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Effects of Cyclic Heat Stress on the Acute Inflammatory Response in Broilers

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Abstract

Heat stress (HS) is a growing concern in broiler production due to increasing environmental temperatures. Little is known of the overall effect of HS on innate immunity. Lipopolysaccharide (LPS), an outer membrane component of Gram-negative bacteria, is commonly used to study the inflammatory response. In avian species, the local tissue and systemic inflammatory activities in response to LPS may be determined concurrently, over time, in an individual, using the growing feather (GF) pulp dermal test along with blood measurements (dual-window approach). To examine the effect of cyclic HS on the local and systemic acute inflammatory responses, Cobb 500 male broiler chicks were reared under thermoneutral (TN) or cyclic HS conditions. Eight environmental chambers were used, four TN and four HS, with each chamber split into two pens. Beginning at 4 d of age, cyclic HS birds were subjected to 35°C from 8 am to 10 pm and TN temperatures from 10 pm to 8 am. At 37 d of age, four groups of broilers were formed: TN-LPS (n = 8 broilers), TN-PBS (phosphate-buffered saline (vehicle) control; n = 4), HS-LPS (n = 8) and HS-PBS (n = 4). The pulps of 12 GF per broiler were each intradermally (i.d). injected with 10 µL of LPS (100 µg/mL) or 10 µL of PBS. Blood and GF were collected before (0 h) and at 6- and 24-h after GF pulp injection. Blood and GF pulp cell suspensions were immunofluorescently stained and leukocyte population profiles analyzed by flow cytometry. Blood also was used to prepare Wright-stained blood smears for differential leukocyte counts. Data were analyzed by ANOVA ($P \leq 0.05$) and Fisher's multiple means comparison tests to determine differences ($P \leq 0.05$) between means. Locally, HS-LPS broilers exhibited lower levels (% pulp cells) of GF pulp infiltrating heterophils at 6- and 24-h, and lower macrophage levels at 24-h post-injection, compared to TN-LPS birds. In the blood, TN and HS broilers had similar baseline line (0 h) concentrations (cells/µL) of heterophils, monocytes,

eosinophils, and basophils, but HS broilers had lower ($P \leq 0.05$) total WBC and T- and B-lymphocyte levels. Concentrations of circulating heterophils and monocytes were greatly elevated ($P \leq 0.05$) at 6- and 24-h, respectively, only in TN-LPS broilers, although a minimal increase ($P = 0.091$) in heterophils also was observed in HS-LPS broilers at 6 h. By 24 h, blood heterophils returned to pre-injection levels in both HS-LPS and TN-LPS broilers. Overall, results indicated that cyclic HS reduced both the local and systemic acute inflammatory response to LPS in broilers, likely impairing their innate defense against microbial infection. With growing concern regarding HS in the poultry industry, further research should be pursued to elucidate the mechanisms by which HS affects the innate immune system of broilers. Application of this approach may be utilized to select individuals expressing greater innate immune robustness while experiencing HS.

Acknowledgements

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Table of Contents

Chapter I. Introduction

Over time, the immune system has continued to develop and evolve due to the intense stress placed upon it by pathogens (Medzhitov & Janeway, 1997). There are two major parts to the immune system, consisting of the innate and adaptive immunity. Innate immunity acts as the body's first line of defense, whereas adaptive immunity is highly specific and protective. One of the innate immune responses developed for dealing with pathogens is the inflammatory response. Inflammation works by accumulating "leukocytes, plasma proteins and fluid derived from blood" to the site of infection to eliminate microbes and repair damaged tissue (Abbas et al., 2018). To recognize infections, the innate immune system has established a method for distinguishing molecular patterns common to groups of pathogens (e.g., lipopolysaccharide (LPS) of Gram-negative bacteria). In doing this, the innate immune system can quickly recognize a pathogen and initiate inflammation (Medzhitov & Janeway, 1997).

This innate immune system is important in the realm of commercial broiler production as chickens are processed at six weeks of ages before their own adaptive immunities have had time to fully develop (French et al., 2020). To examine the effectiveness of the innate immune system, the pulp of growing feathers (GF) was shown to be an effective minimally invasive skin test to monitor local inflammatory responses to bacterial cell structures, such as LPS (Erf & Ramachandran, 2016). Through the simultaneous injection of multiple GF on a bird, followed by periodic sampling of GF for laboratory analyses, we are able to examine leukocyte infiltration profiles and activities taking place *in vivo*. Because these leukocytes are recruited from the blood, changes in the blood cell profiles may be determined from concurrently sampled blood. Recently, one of the first studies using the "GF and blood dual-window approach" in broilers examined the acute inflammatory response to LPS injected into the pulp (French et al., 2020).

For this study, blood and GF were sampled three times – before injection (0 h) and at 6 h and 24 h after LPS injection into GF pulps – to examine the acute phase of the inflammatory response to LPS (French et al., 2020). This study showed extensive recruitment of heterophils and monocytes/macrophages reaching peak levels at 6 and 24 h, respectively, in the dermis of injected GF. Local GF cellular activities included generation of reactive oxygen species (ROS), expression of inflammatory cytokines (e.g., interleukin-1 (IL-1), IL-6, IL-8, IL-10) and antioxidant enzyme activity (superoxide dismutase, SOD) (French et al., 2020). In blood, the concentration, and proportions of heterophils were greatly elevated at 6 h and returned to baseline levels by 24 h, whereas levels of lymphocytes dropped at 6 h and returned to pre-injection levels by 24 h (French et al., 2020). With the successful adaptation of this two-window approach for use in broilers, the influence of environmental conditions or nutrition on innate immune function in broilers may be investigated.

Heat stress is a common environmental issue associated with broiler production, resulting in decreased feed intake and nutrient efficiency, as well as increased water intake (Ruff et al., 2020). Little is known about the effects of heat-stress on the innate immune system of broilers, other than decreased gut barrier functions – allowing for bacterial translocation (Campbell et al., 2019). Examination of the local and systemic inflammatory response to LPS, similar to the study conducted by French et al. (2020), will provide a pertinent, novel understanding of the impacts of heat stress on the acute inflammatory response of broilers.

We hypothesize that birds subjected to cyclic heat stress will exhibit altered inflammatory responses when compared to broilers reared under thermoneutral conditions.

