

**Effects of Induced Moisture Loss on Broiler Chicks Immune Response Post
Salmonella enteritidis Lipopolysaccharide Challenge**

THESIS

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ABSTRACT

It is well understood that above optimum temperatures during incubation can lead to increased moisture loss and dehydrated chicks. For the experiments reported herein, moisture loss was measured without the confounding effects of temperature. Moisture loss was induced in chick embryos from days 11 through hatch by drilling 2 small holes in the air sac of eggs. On the day of hatch, chicks were further divided into three treatment groups, two controls and one treatment consisting of an injection with *Salmonella enteritidis* lipopolysaccharide (LPS). The Salmonella LPS is derived from the outer membrane of this gram-negative bacteria which is commonly found throughout poultry houses (Wang et al., 2003). The LPS injection was used to mimic a potential bacterial challenge to trigger a response from the chick's immune system. The two control treatments consisted of one group receiving a PBS injection alone (no LPS) and a non-injected control group of eggs. At 24 and 48 hours post injection, tissue samples were collected for RT-PCR analysis of the interleukin 1 (IL1) and interleukin 10 (IL10) response in chicks with normal and increased moisture loss during incubation.

The objectives of this study were to 1.) Measure moisture loss differences in egg and chick weights between the treatment groups and 2.) Study effects of induced moisture loss on the immune response when a potential pathogen is introduced.

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CHAPTER 1: INTRODUCTION

The poultry industry, in particular the broiler industry, is always looking for ways to reduce embryonic stress during incubation to facilitate the hatching of high quality, healthy chicks. Intensive genetic selection for traits of commercial importance (i.e. body weight, breast muscle yield) together with improvements in nutrition and management have contributed to the continual improvement in the overall efficiency of broiler production. From 1957 to 2005, broiler growth potential has increased over 400% while the feed conversion ratio has decreased by 50% (Zuidhof et al., 2014). Much of the improvement in feed conversion is due to the reduction in the average number of days for birds to reach similar market weights, approximately 1 day per year over the last 40 years (Nir and Levanon, 1993). The overall efficiency of the production cycle has now been extended to embryonic development with the recognition that the incubation period and early post-hatch period represent an increasing proportion of the overall production cycle (Wolanski et al, 2006).

In the experiments reported herein, embryonic moisture loss was accelerated beginning at either 8 or 11 days of incubation depending upon the experiment. This was done by drilling 2 small holes in the air sac of 50% of the total eggs used in each study. At 18 days of incubation in Experiments 1 and 2, a sample of eggs and embryos were sampled for moisture loss and determination of thymus cell proliferation potential. In Experiments 3 and 4, newly hatched chicks were randomly divided into three groups and challenged with *Salmonella enteritidis* lipopolysaccharide (LPS) administered via intra-peritoneal injection. Salmonella LPS is found in

the outer membrane of gram-negative bacteria that broilers are routinely exposed to during a production cycle (Wang et al., 2003). The LPS injection was used to mimic a potential bacterial challenge and the goal was to see if differences in embryonic moisture loss would influence a chick's immune response. Chicks were sampled at both 24 and 48 hour post-injection to determine the IL-1 and IL-10 cytokine responses by the spleen.

The objectives of the present study were to induce moisture loss without changing temperature to measure the level of stress created. The specific aims are as follows:

Specific Aim 1

To measure moisture loss differences in egg and chick weights between the treatment groups

Specific Aim 2

To identify Treg numbers and immunosuppressive properties between treatment groups after an LPS *Salmonella enteritidis* challenge

CHAPTER 2: REVIEW OF LITERATURE

Avian Incubation Practices

The incubation length for a chick is approximately 21 days from the day of setting until hatch. Normal embryo development occurs at a temperature (99.5°F; 37.5°C) which is cooler than a hen's normal body temperature that is approximately (41°C; 106°F). Embryonic development is initiated as soon as the egg is laid and will be reduced at temperatures less than 37.5°C. Development will stall at temperatures lower than 21°C and this point of near cessation in development is referred to as the physiological zero (Patten and Carlson, 1958). In industry practice, the use of lower storage temperatures near the cessation point of embryonic development is used for short-term storage of fertile eggs prior to setting with no long-term effects on hatchability through approximately 7 days of storage. This allows the commercial industry to better manage and balance the production and setting of fertile hatching eggs.

Under conditions of optimal incubation, eggs are set to achieve a constant eggshell temperature of approximately 37.8°C (100°F; Lundy, 1969). As internal temperatures cannot be taken without physically penetrating the egg, eggshell temperature has been shown to best represent the temperature within the immediate environment experienced by the embryo (Lourens et al., 2005). Previous research has shown that during the first half of incubation, the embryo/eggshell temperatures are slightly lower than machine temperatures suggesting the egg absorbs heat from the surrounding air (Joseph et al., 2006). During the second half of incubation, embryonic metabolism increases and heat is released from the egg with the potential for

incubation air temperature to also be increased. To this end, it is important to gradually reduce the incubation temperature in order to maintain a consistent eggshell temperature and reduce the possibility of embryonic overheating (French, 1997). Adjusting relative humidity within an incubator is also a means of moderating eggshell temperature. Eggs incubated at a lower humidity level will experience an increase in heat loss while the opposite is true for eggs incubated at high humidity (Van der Pol, 2013).

There are two types of incubation systems used in commercial practice, multi-stage and single-stage. In multi-stage incubation systems, new eggs are continuously being added to the incubator as older eggs/embryos are moved to hatching cabinets. This results in multiple age embryos within the incubator at varying stages of incubation and heat production. The metabolic heat produced by older embryos is used to maintain an average incubator temperature of 37.5°C-37.8°C. (Fernandes et al., 2017). Based on egg age and location within the incubator, warm and/or cool spots are more likely to occur in multi-stage incubation. Previous experiments have shown that when large and small eggs are incubated under similar conditions, the larger eggs had increased shell temperatures during the later stages of incubation (El-Hanoun, et al., 2012). The single-stage incubation system is an all in—all out system in which all the eggs within the incubator are set and removed at the same time. This makes incubator and eggshell temperature regulation an easier process to manage.

The “hatch window” refers to the period between when the first and last chicks hatch. Several factors can influence the “hatch window” time spread but the most important factor is keeping temperature variation to a minimum (Lourens et al., 2005). In commercial practice, the total number of chicks hatched is a critical economic metric and chicks are often held in the hatch cabinets longer to give ample time for all eggs to hatch. This can result in the earliest hatching chicks being held without access to feed and especially water for a longer than optimal period and

can often vary from 24 to 48 hours in length (Careghi et al., 2005). The most common factors contributing to variability in the hatch window includes breed differences, egg storage conditions, egg size, and varying egg shell temperatures. Misra and Fanguy (1978) reported that chicks held in hatcher cabinets for 32 to 48 hours after hatching had reduced body weights at placement. Kingston (1979) subsequently reported that when chicks were held in the hatch cabinets more than 48 hour, dehydration was significantly increased resulting in an increased percentage 10 day mortality compared with chicks placed shortly after hatch. An optimized, reduced hatch window will often lead to a broiler flock with increased uniformity (van de Ven et al., 2012). These authors went on to show that in those chicks which hatched later, the percentage lung, heart, stomach and intestine weights were increased suggesting that these chicks were somewhat more mature. In commercial practice, the factor that contributes most to variation in hatch time is incubation temperature, particularly low eggshell temperatures during the first 10 days after setting. This is often due to an incubator not being sufficiently warmed for the newly developing embryos, which as stated previously, have not yet initiated de novo heat production and are subsequently most sensitive to the outside environmental temperature (Ipek et al., 2014).

Several “chick quality” factors can be influenced by a less than optimal incubation environment and these would lead to chicks being be culled or categorized as being of inferior quality. These quality factors would include dehydration, red hocks, open navels, and residual yolk sacs. The “red hock” characteristic is commonly assumed to occur in chicks which have had to work harder to break out of the shell. The hocks have a red, swollen appearance and in some instances may even have open wounds. This condition may restrict feed and water intake by afflicted chicks once they get to a farm resulting in a slow start, increased mortality or both. Dehydration is common in chicks hatched from young breeder hens as the eggs and chicks from these flocks are often smaller and more prone to have higher mortality and reduced body weights at market age (Wyatt et al, 1985). Embryos with an increased metabolic rate due to increased

incubation temperatures are also observed to often have reduced incubation times (Ar and Rahn, 1980). The same effect can be observed with lower humidity levels that will also decrease incubation length via reduced water availability and subsequent increases (Peebles et al, 1986).

Proper Egg Storage Techniques

There is conflicting research on the ideal storage temperature of eggs prior to incubation. Some research has suggested that eggs stored for up to two days prior to setting had higher hatchability when stored at warmer temperatures whereas the opposite was true for eggs stored over a week (Brake et al., 1997). The optimum storage temperature of hatching eggs is 16-17°C (60.8-62.6°F) for the first 3 to 7 days but to minimize the negative effects of long term storage, temperatures can be dropped to as low as 10-12°C (50-53.6°F) if eggs are to be kept for more than 7 days (Butler, 1991). Hatch percentages drop as storage time increases due to cell death and slowed embryo development after incubation temperatures are increased to normal (Fasenko, 2006). Wilson (1991) reported that after the initial 6-7 days of storage, hatchability will decrease by 0.5-1.5% for each additional day of egg storage.

