulation) and therefore, reduced iNOS expression in the bursa. The MDV+NDV treatment likely resulted in greater cytokine release in the bursa in response to vaccination for both viruses. Gene expression of iNOS in the thymus remained relatively unchanged in this study. A study evaluating iNOS

expression in the thymus of mice suggested that the observed response may have been due to the involvement of IFN-γ, since iNOS expression is induced by IFN-γ (Tai *et al.,* 1997). Tai *et al.* (1997) further suggested the effects of nitric oxide (NO) on thymocytes that are at different maturation and activation stages. It is possible that incubation temperature and vaccination stressors may impact the population of thymocytes and therefore influence the induction of iNOS expression and NO activity.

The observations from this study suggested that incubation temperature and vaccination stress may influence the development of the immune system and affect response parameters both systemically and in individual organ systems. Similar to results that we have seen previously, incubation temperature stress alone or in combination with additional stress may cause differential responses. In some cases, these stressors may result in suppression, but they may also induce an enhanced response depending on the combination and timing of stressors, as well as the tissue evaluated. Collectively, the series of conducted studies evaluating incubation temperature and other post-hatch stressors on immune indicators and bird development have provided valuable insight into how the birds respond to environmental stressors, although the responses do appear somewhat variable. As these stress responses still remain somewhat elusive, additional research would prove beneficial to further evaluate the effects of embryonic temperature stress on the post-hatch response to vaccination.

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Incubation Treatment	Vaccine Treatment
LH	Control MDV NDV MDV + NDV
LS	Control MDV NDV MDV + NDV
SH	Control MDV NDV MDV + NDV
SS	Control MDV NDV MDV + NDV

Table 4.1 Four incubation temperature profiles were established to simulate commercial multi-stage incubation conditions. Temperatures were established as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21 (n=500/incubation treatment). Vaccination treatments (n=125/incubation temperature treatment) were designated as Control (no vaccine), Marek's disease virus vaccine (MDV) administered *in ovo* at d 18 of incubation, Newcastle disease virus vaccine (NDV) administered subcutaneously at hatch, and the combination of MDV+NDV.

Gene	GenBank ID	Description	Sequence: forward/reverse
GAPDH	NM_204305	Glyceraldehyde-3-phosphate dehydrogenase	GCCGTCCTCTCTGGCAAAG/ TGTAAACCATGTAGTTCA
hsp70	NM_001006685	Heat shock protein 70	ACTGCTCTCATCAAGCGTAACAC/ GTCTGAGTAGGTGGTGAAGGTCTG
iNOS	D85422	Inducible nitric oxide synthase	CCTGTACTGAAGGTGGCTATTGG/ AGGCCTGTGAGAGTGTGCAA

Table 4.2 Forward and reverse primers used for relative quantification real-time PCR. Primers were designed by Primer Express software (Applied Biosystems, Foster City, CA).

	Incubation Temperature				
	LH	LS	SH	SS	
Yolk-Free Body Weight					***P***
DOH	36.39 ± 0.53	36.87 ± 0.53	36.94 ± 0.53	36.39 ± 0.53	0.0997
d 2 to d 4	70.57 ± 0.55[b]	67.19 ± 0.56[c]	73.06 ± 0.55[a]	73.41 ± 0.55[a]	0.0001

Table 4.3 Effect of incubation temperature on yolk-free body weight (YFBW) at DOH and from d 2 to d 4 post-hatch of Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Data are presented as LS means ± SEM (n=15/treatment). There was a main effect of incubation temperature.

[a,b] Data within a row lacking a common superscript differ significantly

	d 15	**d 21**
Control	0[b]	0[b]
NDV	267.4 ± 47.0[a]	1246.5 ± 123.0[a]
MDV+NDV	202.9 ± 49.2[a]	1164.8 ± 126.2[a]
P	0.0005	< 0.0001

Table 4.4 Effect of vaccination on antibody titers to Newcastle disease virus (NDV) at d 15 and d 21 in Cobb 500 broilers. Birds were administered Marek's disease virus (MDV) vaccine *in ovo* at ED18 and NDV vaccine subcutaneously at hatch. Antibody titers were determined using a commercial NDV ELISA kit[1], and the titer values presented were calculated as the antilog of $\log_{10}$(titer). Data are presented as LS means ± SEM (n=60/treatment). There was a main effect of vaccination.

[a,b] Data lacking a common superscript differ significantly
[1]ProFLOCK® PLUS NDV ELISA Kit, Synbiotics, San Diego, CA

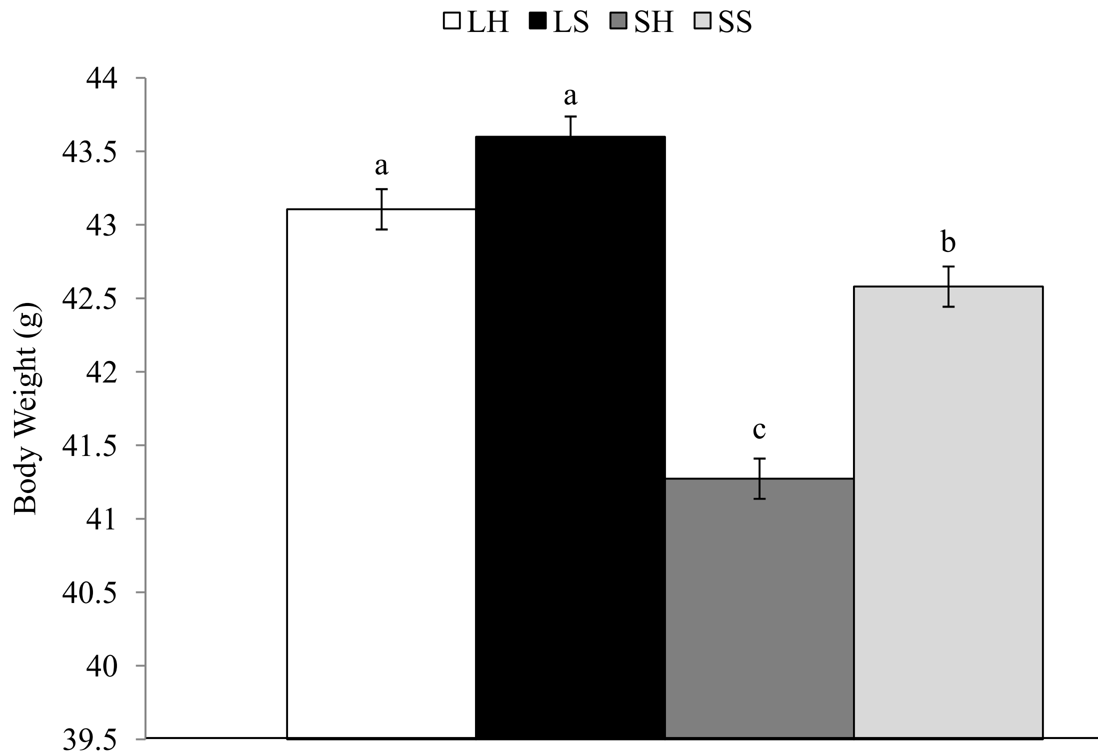

Figure 4.1 Effect of incubation temperature on body weight at DOH of Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Data are presented as LS means ± SEM (n=500/treatment). There was a main effect of temperature ($P<0.0001$).