Chapter II. Literature Review

Innate Immune System

The immune system is comprised of two major categories – the innate and adaptive immune systems. These systems have developed into structured and coordinated responses, integral for maintaining bodily health by preventing microbes from entering the body and defending against infections. The innate immune response works by recognizing "molecules shared by larger groups of microbes," and compounds produced by damaged host cells (Abbas et al., 2018). Contrarily, the adaptive immunity has much greater specificity, adapting as a part of the immune system to recognize distinct epitopes on a variety of microbes and non-microbial antigens to generate stronger and specialized immune responses (Abbas et al., 2018).

The two main protective functions of the innate immune system are inflammation and antiviral defense (Abbas et al., 2018). Innate immunity has evolved over time to serve as the first line of defense against infections (Abbas et al., 2018). Previously, the innate immune system had been deemed to be of lesser importance and function than the adaptive immune system, with respect to protecting the body. This has been disproven, showing that the innate immune system is not only able to rapidly respond to infection and prevent the onset of some infections but is also essential for the development of adaptive immunity (Medzhitov & Janeway Jr., 1997). This discovery has helped place a greater emphasis upon researching innate immunity alongside adaptive immunity (Abbas et al., 2018).

The recognition system of the innate immune system works by identifying molecules of microbial products and damaged cells also known as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), respectively (Abbas et al., 2018). Pattern recognition receptors (PRRs) are strategically placed on extracellular membranes, in the

cytosol, or on endosomal membranes based on function. These PRRs are used to identify various ligands including viral and bacterial nucleic acids, peptides, carbohydrates, and glycerides. One of the more well researched PAMPs are lipopolysaccharides (LPS), an outer membrane component of Gram-negative bacteria. LPS binds to myeloid differentiation protein 2 (MD2) forming a complex able to interact with toll-like receptor 4 (TLR4) on the extracellular surface of responding cells (Abbas et al., 2018). The interaction with TLR4 activates acute inflammation setting forth the expression of inflammatory genes. The inflammatory genes then produce cytokines (tumor necrosis factor (TNF) and interleukin one and 6 (IL-1 & IL-6), chemokines, and E-selectins (endothelial adhesion molecules) responsible for the function of the acute inflammatory response.

Inflammatory Response

The inflammatory response is the hallmark of innate immune system. The acute inflammatory response serves as the primary method used by the innate immune system to handle infections and injured tissues (Abbas et al., 2018). It functions by gathering leukocytes, plasma proteins and fluid from the blood to the site of infection. In response to LPS, neutrophils (or heterophils in avian species) are the first leukocytes to be recruited from the blood due to their rapid response to chemotactic signals (Abbas et al., 2018). Monocytes are soon to follow functioning in antigen elimination and tissue repair and turning into macrophages upon entering the tissue (Abbas et al., 2018). The plasma proteins that are gathered include "complement proteins, antibodies, and acute-phase reactants," (Abbas et al., 2018). These proteins function in inducing an increased inflammatory response, opsonizing microbes, or they are produced as a result of an acute phase response (Abbas et al., 2018).

The activation of the acute inflammatory response leads to local changes at the site of infection and systemic protective effects as well. With the activation of TLR4 interacting with LPS, inflammatory cytokines TNF and IL-1 are produced inducing endothelial cells to express E-selectin and L-selectin ligands (Abbas et al., 2018). These cytokines also stimulate the secretion of chemokines CXCL8 and CCL2, to increase integrin affinities for heterophils/neutrophils and monocytes, respectively (Abbas et al., 2018). The cytokines work to increase leukocyte migration to the site of infection or LPS injection by increasing the adhesion affinities of these leukocytes as they roll across endothelial cells. The leukocytes may then adhere to the endothelial surfaces and exit venules into the tissue. From there they may migrate to the site of infection through chemotaxis. Leukocytes are then also activated by TNF and IL-1 to produce IL-1 and IL-6 which is secreted into the blood to produce systemic effects. Systemically, the cytokines may stimulate the brain to induce fever, the bone marrow for leukocyte production and the liver for acute phase reactant production.

These physiological changes caused by the acute inflammatory response may be measured in an organism. This allows us to evaluate how different treatments or changes in the body may affect the acute inflammatory response. Most notably, to measure the local responses in an organism we may perform leukocyte population analyses to see leukocyte tissue infiltration, evaluate the generation of reactive oxygen species (ROS), and isolate RNA for cytokine gene expression. For systemic changes, we may use whole blood to evaluate leukocyte cell profile changes and isolated plasma to see acute-phase protein production from the liver.

Growing Feather & Blood Model

This inflammatory response is of particular importance for the broiler industry as chickens are processed at about six weeks of age before they have been able to fully develop

their adaptive immunities (French et al., 2020). Therefore, it is imperative that these chickens have a strong innate immunity. Thankfully, in avian species we are granted a special window for evaluating local immunological changes, particularly for the acute inflammatory response. This approach is known as the "growing feather (GF) pulp, a skin derivative and blood dual-window approach" (French et al., 2020). The pulp of growing feathers (GF) of birds may be used as a minimally invasive skin test to monitor inflammatory responses (Erf & Ramachandran, 2016). Simultaneously injecting multiple growing feathers allows us to examine leukocyte infiltration profiles and activities in *vivo* over a time course in an individual. Birds may be sampled with relative ease while causing them minimal bodily harm. Systemic changes in response to intradermal pulp injection may also be evaluated through concurrent sampling of blood at the same time points.

Previously, this approach had been utilized to evaluate the immune responses of egg-type chickens. Recently, French used this approach for the first time as a diagnostic tool for broiler chickens as well (French et al., 2020). From the experiment they were able to determine that using a GF-blood dual-window can be an effective method for examining the acute inflammatory response in broilers (French et al., 2020). In the French study, a row of 12 GF on each breast tract were plucked and allowed 18 days to regenerate to produce uniform GF. From there, 16 GF/bird (n=24) 10 µL of 100 µg LPS/mL (1 µg/GF) were intradermally (i.d.) injected to induce a local acute inflammatory response in the GF (French et al., 2020). Additional birds (n=8) were injected with 10 µL of PBS as a control. Blood and GF were concurrently collected at three time points – before injection (0 h) and at 6 h and 24 h after LPS injection into GF pulps. GF pulps were used to create pulp cell suspensions for cell population analysis, ROS generation, and superoxide dismutase (SOD) activity – pulps were also used for RNA isolation to examine

relative gene expression by qRT-PCR (French et al., 2020). Whole blood was used for blood cell profile analysis and plasma was also used to evaluate SOD activity (French et al., 2020). Pulp cell population changes revealed maximal heterophil and monocyte recruitment into the tissue at 6 h and 24 h post-GF injection with LPS, respectively (French et al., 2020). Blood cell populations showed increases in both heterophil and monocytes at 6h with a return to pre-injection levels at 24 h post-injection (French et al., 2020). ROS generation assay results followed trends similar to heterophil concentrations of pulp cell populations. Gene expression analyses revealed showed that "proinflammatory cytokines IL-1 β and IL-6, as well as chemokine IL-8," were all upregulated post-injection (French et al., 2020). The results produced in this study aligned well with the local and systemic physiological changes one would expect from the activation of the acute inflammatory response. Therefore, this GF dual-window test could find application in identifying help examine the effects of different treatments on broilers (French et al., 2020).

