Research Note: Evaluation of a heat stress model to induce gastrointestinal leakage in broiler chickens

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ABSTRACT The purpose of this study was to evaluate heat stress as a model to induce gastrointestinal leakage in broiler chickens. On the day of hatch, 320 chicks were allocated into 8 environmental chambers, 4 thermoneutral (**TN**) and 4 continuous heat stress (**HS**). Each chamber was divided into 2 pens containing separate feeders and water jugs (8 replicates per treatment, 20 birds/pen). The environment was established to simulate production setting as best possible for the first 21 D. A gradual reduction of temperature from 32°C to 24°C with relative humidity at 55 ± 5% was adopted for the first 21 D. At the time of HS, the HS groups were exposed to 35°C from Day 21 to 42, while thermoneutral ones were maintained at 24°C from Day 21 to 42. Chickens were equipped with a Thermochron temperature logger for continuous monitoring of core body temperature. The environmental temperature and relative humidity were continuously recorded. Fluorescein isothiocyanate–dextran (**FITC-d**) was orally gavaged to 2 chickens/replicate ($n = 16$) randomly selected on days 21, 28, 35, and 42. After 1 h of oral gavage, blood samples were collected to determine the passage of FITC-d. Tibias were removed from all chickens to evaluate break strength only on 21 D and 42 D (before HS and at the end of the trial). Performance parameters were evaluated weekly from 21 D to the end of the trial. Body temperature was significantly ($P < 0.05$) increased after 2 h of starting HS and remained that way until the end of the study. Chronic HS caused an increase in core body temperature which decreased feed intake, body weight, and feed efficiency (28, 35, and 42 D) when compared with control TN chickens. Similarly, serum FITC-d was significantly increased in HS chickens at all points of evaluation. Chronic HS also caused a significant reduction of bone strength at 42 D when compared with the control chickens. The results from the present study suggest that HS can be a robust model to induce gut leakage in broiler chickens.

Key words: chickens, enteric inflammation, heat stress, performance, serum FITC-d

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INTRODUCTION

Poultry production is one of the livestock industries mostly affected by heat stress due to the lack of sweat glands and high metabolic activity of poultry (Abu-Dieyeh, 2006; Prieto and Campo, 2010). It is estimated that heat stress alone costs the U.S. broiler poultry industry 125-165 million dollars per year (St-Pierre et al., 2003). Optimal environmental conditions for performance range from 18°C to 22°C, with the internal (body) temperature of modern broiler (meat-type) chickens between 40.6°C and 41.7°C (Borges et al., 2003). However, under acute or chronic heat stress conditions, their body temperature may reach up to 45°C to 47.2°C, which is the lethal limit (Mohanaselvan and Bhaskar, 2014). Heat stress (**HS**) results from unsuccessful thermoregulation that occurs when animals produce or absorb more temperature dispersed (Lara and Rostagno, 2013). The adverse effects of HS can range from discomfort to multiple organ damage and, under severe stress, to death by spiraling hyperthermia (Lara and Rostagno, 2013). Avian species have several mechanisms to maintain homeostasis and reduce body temperature such as convection, evaporative, and radiant cooling through vasodilation and perspiration (Richards, 1970). However, HS induces a multitude of metabolic problems that impact the productivity of the

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Table 1. Ingredient composition and nutrient content of a corn-soybean starter diet and a corn-soybean grower diet used on as-is basis.