[a,b] Data lacking a common superscript differ significantly

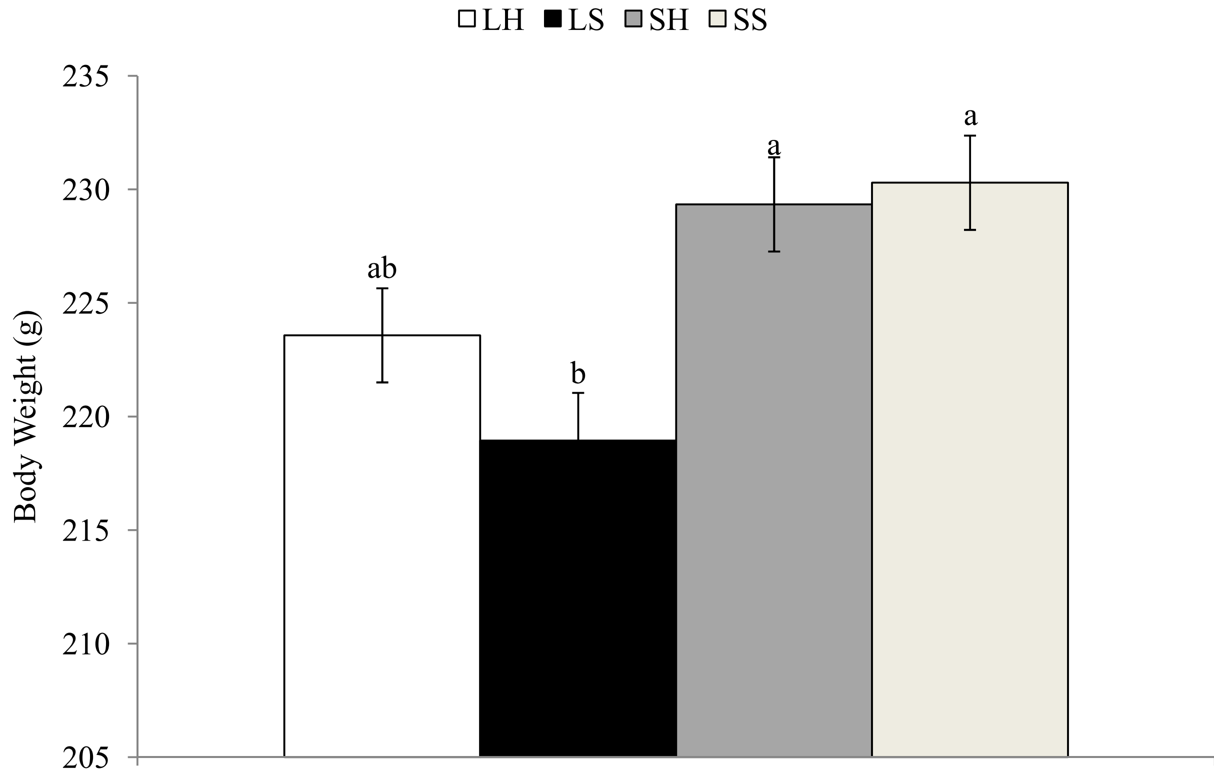

Figure 4.2 Effect of incubation temperature on body weight (d 2 to d 14) of Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Data are presented as LS means ± SEM (n=240/treatment). There was a main effect of temperature (P=0.0003).

[a,b] Data lacking a common superscript differ significantly

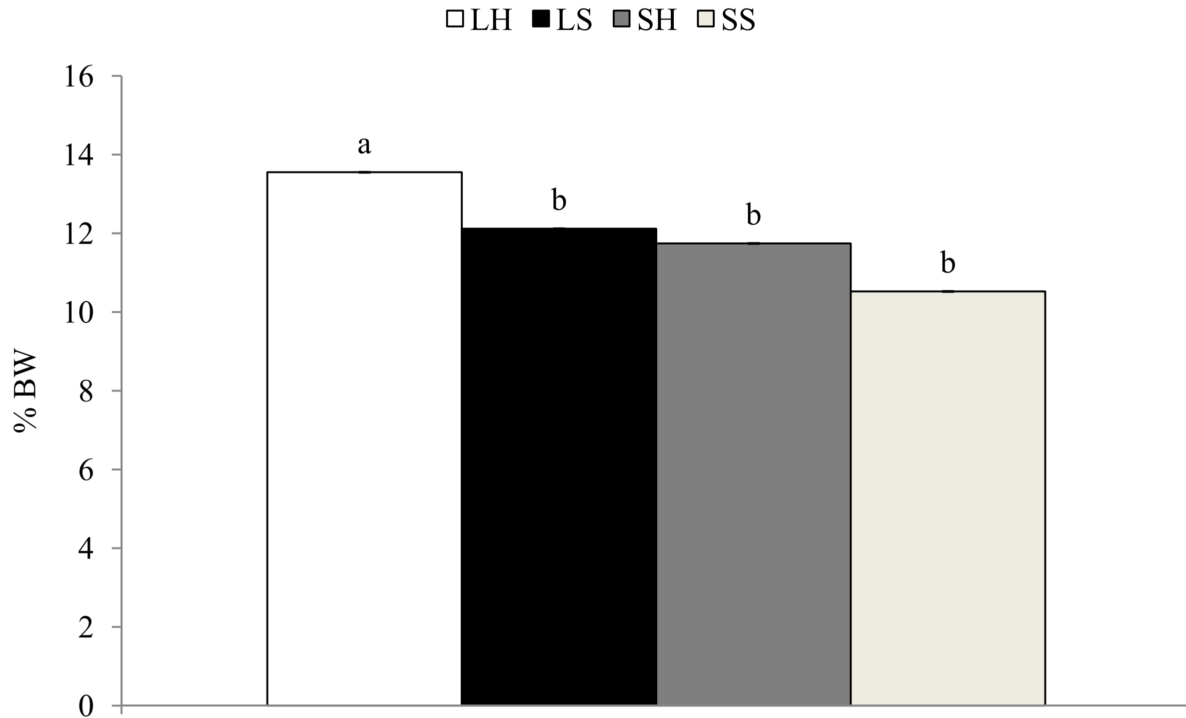

Figure 4.3 Effect of incubation temperature on yolk sac weight (% BW) at DOH in Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Data are presented as LS means ± SEM (n=30/treatment). There was a main effect of temperature ($P<0.0001$).

