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Original Article

Effect of In Ovo Ascorbic Acid Injection and Thermal Manipulation During Incubation on Intestine Morphology of Broilers Reared under Cold and Heat Stress

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ABSTRACT

It was investigated if pre-incubation ascorbic acid (AA) injection in fertile eggs incubated at high temperature impacts the performance, the yield of carcass and parts, and the intestine morphology of broilers reared under heat stress. Three thousand Cobb® fertile broiler eggs were randomly distributed according to weight into three incubations treatments (eggs not injected with AA and incubated at 37.5°C; eggs not injected with AA and incubated at 39°C; and eggs injected with 6 µg AA/100 µL water prior to incubation and incubated at 39°C). The hatched birds were reared at thermoneutral, cold, and hot house temperatures. Broilers reared under hot temperature presented lower feed intake and weight gain than the broilers of the different rearing temperatures. Egg incubation at 39.0 °C and 39.0 °C + AA reduced broiler viability. Carcass and cut yields were not influenced by incubation and rearing procedures. Duodenal goblet cell count was lower in broilers from eggs of the treatment 39°C + AA than in broilers from the other incubation treatments and in broiler rearing in hot temperature. In the jejunum, the goblet cell counts were higher in broilers that were reared under hot than thermoneutral temperatures. The incubation treatment of 39 °C+AA increased the goblet cell counts in the ileum of broilers reared under cold temperatures. Rearing temperature influenced the duodenal villi counts, which were lower under cold rearing conditions than in the two other rearing temperatures. The results showed that egg incubation at 39°C, independently of ascorbic acid injection, did not produce an effective epigenetic heat adaptation in broilers.

INTRODUCTION

High environmental heat exposure results in the high mortality of rapid growth commercial broiler strains. When broilers are subjected to high environmental temperature, the body heat rises to activate physiological and behavioral mechanisms to maintain a proper thermal balance. Furthermore, extreme cases of heat exposure might induce broilers to die due to their deficiency in losing the excess metabolic heat (Macari & Maiorka, 2017).

Thus, increased metabolic heat production resulting from the improved growth rate, coupled with the high ambient temperatures that characterize wide areas of the world, leads to severe difficulties for broilers coping with the heat. This can result in high economic losses from increased morbidity, mortality, decreased quantity and quality of meat production (Imik *et al.* 2012). However, the improvement of the physiological systems that support energy balance (e.g., the cardiovascular and respiratory systems) is not well established.

Therefore, new methods of broiler management are searched to avoid or minimize the heat stress effect during the grow-out period.

The egg nutritional manipulation associated with the thermal manipulation during the incubation process is a strategy that has been used to develop the potential response of embryos to the increase in the environmental temperature building up a heat resistance (Almeida *et al.*, 2015; Ferreira *et al.*, 2015; Sgavioli *et al.*, 2015; Morita *et al.*, 2016; Sgavioli *et al.*, 2016). Studies have shown that *in ovo* inoculation of antioxidants may improve hatching conditions reflecting chick quality (Araújo *et al.*, 2018; Peebles, 2018). Thus, it may improve the physiological and biochemical stages of embryo development, benefiting the poultry industry (Peebles, 2018).

Even though AA is synthesized inside the body, its dietary supplementation is still beneficial. The reason behind this could be the insufficient availability of plasma AA due to a reduction in the bird's capacity to synthesize under heat stress (Goel, 2021). This suggests a beneficial effect of the vitamin associated with a balance between the demand and the availability of the AA by the bird during heat stress. AA addition showed decreased effects of heat stress during broiler grow-out (Sgavioli *et al.*, 2013; Ferreira *et al.*, 2015).

Previous studies suggested that heat stress affects gut health by modulating intestinal morphology in terms of increased crypt depth, decreased villus height, and the relation of villus height to crypt depth (Liu *et al.*, 2016). The intestinal villi play a fundamental role in the final degradation of proteins and carbohydrates (Macari & Maiorka, 2017). According to Goel (2021), reducing the height of the intestinal villi of birds subjected to heat stress due to a hot environment is directly associated with a decrease in feed intake.