It is also suggested that the length of time eggs are stored can also increase the length of the incubation period. Industry data averages suggest that for each day of egg storage, there is an additional hour of incubation time required prior to hatch (Cobb-Vantress Management Guide, 2008). When transporting eggs from the breeder farm to the hatchery, temperature changes need to be closely monitored so that large differentials do not occur. This could lead to the “sweating” of eggs or moisture accumulation on the shell. This could result in bacteria on the shell being drawn inside the egg via the pores in the eggshell resulting in an increased bacterial load inside the egg (Fromm and Margolf, 1958). Proper ventilation of eggs during storage and transport will also reduce the incidence of sweating. The now common practice of “pre-incubating” hatching eggs before or during storage to get the embryo to a more storage resilient hypoblast stage, has

been shown to reduce the negative effects of egg storage periods longer than 7 days (Fasenko et al., 2001). In commercial hatcheries, it is common to use a “V shaped curve” concept in which the core temperature of eggs are gradually reduced from the hen’s body temperature (40°C to 41.1°C), to a truck transport temperature of (20°C-22.7°C) and down finally to the egg storage room at the hatchery (18.9°C-21.1°C). The opposite temperature regimen then occurs prior to incubation.

Embryo Development

The yolk, albumen, and shell membranes function in both protecting and nourishing the developing embryo. By weight, the egg is roughly six parts albumen, 3 parts yolk and one part shell (Romanoff, 1949). The yolk provides the majority of the nutrients needed for embryonic growth and development (Romanoff, 1967). The eggshell is comprised of an outer shell, shell membranes and an inner thin film. These layers act as a filter for gas exchange between the embryo and incubator environment (Vick et al., 1993). All embryonic development nutrient requirements are stored in the yolk and albumen (Romanoff, 1960). Yolk components can change slightly based on egg size, breed and age but in general is approximately 50% water, 33% fat, 15% protein and less than 1% carbohydrate (Shenstone and Carter, 1968). As a hen ages, egg size will naturally increase and the percentage yolk will also increase resulting in a potentially higher nutrient content for developing embryos (Nangsuay et al, 2011). Embryo growth, especially during the last few days of incubation, is influenced by egg weight (Wiley, 1950). Broiler chick weight at hatch is approximately 68% of the egg weight at the start of incubation (Jull and Heywang, 1930).

A hen can ovulate from 7 to 74 minutes (average 32 minutes) after a previous egg has been laid with fertilization normally taking place shortly after ovulation (Phillips, 1936; Olsen and Neher, 1948). The process of segmentation, also called cleavage, is a sequence of cell

divisions that initiate the process of embryonic development and normally begins approximately 5 hours after fertilization (Olsen, 1942). The initial stage of embryonic development results in the formation of the “primitive streak” which is first visible after 8-9 hours of incubation and fully developed by 16-20 hours (Wetzel, 1929). Withdrawal of the yolk sac into the body cavity begins on day 19 and is complete by the time of hatch (Yadgary et al., 2010).

The egg shell itself has pores which facilitate the passage of O₂, CO₂, and water vapor into and out of the egg during incubation (Rahn, 1981). The ideal water loss for optimal hatchability during incubation is approximately 12% (Ar and Rahn, 1980) as evaporation is essential for the chick to create an air cell and successfully hatch. The largest concentration of pores in the eggshell are located at the large, round end of the egg over the air sac. Young breeder flocks with their smaller eggs have a thicker shell and a reduced number of pores. As a breeder hen ages and egg size increases, the amount of shell deposited stays approximately the same resulting in a thinner shell, increased pore size and a subsequent decline in albumen quality (Vick et al., 1993; Lourens et al., 2006). Lourens (2006) reported an increased rate of gas exchange in eggs from older breeder hens which resulted in differences in embryonic incubation needs due to the age of the hen. Broiler breeder hen’s peak hatch of eggs set and fertility occur around week 33 of age at 70% and decline steadily to 54% at 54 weeks of age (Wolc et al., 2010).

Genetic selection along with improved nutrition and management practices, continue to push breed performance limits. Genetic selection over many years has focused on feed conversion and egg production in broiler and layer strains, respectively. This has resulted in commercial broilers achieving a feed conversion ratio of less than 2:1 and commercial layers capable of producing over 300 eggs per year (Druyan, 2010). Due to obvious differences in genetic selection goals between layers and broilers, embryonic development and metabolism differences have also occurred (Janke et al., 2004). Janke et al., (2004) reported that due to embryonic

growth rate differences, commercial broiler embryos have elevated heat production and body temperature when compared to commercial layer embryos during incubation. An additional factor includes broiler breeders producing eggs weighing approximately 10 grams more on average than those eggs produced by laying hens of the same age (Ho et al., 2011).

Immunology

Innate Immunity

The avian immune system is similar to mammals but with a few key differences. The first lines of defense are physical and chemical barriers such as skin and mucus membranes (Zhang et al., 2015). If a pathogen makes it past these initial barriers, it will encounter a host's initial immune response, the innate immune system (Kumar et al., 2011). The innate immune system is non-specific and quick to respond. It serves as a mechanism for causing maximal damage to pathogens but exacts a high metabolic energy cost to the host and can potentially damage normal tissues (Erf, 2004). The four major components of innate immunity are anatomic and physiologic, phagocytic, inflammatory, and cytotoxicity from natural killer cells (Felsburg, 1994).

The response cells comprising the innate immune response (i.e. macrophages, dendritic cells) recognize extracellular pathogens by their particular molecular patterns and this triggers a more specific adaptive immune response (Lillehoj et al., 2012). The response cells, referred to as antigen-presenting cells, result in host lymphocytes recognizing infected cells and presenting peptides on the cell's surface (Felsburg, 1994). The antigen-presenting cells express Toll-like receptors that recognize foreign pathogens that have passed through the initial barriers and activated an inflammatory response (Takeda and Akira, 2005). Antigen-presenting cells present foreign antigens to T helper or cytotoxic T cells which facilitate the immune response, in essence bridging the innate and adaptive immune systems (Cederbom et al., 2000).

Adaptive Immunity

Adaptive or acquired immunity is antigen specific in that it recognizes the unique structure of a particular pathogen and commits it to “memory”. This minimizes the use of the body’s resources to respond to a challenge but the process requires more time and if not for the innate immune response, the pathogen might have already overwhelmed the host.

(Felsburg, 1994). Adaptive immunity has two components, humoral immunity which involves B-lymphocytes that produce antibodies specific for extracellular pathogens and a cell mediated immune response involving T-lymphocytes (Tc) which are specialized cells that interact with intracellular pathogens (Erf, 2004). The primary immune response of the T and B cells was initially observed after the discovery of the importance of the Bursa of Fabricius in avian immunology (Glick, 1956). B-lymphocytes, so named for their maturation in the bursa, proliferate and mature in a similar fashion to what is observed for T-lymphocytes that mature in the thymus (Roitt et al, 1969; Rudrappa and Humphrey, 2007).

There are two major cell types involved in T-lymphocyte activation. Th (CD4⁺) cells, commonly known as T helper cells, help direct the immune response of both B cells and Tc (CD8⁺) cells, commonly called cytotoxic T cells which kill pathogens (Nii, et al, 2011). Nii et al. (2011) went on to describe the importance of the balance of cytotoxic and T helper cells in maintaining a healthy immune system as they regulate anti- and pro-inflammatory immune responses, respectively. With so many pro-inflammatory cytokines and chemokines flooding to a site of infection, there could be significant inflammation and tissue damage if the process is not controlled. Developing thymocytes express both CD4⁺CD8⁺ positive cells until they go through the process of positive selection where thymocytes differentiate into either the CD4⁺ T helper cells or CD8⁺ cytotoxic T cells (Singer et al., 2008). The CD4⁺ T cells, including T helper (Th1), TH17, and FoxP3 regulatory T cells, are important modulators of immune balance (Barnes and

Powrie, 2009). Regulatory T cells (Tregs) express biomarkers, CD4⁺ and CD25⁺ which maintain tolerance to self-antigens and prevent autoimmune disease (Mills, 2004). Mammalian Tregs express the transcription factor, FoxP3⁺, which is generally considered analogous to the avian CD4⁺CD25⁺ marker where FoxP3⁺ is absent and autoimmune disease can occur (Sun et al, 2010). The CD4⁺ CD25⁺ Tregs produce high amounts of the anti-inflammatory cytokine IL-10 (Dieckmann, 2001; Selvaraj, 2011). In the current study, we measured the immune suppressive (Interleukin 10; IL10) and pro-inflammatory (Interleukin-1; IL1) cytokines in the spleen to determine if induced moisture loss would stimulate an immune response in chicks during incubation coupled with a post-hatch lipopolysaccharide (LPS) challenge. The IL-1 family has 11 cytokines with IL-1 α and IL-1 β being the most studied because of their strong inflammatory response (Dinarello, 2011).