Heat stress (HS) is a growing issue within the poultry industry, rendering broilers unable to regulate their body temperatures and causing them to experience reduced feed intake, nutrient efficiency, and increased water intake (Santos et al., 2015; Ruff et al., 2020; Rocchi et al., 2022). A reduction in gut barrier function due to chronic stress placed upon the broilers from heat stress may also be seen (Quinteiro-Filho et al., 2010). The disruption of tight junction proteins found within the gut increases permeability and allows for paracellular translocation of toxins and bacteria inside the body (Farhadi et al., 2003). This chronic stress placed upon the chickens, leads to increased corticosterone serum levels (Quinteiro-Filho et al., 2010). Paired with this, we also see decreases in the relative weights of both primary lymphoid organs, the thymus and bursa of Fabricius, along with secondary lymphoid organs, like the spleen from HS (Quinteiro-Filho et

al., 2010). There is a current lack of information concerning how these physiological changes in broilers may affect their ability to respond to stimulation by LPS. Therefore, evaluation of the acute inflammatory response to LPS in HS broilers could provide some insight as to how HS affects the innate defenses of these birds.

MATERIALS AND METHODS

Experimental Animals and Rearing Conditions

For this study, a quantitative design was used in the form of a true experimental methodology. True experimental methodology involves using multiple created groups with similar measured outcomes where the individuals of each group are randomly assigned and not manipulated in any way (Gribbons & Herman, 1996). Newly hatched Cobb 500 broiler chicks were tagged at hatch and randomly assigned based upon their tag number to two different temperature treatment groups, thermoneutral or cyclic heat (TN or HS) (Gribbons & Herman, 1996). In total, eight environmental chambers were used, four TN and four HS. Each chamber was evenly split into two pens to produce eight pens per treatment (16 pens total). Twenty-three birds were placed into each pen on wood shavings with a stocking density of 10 birds/m^2. This study was carried out at the UA Poultry Environmental Research Laboratory (PERL). All protocols and procedures involving animals used in this trial were approved by the University of Arkansas System, Division of Agriculture, Institutional Animal Care and Use Committee (IACUC; protocol #21018-2).

From D 0 - 3 all birds were grown under the same temperature conditions of 32°C. Cyclic HS conditions began on D 4 for HS birds. HS birds were subjected to 35°C from 8 am to 10 pm (14 hours) and TN temperatures from 10 pm to 8 am. Temperature conditions for TN birds followed normal industry settings. Diets consisted of Rochell Starter D 0-10, Rochell Grower D 11-28, and Rochell Finisher D 28-42 for all treatments. Lighting schedules followed industry standards for broilers with 24 h of light D 0-1; 23 h of light with 1 h of dark D 2-7; 20 h of light with 4 h of dark D 8-14; and 18 h of light with 6 h of dark D 15-42 for all treatments.

Experimental Induction of the Inflammatory Response

There were four treatment groups based on injection and temperature conditions: TN-LPS, TN-phosphate-buffered-saline (PBS), HS-LPS, and HS-PBS. Eight broilers per temperature group were used for LPS injection and four for PBS injection. Three broilers were randomly selected from each chamber, two for LPS (one per pen) and one for PBS (vehicle) injection. When the broilers were 37 days of age, 6 GF from each breast tract were injected with 10 µL of LPS (100 µg/mL of PBS) or 10 µL of PBS (French et al., 2020).

Pulp Sample Collection and Processing

GF (6) were collected before (0 h) and at 6 and 24 h post GF pulp injection (French et al., 2020). Two GF were used to prepare pulp cell suspensions for immunofluorescent staining and cell population analysis by flow cytometry (French et al., 2020). Cell populations were identified using fluorescently labeled (FITC or PE) mouse monoclonal antibodies (mAb) for chicken leukocyte markers. Pulp cell suspensions were dual labeled for total leukocytes and macrophages using mAb CD45-PE and KUL01-FITC, respectively. A second dual labeling was used for B and T cell determination using Bu-1-FITC and CD3-PE, respectively (French et al., 2020). Data were expressed as percent of a leukocyte population in the pulp suspension.

Blood Sample Collection and Processing

At each time point, 1 mL of blood was collected from the wing vein using heparinized 3 mL syringes with 25-gauge x 1-inch needles (French et al., 2020). The blood was used for preparation of Wright-stained blood smears to determine the proportions of lymphocytes, heterophils, monocytes, basophils, and eosinophils by microscopic evaluation of at least 300

white blood cells (WBC) per blood smear. Blood also was used for whole blood assays for immunofluorescent staining and cell population analysis by flow cytometry following a modified methodology of Seliger et al. (2012).

Statistical Analyses

Three-way analysis of variance (ANOVA) was conducted to determine the effect of temperature (TN, HS), time (0, 6, 24 h), and injection (PBS, LPS) and their interactions. Due to significant interactions, data were separated into 10 treatment groups, TN-0h, HS-0h, TN-PBS-6h, TN-PBS-24h, TN-LPS-6h, TN-LPS-24h, HS-PBS-6h, HS-PBS-24h, HS-LPS-6h, HS-LPS-24h and subjected to one-way ANOVA to detect treatment effects. In the presence of significant treatment effects with $P \leq 0.05$, Fisher's multiple means comparison analysis was conducted to determine differences between individual treatment groups. Treatment groups were considered different at $P \leq 0.05$.