High incubation temperatures (Sgavioli *et al.*, 2015) and the injection of AA *in ovo* during chick development have been widely studied (Nowaczewski *et al.*, 2012). However, there is little knowledge on the potential use of nutritional additives as anti-stress *in ovo*, such as the use of AA, using an epigenetic adaptation to the broiler exposed to heat stress during the grow-out period.

In this context, the study aimed to analyze the effect of high incubation temperature associated or not with an intra-egg injection of ascorbic acid on performance, carcass yield, and intestinal morphological characteristics of broilers reared under cold and heat stress.

MATERIAL AND METHODS

The experimental procedures of this study were approved by the local Committee for Ethical Animal Use (CEUA - protocol n. 7377/10) of the College of Agricultural and Veterinary Sciences, São Paulo State University, Jaboticabal, SP, Brazil.

Egg incubation and broiler management

Three thousand fertile eggs of 47-week-old broiler breeders (Cobb 500®) were obtained from a commercial hatchery (Globoaves, Itirapina, SP, Brazil). The eggs were individually weighed (67±2 g) and used in three incubation treatments: eggs incubated at 37.5 °C and not injected (control), eggs incubated at 39°C and not injected (39 °C), and eggs incubated at 39 °C and injected with 6 µg of ascorbic acid/100µl water (39 °C+AA). Under these three incubation conditions, eggshell temperatures (measured every 30 min by Pt100 thermoresistance attached to the eggshells of 5 individual fertile eggs per incubator and the data stored in field loggers connected to a computer) were 37.35±0.15 °C, 38.65±0.45 °C, and 38.25±0.25 °C until day 12 of incubation and 37.95±0.45 °C, 38.90±0.10 °C, and 38.40±0.10 °C during the temperature plateau phase (from day 14 to 20), respectively.

The eggs were distributed homogeneously by weight in five incubators per treatment (200 eggs each) (Premium Ecológica, Belo Horizonte, MG, Brazil), all equipped with automatic control of temperature and egg turning (1 turn every 2 hours until day 18 of incubation). The relative humidity was maintained at 60% until day 18 of incubation and 70% from day 18 until hatching.

An aqueous solution of ascorbic acid (AA) (Synth, 99% purity) was prepared with autoclaved milli-Q water (6 µg AA/100 µL water) using dark bottles and environment due to the high photosensitivity of the vitamin. Eggs were injected with a fresh solution of ascorbic acid prior to incubation. For the injection, eggs were placed horizontally, cleaned with 100% ethanol (area: 1 cm^2), perforated near their thinnest end (opposite end to the air chamber) with a sterile needle [Injex, 0.38 x 13 (27.5 G1/2")] and injected with the solution (100 µL) in the albumen about 6 mm beneath the eggshell. After injection, the hole was closed with a label containing information on the treatment and replicate number.

At hatching, five hundred and forty male chicks (180 chicks per incubation treatment) were distributed homogeneously by body weight in three rearing temperature treatments: control (recommended for this broiler line by the Cobb Broiler Management Guide), cold and hot. Broilers were housed in three

climatic chambers with automatic temperature control and dark/light regime (2h:22h, D:L for all rearing treatments) containing 15 boxes. Thus, five replicates (boxes) with 12 broilers per incubation temperature were housed in each climatic chamber, totalizing 45 boxes (1.5 x 1 m) with the floor covered with wood shavings. Five replicates of 12 broilers per incubation temperature were housed in each climatic chamber in boxes (1.5 x 1 m) with the floor covered with wood shavings.

Only one climatic chamber was used for each rearing temperature treatment. This may involve the risk that rearing temperature, in reality, could be not a temperature effect but a chamber effect. However, we think that the experimental design used is justified since the chamber's environmental conditions were controlled, and the experimental repetitions were uniformly distributed within the chamber. The climatic chambers were not considered experimental units. To consider each chamber as an experimental unit, we would need 45 climatic chambers (considering that the present study has nine treatments with five repetitions each), which is unfeasible from the experimental and economic viewpoint.