Lipopolysaccharide (LPS)

Lipopolysaccharides (LPS) are active components of a bacterial cell wall outer membrane from gram-negative bacteria (i.e. Salmonella, E.coli). These bacteria are commonly found in broiler house environments and have been shown to induce an inflammatory response (Xie et al, 2000). LPS has been linked as the major cause of the harmful effects linked to gram-negative bacteria (Abbas et al, 2000). Once the body recognizes a particular LPS, it initiates a signaling pathway of IL-1 resulting in the release of pro-inflammatory mediators such as fever and inflammation (Heumann, Roger, 2002). The immune response, in an attempt to maintain balance, will activate anti-inflammatory cytokines (Baumgartner et al., 1999; Wang et al., 2003). Gram-negative bacteria effect poultry predominantly by invading the gut epithelium causing inflammation and damage. Salmonella can colonize in the gastrointestinal tract and in newly hatched chicks and spread to other organs such as liver and spleen causing a systemic infection (Barrow et al, 1987).

Spleen

The secondary lymphoid tissues include the spleen, lymph nodes and bone marrow where immunologically mature cells reside and enter the circulation (Davison et al, 2008). In the current study we sampled the spleen because it is the home of mature B and T lymphocytes that are surveying potential pathogens. This results in the spleen serving a compensatory role in immunity if the bursa were to be compromised at a younger age (Chang, 1958). With increasing embryonic and post-hatch age, there is an increasing population of thymus derived T cells found in the spleen (Abdul-Careem et al, 2007). The composition of the spleen consists of periarteriolar (T cells) and periellipsoid (B cells) lymphocyte sheaths and follicles (Jeurissen, 1991). These are essential for initiating the antibody-mediated immune response that is thymus dependent.

Housekeeping genes are essential for a cell to survive so they are present in all conditions therefore can be used as internal controls as they have the ability to maintain constant expression as which to compare cytokine expression in Real Time PCR analysis (Theillin et al, 1999).

CHAPTER 3: MATERIALS AND METHODS

Animal Welfare

All animal protocols were approved by the Institutional Animal Care and Use Committee at The Ohio State University (IACUC # 20140090).

Experimental Procedures

In each of two trials in Experiment 1, 100 fertile eggs from leghorn hens were individually weighed and set at 37.8°C (100°F) and 65% relative humidity. At embryonic day 8 in each trial, all eggs were reweighed and candled for fertility. One-half of the remaining fertile eggs had either 0 or 2 holes drilled in the egg shell above the air cell. For all experiments, a dremel was used to drill two, 1 mm holes at the top most part of the air cell approximately 3 mm apart. On day 18, each egg was reweighed for moisture loss determination and 18 embryos were randomly selected from each treatment group (0 hole; 2 hole). Thymus samples were pooled from 3 embryos (n = 6 replicates per treatment), put in Roswell Park Memorial (RPMI) growth media and immediately taken to the lab to conduct a Methylthiazol Tetrazolium (MTT) proliferation assay.

In Experiment 2, broiler hatching eggs (n=354) were individually weighed and numbered. At eleven days of incubation, the eggs were candled and any infertile or early dead embryos were removed. The remaining eggs were weighed and divided with half the eggs having 2 holes drilled in the air cell and the other half (0 holes) serving as the controls, similar to what was describe for Experiment 1. All eggs were subsequently returned to the incubator. On day 18,

all eggs were reweighed and a sample of embryos (n=18) were randomly selected from each treatment group (0 holes; 2 holes) for a repeat MTT thymocyte proliferation assay.

The same protocol during incubation was followed for Experiment 3 with a smaller number of fertile broiler hatching eggs (n = 300) obtained from the Orrville Chick Hatchery. On embryonic day 18, all eggs were reweighed. The exception for Experiment 3 was that after weighing on embryonic day 18, a sample of eggs from each treatment group (0 hole; 2 hole) was transferred to the hatcher. Thymus and spleen samples were pooled from 3 embryos to get enough replication (n=6). The spleen and thymus were removed from 18 embryos in treatment group (0 hole; 2 hole). Thymus samples were put in Roswell Park Memorial (RPMI) growth media, pooled with 3 samples per tube and taken to the lab immediately to conduct the Methylthiazol Tetrazolium (MTT) Assay. Spleen samples from embryos were pooled with 3 samples per 1 ml tube filled with RNAlater and stored in a -70°C freezer until the time they could be further analyzed.

On Day of hatch (day 21) of experiment 3, chicks (n = 215) were weighed and transferred to battery pens at the OARDC Poultry Research Farm. Chicks from each incubation treatment (0 hole, 2 hole) were randomly separated into 3 additional treatment groups with two groups receiving a 25µl per chick injection and one group acting as a non-injected control group. The chicks from the 0 and 2 hole injected treatment groups were challenged with either Salmonella enteritidis lipopolysaccharide (LPS; 500 ug/kg body weight) or PBS injected intraperitoneally. The phosphate buffered saline (PBS) injection served as a placebo to measure effectiveness of injection administration.

At 24 and 48 hour post-injection (post-hatch), chicks were euthanized by CO₂ inhalation. A topical application of 70% ethanol was applied to the skin prior to tissue sample collection. Spleen and thymus samples were collected at both the 24 and 48 hour sampling times

from 18 birds per treatment for evaluation of thymocyte proliferation, IL1- β and IL-10 gene expression. Because of the smaller number of chicks, there were only 16 chicks used in the 0 and 2 hole control groups at both sampling time periods. Spleen samples were stored in RNA later for later analysis and thymus samples were placed in RPMI for use in an MTT thymocyte proliferation assay.

Effect of Moisture Loss on T-lymphocytes Proliferation

At embryonic day 18, 6 thymus pools of 3 chicks per treatment were used for a thymocyte proliferation assay. This assay utilized a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay as described previously (Breed et al., 1997). The pooled thymus samples were ground over a 0.4- μ m cell strainer (Sigma-Aldrich, St. Louis, MO) with RPMI complete media. Thymocytes were collected after density centrifugation by histopaque (1.077 g/mL; Sigma-Aldrich, St. Louis, MO.) to get a single-cell suspension of thymocytes. Live cells were determined using trypan blue staining (Sigma-Aldrich, St. Louis, MO). Thymocytes (91×10^6) were cultured in 200 μ l complete media supplemented with chicken serum in round bottom 96 well plates for 72 hour in a 5% CO₂ incubator set at 37°C. Each assay was performed in triplicate. Cells were stimulated with 200 ng/ml Phorbol 12-myristate 13-acetate (PMA), 50ng/ml Ionomycin, and 1 μ g/ml of CD28⁺. After 48 and 72 hour of cell culture, 20 μ l of 5 mg/ml MTT solution (Sigma-Aldrich, Sigma Aldrich, St. Louis, MO) was added to the cell culture medium and incubated for an additional 4 hour. The supernatant was subsequently removed by centrifugation and cells were re-suspended in 200 μ L of isopropanol plus 10% dimethyl sulfoxide and 0.04 NHCl and allowed to stand for 1 hour at room temperature. The concentration of MTT formazan formed in the 96-well plates was read on a microplate ELISA reader at 562 nm. The proliferation efficiency was reported as the mean optical density.

Effect of Moisture Loss on IL-1B and IL-10

Total RNA was extracted from all experimental groups using the TRI reagent (Molecular research center, Cincinnati, OH) following manufacturer's instructions. RNA concentration and purity was determined by NanoDrop (Thermo Scientific) using the 260/280 and 260/230 ratios. The RNA was reverse transcribed into cDNA and analyzed for IL-1 β and IL-10 expression by real-time PCR (iCycler, BioRad, Hercules, CA) using SYBR Green. The mRNA analyzed for IL-10 and IL-1 was normalized with β -actin (primer sequences found on Table 1; (Shanmugasundaram and Selvaraj, 2012b). The $2^{-\Delta\Delta Ct}$ method previously described (Livak and Schmittgen, 2001) using the Ct as the threshold cycle to calculate mRNA expression and the fold change calculated as $2^{(Ct_{Sample} - housekeeping) / (Ct_{Reference} - housekeeping)}$. The reference group was the non-injected or non-challenged 0 hole control group.

Each well contained 10 μ l SYBR Green PCR master mix, 9 μ l RNase-free water using IQ5 Cycler (iCycler, BioRad, Hercules, CA), 2 μ l cDNA, 0.5 μ l forward primer (5 μ M), and 0.5 μ l reverse primer (5 μ M). To perform real-time PCR, the following machine settings were used: 95°C for 10 min. (1 cycle), 95°C for 15 seconds and 60°C for 45 seconds (45 cycles) with temperatures of 10°C.