Item	Starter diet	Grower diet
Ingredients (%)		
Corn	57.34	56.68
Soybean meal	34.66	27.05
Poultry fat	3.45	4.09
Dicalcium phosphate	1.86	1.59
Calcium carbonate[1]	0.99	1.03
Salt	0.38	0.34
DL-Methionine	0.33	0.26
L-Lysine HCl	0.31	0.32
Threonine	0.16	0.12
Vitamin premix[2]	0.20	0.20
Mineral premix[3]	0.10	0.10
Choline chloride 60%	0.20	0.20
Antioxidant[4]	0.02	0.02
Calculated analysis		
Metabolizable energy (kcal/kg)	3,035	3,108
Crude protein (%)	22.16	20.73
Ether extract (%)	5.68	7.11
Lysine (%)	1.35	1.20
Methionine (%)	0.64	0.57
Methionine + cystine (%)	0.99	0.91
Threonine (%)	0.92	0.82
Tryptophan (%)	0.28	0.24
Total calcium	0.90	0.84
Available phosphorus	0.45	0.42
Determined analysis		
Crude protein (%)	21.15	20.30
Ether extract (%)	6.05	6.78
Calcium (%)	0.94	0.90
Phosphorus (%)	0.73	0.69

[1]Inclusion of 10^6 spores/g of feed mixed with calcium carbonate.
[2]Vitamin premix supplied the following per kg: vitamin A, 20,000 IU; vitamin D3, 6,000 IU; vitamin E, 75 IU; vitamin K3, 6.0 mg; thiamine, 3.0 mg; riboflavin, 8.0 mg; pantothenic acid, 18 mg; niacin, 60 mg; pyridoxine, 5 mg; folic acid, 2 mg; biotin, 0.2 mg; cyanocobalamin, 16 µg; and ascorbic acid, 200 mg (Nutra Blend LLC, Neosho, MO 64850).
[3]Mineral premix supplied the following per kg: manganese, 120 mg; zinc, 100 mg; iron, 120 mg; copper, 10 to 15 mg; iodine, 0.7 mg; selenium, 0.4 mg; and cobalt, 0.2 mg (Nutra Blend LLC, Neosho, MO 64850).
[4]Ethoxyquin.

birds, such as lower eggshell quality, high mortality, a significant increase in feed conversion, immunosuppression, bacterial translocation, and leaky gut syndrome because the gastrointestinal tract (**GIT**) is very responsive and susceptible to HS (Zeng et al., 2014; Huang et al., 2015). Under thermoneutral conditions, the GIT can efficiently digest and absorb most nutrients through cell plasma membranes (transcellular transport) that involves specific receptors and energy expenditure (Salzman, 2011). However, epithelial cells in the intestine additionally provide a barrier isolating the external environment from the internal body, yet providing tolerance to water and digested nutrients (Salminen and Isolauri, 2006; Elson and Cong, 2012). Any damage in this fragile epithelium results in gut permeability and translocation of microorganisms to the portal vein leading to systemic infections and chronic inflammation (Ilan, 2012). Furthermore, stress is known to have a significant impact on the gastrointestinal tract (Alverdy and Aoys, 1991; Collins and Bercik, 2009;; Verbrugghe et al., 2011; Karavolos et al., 2013). Several studies indicate that acute or chronic stress modifies gut permeability by disruption of tight junction (**TJ**) proteins (Maejima et al., 1984; Koh et al., 1996; Matter and Balda, 2007; Assimakopoulos et al., 2011). Some of these alterations caused by any kind of stress are associated with secretion of neurotransmitters and proinflammatory cytokines in the brain and the gut, with profound effects on the gastric and intestinal physiologies (Groschwitz and Hogan, 2009; Bailey et al., 2011; Lamprecht and Frauwallner, 2012).

As a result, enteric inflammation models can help researchers' study methods to improve health and performance and evaluate various growth promoters and dietary formulations targeted to improve performance in poultry. Our laboratory has previously demonstrated that oral administration of fluorescein isothiocyanate-dextran (**FITC-d**; 3–5k Da) and its paracellular mucosal epithelial leakage are an established marker to evaluate enteric inflammation using different chemical or nutritional models (Tellez et al., 2014; Kuttappan et al., 2015; Vicuña et al., 2015; Galarza-Seeber et al., 2016; Baxter et al., 2017). The purpose of this study was to evaluate continuous HS as an alternative model to induce gastrointestinal leakage in broiler chickens.