The weekly average temperatures inside the chambers during the experimental period were: cold temperatures, 32 °C, 30 °C, 26 °C, 22 °C, 18 °C, and 14 °C; control temperatures, 32 °C, 31 °C, 29 °C, 27 °C, 25 °C, and 23 °C; and hot temperatures, 32 °C, 32 °C, 32 °C, 32 °C, 32 °C, and 32 °C, from the first to the sixth week of age.

Broilers were raised up to 42 days of age receiving water and diet *ad libitum,* and were fed with two diets formulated on corn and soybean meal, adjusted for two phases: starter diet (1-21 days: 12.06 MJ/kg metabolizable energy, 21.27% crude protein (CP), 0.88% digestible methionine + cysteine, 0.56% digestible methionine, 1.22% digestible lysine, 0.85% Ca, 0.19% Na, 0.42% P available) and grower diet (22-42 days old: 13.07 MJ/kg metabolizable energy, 18.86% CP, 0.77% digestible methionine + cysteine, 0.49% digestible methionine, 1.05% digestible lysine, 0.69% Ca, 0.20% Na, 0.32% P available), following the nutritional requirements established by Rostagno *et al.* (2011).

Broilers were vaccinated against Marek's Disease according to the recommended immunization schedule. The following vaccination program was completed during the experimental period: infectious bursal disease (IBD) (mild strain) on day 7 via eye drops; Newcastle disease and IBD (hot strain) via drinking water using powdered milk as a vehicle (2 g L^{-1}) on day 14.

Performance

Weight gain, feed intake, and conversion were evaluated for the total rearing period (1-42 days). Mortality was recorded daily for the correction of performance parameters and to evaluate viability.

Carcass yield and parts

At the end of the experimental period (42 d), two birds per replicate, totaling 90 birds (ten birds per treatment), were selected by the mean weight to evaluate carcass yield and parts. The broilers were individually identified, and after a period of 24h fasting, the birds were slaughtered by cervical displacement followed by jugular bleeding. Afterward, the birds were feathered and eviscerated, and the entire carcass without the feet and head were weighed. The carcass was subjected to the cuts (breast, thigh + overthigh; wing + overwing and back), and the carcass yield was calculated. The pieces were weighted, and the individual weight of each broiler at slaughter was taken as a basis to access the carcass yield.

Intestine morphological characteristics

Forty-five 42-d old broilers, five per treatment (1 broilers/replicate), were selected for intestinal morphological analysis. The broilers were slaughtered by cervical displacement followed by jugular bleeding to collect amounts from the three intestine segments. Duodenum samples were taken from its distal loop, and jejunum and ileum samples were collected before Meckel's diverticulum and cecal insertion. All samples were 2.5 cm long and submitted to a routine procedure for light microscopy. The samples were opened longitudinally and secured on cardboard, washed quickly using distilled water, and then fixed in formaldehyde (10%) for 24 hours at ambient temperature. Subsequently, the samples were dehydrated in a series of increasing ethanol concentrations [70, 80, 90 e 100% (3x)], diaphanized in the ethanol-xylol mixture (1: 1), and xylol (100%) and infiltrated and included in histosec. From each sample, five 6µm thick semi-serial cross-sections were obtained and stained with Schiff periodic acid (PAS) followed by hematoxylin.

These sections were used to analyze villus height and perimeter, crypt depth (40 measurements of each variable per sample), villus number (number of villi along 372 µm µm intestinal wall, five sections per

sample), and number of goblet cells (number of goblet cells over 372 µm2 epithelium. All data were obtained using image capture and analysis system (Leica Q win V3) connected to a microscope (Leica-DM 2500).

Statistical analysis

The effects of incubation treatments (IT: 37.5°C, 39°C, and 39°C+Vit C), the rearing temperatures (RT) (cold, control, and hot), and their interaction (IT x RT) were analyzed according to the experimental model: Yijk = µ + (IT)i + (RT)J + (IT x RT) ij + eijk, where Y is the dependent variables, µ is the overall mean, and e_{ijk} is the error term. Distributions of the means and residuals were examined to check model assumptions. After they were found not to violate these assumptions, the data were submitted for analysis of variance by General Linear Model (GLM) procedure of SAS® statistical package (SAS Institute, 2002). In the case of significant effect (5%), the comparison among means were performed by Tukey test.