Effect of Moisture Loss on CD4⁺, CD8⁺ and Treg Cell Percentages

Single-cell suspensions of thymocytes (n = 6) were concentrated for lymphocytes by density centrifugation over Histopaque (1.077 g/ml; Sigma-Aldrich). The single cell thymocyte suspensions were incubated with a 1:250 dilution of fluorescent-isothiocyanate-conjugated mouse anti-chicken CD4⁺ (Southern Biotech, Birmingham, AL) and 1:400 dilution of phycoerythrin-conjugated mouse anti-chicken CD8⁺ (Southern Biotech, Birmingham, AL) for 15 min. The unbound antibodies were removed by centrifugation, and the percentages of CD4⁺ and CD8⁺ cells were analyzed using a flow cytometer (Guava EasyCyte, Millipore, Billerica, MA) as described previously (Shanmugasundaram et al., 2015).

For the determination of Tregulatory cell percentages, cells (1×10^6) were incubated with 10 $\mu\text{g/ml}$ of primary fluorescent linked mouse anti-chicken CD25⁺, 1:200 fluorescent-conjugated mouse anti-chicken CD4⁺ (Southern Biotechnology Associates, Birmingham, AL), and 1:200 dilution of unlabeled mouse IgG for 45 min. The unbound primary antibodies were removed by centrifugation. The percentage of CD4⁺CD25⁺ cells in different organs were analyzed in a flow cytometer (Guava Easycyte; Millipore) and expressed as a percentage of total CD4⁺ cells.

Statistical Analysis

The General Linear Model (PROC GLM) procedure of SAS was used to exam the main effects of 0 or 2 holes on moisture loss and hatchling body weight. Main effect means were considered significant at $P < .05$. For comparing the means of 18 day embryo IL-1 and IL-10 transcription, a Student T-test was used.

A 2X3 factorial arrangement of treatments (JMP Software, Carey, NC) was used to examine the effect of induced moisture loss during incubation on CD4⁺, CD8⁺ and CD4⁺CD25⁺ concentrations in control chicks post hatch in comparison to those injected with LPS and PBS at 24 and 48 hour time points.

A 2X2 factorial arrangement of treatments (JMP Software, Carey, NC) was used to examine the effect of induced moisture loss during incubation on IL-1 and IL-10 cytokine expression in Control chicks and those injected with LPS at the 24 and 48 hour time points. ΔCt values were used to determine statistical significance with ($P < 0.05$). For significant effects, differences between means were analyzed using Student's T test for least-square means comparison.

CHAPTER 4: RESULTS

Layer Egg Data

In Experiment 1, two trials were conducted using 100 layer eggs for each. On embryonic day 8, 50 eggs had 2 holes drilled in to the air sac to induce moisture loss while the remaining 50 eggs served as 0 hole control eggs/embryos. All eggs were re-weighed on day 18. The egg weight and moisture loss this experiment 1 (Trials 1, 2) are in Tables 2 and 3.

In Trial 1, the eggs were randomly assigned to each treatment prior to weighing with no stratification of the eggs to equalize initial egg weights. This resulted in a significant difference in initial egg weights (Table 2; $P < .008$) in favor of the 2 hole (52.9 g) versus 0 hole eggs (50.1 g). The eggs within the 2 hole treatment were significantly heavier at all subsequent ages (day 8, day 18). When the holes were drilled at day 8, initial moisture loss in the 2 hole treatment was already higher (3.78% versus 2.79%; $P < .001$) and stayed significantly higher at 18 days (9.47% versus 8.76%; $P < 0.025$).

In Trial 2 (Experiment 1), eggs were from an older leghorn flock and were heavier than those in Trial 1. These eggs were stratified to equalize egg weights at the start of the trial (0 holes, 61.7 g; 2 holes, 61.6 g; Table 3). At day 8 when the holes were drilled, eggs in both treatment groups had already lost 4 to 5% moisture (0 hole, 4.23%; 2 holes, 5.14%; $P < .01$) even though the treatments had not yet been administered. At day 18, moisture loss differences were even greater in the 2 hole treatment (2 hole, 10.06%; 0 hole, 8.20%; $P < .01$) which resulted in an approximate 1% increase in moisture loss in the 2 hole treatment between 8 and 18 days.

Broiler Egg Data

In Experiment 2, broiler hatching eggs (n = 354) were individually weighed (mean 69.2 g) prior to setting. On day 11, all eggs were candled and there were only 164 fertile eggs, half of which had 2 small holes drilled in to the air cell. There were sufficient embryos/chicks to measure thymocyte proliferation but not enough the hatch the chicks needed for post-hatch RNA extraction. On day 11, the average egg weight was 65.3 g resulting in a moisture loss of 5.67% from the time of set (data not shown). At the time of transfer on, day 18, the average weight per egg was 62.8g resulting in an additional 3.75% moisture loss for an overall moisture loss of 9.42%.

For Experiment 3, the egg weights were equalized in both treatments after the eggs were initially weighed (Table 4). At 11 days, prior to when the treatments (holes) were initiated; there were no differences in moisture loss between treatments (0 hole, 4.30%; 2 hole, 4.40%; $P < 0.47$; Table 4). From the start of the study until the time of transfer (11 to 18 days), the eggs in the 2 hole treatment lost significantly more moisture (2 holes, 5.13%; 0 holes, 4.06%; $P < .01$) resulting in significant treatment differences in total moisture loss from 0 to 18 days (2 holes, 9.54%; 0 holes, 8.36 %; $P < .01$). All moisture loss results can also be found on Table 4. When chicks were weighed at hatch, the average chick weight of the 0 hole control group was 43.39 g while the average hatch weigh of chicks in the 2 hole treatment group was 42.65 g resulting in a .74g reduction in body weight (-1.71%).

Thymocytes Proliferation Assay

In Trial 1 of Experiment 1, there was a significant, 3-fold increase in the thymocytes proliferation response in embryos from eggs with 2 holes versus 0 holes in both the control (unstimulated) and stimulated cell treatments (Table 5). In Experiment 2 with broiler embryos,

there were no significant differences between treatments (0, 2 holes) in terms of thymocyte response to CD28⁺ stimulation (Table 6).

In Experiment 3, there was no significant difference between treatments (0, 2 holes) in thymocytes proliferation in day 18 embryos (Table 7). The chicks hatched in Experiment 3 were used to study the effects of differences in embryonic moisture loss on post-hatch indices of immunological stress. The chicks in the 2 hole treatment were non-significantly lighter than those in the 0 hole control treatment (2 hole, 42.6 g; 0 hole, 43.4 g; Table 8). At hatch, the chicks in each incubation treatment were randomly divided into three treatment groups and were given an intraperitoneal injection of solutions containing Salmonella LPS or PBS while a third, control treatment group was not injected. A thymocyte proliferation assay similar to what described for Experiments 1 and 2 was conducted. The LPS injection increased thymocyte proliferation compared with both the PBS and non-injected control treatments but within each treatment there was no difference due to embryonic treatment (Table 8).

Effects of Salmonella enteritidis LPS on CD4⁺, CD8⁺ and Treg Cell Percentages

The dispersed thymus was used to generate embryonic CD4⁺CD8⁺ flow cytometry plots to determine the proportion of individual cell types that may have been influenced by the post-hatch treatments. As shown in Figure 1, there was an increased proportion of CD4⁺ positive cells at day 18 of incubation in the 2 hole treatment. In the 0 hole treatment, only .89% of gated cells representing maturation of CD4⁺ (upper left quadrant) and 9.73% (lower left quadrant). In the 2 hole treatment, the maturation of CD4⁺CD8⁺ cells to CD4⁺ positive cells alone accounted for 15.81% of the gated cells in the upper left quadrant and 15.91% in the lower left quadrant.

At 24 hour post-injection, there was no significant difference due to the main effect of moisture loss in the percentage of CD8⁺ positive cells ($P < .81$). There was an increase in the percentage of CD8⁺ positive cells in response to an LPS challenge in the 2 hole treatment (5.74%)

compared with the non-injected control treatment (4.14 %). There was a significant interaction among treatment groups ($P < .001$). There was an increase in the LPS treated cells compared with the non-injected and PBS treatments but the non-injected and PBS control groups were similar in both the shell treatments (0 hole Ctrl, 4.14%; PBS, 4.00%; 2 hole Ctrl, 3.88%, PBS, 4.00%). The interaction of moisture loss and treatment group was not significant (Table 9).

At 48 hour post-injection, the main effect of moisture loss on the percentage of CD8⁺ positive cells significantly decreased in the 2 hole treatment thymus samples (5.34%) compared with the thymus tissue sampled from chicks in the 0 hole treatment (6.32%; $P = .04$). There was a significant interaction between treatment groups in that CD8⁺ positive cells were decreased in the LPS injected group (4.42) when compared to the PBS injected control (5.76) and the non-injected control group (7.31; $P < .001$; Table 9). At 48 hour post injection, there was a significant interaction found between moisture loss and treatment group ($P < .029$; Table 9).