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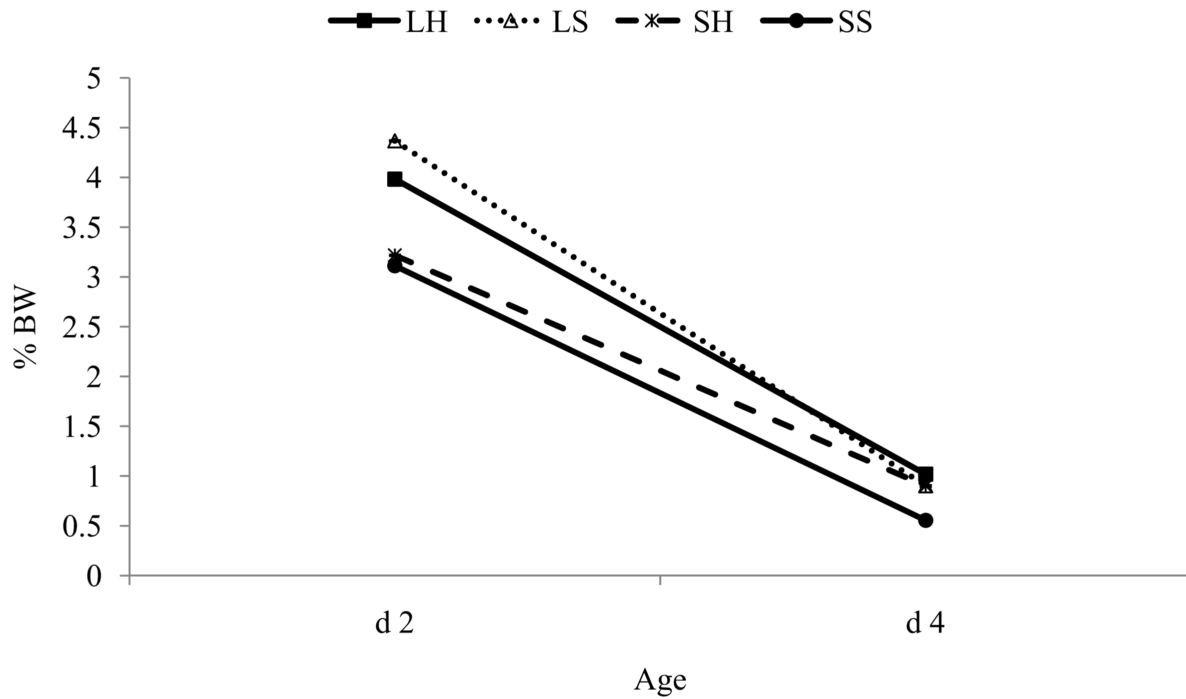

Figure 4.4 Effect of incubation temperature and age on yolk sac weight (% BW) in Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Data are presented as LS means ± SEM (n=60/treatment). There was a two-way interaction of temperature and age (P=0.0011).

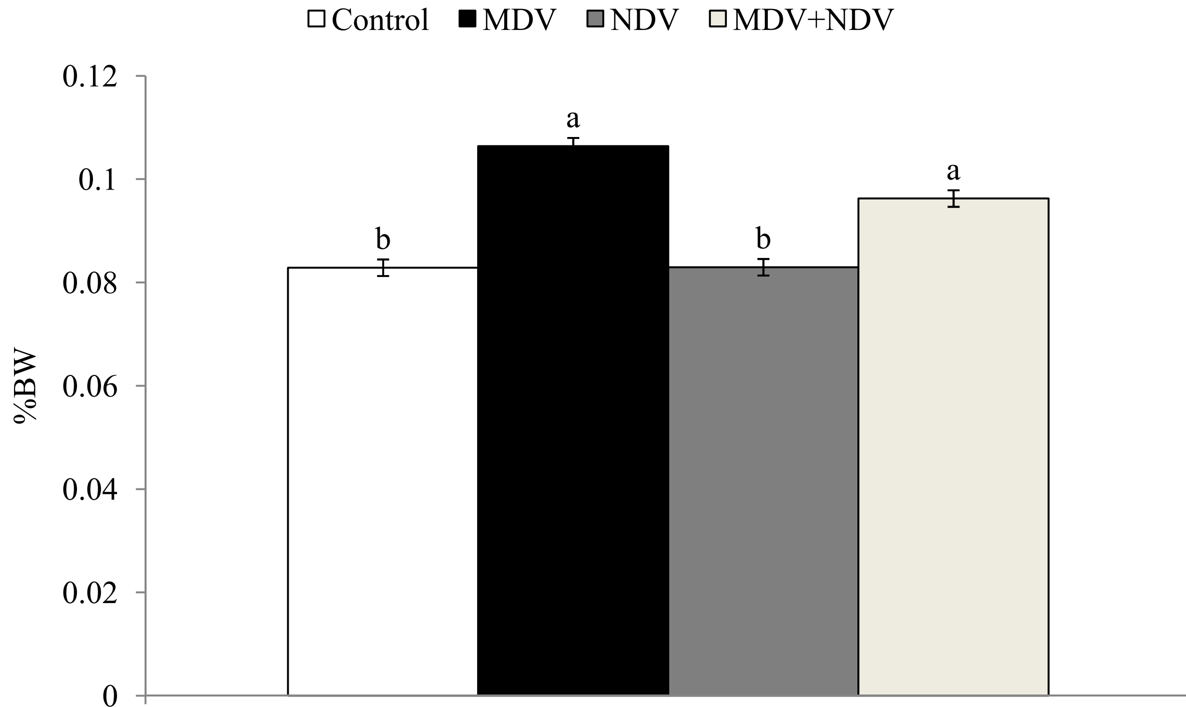

Figure 4.5 Effect of vaccination on spleen weight (% BW) from d 2 to d 14 post-hatch in Cobb 500 broilers. Vaccination treatments were designated as Control (no vaccine), Marek's disease virus (MDV) administered *in ovo* at d 18 of incubation, Newcastle disease virus (NDV) administered subcutaneously at hatch, and the combination of MDV+NDV. Data are presented as LS means ± SEM (n=160/treatment). There was a main effect of vaccination ($P<0.0001$).