RESULTS

Growing Feather Leukocyte Population Profiles

Both TN- and HS-birds intradermally injected with 10 µL of LPS exhibited higher (both $P < 0.001$) levels of leukocytes (% pulp cells) at 6 h post-LPS injection compared to pre-injection levels, increasing from 5.89% to 19.50% and 6.06% to 16.50%, respectively. By 24 h leukocyte levels in GF pulps of TN- and HS-LPS injected birds dropped ($P = 0.009$, $P < 0.001$, respectively) to 16.24% and10.93%, respectively, at 24 h (Figure 1a.) At 6 h and 24 h post-injection, leukocyte levels in HS-LPS injected GF pulps were lower ($P = 0.020$ and $P < 0.001$, respectively) compared to those of TN-LPS birds. Heterophil levels (% pulp cells) of TN- and

HS-LPS injected birds were elevated (both $P < 0.001$) at 6 h post injection, increasing from 0.36% to 13.53% and 0.32% to 11.16%, respectively. Heterophil levels then dropped ($P < 0.001$) at 24 h to 5.89% and 3.79%, respectively (Figure 1b). At 6 h and 24 h post-injection, heterophil levels in the pulp of HS-LPS injected birds were lower ($P = 0.008$, $P = 0.017$) compared to those of TN-LPS birds. In TN- and HS-LPS injected GF pulps, macrophage levels were elevated ($P = 0.038$ and $P = 0.050$) at 6 h post-injection from 1.12% to 2.349% and 1.18% to 2.39%, respectively, and further increased ($P < 0.001$ and $P = 0.038$, respectively) to 5.74% and 3.83%, respectively (Figure 1c). At 24 h post-injection, macrophage levels in HS-LPS GF pulps were lower ($P = 0.005$) compared to those of TN-LPS birds. There was an overall treatment main effect difference ($P = 0.013$) in T cell levels, although no differences between means were detected (Figure 2a). B cell proportions had no main effect treatment differences ($P = 0.575$) (Figures 2b).

Peripheral Blood Cell Population Profiles

For blood erythrocyte concentrations (RBC) (cells/µL), only changed in HS-LPS injected birds decreasing ($P = 0.015$) at 6 h post-injection from 2.56×10^6 to 2.08×10^6 (Figure 3a). Thrombocyte concentrations (cells/µL) of TN- and HS-LPS injected birds increased ($P < 0.001$ and $P = 0.007$, respectively) at 6 h post-injection from 8,185 to 13,420 and 5,989 to 9,663, respectively. In TN-LPS thrombocyte levels dropped ($P = 0.021$) to baseline levels at 24 h to 9,663 (Figure 3b). White blood cell (WBC) concentrations (cells/µL) of HS-PBS injected birds decreased ($P < 0.001$) at 6 h post-injection from 17,980 to 9,376, respectively, followed by a return ($P = 0.035$) to pre-injection levels at 24 h to 15,851 (Figure 3c). WBC concentrations of HS-LPS injected birds also decreased ($P < 0.001$) from 0 h to 6 h post injection from 17,980 to

8,186, respectively, followed by an elevation ($P < 0.001$) at 24 h to 16,137 (Figure 3c). In both TN-PBS and TN-LPS injected birds, WBC concentrations decreased ($P = 0.002$ and $P = 0.004$, respectively) at 6 h post-injection from 24,725 to 16,817 and 24,725 to 18,879, respectively (Figure 3c). HS birds, regardless of injection type, exhibited lowered ($P < 0.001$) WBC concentrations at 0 h compared to TN birds (Figure 3c).

Heterophil counts (cells/µL) increased ($P < 0.001$) only in TN-LPS birds from 0 h to 6 h from 4,610 to 11,383, respectively, followed by a decrease ($P < 0.001$) at 24 h to 6,226 (Figure 4a). T cell counts (cells/µL) for all treatment and temperature groups decreased from 0 h to 6 h and returned to pre-injection (0 h) levels at 24 h (Figure 4b). TN-LPS, HS-PBS, and HS-LPS injected birds had lower (all $P < 0.001$) T cell levels than TN-PBS injected birds at 6 h post injection (Figure 4b). HS birds showed lower ($P < 0.001$) T cell levels compared to TN birds at 0 h, regardless of injection treatment (Figure 4b). B cell counts (cells/µL) for all treatment and temperature groups exhibited decreasing levels from 0 h to 6 h post injection with no return to pre-injection levels at 24 h (Figure 4c). Before injection (0 h), HS birds had lower ($P < 0.001$) B cell concentrations than TN birds (Figure 4c). Blood heterophil to lymphocyte (H:L) ratios increased ($P < 0.001$) in TN-LPS birds from 0 h to 6 h post-injection from 0.25 to 2.57, respectively, followed by a decrease ($P < 0.001$) at 24 h to 0.45 (Figure 4d). H:L ratios in HS-LPS broilers increased ($P < 0.001$) from 0 h to 6 h post-injection from 0.28 to 2.27, respectively, with a decrease ($P < 0.001$) at 24 h to 0.43 (Figure 4d). H:L ratios for HS-PBS injected broilers also increased ($P = 0.010$) from 0.28 at 0 h to 1.22 at 6 h (Figure 4d).

Monocyte concentrations (cells/µL) of TN-LPS injected birds increased ($P < 0.001$) from 0 h to 6 h post injection from 561 to 1,572, respectively, followed by a decrease ($P < 0.001$) at 24 h to 476 (Figure 5a). Monocyte levels in HS-LPS injected birds did not change from 0 h to 6 h

but decreased (P = 0.017) from 6 h to 24 h post-injection from 851 to 340, respectively. TN-PBS, HS-PBS, and HS-LPS injected birds exhibited lower (P = 0.001, P = 0.018. and P < 0.001, respectively) monocyte concentrations than TN-LPS injected birds at 6 h post-injection (Figure 5a). Eosinophil concentrations (cells/μL) in TN-LPS injected birds decreased (P < 0.001) from 0 h to 6 h post-injection from 536 to 223, respectively, followed by a return to baseline levels (P = 0.001) at 24 h to 548 (Figure 5b). Eosinophil levels in HS-LPS injected birds decreased (P = 0.002) from 0 h to 6 h post-injection from 416 to 122, respectively, followed by a return to baseline levels (P = 0.016) at 24 h to 387 (Figure 5b). There were no main treatment effects (P = 0.129) on basophil concentrations (Figure 5c).