The 18 day embryonic CD4⁺CD25⁺ flow cytometry plot charts suggests that there was an increased production of anti-inflammatory cytokine producing T regulatory cells (CD4⁺CD25⁺; Tregs) in response to the small but significant increase in moisture loss. In the 0 hole treatment, there were 1.41% of gated CD25⁺ cells in the upper right quadrant and 0.44% in the lower right quadrant versus 2.31% and 1.38% in the 2 hole treatment, respectively. In both the 0 and 2 hole LPS challenge groups, there was an increase in CD25⁺ lymphocyte proliferation compared with the non-injected control chicks (Figure 2).

At 24 hours post LPS injection, there was no significant difference in main effect of percentage of CD4⁺CD25⁺ Tregs in moisture loss between 0 hole (2.45) and 2 hole (2.59) treatment groups ($P = .48$). There was a significant interaction in the percentage of Tregs in the LPS treatment group (3.57) when compared to the PBS injected control (2.00) and non-injected

control group (1.99; $P = .001$). There was no significant effect in the overall interaction of moisture loss effect on treatment groups ($P = .217$; Table 9).

At 48 hour post-injection, the main effect of moisture loss did have a significant effect on the percentage of $CD4^+CD25^+$ Tregs in the 2 hole treatment group (2.84) versus the 0 hole group (2.23; $P = .001$) There was a significant interaction between treatment groups ($P = .005$; Table 9). At 48 hours post injection, there was a significant interaction between moisture loss and treatment groups with the 2 hole treatments having increased percentages of $CD4^+CD25^+$ Tregs in the thymus versus the 0 hole samples ($P = .001$; Table 9).

Effects on Anti-Inflammatory Cytokine IL-10 Transcription

At 18 days of incubation, 7 days after the treatments (0, 2 holes) were initiated, there was a non-significant increase in IL-10 transcription in the spleen from the 2 hole versus 0 hole embryos (Figure 3). At 24 hours post-injection, the main effect of induced moisture loss in eggs drilled with 2 holes versus the 0 hole eggs was not significantly different. There was a 3.68 fold decrease in LPS IL-10 transcription in spleen samples from the 0 hole embryos and a 11.53 fold decrease in the 2 hole LPS spleen samples ($P < .01$). At 24 hours post injection, there was no significant interaction between moisture loss and treatment groups ($P < .07$; Figure 4).

At 48 hours post LPS injection, the main effect of induced moisture loss resulted in a 7.44 fold increase in IL-10 transcription in spleen samples from the 2 hole versus 0 hole non-injected control treatments although the difference was not significant ($P < .76$). There was a 4.99 fold increase of IL-10 transcription and 7.64 fold increase in the 0 and 2 hole LPS treatments, respectively, when compared with the respective control groups. The interaction of spleen samples from LPS injected chicks and the non-inject controls was not significant ($P < .0518$). The 0 hole non-injected group had increased IL-10 transcription compared with the other treatment

groups at 48 hours. However, there were no significant overall interaction of treatment and moisture loss on IL-10 transcription ($P < .29$; Figure 5).

Effects on Pro-Inflammatory Cytokine IL-1 Transcription

There was no significant difference due to treatment (0, 2 holes) in IL-1 transcription in the 18 day embryos (Figure 6). The main effect of moisture loss on spleen tissue 24 hours post injection had a 1.79 fold increase in IL-1 transcription in the 2 hole non-injected birds. Spleen tissue from chicks in the 2 hole LPS treatment group experienced a 1.53 fold increase of IL-1 transcription of mRNA content. The main effect at 24 hours post injection did not result in a significant effect ($P < .61$). Although the overall interaction between moisture loss and immune response after LPS injection was not significant ($P < .23$), the 0 hole non-inject group had a lower IL-1 transcription than the 2 hole treatment groups (Figure 7).

For IL-1 transcription by 48 hours post challenge, there was a 1.79 fold increase in spleen samples from the chicks in the 2 hole non-inject group over the 0 hole non-inject control group. with no significant difference in the main effect of moisture loss ($P = .75$). There was a 1.51 fold increase and 1.53 fold increase in IL-1 mRNA content in spleen tissue from the 0 hole and 2 hole ,respectively, LPS *S. enteritidis* challenged birds when compared to the control group. No significant differences were found in interactions amongst treatment groups of non-injected verses LPS injected chicks ($P < .2631$). Finally, there were no significant interactions of moisture loss on IL-1 transcription in mRNA content of spleen tissue from non-injected or LPS *S. enteritidis* injected chicks ($P < .66$; Figure 8).

CHAPTER 5: DISCUSSION

In the literature, there are many reports that discuss the importance of moisture loss as a consequence of treatment effects during the course of incubation experiments. The majority of these studies, however, cannot differentiate between moisture loss effects on embryonic development independent of temperature differences or other main effects on embryonic metabolism. There was one study, Molenaar et al. (2010), in which a single hole was drilled in the air cell. While this resulted in a significant increase in moisture loss, there were no significant effects on their measured indices of chick quality. In Experiments 1 and 2, the 2 hole treatment did result in small but significant increases in moisture loss. In Trials 1 and 2 (Experiment 1) with leghorn eggs, the 2 hole treatment was initiated 3 days earlier (8 d) than in the broiler studies (11 d) and this may have contributed to the treatment effects observed at 18 days. We would have expected to see a moisture loss difference closer to 11-12 % but the humidity levels during incubation (65%) were slightly higher than what is used in commercial practice. Larger eggs are also expected to require a slightly longer period to achieve an optimal, initial embryonic temperature as well as modulating embryonic heat loss during the latter stages of incubation (Lourens et al., 2005).

When measuring thymocyte cell proliferation by the MTT assay described by Peters et al. (2003), the shorter time frame from onset of treatment to time of hatch in the broiler embryos (11-18 versus 8-18 d) could have contributed to 18 day thymocytes proliferation assay results in the broiler embryos showing minimal treatment differences. While the 18 day embryos did exhibit a slight increase in moisture loss, the thymocyte proliferation assay results suggested little evidence of immunological stress.

The flow cytometry plots of thymocyte CD4⁺ maturation in the 18 day embryos suggests that there was increased cell maturation in the 2 hole treatment group. This suggests that either the slight increase in moisture loss or an increased exposure to external environmental mitogens via the holes elicited a stimulatory response. At 24 hours post *S. enteritidis* LPS injection, the significant increase in CD8⁺ transcription only in the LPS treatments supports the hypothesis that the embryonic treatments alone were not sufficient to elicit a response. At 48 hours post *S. enteritidis* LPS injection, the CD8⁺ response to treatment with LPS alone was the only effect observed, in this case a lower concentration of these cells. This is probably the result of an increase in Tregs which contributed to the decrease in cytotoxic CD8⁺ levels.

When looking at the flow cytometry plots for the 18 day embryo thymus samples, the upregulation of CD25⁺ cells, or Tregs, represent a slight increase in the anti-inflammatory immune response. We had similar findings in the CD25⁺ Treg overall results compared to CD8⁺ findings with significant differences between the control and treatment groups but only slight and non-significant differences due to moisture loss. This suggests that the LPS injection did present an immune challenge as would be expected from previous studies that resulted in up regulation of CD25⁺ with an anti-inflammatory response (Takahashia et al., 1997; Xie et al., 2000). However, the induced moisture loss in the 2 hole LPS and control treatments did not result in significant differences when compared with the 0 hole LPS and control treatments.

The spleen mRNA IL-10 and IL-1 transcription levels in the 18 day embryos was not significant suggesting that the 2 hole treatment was not sufficient to elicit a response. At 24 hours post-hatch and post-injection, transcription of both cytokines was reduced compared with the 0 hole non-injected control samples but the negative effect on IL-10 in the LPS 2 hole samples was far greater than what was observed for IL-1. At 48 hours post-injection, the transcription of both cytokines was increased though again, the fold increases in IL-10 were far greater than what was

observed for IL-1 and this somewhat reciprocal relationship between these two cytokines has been previously shown in other chick challenge studies (Hangalapura et al., 2006). This is likely an effect of an increase in Tregs, which are positively correlated with IL-10 transcription (Shanmugasanduram and Selvaraj, 2011).

CHAPTER 6: CONCLUSIONS

The objectives of this study were to

- 1.) Measure moisture loss differences in egg and chicks weights between the treatment groups
- 2.) To study the effects of induced moisture loss on the immune response when a potential pathogen is introduced.

In both the layer and broiler experiments, slight but significant increases in moisture loss occurred in the eggs with 2 versus 0 drilled holes. The moisture loss that occurred during the first half of incubation was more than expected, approximately half of the total moisture loss occurred a period with minimal embryonic development (i.e. metabolic heat loss).

When measuring thymocyte proliferation with MTT assay, the leghorn eggs (Trials 1,2; Experiment 1) did result in a significant increase in thymocyte proliferation at embryonic day 18 in the treatment group while this was not found in the broiler egg experiments. The three extra days of induced moisture (8 versus 11 d) may have played a roll in these findings.

