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

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Meet the Student-Author

Alessandro Rocchi

Alessandro and Dr. Erf are identifying growing feathers for pulp injection on the breast tract of a broiler chicken.

I graduated Summa Cum Laude in the spring of 2023 with a double major in Poultry Science and Environmental, Soil, and Water Science, and a minor in Soil Science. I am from Bentonville, Arkansas and graduated from Bentonville High School in 2019. I was a member of the Vietnamese Student Association and Crop, Soil, Environmental Science Club while also serving as an officer in both the Poultry Science Club and Bumpers Honors Student Board. These past two years, I worked at the Poultry Health Laboratory under Dr. Billy Hargis and in the Poultry Science Parasitology Lab under Dr. Danielle Graham, where I focused on *Histomonas meleagridis*, a parasitic protozoan in turkeys. I was named the 2023 USPOULTRY Frank Perdue Student of the Year. This fall, I will be attending Clemson University to pursue a M.S. degree in microbiology, focusing on the molecular aspects of *H. meleagridis*. I have always been fascinated by biology, which has played a large role in getting me to where I am today. I truly feel fortunate that I have been able to join the Bumpers family these past four years and have had a wonderful time along the way. Thank you to Dr. Gisela Erf for her excellent mentorship and guidance these past four years, especially throughout my Honors program, and to my committee, Dr. Billy Hargis and Dr. Guillermo Isaias Tellez, for their assistance in conducting my honors project and completing my honors thesis. Thank you to Aaron Forga and the Poultry Health Lab team for their assistance in bird rearing and care.

Research at a Glance

- Heat stress is a growing concern within the poultry industry in both broiler production and animal welfare.
- Cyclic heat stress reduces circulating levels of T- and B-lymphocytes but not other blood cells in broiler chickens.
- Cyclic heat stress attenuates the acute local and systemic inflammatory response to lipopolysaccharide in broilers.

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. Little is known regarding the effect of HS on immune function. To examine the effects of HS on innate immunity, the local- and systemic-inflammatory responses to lipopolysaccharide (LPS) were examined in Cobb 500 male broiler chicks reared under thermoneutral (TN) or cyclic HS conditions. Beginning at four days of age, HS birds were subjected to 35 °C from 8:00 a.m. to 10:00 p.m. and TN temperatures from 10:00 p.m. to 8:00 a.m. At 37 days of age, four groups of broilers were formed: LPS-TN (8 broilers), phosphate-buffered saline (PBS)-TN (4 broilers), LPS-HS (8 broilers), and PBS-HS (4 broilers), with each broiler receiving LPS- (100 µg/mL) or PBS-treatments by intradermal pulp-injection of 12 growing feathers (GF; 10 µL/GF). Blood and GF were collected before (0 h) and at 6 and 24 h post-injection to determine leukocyte population changes. Locally, LPS-HS broilers had lower ($P \leq 0.05$) levels (% pulp cells) of infiltrating heterophils and macrophages in GF-pulps at 6 and 24 h, respectively, compared to LPS-TN birds. In the blood, TN and HS broilers had similar baseline (0 h) concentrations (cells/µL) of heterophils, monocytes, eosinophils, and basophils, but HS broilers had lower ($P \leq 0.05$) T- and B-lymphocyte levels. Concentrations of heterophils and monocytes were greatly elevated ($P \leq 0.05$) at 6 and 24 h, respectively, only in LPS-TN broilers. Overall, results indicated that cyclic HS reduced both the local and systemic acute inflammatory responses to LPS in broilers, likely impairing their innate defense against microbial infection.

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Introduction

There are two major parts to the immune system, consisting of innate and adaptive immunity. Innate immunity acts as the body's first line of defense, developing ways for immediately dealing with pathogens through an inflammatory response. Inflammation works by accumulating "leukocytes, plasma proteins and fluid derived from blood" at the site of infection to eliminate microbes and repair damaged tissue (Abbas et al., 2018). To recognize infections, the innate immune system established a method for distinguishing molecular patterns common to groups of pathogens (e.g., lipopolysaccharide (LPS) of Gram-negative bacteria). With this, the innate immune system can quickly recognize pathogens and initiate inflammation (Medzhitov and Janeway, 1997).

