IMMUNOLOGY, HEALTH, AND DISEASE

Effects of long-term heat stress in an experimental model of avian necrotic enteritis

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ABSTRACT Stressful conditions are predisposing factors for disease development. Heat stress is one of the most important stressors in poultry production. The reemergence of some previously controlled diseases [e.g., avian necrotic enteritis (NE)] has been extensively reported. The combination of bacterial infection and certain environmental factors have been reported to trigger the disease. The aim of this study was to analyze the effects of long-term heat stress (35 ± 1°C) on the development of NE in broiler chickens. For this purpose, 60 male broiler chickens were divided into the following 6 groups: control group (C), heat stressed control group (C/HS35), thioglycolate group (T), thioglycolate heat-stressed group (T/HS35), infected group (I), and infected heat-stressed group (I/HS35). The poultry of groups I and I/HS35 were experimentally infected with *Clostridium perfringens* via their feed from 15 to 21 d of life. Heat stress (35 ± 1°C) was constantly applied to the birds of the stressed groups from 14 to 21 d of life. The infected and heat-stressed broiler chickens presented a trend toward a decrease in gross lesion scores and significantly lower microscopic scores of necrosis in the duodenum and jejunum (*P* < 0.05), lower fusion of villi in the duodenum (*P* < 0.05), and lower congestion scores in the jejunum and ileum (*P* < 0.05) in relation to infected and non-heat-stressed chickens. Broilers of I/HS35 group also exhibited small number of heterophils in the duodenum and jejunum compared with those of the I group (*P* < 0.05). Furthermore, the duodenum and jejunum of infected and heat-stressed broilers showed lower number of clostridia on the intestinal mucosa (*P* < 0.05). Data were discussed in light of a heat stress induced reduction on intestinal inflammation via a decrease in heterophil migration to the intestinal mucosa, which in turn might have reduced tissue damage during inflammation, hence preventing the development of a more severe form of NE.

Key words: avian necrotic enteritis, *Clostridium perfringens*, heat stress

INTRODUCTION

Animal welfare is considered an important component of animal production (Zhao et al., 2013). The decline in an animal’s welfare, particularly when associated with stressful conditions, disrupts the animal’s homeostasis, leading to physiopathological changes (Yang et al., 2011). Therefore, a greater understanding of the effects of stressors on animal health has been sought in several scientific fields including, among others, neuroimmunomodulation, a science that concerns the complex bidirectional interactions between the nervous system and the immune system (del Rey et al., 2012).

Heat stress is one of the most important environmental stressors in poultry production. We have shown that broiler chickens subjected to long-term heat stress exhibited decreased performance parameters, decreased immunity, and increased intestinal injury; these effects were attributed to hypothalamic-pituitary-adrenal axis (HPA) activation (Quinteiro-Filho et al., 2012b). Furthermore, heat stress has been linked to a decrease in intestinal immune activity and to impaired intestinal morphology following pathogenic bacteria invasion of the body through the intestinal epithelium (Burkholder...
et al., 2008; Deng et al., 2012; Quinteiro-Filho et al., 2012a; Verbrugghe et al., 2012).

Experiments conducted using *Clostridium perfringens* type A showed that in cases of experimental infection in which the bacteria were administered orally, no clinical signs of NE were observed in the chickens maintained under controlled environmental climate and proper handling conditions (Helmboldt and Bryant, 1971; Al-Sheikhly and Truscott, 1977a; Cowen et al., 1987; Craven, 2000; Williams et al., 2003). However, the combination of bacterial infection with unbalanced feed; and coinfection with coccidia, environmental stressor application, or both have been reported to trigger this disease (Helmboldt and Bryant, 1971; Al-Sheikhly and Truscott, 1980; Cowen et al., 1987; Craven, 2000; Williams et al., 2003). Thus, to investigate the possible stressor effects on avian necrotic enteritis development, we evaluated the effects of long-term heat stress on broiler chickens infected with *C. perfringens* type A.

**MATERIALS AND METHODS**

**Birds**

One-day-old broiler chicks were housed in climate-controlled rooms at the School of Veterinary Medicine of the University of São Paulo, Brazil. A total of 60 male broiler chickens (Cobb) were maintained in isolator chambers (Alesco, São Paulo, Brazil), containing high efficiency particulate air (HEPA) filters, from the first posthatching day (ED1) until the last experimental day (ED21). The chickens received water ad libitum and were fed as described below. The RH was monitored and controlled (at not less than 45%). The birds were maintained and used in accordance with the guidelines and approval of the Committee on the Care and Use of Laboratory Animal Resources of The School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil (no. 2570/2012).