MATERIAL AND METHODS

Experimental Design

Day-of-hatch Cobb 500 by-product male chicks (320 in total) were randomly assigned to 8 environmental chambers, 4 thermoneutral (**TN**) and 4 continuous (24 h/D) HS. Each chamber was divided into 2 pens (150 × 300 cm) each containing separate feeders and watering systems (8 replicates per treatment, 20 birds/pen). The environment was established to simulate commercial production settings (temperature, light) for the first 21 D. A gradual reduction on temperature from 32°C to 24°C with relative humidity at 55 ± 5% for the first 21 D. At the time of heat stress, the continuous heat stress treatment group was exposed to 35°C from Day 21 to 42, while the thermoneutral treatment group was maintained at 24°C from Day 21 to 42. Before the onset of HS, 4 birds per pen were equipped with a Thermochron temperature logger for continuous monitoring of core body temperature (iButton, DS1922 L, Embedded Data Systems, Lawrenceburg, KY). The data loggers were inserted into the mouth of the bird and swallowed, where it remained in the gizzard. This location provides the most consistent and reliable measure of bird deep body temperature, with no adverse effects on feeding behavior, well-being, and growth (Rajaei-Sharifabadi et al., 2017). Temperature was recorded every minute during acute (the first 2 h after initiating HS). Then, temperature was recorded every hour during chronic HS, until 42 D. At the end of the HS period, relative humidity was continuously recorded. Fluorescein isothiocyanate–dextran was administered only once at 9:00 AM via oral gavage to 2 chickens/replicate (n = 16), and blood was collected on 21 D before HS and then on 28 D, on 35 D, and at the end of the experiment on 42 D to determine serum

Table 2. Evaluation of the effects of heat stress on body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) in broiler chickens.

Item	Control TN	Control HS
BW, g/broiler		
Day 0	41.15 ± 0.25	41.48 ± 0.22
Day 10	224.75 ± 9.52	220.30 ± 10.20
Day 22	878.84 ± 18.45	875.08 ± 15.17
Day 28	1507.63 ± 21.98[a]	1263.88 ± 26.10[b]
Day 35	2285.32 ± 33.39[a]	1517.26 ± 51.29[b]
Day 42	2921.48 ± 63.52[a]	1687.26 ± 80.82[b]
BWG, g/broiler		
Day 0 to 10	183.60 ± 9.62	178.84 ± 10.23
Day 10 to 22	654.22 ± 14.03	654.36 ± 13.35
Day 22 to 28	632.39 ± 12.46[a]	397.89 ± 25.86[b]
Day 28 to 35	798.09 ± 20.48[a]	261.72 ± 38.62[b]
Day 35 to 42	644.89 ± 58.44[a]	163.56 ± 40.91[b]
Accumulated BWG, g/broiler		
Day 0 to 10	183.60 ± 9.62	178.84 ± 10.23
Day 0 to 22	837.7 ± 18.50	833.6 ± 15.08
Day 0 to 28	1466.66 ± 21.96[a]	1219.55 ± 25.43[b]
Day 0 to 35	2244.28 ± 33.46[a]	1474.84 ± 50.98[b]
Day 0 to 42	2880.44 ± 63.50[a]	1647.59 ± 80.38[b]
FI, g/broiler		
Day 0 to 10	141.44 ± 7.85	135.07 ± 8.35
Day 10 to 22	922.69 ± 20.64	898.41 ± 20.03
Day 22 to 28	965.36 ± 20.67[a]	756.06 ± 21.71[b]
Day 28 to 35	1480.98 ± 35.39[a]	1041.39 ± 46.53[b]
Day 35 to 42	1395.75 ± 75.00[a]	631.43 ± 82.04[b]
Accumulated FCR		
Day 0 to 10	0.77 ± 0.03	0.78 ± 0.09
Day 0 to 22	1.26 ± 0.02	1.23 ± 0.02
Day 0 to 28	1.28 ± 0.02[b]	1.37 ± 0.02[a]
Day 0 to 35	1.38 ± 0.02[b]	1.75 ± 0.05[a]
Day 0 to 42	1.48 ± 0.03[b]	1.94 ± 0.06[a]

[a–c]Values within rows with different superscripts differ significantly ($P < 0.05$).