At 24 hours post injection, CD8⁺ and CD4⁺CD25⁺ positive cell percentages were increased in thymus samples from the *S. enteritidis* LPS injected chicks versus the PBS and non-injected control groups. At 48 hours post injection, the opposite was true as the percentage CD8⁺ cells declined while percentage Tregs increased in the 2 hole LPS group. At 48 hours post injection, both CD8⁺ and CD4⁺CD25⁺ responded to the significant main effect of moisture loss and there was also a significant interaction between treatment groups and moisture loss. This suggests an increase in the anti-inflammatory immune response. An anti-inflammatory immune

response is also supported by the increased IL-10 transcription at 48 hours post injection while the pro-inflammatory cytokine, IL-1, stayed relatively consistent at the different sampling periods.

For future studies, measuring immune response later in the chick's life might result in increased levels of immune response measurements. Also, carrying the experiment out to time of process in order to compare those responses to carcass characteristics and time of process.

In conclusion:

1. Significant increase in moisture loss during incubation was achieved in the 2 hole treatment group.
2. The layer trials with longer treatment periods during incubation resulted in significant increase of thymocyte proliferation in the 2 hole treatment group.
3. At 48 hours post injection, there was a significant interaction between the main effect of moisture loss and the treatment groups for CD8⁺ (P=.029) and Tregs (P=<.0001).
4. Induced moisture loss during incubation did result in an anti-inflammatory immune response post-hatch (increased IL-10).
5. There was no significant pro-inflammatory response post hatch (no change in IL-1 response).
6. Significant differences between control and treatment groups but only slight and non-significant differences due to moisture loss.

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APPENDIX A: TABLES

Gene	Primer Sequence ¹
<i>IL-10</i>	F 5'-CATGCTGCTGGGCCTGAA-3' R 5'-CGTCTCCTTGATCTGCTTGATG-3'
IL-1	F 5'-TCCTCCAGCCAGAAAGTGA-3' R 5'-CAGGCGGTAGAAGATGAAGC-3'
β -actin	F 5'-ACCGGACTGTTACCAACACC-3' R 5'-GACTGCTGCTGACACCTTCA-3'

Table 1. Primer sequences for genes under study. ¹ F, Forward; R, Reverse.

Treatment (Holes)	Initial Egg Wt.	Egg Weight (gm)		Moisture Loss (%)		
		Day 8	Day 18	Day 0-8	Day 0-18	Day 8-18
0	50.1	48.7	45.7	2.79	8.76	3.96
2	52.9	51.0	47.9	3.78	9.47	4.91
Pooled SEM	0.7	0.73	0.7	0.11	0.22	0.09
Probability	0.007	0.03	0.03	0.01	0.025	0.01

Table 2. The effect of 0 or 2 holes in the eggshell administered at 8 days in incubation on egg weight and moisture in eggs from leghorn hens in Experiment 1 (Trial 1). Eggs were individually weighed at day of set. On day 8, eggs were reweighed and candled for fertility. One-half of the remaining fertile eggs had either 0 or 2 holes drilled in the egg shell above the air cell.

Treatment (Holes)	Initial Egg Wt.	Egg Weight (gm)		Moisture Loss (%)		
		Day 8	Day 18	Day 0-8	Day 0-18	Day 8-18
0	61.7	59.13	56.7	4.23	8.20	3.96
2	61.6	58.43	55.4	5.14	10.06	4.91
Pooled SEM	1.1	1.1	1.1	0.10	0.19	0.09
Probability	0.91	0.65	0.40	0.01	0.01	0.01

Table 3. The effect of 0 or 2 holes in the eggshell administered at 8 days of incubation on egg weight and moisture loss in leghorn eggs in Experiment 1 (Trial 2). Eggs were individually weighed at day of set. On day 8, eggs were reweighed and candled for fertility. One-half of the remaining fertile eggs had either 0 or 2 holes drilled in the egg shell above the air cell. Lastly, eggs were weighed when transferred to hatch baskets on day 18.

Treatment (Holes)	Initial Egg Wt.	Egg Weight (gm)		Moisture Loss (%)		
		Day 11	Day 18	Day 0-11	Day 0-18	Day 11-18
0	62.98	60.26	57.71	4.30	8.36	4.06
2	63.27	60.47	57.16	4.40	9.54	5.13
Pooled SEM	0.37	0.36	0.35	0.09	0.12	0.06
Probability	P < .58	P < .67	P < .27	P < .43	P < .001	P < .001

Table 4. The effect of 0 or 2 holes drilled on Day 11 on moisture loss at day 18 from broiler eggs (Experiment 3). Eggs were individually weighed at day of set. On day 11, eggs were reweighed and candled for fertility. One-half of the remaining fertile eggs had either 0 or 2 holes drilled in the egg shell above the air cell. Lastly, eggs were reweighed when transferred to hatch baskets on day 18.

Treatment (Holes)	Trial 1	
	Unstimulated	Stimulated
0	0.167	0.563
2	0.458	1.153
Pooled SEM	0.071	0.145
Probability	0.018	0.016

Table 5. The effect of inducted moisture loss on thymocyte proliferation in leghorn embryos at 18 days of incubation (Experiment 1). Onset of treatment began on day 8 when eggs were weighed and candled for fertility. One-half of the remaining fertile eggs had either 0 or 2 holes drilled in the egg shell above the air cell. Thymus samples were pooled from 3 embryos (n=6 replicates per treatment), put in Roswell Park Memorial (RPMI) growth media and immediately taken to the lab to conduct a Methylthiazol Tetrazolium (MTT) proliferation assay.

Treatment (Holes)	Day 18 Thymocyte Proliferation
0	0.557
2	0.902
Pooled SEM	0.13
Probability	0.116

Table 6. The effect of induced moisture loss on thymocyte proliferation in broiler embryos at 18 days of incubation (Experiment 2). Onset of treatment began on day 11 when eggs were weighed and candled for fertility. One-half of the remaining fertile eggs had either 0 or 2 holes drilled in the egg shell above the air cell. Thymus samples were pooled from 3 embryos (n=6 replicates per treatment), put in Roswell Park Memorial (RPMI) growth media and immediately taken to the lab to conduct a Methylthiazol Tetrazolium (MTT) proliferation assay.

Treatment (Holes)	Day 18 Thymocyte Proliferation
0	1.312
2	1.238
Pooled SEM	0.96
Probability	0.599

Table 7. The effect of induced moisture loss on thymocyte proliferation in broiler embryos at 18 days of incubation (Experiment 3). Onset of treatment began day 11 when eggs were weighed and candled for fertility. One-half of the remaining fertile eggs had either 0 or 2 holes drilled in the egg shell above the air cell. Thymus samples were pooled from 3 embryos (n=6 replicates per treatment), put in Roswell Park Memorial (RPMI) growth media and immediately taken to the lab to conduct a Methylthiazol Tetrazolium (MTT) proliferation assay.

Treatment (Holes)	Body Weight (g)	24 hr. Post LPS Injection		
		Non-injected	LPS	PBS
0	43.39	0.962	1.869	0.781
2	42.65	0.984	1.555	0.779
Pooled SEM	0.3	0.127	0.360	0.201
Probability	0.09	0.902	0.555	0.201

Table 8. The effect of inducted moisture loss on thymocyte proliferation in stimulated cells from chicks that were non-injected or injected with PBS or LPS (Experiment 3). On embryo day 11, one-half of the remaining fertile eggs had either 0 or 2 holes drilled in the egg shell above the air cell. On Day of hatch (day 21), chicks from each incubation treatment (0 hole, 2 hole) were randomly separated further into 3 additional treatment groups. There was a non-injected control group, injection of lipopolysaccharide from *Salmonella enteritidis* (500ug/kg body weight), and a phosphate buffered saline (PBS) injection group. Thymus samples were pooled from 3 embryos (n=6 replicates per treatment), put in Roswell Park Memorial (RPMI) growth media and immediately taken to the lab to conduct a Methylthiazol Tetrazolium (MTT) proliferation assay.

Treatment	Holes	24 Hours Post-injection		48 Hours Post-injection	
		% CD8 ⁺	% Tregs	% CD8 ⁺	% Tregs
-----	0	4.62	2.45	6.32	2.23
	2	4.53	2.59	5.34	2.84
Control		4.01	1.99	7.31	2.83
PBS		4.00	2.00	5.76	2.20
LPS		5.72	3.57	4.42	2.58
Pooled SEM		0.26	0.16	0.35	0.11
Analysis of Variance		Probability			
Holes		0.811	0.483	0.044	<0.001
Treatment		<0.001	<0.001	<0.001	0.005
Holes * Treatment		0.922	0.217	0.029	<0.001

Table 9. Effect of a challenge with *Salmonella enteritidis* lipopolysaccharide in day old broilers, (500ug/kg body weight) on thymus CD8⁺ and CD4⁺CD25⁺ cell percentages 24 and 48 hours post

injection. On embryo day 11, one-half of the eggs had either 0 or 2 holes drilled in the egg shell above the air cell. On the day of hatch (day 21), chicks from each incubation treatment (0 hole, 2 hole) were randomly separated further into 3 additional treatment groups. There was a non-injected control group, injection of lipopolysaccharide from *Salmonella enteritidis* (500ug/kg body weight) and injection of phosphate buffered saline (PBS). Thymus samples were pooled from 3 embryos (n=6 replicates per treatment) and thymocytes were collected by density centrifugation by histopaque. The percentage of CD4⁺, CD8⁺ and CD4⁺CD25⁺ positive cells were determined using flow cytometry.