**Group Formation and Heat Stress**

From d 1 to 13 of life (ED1 to ED13), the birds were maintained at the recommended environmental temperatures (33 ± 1°C from ED1 to ED7 and 28 ± 1°C from ED7 to ED13). On ED1, the broiler chickens were randomly and equally allocated into the following 6 groups of 10 birds each: control group (C), thioglycolate group (T), infected group (I), control heat-stressed group (C/HS35), thioglycolate heat-stressed group (T/HS35), and *Clostridium*-infected and heat-stressed group (I/HS35). The group treatments are described in Table 1.

From ED14 to ED21, the birds in the non-heat-stressed groups (C, T, and I) were maintained at an environmental temperature of 28 ± 1°C. The chickens in the heat-stressed groups (C/HS35, T/HS35, and I/HS35) were maintained at an environmental temperature of 35 ± 1°C all day from ED14 to ED21 (Quinteiro-Filho et al., 2012a). On ED21, the birds were euthanized in CO₂-containing chambers, and the necropsy and collection of tissues were performed.

**Clostridium perfringens Inoculum and Infection Protocol**

A pathogenic strain of *C. perfringens* type A (strain CP8.2) with a genotype allowing α and Tpel toxin production was used (β2 and netB negative). Toxinotyping was determined according to methodology proposed by Meer and Songer (1997) and Coursodon et al. (2012). This bacterial strain was maintained in glycerol at −80°C. The inoculum was prepared following the procedure described by Cooper and Songer (2010). Briefly, the inoculum was prepared by 2 alternate cultivations in cooked meat medium (Becton, Dickinson and Company, Sparks, MD) and in thioglycolate broth culture medium (Becton, Dickinson and Company) containing yeast extract 2% (Becton, Dickinson and Company) and kept in anaerobic CO₂-containing chambers. The inoculum was prepared by 2 alternate cultivations in cooked meat medium (Becton, Dickinson and Company, Sparks, MD) and in thioglycolate broth culture medium (Becton, Dickinson and Company) containing yeast extract 2% (Becton, Dickinson and Company). All cultures were kept in anaerobiosis at 37°C for 15 h. Numbers of cfu in the inoculum were determined daily from ED15 to ED20 by plating serial dilutions of the inoculum in BHI agar. The bacteria (1 × 10⁶ cfu/mL) were offered to the chickens mixed to the feed (vol/vol; culture medium/feed) in aluminum feeders; the

### Table 1. Schematic presentation of the experimental groups feed treatments

<table>
<thead>
<tr>
<th>Group²</th>
<th>Heat stress²</th>
<th>Feed treatment³</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>−</td>
<td>Thioglycolate broth culture medium with <em>Clostridium perfringens</em> type A</td>
</tr>
<tr>
<td>T</td>
<td>−</td>
<td>Thioglycolate broth culture medium with <em>Clostridium perfringens</em> type A</td>
</tr>
<tr>
<td>I</td>
<td>−</td>
<td>Thioglycolate broth culture medium with <em>Clostridium perfringens</em> type A</td>
</tr>
<tr>
<td>C/HS35</td>
<td>+</td>
<td>Thioglycolate broth culture medium</td>
</tr>
<tr>
<td>T/HS35</td>
<td>+</td>
<td>Thioglycolate broth culture medium</td>
</tr>
<tr>
<td>I/HS35</td>
<td>+</td>
<td>Thioglycolate broth culture medium</td>
</tr>
</tbody>
</table>

¹C = control group; T = thioglycolate group; I = infected group; C/HS35 = control heat-stressed group; T/HS35 = thioglycolate heat-stressed group; I/HS35 = *Clostridium*-infected and heat-stressed group.

²(−) absence, (+) presence.

³From d 14 to 21 of life.

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**LONG-TERM HEAT STRESS**

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mixtures and inoculums offered to the chickens were prepared daily. This feed management method, adapted from the protocol described by Cooper and Songer (2010), was reported to induce an enteric condition necessary to allow *C. perfringens* infection development. The birds of the control groups (C and C/HS35) were fed only with commercial feed without bacteria, fish meal, and thioglycolate broth culture medium.