RESULTS

Performance

Incubation treatments did not influence performance variables (p>0.05), except for viability, which was lower with egg incubation at 39.0 °C and 39.0 °C + AA than at 37.5°C (Figure 1A) (p<0.0001). The rearing temperature affected the viability (Figure 1A) (p=0.0249), weight gain, and feed intake (Figure 1B) (p<0.0001). Viability and weight gain were lower in broilers raised at hot temperature than broilers raised under cold temperature and thermoneutral, which did not differ. Feed intake decreased by increasing the rearing temperature.

Carcass and parts yield

No effect of incubation treatments, rearing temperatures, or interaction between carcass yield and cuts was observed (p>0.05).

Intestinal villi and crypt size

No effect of incubation treatments and rearing temperatures was found in the duodenum. There was no interaction between both variables on the villi height and perimeter and crypt depth (p>0.05). However, the villi count was influenced by rearing temperature and were lower at cold temperatures (p<0.0001). Goblet cell count was affected by incubation treatments (p=0.0416) and rearing temperatures (p=0.211), being lower in the broilers from eggs injected with AA and incubated at high temperature and in broilers raised

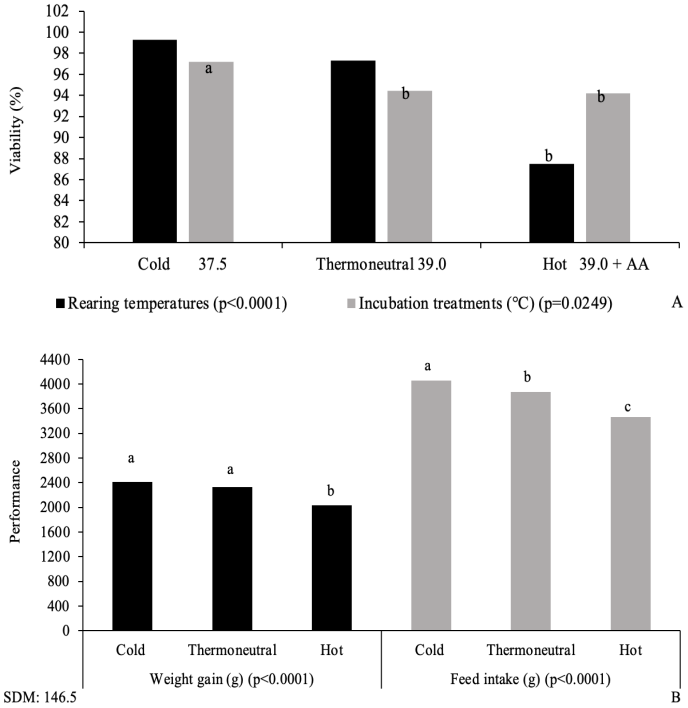

Figure 1 – Effects of incubation treatments and/or rearing temperatures on viability (%) (A) broiler weight gain (g) and feed intake (g) from day 1 to 42 age (B). Bars with distinct letters indicate a difference between means (p<0.05). AA-ascorbic acid. SDM: standard deviation means.

at hot temperature and thermoneutral, which did not differ (Table 1).

In the jejunum, there were no effects of incubation treatments and rearing temperatures, and no interaction between them for any of the variables evaluated (p>0.05), except for the goblet cell count, which was lower in broilers raised at hot and cold temperature, which did not differ (p=0.0226) (Table 2).

In the ileum, the height, perimeter, and crypt depth were not affected by the incubation treatments and rearing temperatures nor by the interaction between the two (p>0.05). An effect (p=0.0489) of incubation treatment for count villi being lower in the broiler from eggs incubated at 39 °C + AA and 39 °C, which did not differ (Table 3). There was a significant interaction between incubation treatments and rearing temperatures for goblet cell count (p=0.0342).

Goblet cell counts were lower in broilers from eggs incubated at 39 °C with and without AA when the rearing temperature was hot (p=0.0137). In broilers from eggs incubated at 39 °C when the rearing temperature was cold (p=0.0059) and in broilers from eggs incubated at 37.5 °C when the rearing temperature was cold or thermoneutral (p=0.0125) (Table 4).