The innate immune system is important for commercial broiler production as chickens are processed at six weeks of age before they can fully develop their adaptive immunities (French et al., 2020). The pulp of growing feathers (GF) has been shown to be an effective, minimally invasive skin test site to monitor local inflammatory responses in order to examine the effectiveness of the innate immune system (Erf and Ramachandran, 2016). Through simultaneous GF injection and periodic collection of injected GF, we may examine local leukocyte infiltration profiles and activities taking place *in vivo*. Changes in the blood may be determined from concurrently sampled peripheral blood.

Recently, a study using the "GF and blood dual-window approach" in broilers examined the acute inflammatory response to LPS injected into the GF pulp (French et al., 2020). This study showed extensive recruitment of heterophils and monocytes/macrophages in the dermis of injected GF, reaching peak levels at 6 and 24 h post-injection, respectively. Local GF cellular activities included the 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 (French et al., 2020). In the blood, concentration and proportions of heterophils were elevated at 6 h and returned to baseline levels by 24 h, whereas the proportions 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 (HS) is an environmental issue associated with broiler production, resulting in reduced feed intake and nutrient efficiency, along with 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.

Materials and Methods

Newly hatched Cobb 500 broiler chicks were tagged at hatch and assigned randomly based upon their tag number to two different temperature treatment groups, thermoneutral or cyclic heat (TN or HS) (Gribbons and 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 conducted 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 #21-018-2).

From Day 0 to 3, all birds were grown under the same temperature conditions of 32 °C. Cyclic HS conditions began on Day 4 for HS birds. HS birds were subjected to 35 °C from 8:00 a.m. to 10:00 p.m. (14 hours) and TN temperatures from 10:00 p.m. to 8:00 a.m. Temperature conditions for TN birds followed industry settings (i.e., Day 4–6, 31 °C; Day 7–10, 29 °C; Day 11–14, 26 °C; and Day 15 onwards, 24 °C). Diets followed industry standards, consisting of Starter from 0 to 10 days, Grower from 11 to 28 days, and Finisher from 28 to 42 days for all treatments. Lighting schedules followed industry standards for broilers with 24 h of light Days 0 to 1; 23 h of light with 1 h of dark Days 2 to 7; 20 h of light with 4 h of dark Days 8 to 14; and 18 h of light with 6 h of dark Days 15 to 42 for all treatments.

There were four treatment groups based on injection and temperature conditions: LPS-TN, phosphate-buffered-saline (PBS)-TN, LPS-HS, and PBS-HS. Eight broilers per temperature group were used for LPS injection and four for PBS injection. Three broilers were selected randomly 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). Six GF were collected before (0 h) and at 6 and 24 h post-GF pulp injection. Two GF were used to prepare pulp cell suspensions for direct immunofluorescent staining and cell population analysis by flow cytometry, as described by French et al. (2020).

Briefly, cell populations were identified using fluorescently labeled mouse monoclonal antibodies (mAb) for chicken leukocyte markers. Pulp cell suspensions were dual labeled for total leukocytes and macrophages using mAb CD45-SR 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). Heterophils were identified based on CD45-expression and granularity (side scatter characteristics) (Seliger et al., 2012). Data were expressed as percentages of leukocytes in the pulp cell suspension (% pulp cells).

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 the 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 was also used to determine concentrations (cells/µL) of RBC, thrombocytes, WBC, heterophils, monocytes, and T- and B-cells in a whole blood assay. In this assay, various cell types were identified by direct immunofluorescent staining, and cell population analysis was conducted following a modified methodology of Seliger et al. (2012) on a BD C6-Plus flow cytometer (Becton Dickinson Biosciences, San Jose, Calif.). For both pulp and whole blood fluorescent staining, controls were included to detect non-specific binding of fluorescently labeled mAb, to determine cut-offs between positive and negative fluorescence, and to set compensations (French et al., 2020). The concentration of eosinophils and basophils was calculated by multiplying the WBC concentration determined by flow cytometry by the percentage of eosinophils and basophils, as determined by manual differential leukocyte counting and dividing the product by 100.