As presented in Table 1, our experimental protocol involved the use of feed formulated with a high concentration of CP and animal protein; this supplementation was reported to disrupt the intestinal microenvironment and consequently to predispose the birds to develop *C. perfringens* infections in their intestinal lumens and mucosa (Truscott and Al-Sheikhly, 1977; Williams et al., 2003). However, it should be noted that the thioglycolate broth medium in which the bacteria were cultivated is a chemotactic substance per se. This substance is commonly used to stimulate mononuclear cells migration to mammalian tissues (Nathan and Root, 1977; Segal et al., 2002). Thus, thioglycolate was also tested in this study (T and THS35 groups).

During the first 7 d of life, the chickens of all of the groups were fed with a poultry feed containing 24% CP without antimicrobials (all groups). From ED8 to ED14, birds of group T, T/HS35, I, and I/HS35 received a high-protein feed (28% CP) mixed with fish meal (55% CP) in a ratio of 1:1 (vol/vol). From ED15 until ED20, the culture medium containing the pathogenic strain of *C. perfringens* type A (groups I and I/HS35; 1 × 10^8 cfu/mL) or the thioglycolate broth culture medium (groups T and T/HS35) was mixed into the feed in the proportion 1:1 (vol/vol) and given to the chickens. The final concentrations of bacteria were about 1 × 10^4 cfu/g of feed with a paste-like consistency. Although different in consistency, feed plus thioglycolate broth culture medium with or without the bacterial load were normally ingested by the chicken; the approximate daily feed mixed with the culture medium intake was 100 g per bird, a value not statistically different from those of feed taken by the chickens of control groups (C and T).

**Gross Intestinal Evaluation**

On ED21, necropsy was performed on all of the broiler chickens immediately after euthanasia. The small intestine (duodenum, jejunum, and ileum) were separated and sectioned for macroscopic examination of the entire mucosa.

The gross aspect of the intestinal mucosa in the infected birds was rated using a lesional scoring system based on its reliability and repeatability for research in pathology (Gibson-Corley et al., 2013). This scoring system is based solely on macroscopic visualization; inferences related to necrosis and inflammation were left to be defined during the microscopy evaluation. Table 2 presents a description of the scoring system used. Two pathologists who were blind to the group affiliation examined all intestines; a high positive correlation was found between their obtained data set.

**Bacterial Identification**

The duodenal, jejunal, and ileal contents were diluted in PBS (pH 7.2, 0.01 M) and plated on BHI agar containing 0.5% yeast extract and 5% bovine citrated blood. The diluted contents were incubated under anaerobic conditions at 37°C for 48 h. After incubation, the bacterial colonies within each group of chickens were evaluated by the type of hemolysis of the culture medium and by the colony morphology. No serial dilution was performed to count cfu. Analysis of PCR was performed on feces of all chickens before *C. perfringens* infection, and they were all negative for *C. perfringens* type A and Tpel producing strains.

**Histopathological Evaluation**

Sections with 2.5 cm length of the inferior duodenal flexure, jejunum (the middle region between the end of duodenum and the Meckel’s diverticulum), and ileum (the middle region between the Meckel’s diverticulum and the ileo-cecal junction) were collected for histopathological examination. The same regions were
collected from all birds, regardless of histopathological changes. Tissues were fixed in 4% formaldehyde for 48 h; subsequently, the materials were embedded in paraffin following a standard procedure for tissue inclusion. Cross sections of 5 μm were stained separately with HE, periodic acid-Schiff (PAS), Gram, or Alcian Blue dyes. Histopathological examination was performed using optical microscopy under 40×, 200×, and 400× magnifications.

The determination of mucin production was performed by qualitative analysis taking into account the birds of group C as normal. A comparison was made between the stressed and unstressed birds. The histochemical techniques used, PAS for neutral mucus and Alcian Blue for acidic mucus, allowed us to infer the differences in the mucus production by the bright red magenta (PAS) and the blue staining (Alcian Blue), respectively.

