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# *Effect of In-Ovo Ascorbic Acid Injection on the Bone Development of Broiler Chickens Submitted to Heat Stress During Incubation and Rearing*

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■Keywords

Ash, mineral density, femoral bone strength, tibia, vitamin C.

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## ABSTRACT

This experiment was conducted to evaluate the effect of *in-ovo* ascorbic acid (AA) injection on the bone development of broilers submitted to heat stress during incubation and rearing. One thousand (1,000) Cobb®fertile broiler eggs were randomly distributed according to the weight into five incubators, with 200 eggs per incubator. The incubation treatments were: 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 three different house temperatures: cold, thermoneutral, or and hot. The high incubation temperature negatively influenced broilers' bone characteristics. The femur of the birds hatched from eggs incubated at 39°C and injected with AA presented lower shaft mineral density, lower maximum force and lower elongation at maximum force. Their tibia presented reduced mineral density at the proximal and distal epiphysis. *In-ovo* AA injection of eggs incubated at high temperature did not minimize the negative effects of high rearing temperature on the performance andbone development of broiler chickens reared until 42 days of age.

## INTRODUCTION

Fast-growing chickens, i.e., genetically-improved broilers breeds, produce high metabolic heat and may not be able to maintain normothermia under hot environmental conditions (Bruno *et al.,* 2000). The resulting heat stress can negatively affect bone development in these broilers breeds. Both manipulations of the incubation temperatures and the *in-ovo* injection of ascorbic acid (AA) have been separately studied for the induction of epigenetic adaptation and the promotion of bone development in heat-stressed broilers. However, the joint effect of high incubation temperature and *in-ovo* AA injection for the mitigation of heat stress during broiler rearing has not been analyzed yet.

During the post-hatching period, ambient temperature has significantly affects broiler performance, including feed intake (Teeter *et al.,* 1984; Quinteiro Filho *et al.,* 2010), weight gain, and feed conversion ratio. Furthermore, broilers under heat stress exhibit changes in the skeletal system development, which negatively influences their performance and consequently, farm productivity (Nääs, 2008).

Anti-stressors, such as ascorbic acid, have been evaluated to try to minimize the effects of heat stress. Thornton (1961) reported a reduction in the blood concentration of ascorbic acid in heat-stressed birds. This suggests that domestic fowl under heat stress should receive vitamin C supplementation. Ascorbic acid participates in several biological processes; it assists collagen synthesis and maintenance; it is essential for formation and maintenance of connective tissue, bone

and cartilage; it, stimulates the immune system and enhances disease resistance (Farquharson *et al.,* 1998; Berzina *et al.,* 2013; Sgavioli *et al.,* 2013; Chand *et al.,* 2014). Finally, ascorbic acid influences the development of the leg bones in stressed birds (Lesson & Summers, 2001).

There are several studies on the effects of high incubation temperatures on *in-ovo* development (Wineland *et al.,* 2000; Lekrisompong *et al.,* 2007; Lourens *et al.,* 2007; Molenaar *et al.,* 2011) and on the *in-ovo* injection of ascorbic acid (Zakaria & Al-Anezi, 1996; Ghonim *et al.,* 2009; Mohammed *et al.,* 2011; Nowaczewski *et al.,* 2012) in broilers. However, there are no studies on the *in-ovo* injection ascorbic acid and the subsequent submission of the eggs to heat stress during incubation. This practice could promote the epigenetic adaptation of broilers to high environmental temperatures during the rearing period and support their, skeletal system development.

This study aimed at evaluating the effect of the pre-incubation injection of ascorbic acid (AA) on the bone development of broilers subjected to heat stress during incubation and rearing.

## MATERIALS AND METHODS

This study was conducted in accordance with the ethical principles for animal experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) and the experimental procedures 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.

One thousand (1,000) Cobb®fertile broiler eggs from 47-week-old breeders were distributed according to average egg weight (67 ± 2g) into five incubators/ treatments (200 eggs each). A completely randomized design in a 3x3 factorial arrangement was applied, with three treatments during the incubation phase (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 before incubation and incubated at 39°C) and three temperatures during rearing (cold, thermoneutral and hot) (Table 1). The eggs were incubated in Ecological Premium IP200, Belo Horizonte, incubators equipped with automatic temperature, humidity, and egg-turning control. The eggs were turned every 2 hours and maintained at 60% relative humidity until the transfer to the hatcher, when humidity was increased to 70% during the last two days of incubation.