[a,b] Data lacking a common superscript differ significantly

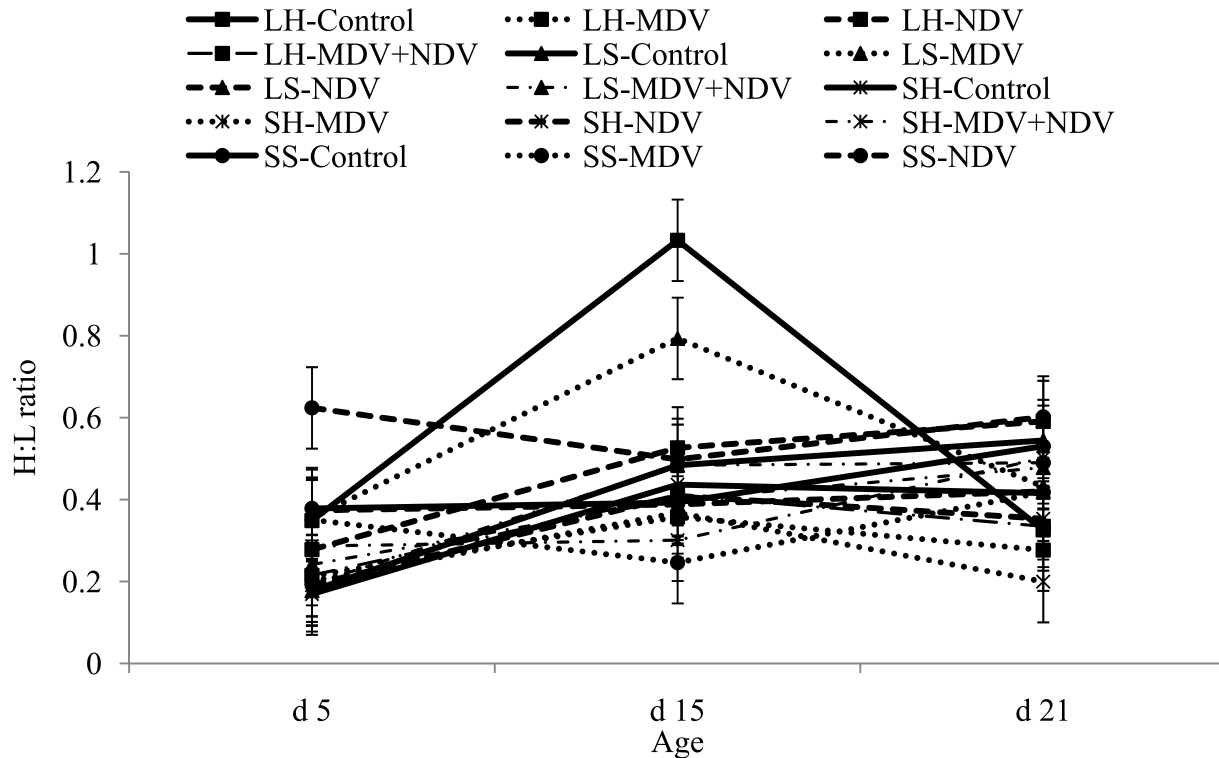

Figure 4.6 Effect of incubation temperature, vaccination, and post-hatch age on H:L ratios in Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Vaccination treatments were designated as Control (no vaccine), Marek's disease virus (MDV) administered *in ovo* at d 18 of incubation, Newcastle disease virus (NDV) administered subcutaneously at hatch, and the combination of MDV+NDV. Data are presented as LS means ± SEM (n=8/treatment). There was a three-way interaction of temperature, vaccination, and age (P=0.0289).

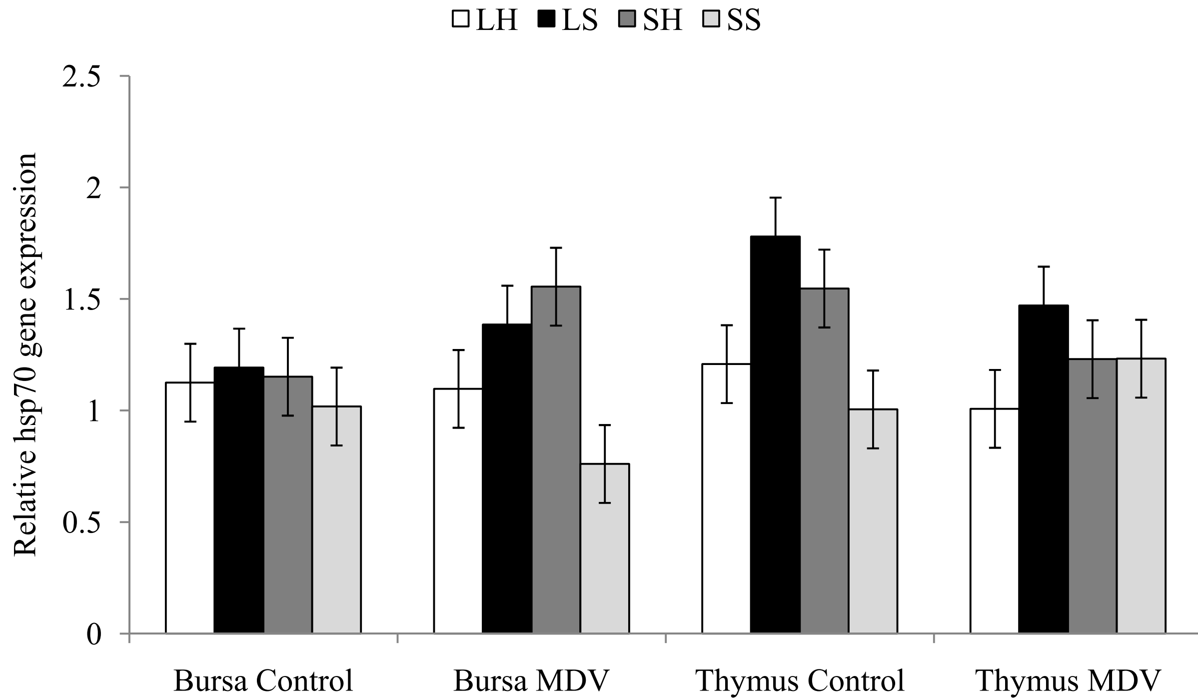

Figure 4.7 Effect of incubation temperature, vaccination, and tissue on relative mRNA expression of hsp70 in the bursa and thymus at DOH in Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Vaccination treatments were designated as Control (no vaccine), Marek's disease virus (MDV) administered *in ovo* at d 18 of incubation, Newcastle disease virus (NDV) administered subcutaneously at hatch, and the combination of MDV+NDV. Relative gene expression ($2^{-\Delta\Delta Ct}$) for hsp70 was calculated using the ΔΔCt method with the average ΔCt values for bursa and thymus in the SS-Control group, respectively, as the calibrator(s). Data are presented as LS means ± SEM (n=4/treatment). There was a three-way interaction of temperature, vaccination, and tissue (P=0.0409).