For diagnosis of the lesions and group data comparisons, the entire area of 3 transversal intestinal sections per intestinal portion (duodenum, jejunum, and ileum) was evaluated for necrosis, inflammation (performed polymorphonuclear and mononuclear cells), fusion of villi, congestion, edema, and hemorrhage. Each lesion type was classified as mild (score 1), moderate (score 2), and marked to severe (score 3) for necrosis, inflammation, congestion, edema, and hemorrhage (Diab et al., 2012). The fusion of villi was also scored as (1) less than 3 fused villi per section, (2) between 3 to 6 fused villi per section, and (3) more than 6 fused villi per section. A total score per bird was performed for statistical analysis by summing the scores of lesions observed.

The intestinal sections subjected to Gram staining were scored for the amount of bacteria they present in both mucous layer and intestinal tissue, excluding the intestinal load. Briefly, within each intestinal portion the bacteria were scored as (1) diffuse presence of gram-positive rods in the mucus layer lining the villi, without bacterial aggregates into the tissue; (2) diffuse distribution of gram-positive rods in the mucus layer lining the villi with multifocal bacterial aggregates, associated with regions of necrosis; and (3) diffuse distribution of gram-positive rods in the mucus layer lining the villi plus the presence of multifocal to coalescing bacterial aggregates associated with necrosis regions.

Two pathologists that were blind to the birds’ group affiliation examined the slides. A high positive agreement was found between their independent evaluations.

### Statistical Analysis

The statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA). All data scored were nonparametric. Thus, Kruskal-Wallis ANOVA for nonparametric data was employed followed by the Mann-Whitney U test for comparisons among groups. The differences were considered significant at \( P \leq 0.05 \). The data are presented as a percentage of scores or median plus limit values.

### RESULTS

#### Gross Evaluation and Lesion Score Determination of the Small Intestine

No gross pathological changes were observed in the intestines of the birds in the control groups (C and C/HS35). However, although the color of the mucosa was not rated and statistically analyzed, a trend for an increase in the reddish color of the mucosa of the duodenum and jejunum was observed in the chickens of the T and T/HS35 groups compared with those of the control groups (C and C/HS35).

The scores attributed to the macroscopic lesion are shown in Table 3. As shown, the macroscopic lesions found in the intestines of the different groups were not significantly different (duodenum: \( P = 0.4726 \); jejunum: \( P = 0.2698 \); ileum: \( P = 0.1698 \)). However, it seems relevant to point out that the all chickens of the nonstressed group presented higher lesions scores in the jejunum (3), a value not found in the broilers of the remaining groups that presented scores equal or smaller than (2).

#### Microscopic Evaluation of the Small Intestine

As shown in Table 4, the small intestines of the chickens in the T and T/HS35 groups presented a trend for an increase in the thickness of the villi, aggregates of lymphoid cells associated with the mucosa (gut-associated lymphoid tissue) and inflammatory infiltrates in the lamina propria compared with those of the chickens in the control groups (C and C/HS35). A clearly increased number of polymorphonuclear heterophils was observed in the lamina propria and villi of the duodenum and jejunum in broilers of infected and nonstressed group (\( P < 0.05 \)). Mild-to-moderate degrees of enteritis and lymphoplasmacytic proliferation were observed in the broilers of the T and T/HS35 groups

<table>
<thead>
<tr>
<th>Intestinal portion</th>
<th>1/HS35</th>
<th>I</th>
<th>1/HS35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>Median</td>
<td>2.5</td>
<td>2</td>
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<tr>
<td></td>
<td>Max/min</td>
<td>3/1</td>
<td>3/1</td>
</tr>
<tr>
<td>Jejunum</td>
<td>Median</td>
<td>2.0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Max/min</td>
<td>3/1</td>
<td>2/1</td>
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<tr>
<td>Ileum</td>
<td>Median</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Max/min</td>
<td>1/0</td>
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</table>

1 Data are median ± SD of birds (n = 10/group). No statistical significance was found using Mann-Whitney U test (\( P < 0.05 \)).
Table 4. Percentage of scores for histologic lesions found in the duodenum, jejunum, and ileum of the broilers of all experimental groups.