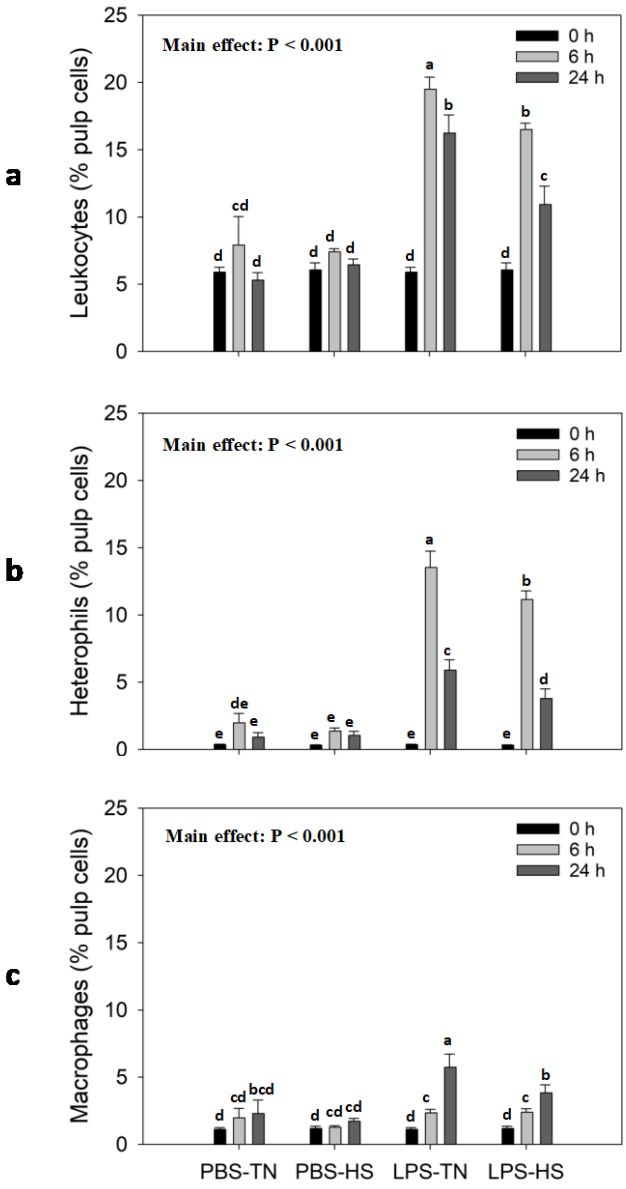

Figure 1a-c. Effects of cyclic heat stress (HS) on the proportions (% pulp cells) of leukocytes, heterophils, and macrophages in the pulp of growing feathers (GF) at 0, 6, and 24 h post-intradermal (i.d.) injection of lipopolysaccharide (LPS). Pulps of 12 GF from each of 16 birds (8 thermoneutral (TN) and 8 HS) were injected i.d. with 10 µL of LPS (1 µg/GF; 12 GF/bird; 12 µg/bird). Pulps of 12 GF from each of 8 additional birds (4 TN and 4 HS) were injected i.d. with 10 µL of PBS. Two GF from each chicken were used to prepare pulp cell suspensions at each time point (0, 6 and 24 h post injection). Cell populations were identified using fluorescently labeled (FITC or PE) mouse monoclonal antibodies (mAb) for chicken leukocyte markers. Suspensions were dual labeled for total leukocytes and macrophages using mAb CD45-PE and KUL01-FITC, respectively. Cell populations were then analyzed by flow cytometry and FlowJo software. Heterophils were distinguished based on size (FSC), granularity (SSC and characteristics of leukocytes (CD45+). All data shown are means ± SEM. For each subfigure, means without a common letter are different ($P \leq 0.05$).