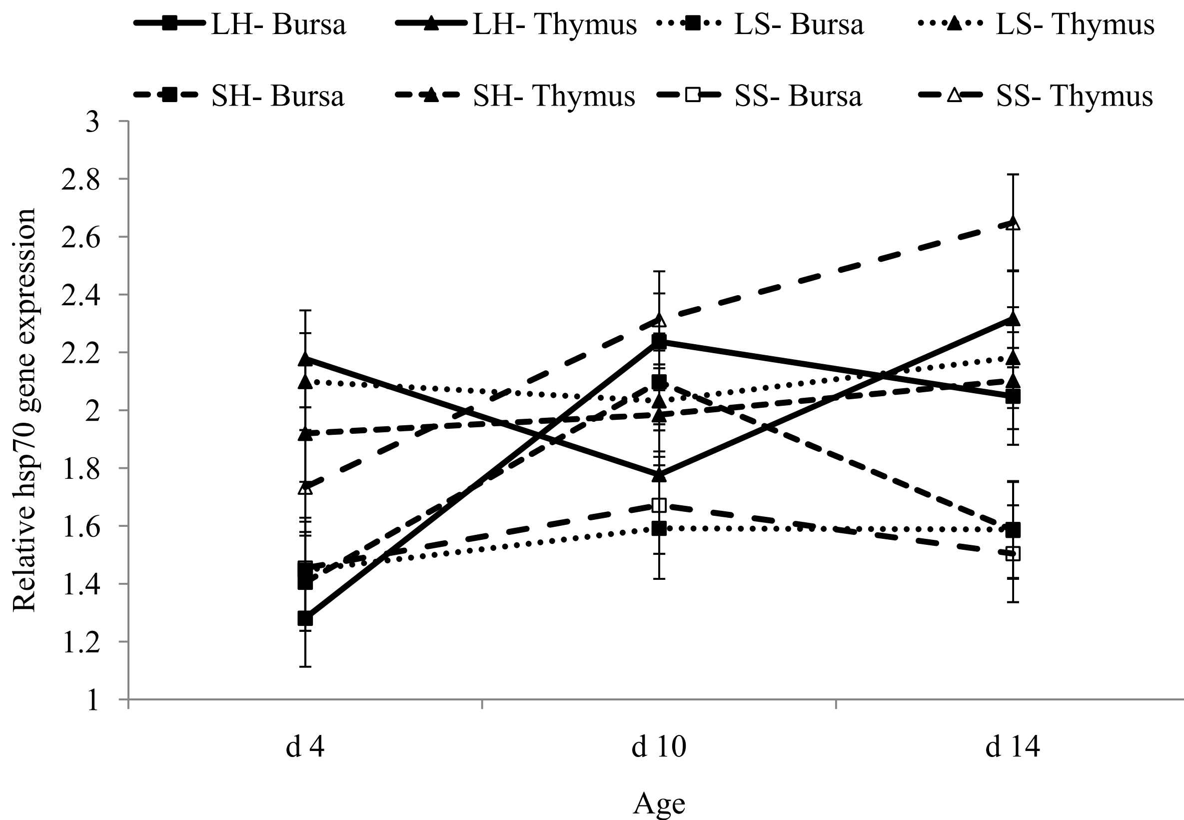

Figure 4.8 Effect of incubation temperature, tissue, and age on relative mRNA expression of hsp70 in the bursa and thymus of Cobb 500 broilers at d 4, d 10, and d 14. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Relative gene expression ($2^{-\Delta\Delta Ct}$) for hsp70 was calculated using the ΔΔCt method with the average ΔCt values for bursa and thymus in the SS-Control group as the calibrator(s). Data are presented as LS means ± SEM (n=16/treatment). There was a three-way interaction of temperature, tissue, and age (P=0.0177).