Table 1 – Size and counts of villi, crypt depth, and goblet cell count in the duodenum of 42-d old broilers, according to incubation treatments and rearing temperatures.

	Villi Height (µm)	Villi Perimeter (µm)	Villi Count (villi number/372 µm)	Goblet Cell Count (cells number/372 µm)	Crypt Depth (µm)
Incubation treatments (IT, °C)					
37.5	1,447±171	3,182±288	11±1	27±4a	196±35
39.0	1,454±130	3,138±241	11±1	27±2a	190±20
39.0+AA	1,469±137	3,215±278	11±1	24±3b	206±36
Rearing temperature (RT)					
Cold	1,476±88	3,198±140	10±0.6b	27±3a	204±33
Thermoneutral	1,395±157	3,117±365	11±0.9a	25±3ab	190±29
Hot	1,503±165	3,224±260	11±1.2a	24±3b	200±34
p-value					
IT	0.9015	0.7698	0.9647	0.0416	0.3898
RT	0.1304	0.6315	<0.0001	0.0211	0.4628
IT x RT	0.2538	0.1419	0.4032	0.1135	0.4478
CV (%)	9.66	8.24	8.54	12.34	16.32

CV = coefficient of variation. AA=ascorbic acid; a-b = means followed by different letters (column) differ by the Tukey test ($p<0.05$, X±SD).

Table 2 – Size and counts of villi, crypt depth, and goblet cell count in the jejunum of 42-d old broilers, according to incubation treatments and rearing temperatures.

	Villi height (µm)	Villi perimeter (µm)	Villi counts (villi/372 µm)	Goblet cell counts (cells number/372 µm²)	Crypt width (µm)
Incubation treatment (IT, °C)					
37.5	965±148	2,063±298	15±2	35±7	138±26
39.0	1,007±105	2,002±246	16±2	37±7	144±27
39.0+ AA	1,021±115	2,090±263	15±1	34±6	146±29
Rearing temperature (RT)					
Cold	991±141	2,085±292	16±2	35±7ab	147±27
Thermoneutral	1,016±109	2,114±243	15±1	32±5b	134±24
Hot	986±126	1,956±251	15±2	39±5a	146±30
p -value					
IT	0.4592	0.6653	0.7100	0.5591	0.6937
RT	0.7892	0.2530	0.2713	0.0226	0.4046
IT x RT	0.4090	0.6445	0.8198	0.6596	0.7241
CV (%)	12.72	13.27	14.31	18.39	20.11

CV = coefficient of variation. AA=ascorbic acid; a-b = means followed by different letters (column) differ by the Tukey test ($p<0.05$, X±SD).

Table 3 – Size and counts of villi, crypt depth, and goblet cell count in the ileum of 42-d old broiler, according to incubation treatments and rearing temperatures.

	Villi height (µm)	Villi perimeter (µm)	Villi count (villi/372 µm)	Goblet cell count (cells number/372 µm²)	Crypt depth (µm)
Incubation treatment (IT, °C)					
37.5	781±96	1,607±181	12±1a	44±6	138±14
39.0	757±73	1,548±158	11±1ab	45±4	153±19
39.0+ AA	729±85	1,536±178	10±1b	46±6	139±21
Rearing temperature (RT)					
Cold	747±84	1,564±150	11±1	45±5	141±26
Thermoneutra	775±81	1,618±168	11±1	42±6	145±16
Hot	744±94	1,512±187	11±1	48±4	143±15
p-value					
IT	0.3276	0.5658	0.0489	0.4783	0.1094
RT	0.6403	0.3026	0.4881	0.0109	0.7836
IT x RT	0.9634	0.9253	0.7258	0.0342	0.5282
CV (%)	11.98	11.44	12.36	11.18	13.52

CV = coefficient of variation. AA=ascorbic acid; a-b = means followed by different letters (column) differ by the Tukey test ($p<0.05$, X±SD).

Table 4 – Interactions between incubation treatments and rearing temperatures for the goblet cells counts in the ileum of 42-d old broilers, according to incubation treatments and rearing temperatures.