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

Results and Discussion

Using the minimally invasive, "two-window approach" in broilers, French et al. (2020) were able to describe local and systemic inflammatory activities in response to intradermal (i.d.) pulp injection of LPS within the same individuals and over time. Similar to French et al. (2020), LPS administration in this study stimulated heterophil and monocyte/macrophage recruitment from the blood into the injected pulps. In TN and HS GF-pulps injected with LPS, heterophils reached peak levels (% pulp cells) at 6 h and remained above pre-injection levels at 24 h, whereas levels of macrophages were elevated at 6 h and continued to increase further by 24 h (Fig. 1). Baseline levels (0 h) of heterophils and macrophages in the pulp were similar in TN- and HS-broilers. However, pulp-infiltration was lower in LPS-HS compared to LPS-TN broilers at 6- and 24-h for heterophils and at 24 h for macrophages, indicating attenuated local inflammatory responses to LPS in broilers reared under cyclic HS conditions. Like in the French et al., 2020 study, lymphocytes were not recruited to the pulp by LPS injection and lymphocyte proportions tended to decrease (time main effect $P = 0.003$) over the 24-hour post-injection period (Fig. 1). There were no changes in individual lymphocytes populations (i.e., T- and B-cells) following LPS injections. In both PBS-TN and -HS broilers, injection of PBS was not associated with significant changes in pulp heterophils or macrophages. For lymphocytes, pre-injection levels were greater than at 24 h only in PBS-TN broilers, although this drop was not significant for individual T- and B- lymphocyte populations (Fig. 1). Apart from the drop in lymphocytes for PBS-TN broilers, these results agree with observations reported by French et al. (2020). The small changes in leukocyte populations in PBS-injected GFs are likely due to inflammatory processes initiated by tissue damage associated with the injection and/or the stress of handling.

In LPS-TN broilers, the local inflammatory response was also reflected in blood cell profile changes (Fig. 1) that were similar to those reported by French et al. (2020). Specifically, concentrations of heterophils and monocytes were greatly elevated at 6 h. However, in LPS-HS broilers, there was no change in heterophil or monocyte concentrations in the blood over the 24-hour period (Fig. 1). The observations that LPS-HS broilers had lower GF-pulp infiltration of heterophils macrophages, together with a lack of elevation in blood heterophil and monocyte concentrations (cells/µL) post-injection, suggests an attenuated ability of LPS-HS broilers to meet demands of the inflammatory response (i.e., increased production and release of heterophils and monocytes from the bone marrow into the blood).

Interestingly, blood lymphocyte concentrations were greatly reduced at 6 h in both LPS-TN and LPS-HS broilers (Fig. 1). The drop in lymphocyte concentrations at 6 h in both groups of broilers was due to lower concentrations of T- and B-cells (Table 1). For both LPS-TN and LPS-HS broilers, T cell levels returned to baseline levels by 24 h, while B cell concentrations remained low. Moreover,