Data expressed as mean ± SE.

concentration of FITC-d post-mortem. One hour after FITC-d gavage, chickens were euthanized by CO_2 inhalation (at respective time point as listed previously) and blood samples were collected from the femoral vein. Blood samples were centrifuged (1000 × *g* for 15 min) to collect serum for FITC-d measurement. The left tibia from each chicken was removed to evaluate break strength (kg) on 21 D and 42 D (before HS and at the end of the trial), as described in the following. Performance parameters were evaluated weekly from 21 D to the end of the trial at 42 D. This study was carried out by the recommendations of the Institutional Animal Care and Use Committee (**IACUC**) at the University of Arkansas, Fayetteville, under IACUC-approved protocol #16084. Starter and grower feed diets (Table 1) used in this experiment were formulated to approximate the nutritional requirements of broiler chickens as recommended by the National Research Council (NRC, 1994) and adjusted to breeder's recommendations (Cobb-Vantress, Inc., 2015).

Serum Determination of FITC-d Gut Leakage

Intestinal leakage of FITC-d (MW 3–5 kDa; Sigma-Aldrich Co., St. Louis, MO) into serum was determined as FITC-d is a marker of paracellular transport and mucosal barrier dysfunction (Kuttappan et al., 2015; Vicuña et al., 2015). One hour before humanely euthanizing the chickens by CO_2 inhalation, 20 broiler chickens from each group were given an oral gavage dose of 8.32 mg/kg FITC-d (Baxter et al., 2017), and 5 broiler chickens per group were used as no FITC-d control. Fluorescein isothiocyanate–dextran concentration from diluted sera was measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm (Synergy HT, multimode microplate reader, Bio-Tek Instruments, Inc., VT).

Table 3. Evaluation of heat stress on intestinal permeability and tibia breaking strength (kg) at Day 21, Day 28, Day 35, and Day 42.

	Control thermoneutral	Heat stress
Serum FITC-D (ng/mL)		
Day 21	231.37 ± 16.29[a]	157.07 ± 7.30[b]
Day 28	240.74 ± 11.28[a]	247.13 ± 12.05[a]
Day 35	177.65 ± 4.71[b]	235.79 ± 12.39[a]
Day 42	218.54 ± 13.22[b]	312.60 ± 20.08[a]
Break strength (kg)		
Day 21	17.93 ± 0.86[a]	18.79 ± 0.94[a]
Day 42	37.71 ± 2.54[a]	24.37 ± 1.37[b]

[a–b]Indicates significant differences between the treatments within the rows ($P < 0.05$).

Data are expressed as the mean ± SE.

Bone Strength

Tibial diaphysis from individual birds was cleaned of adherent tissues, the periosteum was removed, and the biomechanical strength of each bone was measured using an Instron 4,502 material testing machine (Norwood, MA) with a 509 kg load cell. The bones were held in identical positions and the mid-diaphyseal diameter of the tibial midshaft, which was also the site of impact, was measured using a dial caliper. The maximum load at failure was determined in the tibial midsection between epiphyses, using a three-point flexural bend fixture with a total distance of 30 mm between the 2 lower supporting ends. The load, defined as the force in kilograms per square millimeter of cross-sectional area (kg/mm^2), represents bone strength. The rate of loading was kept constant at 20 mm/min collecting 10 data points per second. The data were automatically calculated using Instron's Series IX Software (Norwood, MA).

Data and Statistical Analysis

All data were subjected to analysis of variance as a completely randomized design, using the general linear models procedure of SAS (SAS Institute, 2002). Significant differences among the means were determined by Duncan's multiple range test at $P < 0.05$.