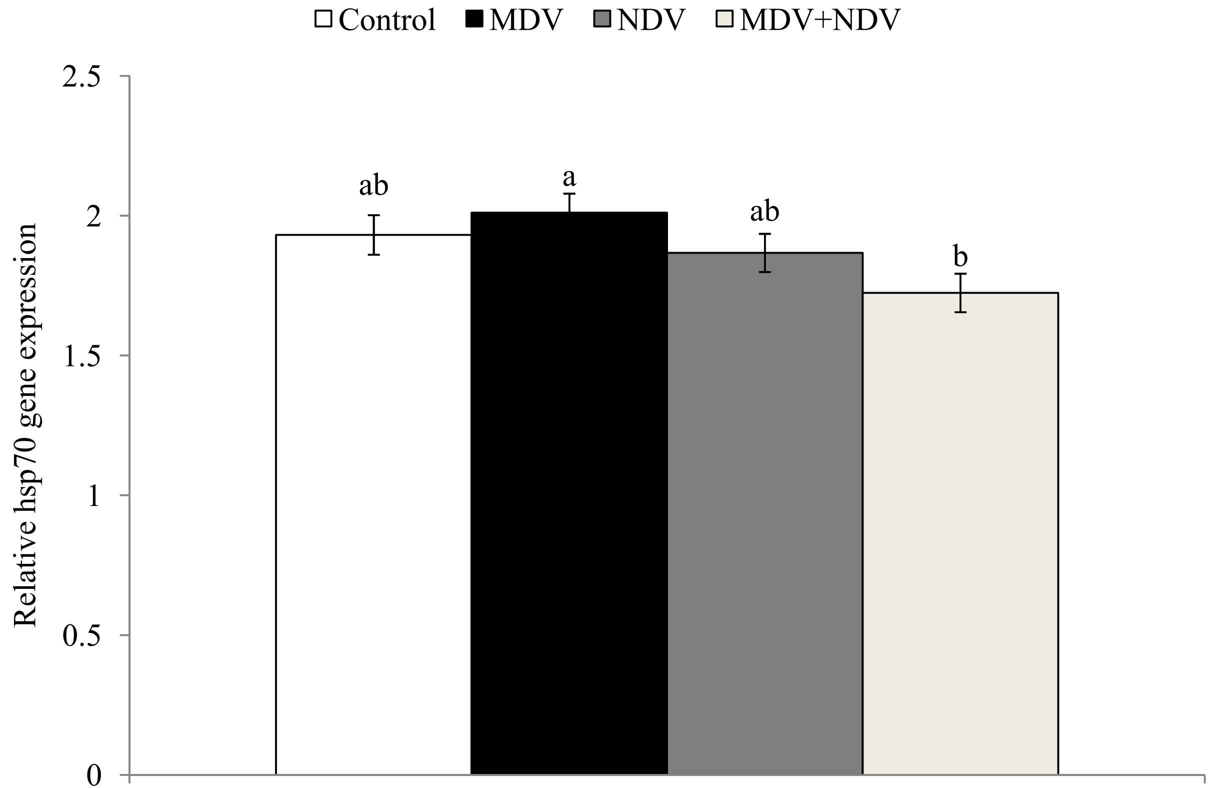

Figure 4.9 Effect of vaccination on relative mRNA expression of hsp70 in the immune organs (bursa and thymus, collectively) from d 4 to d 14 in Cobb 500 broilers. Vaccination treatments were designated as Control (no vaccine), Marek's disease virus (MDV) administered *in ovo* at d 18 of incubation, Newcastle disease virus (NDV) administered subcutaneously at hatch, and the combination of MDV+NDV. Relative gene expression ($2^{-\Delta\Delta Ct}$) for hsp70 was calculated using the ΔΔCt method with the average ΔCt values for bursa and thymus in the SS-Control group, respectively, as the calibrator(s). Data are presented as LS means ± SEM (n=96/treatment). There was a main effect of vaccination (P=0.0049).

[a,b] Data lacking a common superscript differ significantly

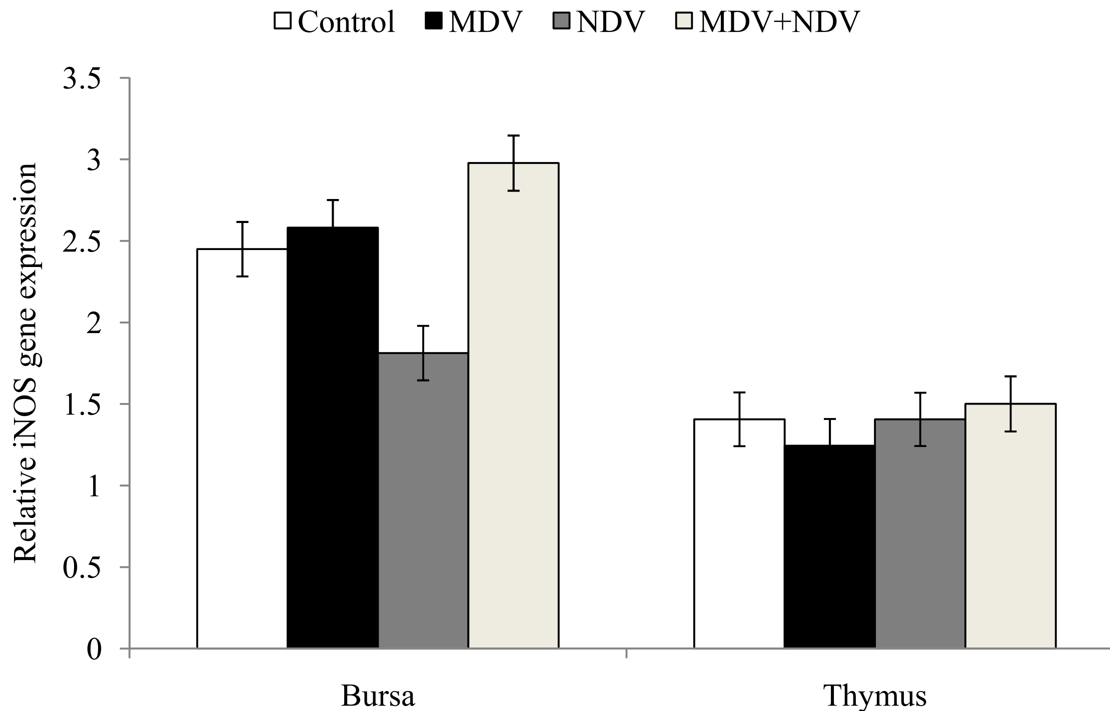

Figure 4.10 Effect of vaccination and tissue on relative mRNA expression of iNOS in the immune organs (bursa and thymus, collectively) from d 4 to d 14 in Cobb 500 broilers. Vaccination treatments were designated as Control (no vaccine), Marek's disease virus (MDV) administered *in ovo* at d 18 of incubation, Newcastle disease virus (NDV) administered subcutaneously at hatch, and the combination of MDV+NDV. Relative gene expression ($2^{-\Delta\Delta Ct}$) for iNOS was calculated using the ΔΔCt method with the average ΔCt values for bursa and thymus in the SS-Control group, respectively, as the calibrator(s). Data are presented as LS means ± SEM (n=48/treatment). There was a two-way interaction of vaccination and tissue (P=0.0250).

CHAPTER V

EPILOGUE

Non-optimal incubation temperatures impose challenges at many production levels in the poultry industry. Incubation temperatures that do not fall within the desired range (37°C to 38°C) required by the embryo have been shown to impact hatchability, chick quality, post-hatch development, and bird performance. This situation in the commercial poultry industry is a reflection of selection for fast growing, high-yield broilers and the associated increase in metabolic heat production by the embryo. Currently, few modifications in hatchery equipment and management have not occurred to compensate for the changed needs of the high-yield embryo, and consequently, many conditions in commercial incubators are reflective of environmental requirements of classic broiler embryos of the past. Studies have been conducted to evaluate the impact of non-optimal incubation temperatures on chick quality, organ development, thermoregulation, performance, and overall bird growth and development. No studies have evaluated the effects of incubation temperature alone on immune parameters or indicators of immune activity, or incubation temperature in combination with other post-hatch stressors, such as transportation from the hatchery or vaccination.

Results from the presented studies showed responses by the spleen to post-hatch transportation and vaccination. Histological evaluation of lymphoid tissue would further elucidate the impacts of incubation temperature or additional stressors on development of the immune system. The spleen serves as a secondary lymphoid organ that provides an environment for interactions between cells, cytokines, and antigens required to elicit an immune response (Mast and Goddeeris, 1999). How are these cellular interactions impacted by stress and how is the structure of the organ affected? The peri-arterior lymphoid sheath (**PALS)** and ellipsoids are

formed around ED20, and the maturation of these structures and the peri-ellipsoid lymphoid sheath (**PELS**), which serves as a critical compartment for B lymphocytes, occurs during the first week post-hatch (Mast and Goddeeris, 1999). Mast and Goddeeris (1999) further suggested that the late embryonic and neonatal spleen may not be fully functionally developed. It is therefore possible that these post-hatch stressors to which the chicks are exposed could be influencing their functional capacity to produce an immune response, and this impact could affect the lifetime immunocompetence of the bird. Morphological evaluation of the bursa would provide further insight as the bursa responded to transportation with decreased organ weight as a percentage of body weight. Could this decrease in organ weight be reflective of a decrease in bursal follicles and associated cells? Is the exposure to stress resulting in decreased lymphocyte production or are the cells responding to stress with apoptosis? Lymphocyte populations in each of the immune organs should be assessed with flow cytometry to further evaluate this theory.