APPENDIX B: FIGURES

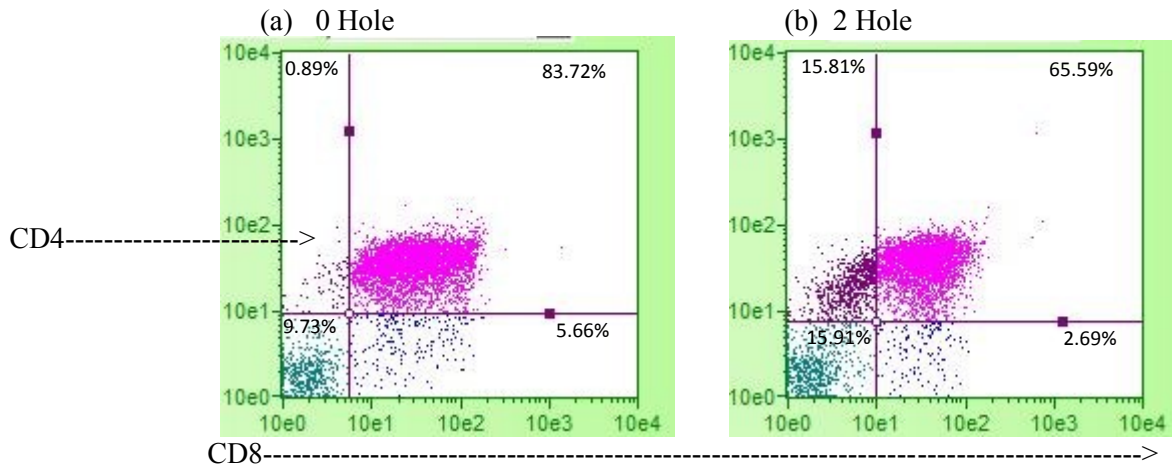


Figure 1. The CD4⁺ CD8⁺ cell percentages of the thymus from chicks with 0 holes or 2 holes in the egg's air sac during incubation. At day 11 of incubation, half the eggs had 2 holes drilled into the air sac to induce moisture loss.

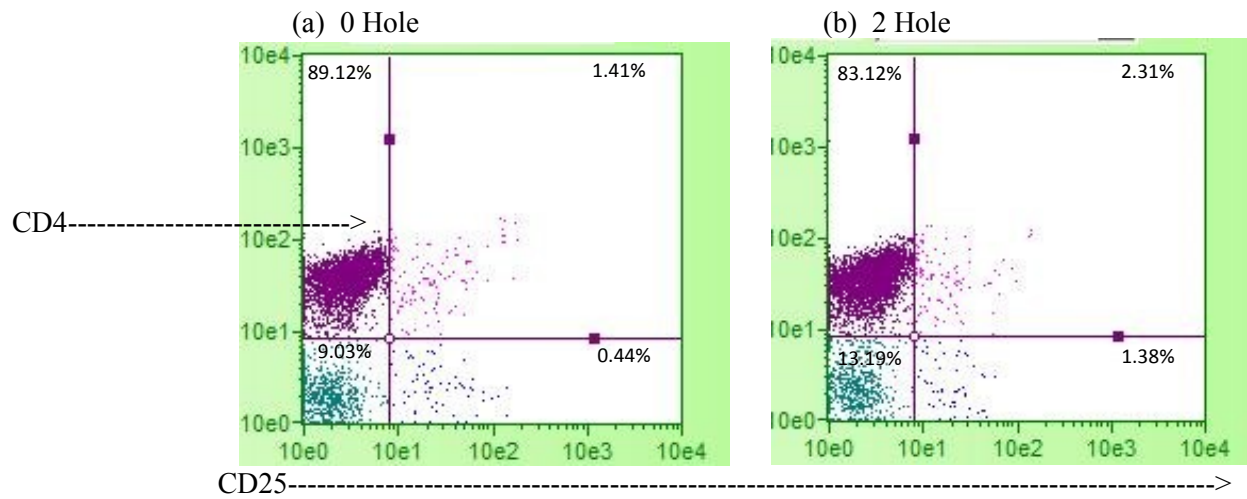


Figure 2. The CD4⁺CD25⁺ cell percentages in the control group of 0 holes and treatment group with 2 holes. Cells were enriched by density gradient centrifuge and stained with a fluorescent-conjugated anti—chicken CD4⁺ and CD25⁺.

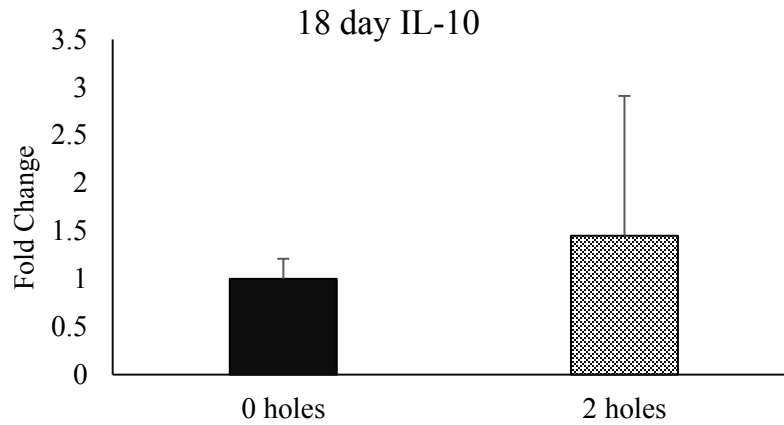


Figure 3. Effect of induced moisture loss during incubation on spleen IL-10 mRNA at Day 18 of incubation, 7 days after onset of treatment. At day 11 of incubation, half the eggs had 2 holes drilled into the air sac. The 2hole bar represents fold change to the control group. Bars (mean of 18 birds pooled by 3 \pm SEM) did not differ significantly (2H-2H= -1.9046 and 0H- 0H=-1.4496)

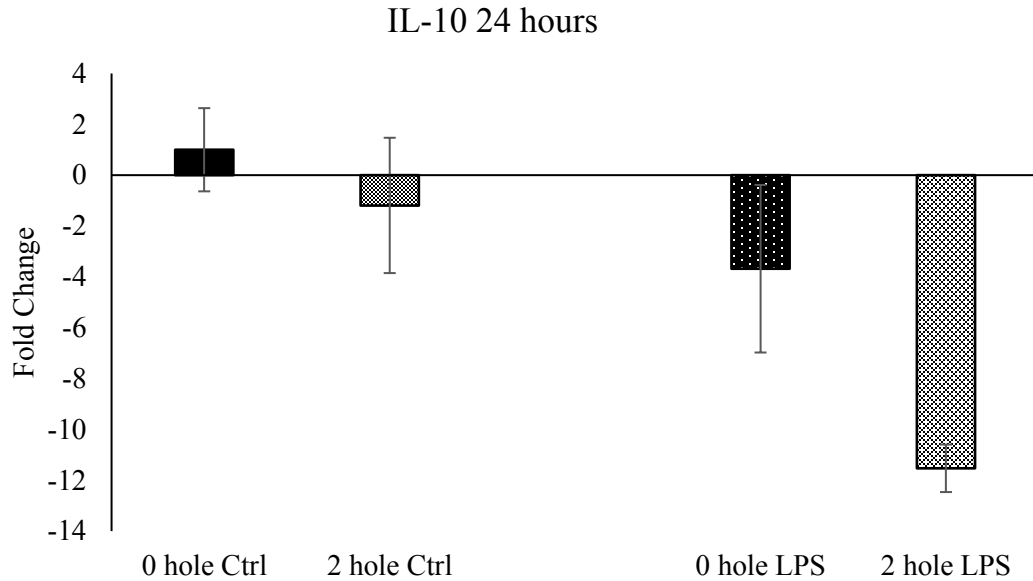


Figure 4. Effect of Lipopolysaccharide from *Salmonella enteritidis* challenge on spleen IL-10 mRNA at 24 hours post challenge. At day 11 of incubation, half the eggs had 2 holes drilled into the air sac to induce moisture loss. Spleen tissues were collected and mRNA was analyzed by real time PCR, corrected for β -actin and normalized to the mRNA content for the uninfected birds. All bars represent fold change compared to the 0 hole, non-inject control group. Main effect of moisture loss had no significant effect. The interaction between the non-inject control group and LPS injected treatment group resulted in a significant increase in IL-10 transcription in spleen samples from the treatment group ($P < .01$). The interaction between moisture loss and treatment groups was not significant. Bars (mean of 18 birds pooled by $3 \pm \text{SEM}$) did not differ significantly ($P < 0.05$).