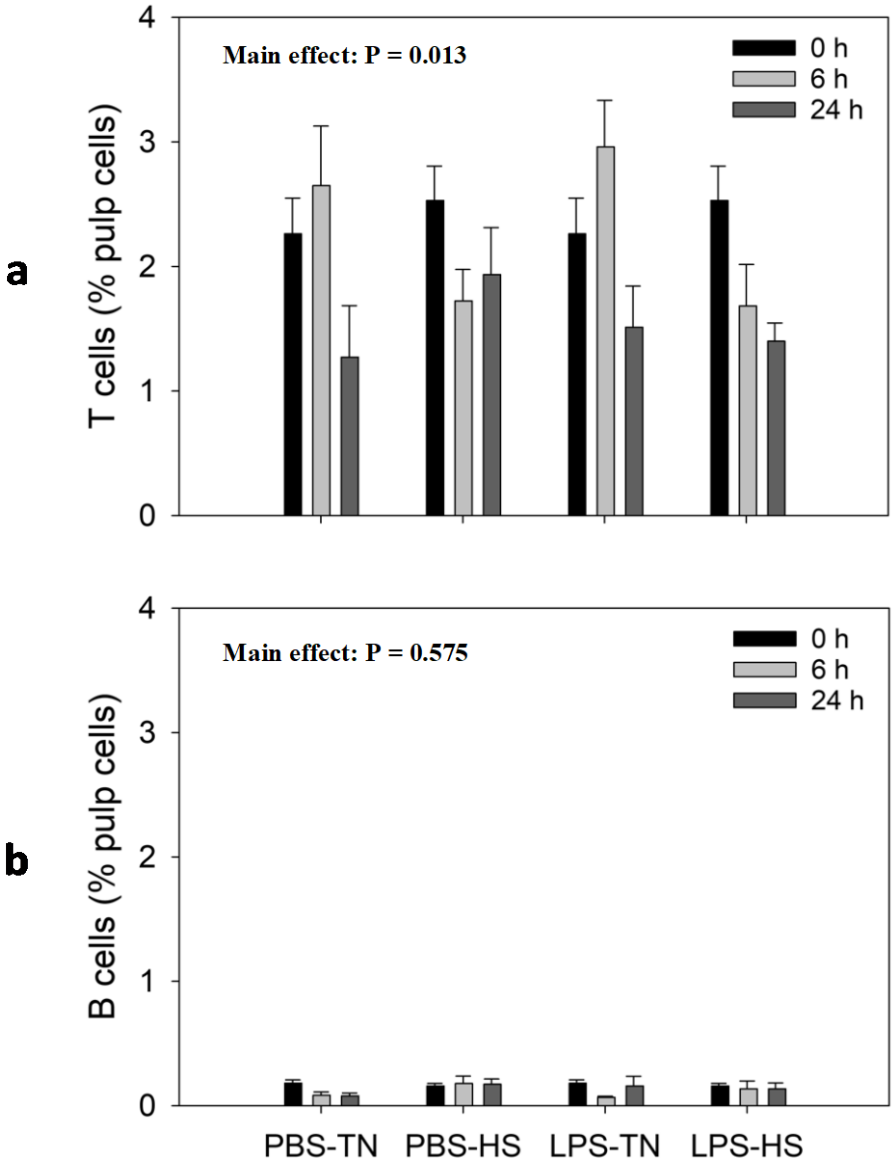

Figure 2a-b. Effects of cyclic heat stress (HS) on the proportions (% pulp cells) of T and B lymphocytes in the pulp of growing feathers (GF) at 0, 6, and 24 h post-intradermal (i.d.) injection of lipopolysaccharide (LPS). Pulps of 12 GF from each of 16 birds (8 thermoneutral (TN) and 8 HS) were injected i.d. with 10 µL of LPS (1 µg/GF; 12 GF/bird; 12 µg/bird). Pulps of 12 GF from each of 8 additional birds (4 TN and 4 HS) were injected i.d. with 10 µL of PBS. Two GF from each chicken were used to prepare pulp cell suspensions at each time point (0, 6 and 24 h post LPS injection). Cell populations were identified using fluorescently labeled (FITC or PE) mouse monoclonal antibodies (mAb) Bu-1-FITC and CD3-PE, respectively, in a dual labeling procedure to identify B- and T-cells respectively. Cell populations were then analyzed by flow cytometry and FlowJo software. All data shown are means ± SEM. For each subfigure, means without a common letter are different ($P \leq 0.05$).

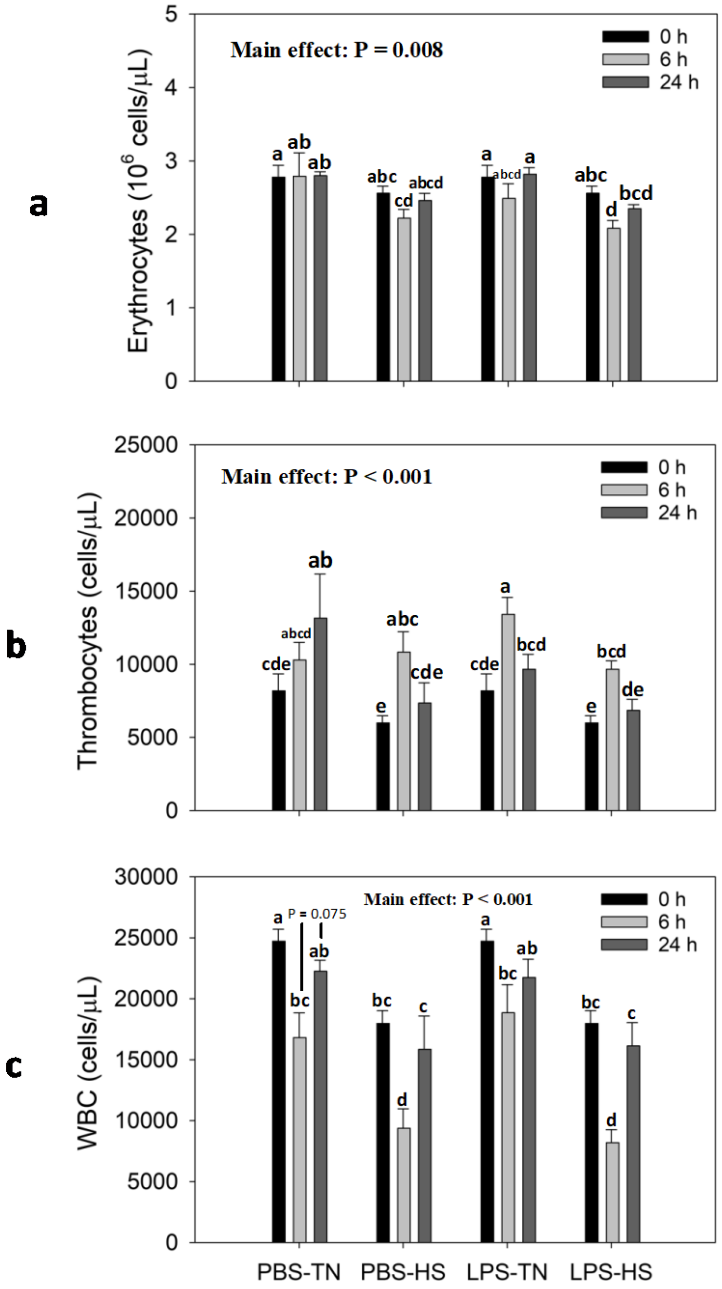

Figure 3a-c. Effects of cyclic heat stress (HS) on blood concentrations (cells/µL) of erythrocytes (10^6 cells/µL), thrombocytes, and white blood cells (WBC) at 0, 6, and 24 h post-intradermal (i.d.) injection of the pulp of growing feathers (GF) with lipopolysaccharide (LPS). Pulps of 12 GF from each of 16 birds (8 thermoneutral (TN) and 8 HS) were injected i.d. with 10 µL of LPS (1 µg/GF; 12 GF/bird; 12 µg/bird). Pulps of 12 GF from each of 8 additional birds (4 TN and 4 HS) were injected i.d. with 10 µL of PBS. One mL of blood was collected from the wing vein using heparinized 3 mL syringes with 25-gauge x 1-inch needles. Whole blood was diluted by a factor of 1600 and fluorescently labeled for thrombocytes and total WBC using CD41/61-FITC and CD45-SR, respectively. Cell populations and concentrations were then analyzed by flow cytometry and FlowJo software. All data shown are means ± SEM. For each subfigure, means without a common letter are different ($P \leq 0.05$).