Expression of pro-inflammatory cytokines should be evaluated to further understand the inflammatory response to these stressors, and cytokine expression should be evaluated in combination with corticosterone production. Stress exposure results in an increase in cytokine production (Ogle *et al.,* 1997), which then ultimately increases corticosteroid production by the adrenal glands. This results in a reduction in immune response mediators (Sapolsky *et al.,* 2000). It would be interesting to examine if there is a decrease in cytokine production with a corresponding increase in production of corticosterone, and if exposure to different stressors results in varying responses or if the bird perceives stress universally and the ratio of corticosterone and cytokine production remains consistent regardless of stress type.

The pro-inflammatory cytokines, IL-1β, TNF-α, and IL-6 have been suggested to stimulate hypothalamic-pituitary-adrenal (HPA) activity (Seguin *et al.,* 2009). If embryos are

stressed during incubation and increase their production of corticosterone, then this may result in a subsequent decrease in cytokine production and impact the development of the HPA axis. The HPA axis is activated at ED16 and stress exposure at this time point could affect the stress response of the embryo and of the neonatal chick due to the influence of the stressor on HPA activation. Activation of both the HPA and hypothalamic-pituitary-thyroid axes is critical for hatching of the chick. The concentrations of thyroid hormones increase significantly prior to hatch (Yahav *et al.,* 2004), which influences the final maturation of tissues and their ultimate function. The roles of these axes and the embryonic responses to temperature stress in the incubator have further implications in terms of acquisition of thermotolerance and the bird's ability to thermoregulate after hatch.

There may be potential for *in ovo* feeding to mediate some of the negative impacts that incubation temperature stress has on gastrointestinal tract development. Elevated incubation temperatures during the latter portion of incubation resulted in decreased gastrointestinal tract weight as a percentage of body weight at hatch compared to those embryos exposed to a standard incubation temperature (Leksrisompong *et al.,* 2007). This decreased organ weight likely relates to delayed maturation in the gut. Administration at ED17.5 of a solution containing maltose, sucrose, dextrin, and β-hydroxy-β-methylbutyrate resulted in increased body weights and higher pectoral muscle weights, both of which were maintained to d 25 post-hatch (Uni *et al.,* 2005). Could *in ovo* feeding help promote intestinal growth and development to compensate for the negative effects of incubation heat stress and therefore allow these temperature-stressed birds to achieve a better start post-hatch? Additionally, we have shown that temperature-stressed embryos utilize their yolk sac less efficiently. If these stressed embryos were in ovo fed, would they still present higher yolk sac weights, or would yolk sac absorption efficiency be improved?

To date, most studies involving incubation temperature stress focus on high-yield broiler lines due to their rapid growth and metabolic heat production and the resulting direct impact on embryonic thermal stress in the incubator. However, there is increased interest in the differences in embryonic development between broiler and layer chickens. Layers have shown increased corticosterone production at external pipping and increased T_4 at internal pipping compared to broilers (Everaert *et al.,* 2008). Layers have been selected for strong egg shells; therefore, chicks may have difficulty hatching (Everaert *et al.,* 2008). If layer embryos were exposed to elevated incubation temperatures and experienced similar responses as broiler embryos to the temperatures stress, would layer hatchability be dramatically reduced? Although elevated temperatures in layer embryo incubation would not be a result of rapid growth or high metabolic heat output, there could be application in terms of thermally manipulating layer embryos in the incubator to attempt to impact thermoregulation after hatch. However, would layer embryos be able to endure the elevated temperatures without a detrimental impact on hatchability or post-hatch chick quality?

Alternative broiler lines and progeny from different breeder flock ages may exhibit differential stress responses. Tona *et al.* (2004) evaluated physiological parameters of embryos from several different broiler lines during incubation, and there was a difference in heat production by the embryos among lines as well as body weight differences at d 7 and d 41 post-hatch. Additionally, different flock ages have been shown to exhibit different levels of oxygen consumption, CO_2 production, and heat output (Hamidu *et al.,* 2007). It is possible that progeny from differing breeder flock ages that produce a greater amount of metabolic heat could be more deleteriously impacted by incubation temperature stress. However, the increased level of stress

imposed upon these embryos during incubation due to higher heat output may cause a more efficient response to additional post-hatch stressors.

Turkeys may respond to incubation temperature stress differently than broilers. Similar to broilers, turkeys are genetically selected for rapid growth and also have a high metabolic heat output. The standard incubation temperature for turkeys is the same as for broilers (37°C to 38°C), and deviations from this temperature range may result in malpositioned embryos, ruptured yolk sacs, edematous heads, and excess albumin (French, 2000). Research by French (2000) also demonstrated increased mortality of embryos due to overheating during the 3rd and 4th weeks of incubation and at pipping. Additionally, oxygen requirements of turkeys during the latter portion of incubation undergo modifications around ED25 and ED26 at which time the embryos utilize more oxygen and dispose of more carbon dioxide than the eggshell is capable of supporting (Dietz *et al.,* 1998). This creates a subsequent plateau in oxygen consumption (Rahn, 1981). The oxygen plateau causes the embryo to continue growth and metabolic processes under hypoxic conditions, and consequently, delays intestinal maturation due to improper supply of energy under these conditions (Christensen *et al.,* 2004). Could the delay in intestinal maturation due to the combination of temperature and hypoxic stress result in increased susceptibility to enteric diseases? How will the poults elicit a response to a disease challenge if they have been previously stressed during incubation?

The results from these presented two studies offer valuable insight as to how the young chick responds to incubation temperature, transportation, and vaccination stressors alone or in combination. We have demonstrated that these evaluated parameters impacted immune organ development, gene expression of innate immunity indicators, antibody production, and bird performance. In order to better understand the mechanisms responsible for these responses,

further research needs to be conducted. It would be helpful to evaluate changes in immune parameters resulting from incubation temperature, transportation, and vaccination stress separately. By evaluating the parameters independently, more attention and focus could be applied on the immune response specific to each condition. Once a better understanding of the individual stressors has been attained, then the combination of stressors, in addition to the other parameters mentioned, should be considered.