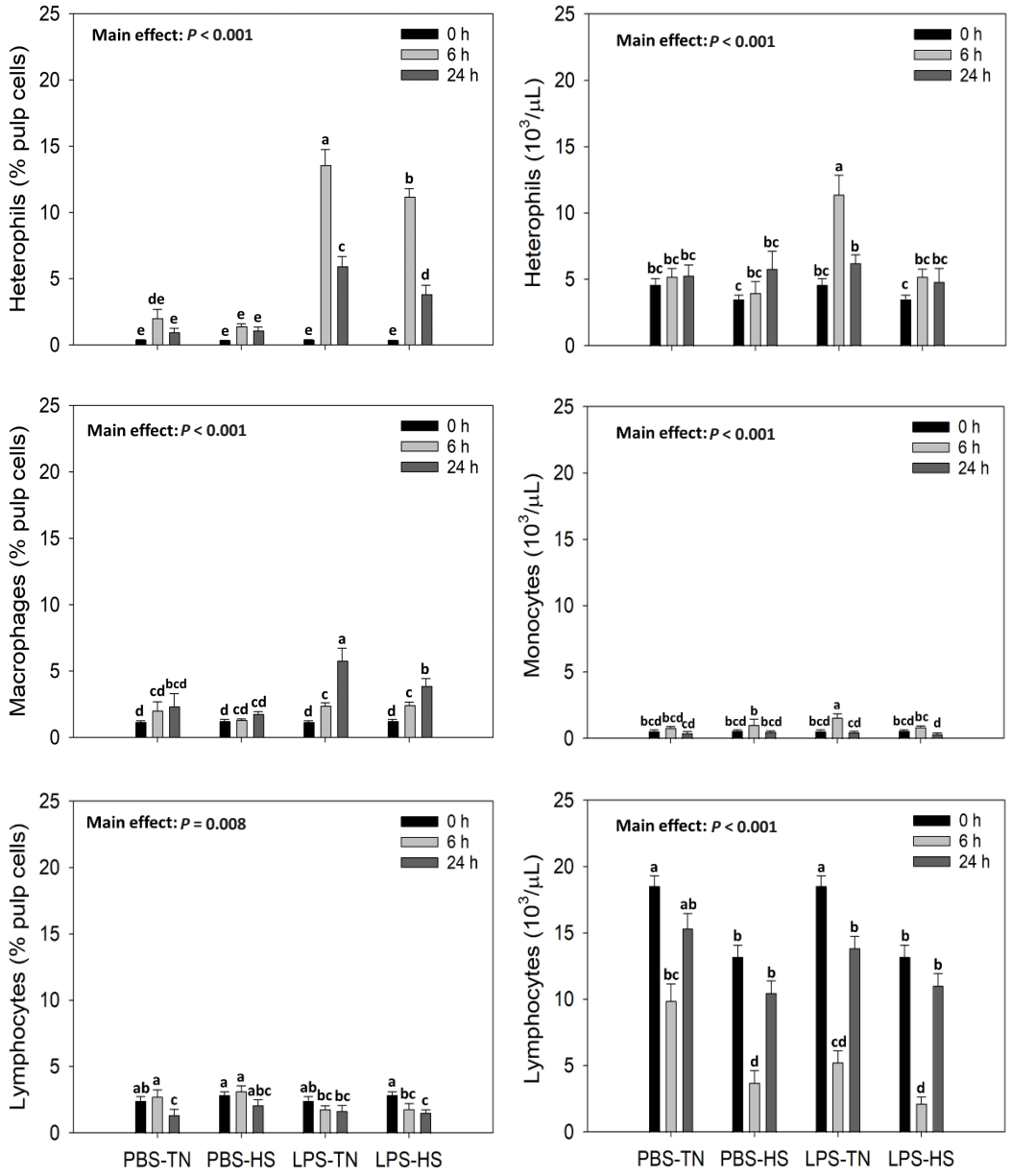

Fig. 1. Effects of cyclic heat stress (HS) on the proportions (% pulp cells) and blood concentrations ($10^3/\mu L$) of heterophils, macrophages, and lymphocytes in the pulp of growing feathers (GF) and blood at 0, 6, and 24 h post-intradermal injection of lipopolysaccharide (LPS). Pulps of 12 GF from each of 16 birds (8 thermoneutral (TN) and 8 HS) were intradermally (i.d.) injected with 10 μL of LPS (1 μg/GF; 12 GF/bird; 12 μg/bird) at 37-days of age. Pulps of 12 GF from each of 8 additional birds (4 TN and 4 HS) were i.d. injected with 10 μL of PBS. Blood (1 mL) and GF (2) were collected before and at 6 and 24 h post-GF injection. Pulp and blood cell suspensions prepared at each time point were used in a direct, dual labeling procedure using a panel of fluorescently labeled (FITC, PE, or SR) chicken leukocyte-specific mouse monoclonal antibodies, i.e., CD45-SR (total leukocytes/WBC), KUL01-FITC (monocytes/macrophages), Bu-1-FITC and CD3-PE (B- and T-cells, respectively). Cell population analysis was conducted using a Becton Dickinson C6-Plus flow cytometer. The lymphocyte population was calculated by adding B- and T-cell data. Heterophils were distinguished based on size (FSC) and granularity (SSC) characteristics of the leukocyte population (CD45+ cells). All data shown are means ± SEM. For each cell population, means without a common letter are different ($P \leq 0.05$).

baseline concentrations (0 h) of T- and B-cells, and hence, lymphocytes, were lower in HS-broilers compared to TN-broilers (Fig. 1; Table 1). Hence, rearing broilers in cyclic HS conditions not only attenuated the expected increases in circulating levels of heterophils and monocytes in response to i.d. LPS injection but also reduced baseline levels of circulating lymphocytes (Table 1).