RESULTS

The results of the evaluation of body weight, body weight gain, feed intake, and feed conversion ratio in broiler chickens under HS are summarized in Table 2. In the present study, heat stress caused a significant

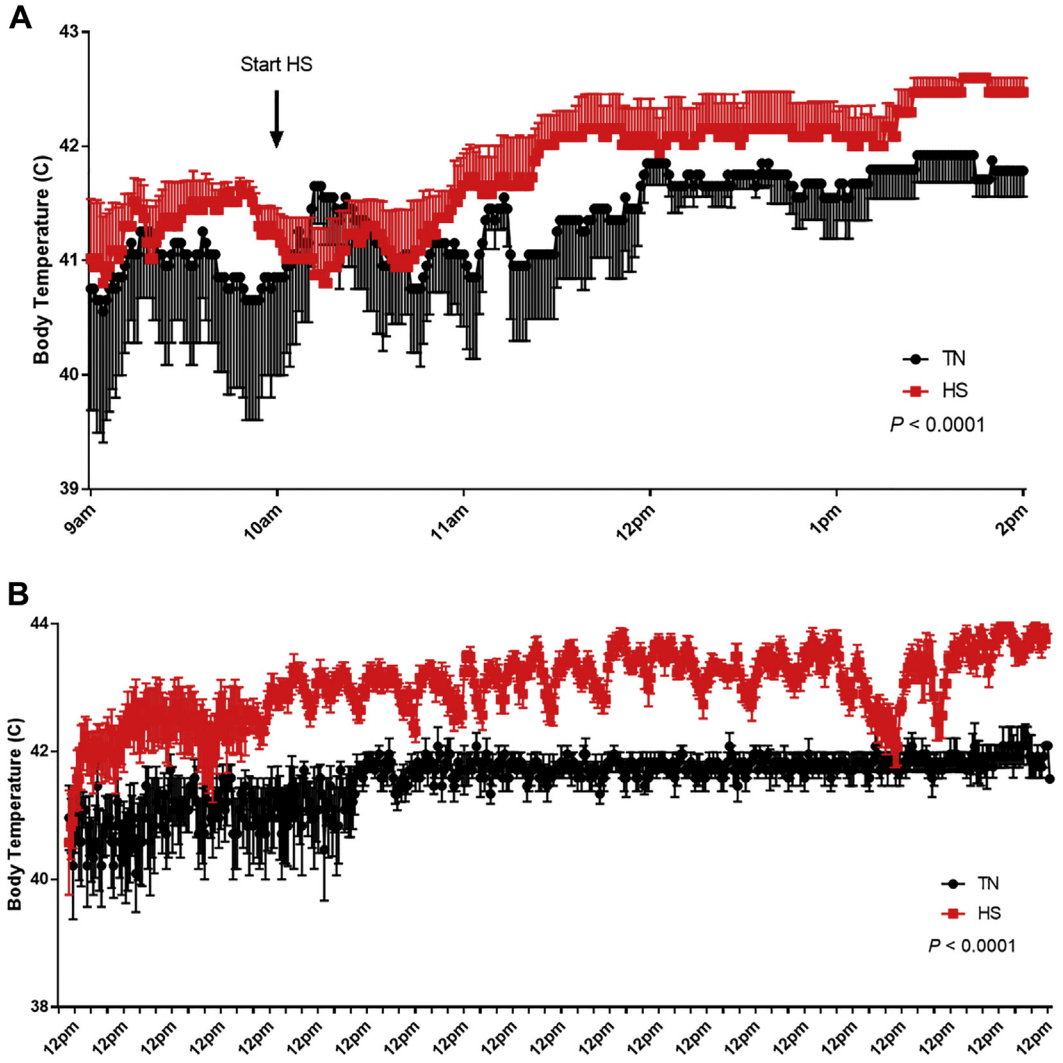

Figure 1. Core body temperature of chicken during (A) acute and (B) chronic heat stress (HS). Temperatures were recorded every minute during acute HS and every hour during chronic HS. $n = 8$ birds per group. Data are reported as means ± SEM.

reduction ($P < 0.05$) in all performance parameters evaluated when compared to TN chickens (Table 2).