	Rearing temperatures (°C)			*p*-value
Incubation treatments	Cold	Thermoneutral	Hot	
37.5	40±3Bb	42±7B	50±2Aa	0.0125
39.0	45±5b	45±5	47±1b	0.3664
39.0+AA	50±4a	40±5	48±7b	0.5518
p-value	0.0059	0.8125	0.0137	

AA=ascorbic acid, a-b, A-B: means followed by different letters (column and line, respectively) differ by the Tukey test ($p<0.05$, X± SD).

DISCUSSION

This study analyzed the potential use of high incubation temperature associated or not with pre-incubation *in ovo* ascorbic acid (AA) on the performance and intestine morphology of broilers reared under cold and hot conditions. The results showed that high incubation temperature with or without AA did not reduce or inhibit the effects of the hot rearing temperatures on the broiler viability, weight gain, and feed intake. The cold temperature did not influence the performance, but the hot temperature reduced viability, weight gain, and feed intake. This result is consistent with the results observed by Awad *et al.* (2019) and appears to be related to body temperature maintenance. Body temperature is conveniently used as a marker for HS, and various reports suggested an enhancement in the body temperature under heat stress in chickens (Barrett *et al.*, 2019).

The hot rearing temperature reduced the performance of the birds, as shown by the lower feed intake and weight gain of the broilers. The current literature also found the effects of heat on broiler performance (Awad *et al.*, 2019; Barrett *et al.*, 2019). Furthermore, none of the incubation treatments prevented or minimized the effects of heat stress during rearing, according to the results for the viability, indicating that egg incubation at 39°C, independently of ascorbic acid (AA) injection, did not produce an effective epigenetic heat adaptation in broilers.

Incubation of eggs at high temperatures with or without ascorbic acid injection did not influence the yield of carcasses and parts, as did cold and hot rearing temperatures. However, Ferreira *et al.* (2015) observed that incubation at 39°C and 39°C plus AA prevented the effects of hot and cold rearing temperatures by diminishing and increasing the muscle fiber area, respectively.

AA injection in eggs before incubation at high incubation temperature had a long-term and reducer effect on the goblet cells counts on the duodenal and the ileum broilers reared under hot conditions. The long–term adverse effect of the high incubation temperature with or without AA on the villi count in the ileum was also shown in broilers. In the present study, the effect of high incubation temperature associated (or not) with the addition of ascorbic acid and high rearing temperature on the counting of caliciform cells needs further interpretation. High rearing temperature lessened the caliciform cells in the duodenum; however, this condition increased the globet cells in the jejunum and the ileum. In the ileum, the high rearing temperature in reducing the number of caliciform cells was not avoided with the incubation of eggs at high temperature (with or without the addition of AA).

High incubation temperatures associated with injection of AA (39°C+AA) led to a similar effect in the duodenum, decreasing the globet cells (Gursu *et al.* 2004). Nevertheless, exposition to 39°C increased the globet cells of the ileum when broilers were raised under cold ambient conditions (Burkholder *et al.* 2008). The results indicate globet cells reduce when birds are exposed to heat stress, either during rearing or during incubation. There is no reduction in these heat stress effects by injecting in-egg AA. Globet cells produce substances associated with selecting the size of the molecules, protection, and the restoration of intestine epithelial (Macari & Maiorka, 2017). These cells also contribute to the appropriate immunological response of the intestine mucous, which regulates the microbiota development and keeps the epithelial barrier and intestinal homeostasis (Macari & Maiorka, 2017). The rise in globet cells under heat stress may be related to microbiota and immunoprotection changes.

In addition, high incubation temperature associated or not with AA injection reduced the number of villi in the ileum of birds, regardless of the temperature where they were reared. The nutrient digestion and absorption area are directly related to the number of intestinal villi (Boleli & Thimotheo, 2017). The jejunum and upper ileum are the main sites of digestion and absorption of minerals in broilers (Mutucumarana *et al.*, 2014). Therefore, high incubation temperature and AA injection may have impaired digestion and absorption of minerals in the ileum.

CONCLUSIONS

Egg incubation at 39°C, independently of ascorbic acid (AA) injection, did not produce an effective epigenetic heat adaptation in broilers that could avoid

the reduction of coliform cells and the decrease in the performance of birds reared under hot conditions.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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