LPS and HS also affected the concentrations of other blood cells. This included a drop in RBC at 6 h in LPS-HS but not in LPS-TN broilers, and an increase in thrombocytes and a drop in eosinophils at 6 h in both LPS-TN and -HS broilers. There was no change in basophil concentrations in LPS-TN and LPS-HS broilers (Fig. 2). PBS injection had no effect on blood cell concentrations in either the PBS-TN or -HS group, except for elevated thrombocyte concentrations at 6 h and 24 h in PBS-HS and PBS-TN broilers, respectively. This change in thrombocyte concentrations may also be due to tissue injury caused by GF-pulp injection and/or handling stress. It should be noted that except for lymphocytes, the baseline levels of all other blood cells (i.e., RBC, thrombocytes, heterophils, monocytes, eosinophils, and basophils) were not different for broilers reared under TN versus HS conditions. Hence, it appears that cyclic HS alone did not affect hematopoiesis of myeloid cells. Rather, the lower concentrations of circulating T- and B-lymphocytes point towards an effect of cyclic HS on their development in the thymus and bursa of Fabricius, respectively.

In a similar study by Quinteiro-Filho et al. (2010), 35-day-old broilers were subjected to 10 h of HS (36 °C),

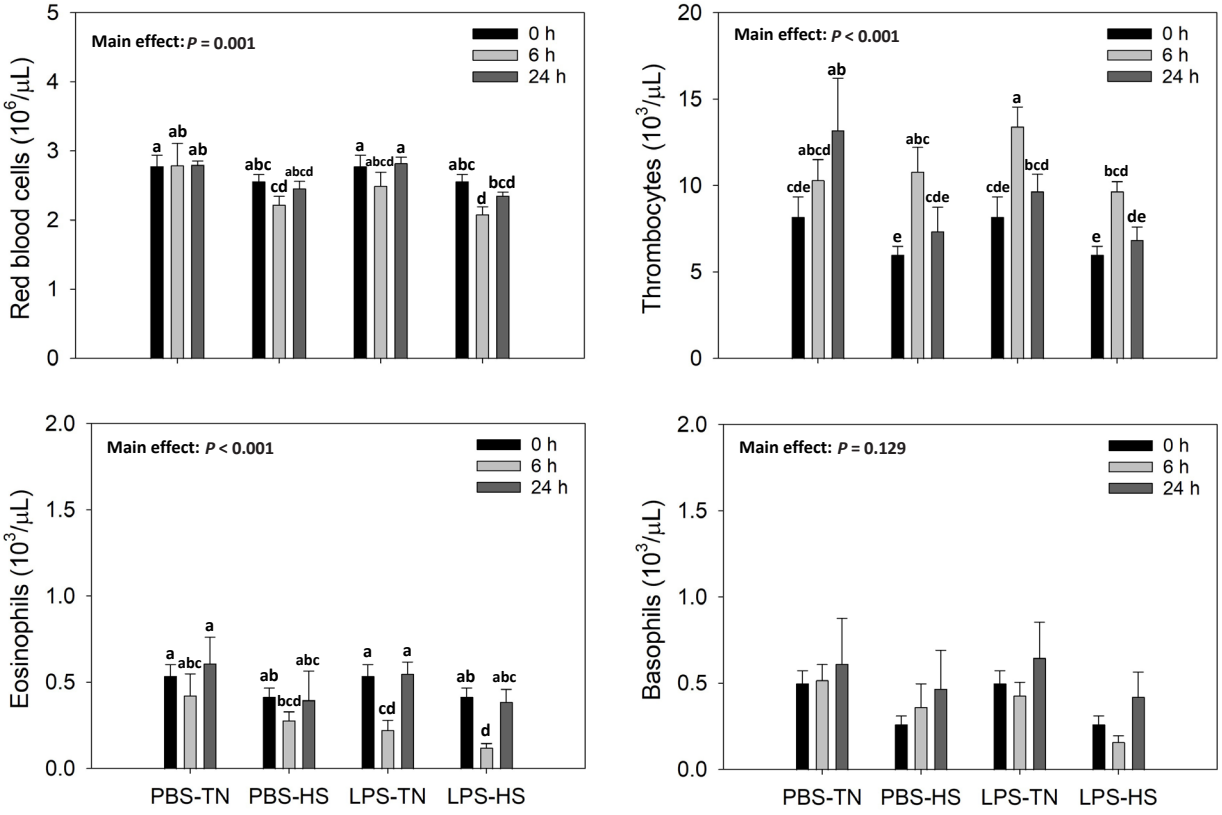

Fig. 2. Effects of cyclic heat stress (HS) on concentrations (cells/μL) of red blood cells, thrombocytes, eosinophils, and basophils in the blood at 0, 6, and 24 h post-intradermal injection of lipopolysaccharide (LPS). Pulps of 12 GF from each of 16 birds (8 thermoneutral (TN) and 8 HS) were intradermally (i.d.) injected with 10 μL of LPS (1 μg/GF; 12 GF/bird; 12 μg/bird) at 37-days of age. Pulps of 12 GF from each of 8 additional birds (4 TN and 4 HS) were i.d. injected with 10 μL of PBS vehicle. 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 and labeled for thrombocytes and total WBC using CD41/61-FITC and CD45-SR, respectively, monoclonal antibodies 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 cell population, means without a common letter are different (P ≤ 0.05).