<table>
<thead>
<tr>
<th>Item²</th>
<th>Necrosis (%)</th>
<th>Inflammation (%poly/%mon)</th>
<th>Villi fusion (%)</th>
<th>Congestion (%)</th>
<th>Edema (%)</th>
<th>Hemorrhage (%)</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
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<td>1</td>
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<td>3</td>
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<tr>
<td>Duodenum</td>
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<td>C</td>
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<tr>
<td>C/HS35</td>
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<tr>
<td>T</td>
<td>50</td>
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<tr>
<td>T/HS35</td>
<td>10</td>
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<td>I</td>
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<tr>
<td>I/HS35</td>
<td>80</td>
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<tr>
<td>Jejunum</td>
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<td>I/HS35</td>
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</table>

¹For necrosis, inflammation, congestion, edema, and hemorrhage: —, not observed; 1, mild; 2, moderate; 3, marked/severe. For villi fusion: 1, small numbers (less than 3 fused villi); 2, moderate numbers (between 3 to 6 fused villi); 3, large numbers (above 6 fused villi); poly, infiltrate of polymorphonuclear cells; mon, infiltrate of mononuclear cells.

²C = control group; T = thioglycolate group; I = infected group; C/HS35 = control heat-stressed group; T/HS35 = thioglycolate heat-stressed group; I/HS35 = Clostridium-infected and heat-stressed group.

*P < 0.05 compared with I group (Mann-Whitney U test).
Figure 1. Effects of infection, heat stress, or both on bacterial scores within histological sections of the duodenum, jejunum, and ileum of chickens. I = infected group; I/HS35 = Clostridium-infected and heat-stressed group. *P < 0.05 (n = 10/group).

Figure 2. Total sum of the microscopic lesion scores in the duodenum, jejunum, and ileum of chickens of the heat-stressed (C/HS35, T/HS35, and I/HS35) and nonstressed (C, T, and I) groups. C = control group; T = thioglycolate group; I = infected group; C/HS35 = control heat-stressed group; T/HS35 = thioglycolate heat-stressed group; I/HS35 = Clostridium-infected and heat-stressed group. Different letters (a-c) above the columns indicate significant differences, at P < 0.05 or less (Kruskal-Wallis and Mann-Whitney’s U tests; n = 10/group).
also found that heat stress decreased the median of significant differences were found between them. It was of the T and T/HS35 groups; however, no statistically thioglycolate induced gut lesions in the broiler chickens that were pooled for interpretation. As can be seen, scores observed in the chickens of the different groups HS35 group presented only edema and congestion, finding, edema, and hemorrhage. The broilers of the C/HS35 for necrosis, inflammation, villi fusion, congestion, 
P < 0.05). These findings showed the presence of gram-positive bacilli in the mucus layer that covered the villi in the nonstressed birds (I). This layer of mucus was reduced in stressed birds (I/HS35), resulting in a significant small number of gram-positive bacilli within the epithelium in the duodenum (P < 0.05) and jejunum (P < 0.05) of these birds, compared with those of the nonstressed group (I; Figure 1). Differences were not found in the production of acid mucus.

The morphology of the bacteria found in the infection sites of the broiler chickens of groups I and I/HS35 was consistent with that of C. perfringens.

The higher polymorphonuclear inflammatory cell infiltrate in the intestines of the infected nonstressed birds (I) compared with those of the stressed birds (I/HS35) was accompanied by a statistically significant greater severity of necrosis in the duodenum (P = 0.0128) and jejunum (P = 0.0356) but not in the ileum (P > 0.05). Further analysis of Table 4 data, showed statistically significant difference between groups I and I/HS35 for the jejunum and ileum congestion (P < 0.05) as well as for villi fusion in the duodenum (P < 0.05). No differences were found between stressed and nonstressed infected groups for hemorrhage and edema in all portions of the small intestine (P > 0.05). These findings were more evident in the duodenum and jejunum of these birds (Table 4). No statistically significant differences were found between birds in groups T and T/HS35 for necrosis, inflammation, villi fusion, congestion, edema, and hemorrhage. The brokers of the C/HS35 group presented only edema and congestion, findings that were not observed in the brokers of C group that displayed no lesions.

Figure 2 depicts the sum of all microscopic lesion scores observed in the chickens of the different groups that were pooled for interpretation. As can be seen, thioglycolate induced gut lesions in the broiler chickens of the T and T/HS35 groups; however, no statistically significant differences were found between them. It was also found that heat stress decreased the median of the total microscopic lesion scores found in the infected and stressed birds (I/HS35) compared with those of the nonstressed chickens (I); however, as a consequence of the higher variation of the data, statistically significant differences were not found between these 2 groups.