Table 3 shows the results of the evaluation of serum concentration of FITC-d and bone strength in broiler chickens under HS. Unexpectedly, at 21 D, and before inducing the HS, chickens that were allocated in the chambers identified as TN show a significant increase ($P < 0.05$) in serum FITC-d when compared with the chickens in chambers identified as HS. Seven days after HS was induced, no significant differences were observed between TN or HS chickens. However, on 35 D and 42 D, a significant increase in serum FITC-d was observed in HS chickens when compared with TN chickens (Table 3). Interestingly, although no differences were observed in tibia break strength at 21 D before HS, at 42 D, a significant reduction in break strength was observed in heat-stressed chickens compared with control TN chickens (Table 3).

Body temperature was significantly increased within 2 h of HS initiation in the HS treatment group which persisted until termination of the study (Figure 1). Chronic HS caused an increase in core body temperature which was associated with a decreased feed intake, body weight, and feed efficiency (28 D, 35 D, and 42 D) when compared with control TN chickens (Figure 1; Table 2).

DISCUSSION

In addition to digestion and absorption of water and nutrients, the GIT plays an essential role for endocrine and paracrine production of hormones (Peterson and Artis, 2014). Furthermore, the intestinal mucosa is a remarkable physical, chemical, and biological barrier that isolates the external environment (Farhadi et al., 2003; Moretó and Pérez-Bosque, 2009). Any stress to the intestinal barrier induces increased permeability of antigens to the blood stimulating inflammation (Berkes et al., 2003; Turner, 2009). Although stress and inflammation are innate responses in living organisms involving hormones, immune cells, and molecular mediators as essential mechanisms for the

survival and the healing process (Konturek et al., 2011), chronic stress and inflammation induces the production of reactive oxygen species causing peroxidation of lipids in cell membranes (Espinosa-Diez et al., 2015). In the GIT, oxidative stress and free radicals also increase disruption of the tight junctions, leading to changes in tyrosine kinase and protein tyrosine-phosphatase activities, and modifying the phosphorylated state of TJ proteins (Sander et al., 2005).

In contrast to transcellular transport, the transfer of molecules through the space between the cells across an epithelium (paracellular transport) is unmediated and passive down a concentration gradient, and this transport is regulated by the TJ (Hu et al., 2013). Hence, stress and inflammation alter gut permeability due to disruption of TJ (Muthusamy et al., 2014; Qin et al., 2015). Under thermoneutral conditions, the paracellular junction is rigorously regulated (Di Pierro et al., 2001). However, under heat stress conditions, the TJ barrier becomes compromised, and luminal substances leak into the bloodstream, explaining the term leaky gut (Bosenberg et al., 1988), a condition that induces chronic systemic inflammation which requires tremendous resources of energy impacting the performance of the animals negatively. Alterations in gut permeability are associated with bacterial translocation in the portal and systemic circulation in several types of leaky gut syndromes (Ilan, 2012). In the present study, HS had a significant negative impact on performance parameters. The reduction in feed intake not only impacted performance parameters but also affected bone mineralization.

Several studies in poultry have shown that acute or heat stress impaired intestinal integrity and increase proinflammatory cytokines (Song et al., 2014; Abdelqader et al., 2017; Alhenaky et al., 2017). However, to our knowledge, this is the first study that evaluates the effect of continuous HS on intestinal permeability using FITC-d as a biomarker in broiler chickens.

FITC-d is a large molecule (3–5 kDa) which does not leak through the intact gastrointestinal tract barrier. However, when there are conditions that disrupt the TJ between epithelial cells, the molecule can enter circulation demonstrating an increase in transmucosal permeability associated with induced disruption of tight junctions, leading to an elevated serum level of FITC-d after oral administration (Baxter et al., 2017). Heat stress chickens showed a significant increase in the levels of FITC-d, suggesting that TJ was disrupted. The results from the present study showed that continuous HS is a consistent method for inducing mucosal leakage that could lead to enteric inflammation in poultry. As in previous publications from our laboratory, we confirm that serum FITC-d measurement is a reliable and noninvasive biomarker useful to evaluate intestinal permeability that can be used in poultry at multiple time points within the same experiment.

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