with control birds kept at TN (21 °C) temperatures for 24 hours per day for one week. At 42-days of age, HS broilers were reported to have elevated serum corticosterone concentrations, decreased relative weights (% BW) of the thymus and bursa of Fabricius (primary lymphoid organs), and reduced *Staphylococcus aureus*-induced reactive oxygen species generation (ROS) by macrophages. These alterations in the thymic and bursal weights, as well as 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 primary lymphoid organs by reducing the levels of immature lymphocytes (e.g., CD4+CD8+ thymocytes) and hence, the weight of these organs. 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 concentrations 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 are likely due to elevated levels of HS-associated stress hormones. To gain a 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.

Conclusions

Overall, the reduction in the LPS-stimulated, local and systemic acute inflammatory responses and in circulating concentrations of lymphocytes in HS broilers suggest that HS conditions impair the ability of broilers to mount effective innate immune responses. This may prove to be problematic for maintaining flock health as HS continues to grow as a major environmental concern. Further research should be done to elucidate the mechanisms and extent of this impaired immune function in HS conditions. Application of the "dual-window approach" could prove to be useful in selecting broilers exhibiting greater immune robustness while under the effects of HS.

Acknowledgments

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Table 1. Effects of intradermal injection with phosphate buffered saline (PBS) or lipopolysaccharide (LPS) on T- and B-cell concentrations in peripheral blood of broilers reared in thermoneutral or cyclic heat stress conditions.[†]

Lymphocytes	PBS-TN[‡]	PBS-HS[§]	LPS-TN[‡]	LPS-HS[§]
T cells (10^3 cell/μl)[¶]				
0 h	$12.72 \pm 0.58^{a, x}$	$9.07 \pm 0.59^{b, x}$	$12.72 \pm 0.58^{a, x}$	$9.07 \pm 0.59^{b, x}$
6 h	$8.59 \pm 0.98^{a, y}$	$3.07 \pm 0.81^{b, y}$	$4.34 \pm 0.78^{b, y}$	$1.97 \pm 0.28^{b, y}$
24 h	$13.73 \pm 0.99^{a, x}$	$9.09 \pm 1.16^{b, x}$	$12.08 \pm 0.70^{ab, x}$	$9.35 \pm 0.70^{b, x}$
B cells (10^3 cell/μl)[¶]				
0 h	$5.80 \pm 0.26^{a, x}$	$4.13 \pm 0.32^{b, x}$	$5.80 \pm 0.26^{a, x}$	$4.13 \pm 0.32^{b, x}$
6 h	1.28 ± 0.31^{y}	0.66 ± 0.18^{y}	0.90 ± 0.13^{y}	0.61 ± 0.06^{y}
24 h	1.59 ± 0.29^{y}	1.38 ± 0.25^{y}	1.77 ± 0.22^{y}	1.67 ± 0.31^{y}

[†] One mL of heparinized blood was collected from the wing vein of 37-day old broilers before (0 h) and at 6 and 24 h post GF-pulp injection of PBS or LPS. Whole blood cell suspensions were prepared, and T- and B-cell populations were identified using fluorescently labeled mouse monoclonal antibodies CD3-PE and Bu-1-FITC to identify chicken T- and B-cells, respectively, in a dual direct labeling procedure. The proportions and concentrations of T- and B-cell populations were determined by flow cytometry.

[‡] TN = Thermoneutral

[§] HS = Heat stress

[¶] Data shown are mean ± SEM; at 0 h, n = 12 broilers for TN/HS groups; at 6- and 24-h, n = 4 broilers for PBS-TN/-HS and 8 broilers for LPS-TN/-HS groups.

a, b: Lymphocyte concentration means within a row without a common letter are different ($P \leq 0.05$).

x, y: For each type of lymphocyte, concentration means within a column without a common letter are different ($P \leq 0.05$).

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