Bacterial Identification

Clostridium perfringens was not found in the blood agar microbiological cultivation in the duodenum, jejunum, and ileum contents of the birds that were not infected (C, C/HS35, T, and T/HS35). On the contrary, colonies consistent with C. perfringens were isolated from contents of the duodenum, jejunum, and ileum of all infected birds (I and I/HS35). These isolated C. perfringens colonies were later confirmed as being type A by the multiplex PCR method.

DISCUSSION

The majority of the experimental models of avian necrotic enteritis use a combination of bacterial infection and coccidial infection. This co-infection is known to facilitate C. perfringens settlement and the development of a clinical infection (Al-Sheikhly and Al-Saieg, 1980; Shane et al., 1985). Cooper and Songer (2010) proposed an experimental model of C. perfringens infection that did not use coinfection. This approach appeared to be ideal for the present study to investigate the effects of heat stress on C. perfringens infection per se. Thus, the method proposed by Cooper and Songer (2010) was applied in this study with small adaptations; it was necessary to increase the experimental period from ED19 to ED21. This model proved to be appropriate because signs of NE infection were observed in all of the infected birds (I and I/HS35).

The potential inflammatory effect of the thioglycolate and its irritative actions on the intestinal mucosa were clearly observed in the current study, which agrees with data reported in other experimental conditions (Nathan and Root, 1977; Segal et al., 2002). Gross and histological examinations revealed mild enteritis in the chickens in the T and T/HS35 groups. Thus, thioglycolate is an inflammatory agent per se in the intestinal lumen that is able to induce tissue damage. In the course of this study, this type of epithelial lesion was desirable because it would facilitate C. perfringens proliferation in the intestinal mucosa, as well as bacterial infection and toxin production (McClane, 1996; Craven, 2000; Cooper and Songer, 2010). Moreover, an irritation-induced lesion was reported to impair the intestinal physical barriers, a condition that would also be desirable within the context of this experimental study (McClane, 1996; Craven, 2000; Cooper and Songer, 2010).

Epithelial changes such as those described here in the broiler chickens of the infected groups (I and I/HS35) have been reported to trigger an imbalance in the intestinal commensal microbiota (Lutgendorff et al., 2008). In this situation, there is a higher probability of compe-
tion between a pathogenic bacteria (e.g., \textit{Clostridium}) and commensal bacteria for adherence and proliferation in the mucosa (Grenham et al., 2011).

In our study, the birds were housed in controlled isolator chambers during the entire experimental period thus, protected from external contaminants. The broiler chicks used were not challenged with a diversity of ambient bacteria, as confirmed by the microbiological analysis performed in their feces (data not shown). As a matter of fact, \textit{C. perfringens} was the only strict anaerobic bacterium isolated in the small intestinal load of the infected chickens (I and I/HS35) on ED21. This condition contrasts with the multiple anaerobic bacteria known to exist in birds raised under ordinary industrial production conditions (Singh et al., 2012). Germ-free animals were reported to exhibit deficient immune system maturation, central nervous system abnormalities, and intestine absorptive problems (McCracken and Lorenz, 2001). In the present set of experiments, the birds were placed into microbially controlled isolator chambers on ED1; thus, their immune-system maturation, development, or both, as well as their intestinal absorption, might be different from those of birds raised under conventional conditions. It should not be forgotten, however, that birds were not specific pathogen free and might have their intestines contaminated by bacteria other than \textit{C. perfringens} that might have been located on the egg surface.

The presence of thioglycolate in the chickens’ feed (T and T/HS35) might be related to the development of bowel inflammation. This irritating substance could be responsible for the migration of polymorphonuclear cells and macrophages into the intestine, where they might have released proinflammatory mediators, such as cytokines and enzymes, as reported by Gautier et al. (2013) for different experimental conditions. This inflammatory process together with the release of inflammatory mediators into the intestine and the development of congestion and necrotic foci in the intestinal mucosa might have contributed to the observed increase in goblet cells within the villous surface.

The difference in the production of mucus between stressed (I/HS35) and nonstressed (I) groups may have been a major modulating factor for intestinal proliferation of \textit{C. perfringens}. It seems reliable that the increased epithelial cell turnover and number of the goblet cells and the reduction in the mucus layer prevented bacilli proliferation near the mucosa because they were unable to find the primary substrate for adherence and proliferation. Indeed, a significant small number of colonies of bacteria was found close to the epithelium of the heat-stressed and infected chickens (Figure 1).