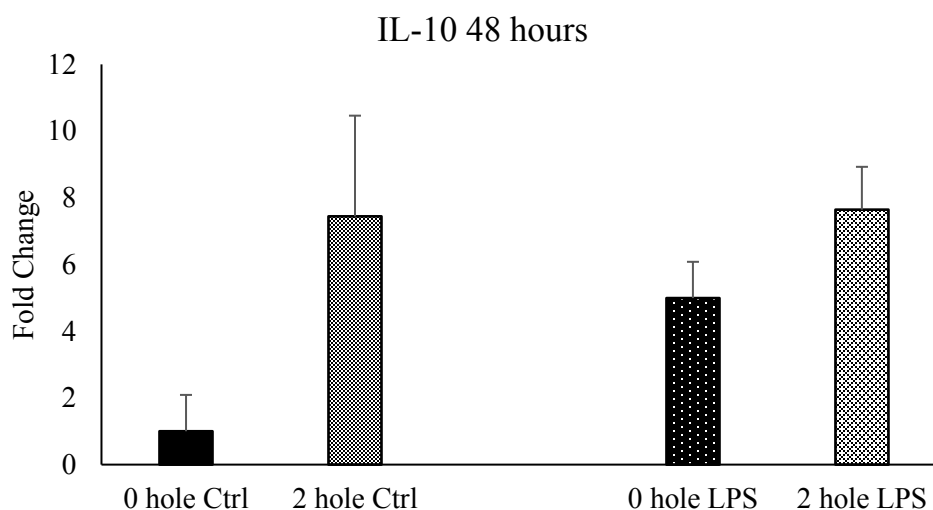


Figure 5. Effect of a *Salmonella enteritidis* lipopolysaccharide challenge on spleen IL-10 mRNA at 48 hours post challenge. At day 11 of incubation, half the eggs had 2 holes drilled into the air sac to induce moisture loss. Spleen tissues were collected and mRNA was analyzed by real time PCR, corrected for β -actin and normalized to the mRNA content for the uninfected birds. All bars represent fold change compared to the 0 Hole, non-inject control group. The main effect of moisture loss had no significant effect. The interaction between the non-inject control and LPS injected treatment group resulted in a non-significant decrease in IL-10 transcription in spleen samples from the treatment group ($P < .052$). The interaction between moisture loss and treatment groups was not significant effect ($P < .29$). Bars (mean of 18 birds pooled by $3 \pm$ SEM) did not differ significantly ($P < 0.05$).

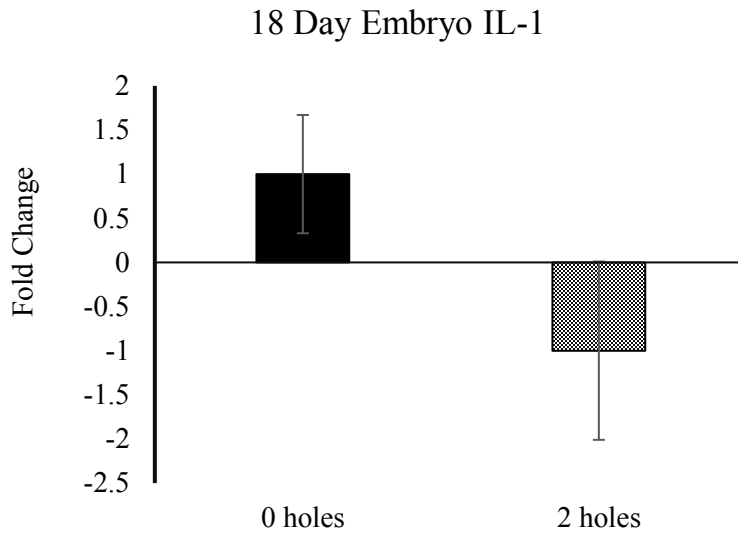


Figure 6. Effect of induced moisture loss during incubation on spleen IL-1 mRNA at day 18 of incubation, 7 days after treatment onset. At day 11 of incubation, half the eggs had 2 holes drilled into the air sac and the 2 hole bar the represents fold change when compared to the control treatment. The bars (mean of 18 birds pooled into groups of 3 \pm SEM) did not differ significantly (2H-2H= -1.1059 and 0H- 0H=-0.8351).

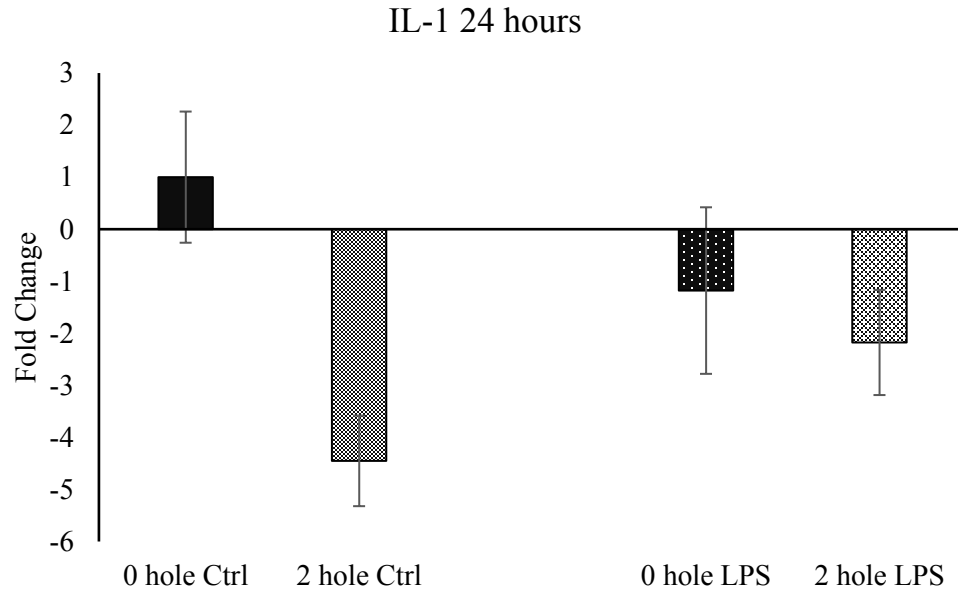


Figure 7. Effect of a *Salmonella enteritidis* lipopolysaccharide challenge on spleen IL-1 mRNA transcription at 24 hours post challenge. At day 11 of incubation, half the eggs had 2 holes drilled into the air sac to induce moisture loss. Spleens were collected and mRNA was analyzed by real time PCR, corrected for β -actin and normalized to the mRNA content for the unchallenged control chicks. All bars represent the fold change response when compared to the 0 hole, non-injected control group. The main effect of moisture loss was not significant effect. The interaction between the non-injected and LPS injected treatments resulted in a significant increase in IL-1 transcription in spleen samples from the LPS treatment group ($P < .02$). The interaction between moisture loss and treatment groups was not significant ($P < .23$). Bars (mean of 18 birds pooled by $3 \pm$ SEM) did not differ significantly ($P < 0.05$).

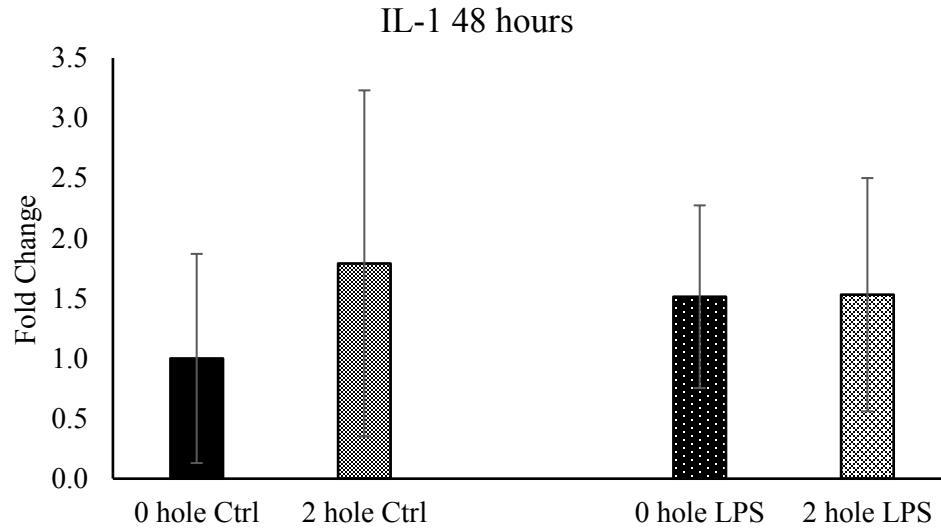


Figure 8. The effect of a *Salmonella enteritidis* challenge on spleen IL-1 mRNA at 48 hours post challenge. At day 11 of incubation, half the eggs had 2 holes drilled into the air sac to induce moisture loss. Spleens were collected and mRNA was analyzed by real time PCR, corrected for β -actin and normalized to the mRNA content for the uninfected birds. All bars represent fold change compared to the 0 hole, non-inject control group. The main effect of moisture loss was not significant effect. The interaction between the non-injected control group and LPS injected treatment group was not significant. The interaction between moisture loss and post hatch treatments was also not significant (mean of 18 birds pooled by $3 \pm$ SEM) did not differ significantly ($P < 0.05$).