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APPENDIX A

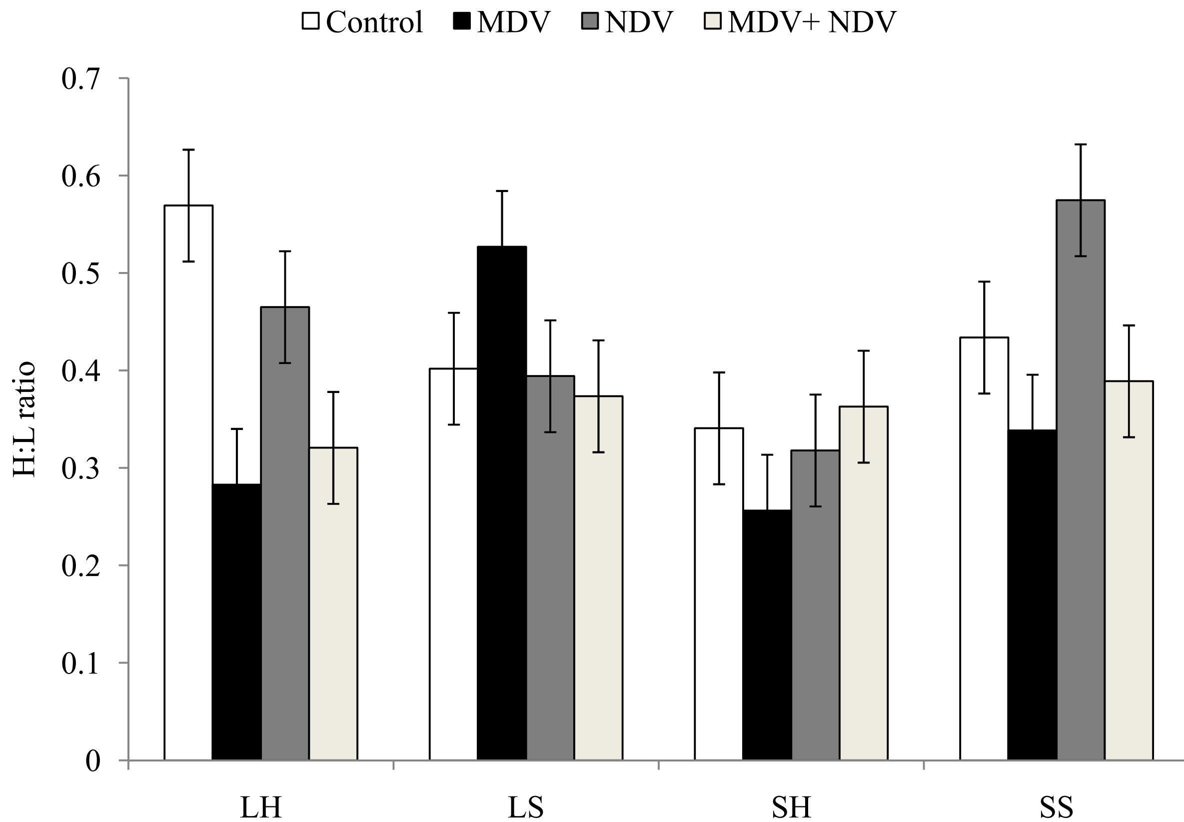

Figure A.1 Effect of incubation temperature and vaccination on H:L ratios in Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Vaccination treatments were designated as Control (no vaccine), Marek's disease virus (MDV) administered *in ovo* at d 18 of incubation, Newcastle disease virus (NDV) administered subcutaneously at hatch, and the combination of MDV+NDV. Data are presented as LS means ± SEM (n=24/treatment). There was a two-way interaction of temperature and vaccination (P=0.0060).

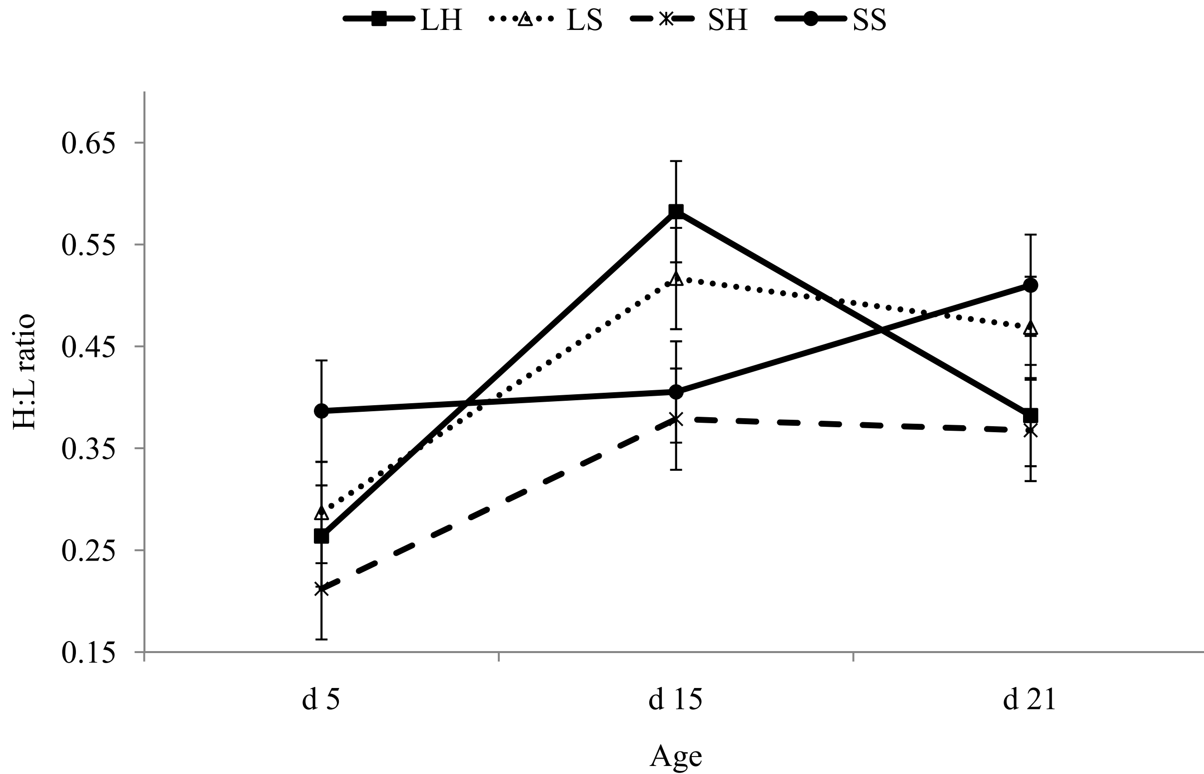

Figure A.2 Effect of incubation temperature and age on H:L ratios in Cobb 500 broilers. Incubation temperature treatments were designated as Low (L: 36.7°C) or Standard (S: 37.5°C) from ED0 to ED7, S from ED8 to ED14, and S or High (H: 39.0°C) from ED15 to ED21. Data are presented as LS means ± SEM (n=32/treatment). There was a two-way interaction of temperature and age (P=0.0425).