A significant reduction in the number of polymorphonuclear leukocytes, particularly the heterophils in the villi and intestinal lamina propria, was observed in the infected stressed birds (I/HS35) compared with the infected nonstressed birds (I). Heat stress was shown to increase corticosterone serum levels (Quinteiro-Filho et al., 2012a), and corticosterone is known to have immunomodulatory effects (Shini et al., 2008, 2010). Thus, the reduction of the inflammatory process triggered by thioglycolate might be related to a corticosterone-induced decrease in the number of heterophils present within the intestines of the stressed birds. The inflammatory reduction was also characterized by the significant decrease in the number of necrotic regions, as well as by the villous fusion and congestion found in the birds of group I (Table 3). In fact, heat stress through alteration of the HPA axis might have modulated the response of the intestinal lymphocytes, as reported elsewhere in a different context (Domingues-Junior et al., 2000). The significant differences in the heterophil distribution in the intestinal mucosa might have been mediated by the amount and quality of the cytokines or chemokines released by the epithelial or immune cells, or both, under the different experimental conditions (Harmon, 1998; Bojesen et al., 2004; Zekarias et al., 2005).

Accordingly, a trend toward lower values of gross lesion scores was reported in the stressed and infected birds (I/HS35) in relation to the infected ones of the nonstressed group (I). The results of the microscopic analysis of the infected birds (I and I/HS35) also reinforce and agree with the data reported in the gross analysis. Notably, we observed significant lower scores of necrosis, congestion, and villi fusion in the stressed birds (I/HS35) compared with those of the nonstressed (I), accompanied by increased neutral and acid mucus production, as demonstrated using the specific histochemical stains. The outbreaks of bacterial colonization in the villi were significant milder in the stressed birds (I/HS35), with the inflammatory reactions observed in this group being mainly characterized by a small number of heterophils in the intestinal mucosa (Figure 2 and Table 4). Moreover, the large numbers of villi fusion observed in the mucosa of the stressed birds (I/HS35) was not observed in the duodenum of the nonstressed broilers (I), which showed predominantly mild to moderate numbers of villous fusion instead.

Our findings showed that the broiler chickens of group I/HS35 had discretely distributed heterophils in their intestinal regions. In contrast, widely distributed heterophils were detected in the broiler chickens of group I. One of the main findings of the histopathological examination of the intestines of the broiler chickens studied was the observed association between the lower number of polymorphonuclear heterophils within the mucosa and the lower scores of lesions present (Table 4 and Figure 2). Thus, it is feasible to suggest that heterophils might be requisite for the tissue damage observed in the unstressed birds.

Most of the histopathological findings in the nonstressed birds (T and I) might be related to the immune system effects on the gut. More specifically, in an attempt to fight infection, the immune system could have damaged chicken tissues as a consequence of the release of free radicals and enzymes that are known to contribute to the development of infectious processes (Dylag
et al., 2014). We hypothesize that the better clinical conditions found in the heat-stressed birds (1/HS35) in the present study might be related to the stress-induced regulation of intestinal inflammation, specifically the stress-induced decrease in intestinal polymorphonuclear cell infiltration. Indeed, this phenomenon would allow the birds to better overcome the induced infection and consequently, for the lesions caused by the bacteria to recover in an environment with no inflammation-induced tissue damage.

Based on the data hitherto reported, experimentally induced heat stress appears to have prevented the development of C. perfringens type A-mediated necrotic enteritis. However, it is feasible to hypothesize that heat stress could have the opposite effects under field conditions (i.e., would contribute to the emergence of avian necrotic enteritis). It is possible that specifically through corticosterone release, heat stress could decrease the chickens’ innate immune response in commercial industrial production, thus decreasing their resistance to other pathogens, such as coccidia, that in turn would predispose the birds to NE. The data provided by studies of other stressors and laboratory animals’ host resistance support this assumption (Righi et al., 1999: Palermo-Neto et al., 2001, 2003; Costa-Pinto and Palermo-Neto, 2010). Altogether, the present data suggest that central nervous system activation by heat stress is responsible for the changes in intestinal immunity reported in this study, which reinforces the idea of the existence of a link between the nervous and immune systems in maintaining intestinal homeostasis.

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