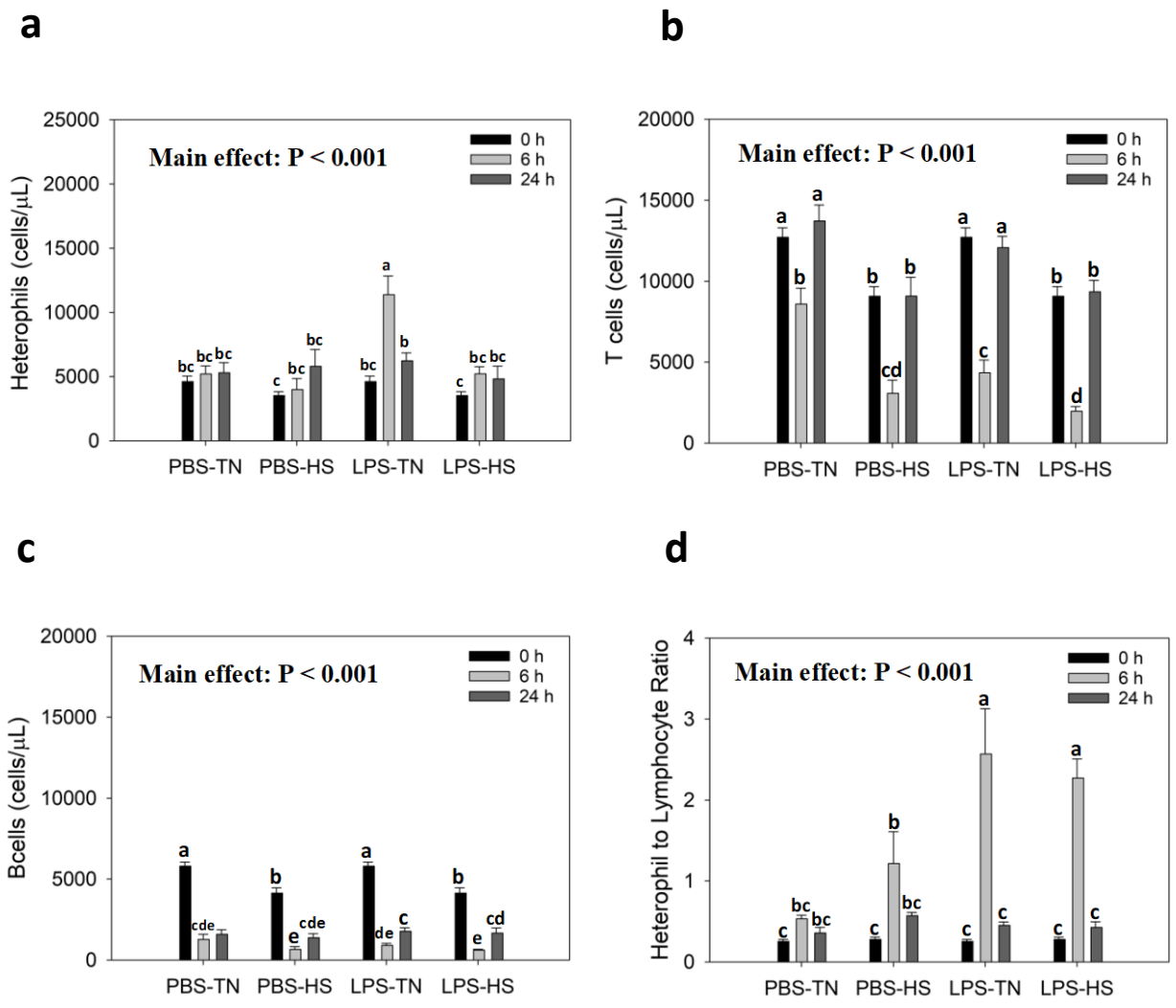

Figure 4a-d. Effects of cyclic heat stress (HS) on blood concentrations (cells/µL) of heterophils, heterophil to lymphocyte (H:L) ratios, T lymphocytes, and B lymphocytes at 0, 6, and 24 h post-intradermal (i.d.) injection of the pulp of growing feathers (GF) with lipopolysaccharide. Pulps of 12 GF from each of 16 birds (8 thermoneutral (TN) and 8 HS) were injected i.d. with 10 µL of LPS (1 µg/GF; 12 GF/bird; 12 µg/bird). Pulps of 12 GF from each of 8 additional birds (4 TN and 4 HS) were injected i.d. with 10 µL of PBS. One mL of blood was collected from the wing vein using heparinized 3 mL syringes with 25-gauge x 1-inch needles. Whole blood was diluted by a factor of 1600 and fluorescently labeled for total WBC using CD45-SR monoclonal antibody (mAb). Levels of B- and T-cell were determined using Bu-1-FITC and CD3-PE, respectively, in a dual staining procedure. Cell populations were then analyzed by flow cytometry and FlowJo software. Heterophil concentrations were determined using % heterophil data derived by differential leukocyte counts, dividing the % heterophils by 100 and multiplying it by the concentration of total WBC. All data shown are means ± SEM. For each subfigure, means without a common letter are different ($P \leq 0.05$).

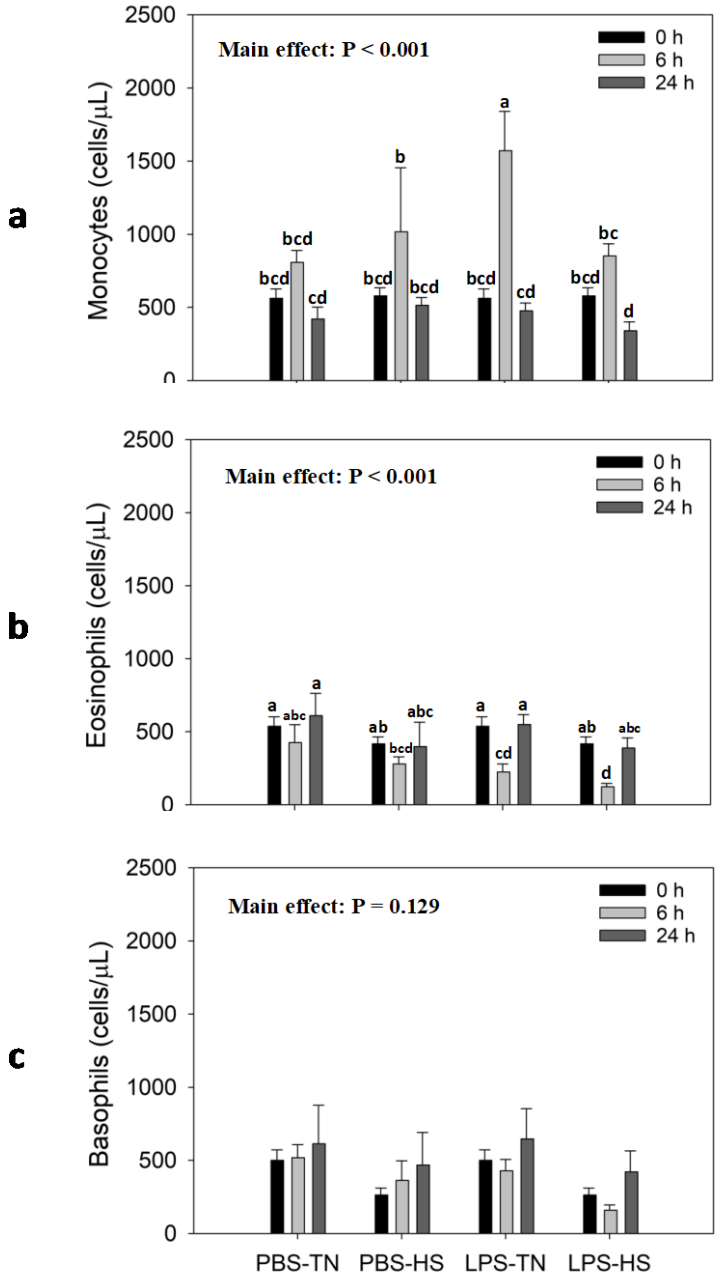

Figure 5a-c. Effects of cyclic heat stress (HS) on concentrations (cells/µL) of monocytes, eosinophils, and basophils at 0, 6, and 24 h post-intradermal (i.d.) injection of the pulp of growing feathers (GF) with lipopolysaccharide (LPS). Pulps of 12 GF from each of 16 birds (8 thermoneutral (TN) and 8 HS) were injected i.d. with 10 µL of LPS (1 µg/GF; 12 GF/bird; 12 µg/bird). Pulps of 12 GF from each of 8 additional birds (4 TN and 4 HS) were injected i.d. with 10 µL of PBS. One mL of blood was collected from the wing vein using heparinized 3 mL syringes with 25-gauge x 1-inch needles. Whole blood was diluted by a factor of 1600 and fluorescently labeled for total WBC using CD45-SR monoclonal antibody (mAb) and KUL01-FITC mAb to identify monocytes in a dual labeling procedure. Cell populations were then analyzed by flow cytometry and FlowJo software. Eosinophil and basophil concentrations were determined using differential leukocyte counts to determine their percentages, dividing the % by 100 and multiplying by the concentration of total WBC All data shown are means ± SEM. For each subfigure, means without a common letter are different. ($P \leq 0.05$).

DISCUSSION

Inflammation is a highly conserved, innate immune system response in animals, and constitutes a major first line defense in the fight against microbial infection. Inflammation involves the recruitment of leukocytes, plasma proteins, and fluids from the blood to the infected tissue, followed by activation of the recruited cells to fight the infection, and resolution of the response once the infection is eliminated (Abbas et al., 2018). As shown by French et al. (2020), intradermal (i.d.) GF-pulp injection of LPS in broilers resulted in a local acute inflammatory response characterized by rapid infiltration of heterophils at 6 h post-injection, that was accompanied and followed by monocyte/macrophage recruitment reaching maximal levels by 24 h. The local inflammatory activity was also reflected by increased heterophil and monocyte concentrations in the blood circulation at 6 h. Additionally, levels of lymphocytes at the site of LPS injection (GF-pulp) and in the blood dropped in response to i.d. LPS injection.

In the current study, we observed similar changes in local and systemic leukocyte profiles in response to GF-pulp injection of LPS in broilers reared in conventional, TN conditions. However, while cyclic HS had no noticeable effects on baseline (0 h) levels of leukocytes in GF pulps, cyclic HS negatively impacted the local and systemic acute inflammatory response to i.d. injection of LPS. Specifically, compared to TN-LPS broilers, HS-LPS broilers had lower GF-pulp infiltration of heterophils at 6- and 24-h and lower macrophage levels at 24 h post-injection. Moreover, in the blood, heterophil and monocytes concentrations (cells/μL) were not elevated in response to i.d. LPS injection in HS-LPS broilers, suggesting a lower ability of HS-LPS broilers to meet demands of the local LPS-induced inflammatory response by increasing production and release of heterophils and monocytes from the bone marrow into the blood.

Also notable are the pre-injection differences in the blood in broilers reared in cyclic HS compared to TN conditions, suggesting effects of HS on leukocyte development. As there were no differences between TN and HS broilers before LPS administrations in the blood concentrations of RBC, thrombocytes, heterophils, monocytes, basophils, and eosinophils, it does not appear that HS affected hematopoiesis of myeloid cells. Rather, the lower concentrations of circulating T- and B-lymphocytes point towards an effect of cyclic HS on development of lymphocytes in the generative organs, the thymus and bursa of Fabricius, respectively. The lower concentrations of circulating lymphocytes also explain the lower levels of WBC observed in HS- compared to TN-broilers in this study.

In a similar study by Quinteiro-Filho et al. (2010), 35-day-old broilers were subjected to 10 h of 36 ºC (HS) from 8:00 am to 6:00 pm, and at 21 ºC (TN) from 6:00 pm to 8:00 am for one week, with control birds kept at TN temperatures 24 h per day. HS broilers were reported to have greatly elevated serum corticosterone levels, decreased relative weights (% BW) of thymus, bursa of Fabricius, and spleen, as well as reduced *Staphylococcus aureus*-induced reactive species generation (ROS) by macrophages. These alterations in primary and secondary lymphoid organ weight as well as in macrophage ROS generation were attributed to the elevated levels of the stress hormone corticosterone (Quinteiro-Filho et al., 2010). Corticosterone, as well as sex steroids, are known to drive regression of the thymus and bursa glands, particularly by reducing the levels of immature lymphocytes (e.g., CD4+CD8+ thymocytes) and, hence the weight of the primary lymphoid organs will also be reduced. The decreased output of T- and B- cells by the primary lymphoid organs in HS birds, also may explain the reduction in relative spleen weights, likely due to fewer lymphocytes in the splenic white-pulp. Moreover, corticosterone and other glucocorticoids are known to have anti-inflammatory properties, explaining the reduced ROS

generation in response to *S. aureus* stimulation of macrophages in the Quinteiro-Filho et al. (2010) study.

While corticosterone levels were not measured in the current HS study, our observations of reduced local and systemic acute inflammatory responses and reduced circulating levels of T- and B-lymphocytes in HS broilers likely are effects mediated by elevated levels of HS associated stress hormones. To gain a more complete picture of the effects of cyclic HS stress on the LPS-induced inflammatory response, further studies are underway to examine functional activities of the cells recruited to the site of LPS injection, i.e., expression of cytokines and generation of ROS in GF-pulps, as well as changes in plasma proteins in response to i.d. LPS injection.

Overall, the observed reduction in the LPS-stimulated, local and systemic acute inflammatory responses and in circulating levels of lymphocytes in HS broilers, suggest that HS conditions may impair the ability of broilers to mount effective innate immune responses, which may prove to be problematic for maintaining flock health as HS continues to grow as an issue. Further research should be done to elucidate the mechanisms and extent of this impaired immune function in HS conditions. Application of this dual-window approach could prove to be useful in selecting individuals exhibiting greater immune robustness while experiencing HS.

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