**Table 1 –** Environmental temperature throughout the rearing period.

| Week | Environmental temperature (ºC) | | |
|---|---|---|---|
| | Cold | Thermoneutral | Hot |
| 1st | 32±4 | 32±5 | 32±5 |
| 2nd | 30±4 | 31±5 | 32±7 |
| 3rd | 26±4 | 29±4 | 32±7 |
| 4th | 22±5 | 27±3 | 32±5 |
| 5th | 18±2 | 25±2 | 32±7 |
| 6th | 14±4 | 23±2 | 32±7 |

For the AA injection, the eggs were held horizontally and after cleaning with 100% ethanol, the shell was perforated near the thin end (the end opposite to the air cell) with a sterile needle [Injex, 13 x 0.38 (27.5 G1/2")]. Then, 100 µL of aqueous AA solution (6 µg) (Synth, 99% pure) was injected in the albumen, about 6 mm below the membrane. The AA solution was prepared with ultra pure water autoclaved in the dark due to its photosensitivity. After injection, the hole was sealed with a label identifying the treatment and repetition.

After hatching, the male and female one-day-old chicks were divided into three environmentally-controlled houses with 15 pens of12 birds each, resulting in five replicates per treatment. The chicks were reared until 42 days of age. Temperatures were controlled with brooders and HVAC equipment.

The chicks were vaccinated against Marek's disease and avian pox, and later, against infectious bursal disease (IBD) and Newcastle disease, according to the Cobb® vaccination program. Feed and water were provided *ad libitum*. The corn and soybean-meal based diets (Table 2) were formulated according to rearing phase (starter: 1-21 days; grower: 22 to 42 days old), following the nutritional requirements established by Rostagno *et al.* (2011).

### Hatchability, weight gain, feed intake, and bone collection

Hatchability was determined as a function of the total number of fertile eggs, according to embryo diagnosis, and calculated as: hatchability=100*number of hatched birds/number fertile eggs incubated. Weight gain and feed intake were evaluated for the total rearing period (1-42 days). At 42 days of age, eight birds per treatment were stunned with $CO_2$ and killed by exsanguination by cutting the jugular vein for blood sample collection.

### Bone length, width and weight

The left femur and tibia of the birds were weighed on a digital scale. A digital caliper was used to measure bone length and width (middle portion of the shaft).

**Table 2 –** Ingredients and calculated nutritional composition of the starter (1-21 days of age) and grower (21-42 days of age) diets.

| Ingredients (%) | Starter | Grower |
|---|---|---|
| Corn | 60.82 | 63.74 |
| Soybean meal 45% | 35.15 | 29.79 |
| Soybean oil | - | 3.12 |
| Dicalcium phosphate | 1.63 | 1.16 |
| Limestone particulate | 0.84 | 0.76 |
| Salt | 0.42 | 0.44 |
| L- Lysine HCl (78%) | 0.25 | 0.21 |
| DL- Methionine (99%) | 0.29 | 0.23 |
| L- Threonine | 0.08 | 0.04 |
| Butylated hydroxytoluene (BHT) | 0.01 | 0.01 |
| Vitamin and mineral supplement* | 0.50 | 0.50 |
| TOTAL | 100.00 | 100.00 |
| Nutritional Composition | | |
| Metabolizable energy (kcal/kg) | 2.883 | 3.121 |
| Crude protein (%) | 21.27 | 18.86 |
| Ca (%) | 0.85 | 0.69 |
| Na (%) | 0.19 | 0.20 |
| Available phosphorus (%) | 0.42 | 0.32 |
| Digestible methionine + cystine (%) | 0.88 | 0.77 |
| Digestible methionine (%) | 0.56 | 0.49 |
| Digestible lysine (%) | 1.22 | 1.05 |
| Digestible threonine (%) | 0.79 | 0.68 |
| Digestible tryptophan (%) | 0.24 | 0.21 |
| Digestible arginine (%) | 1.32 | 1.16 |

* Nutrients per kilogram of diet: From 1 to 21 days of age - Vit. A 7,000 U.I., Vit. D3 3,000 U.I., Vit. E 25 U.I., Vit. K 0.98 mg, Vit. B1 1.78 mg, Vit. B2 9.6 mg, Vit. B6 3.5 mg, Vit. B12 10 µg, Folic Acid 0.57 mg, Biotin 0,16 mg, Niacin 34.5 mg, Calcium Pantothenate 9.8 mg, Copper 0.12 g, Cobalt 0.02 mg, Iodine 1.3 mg, Iron 0.05 g, Manganese 0.07 g, Zinc 0.09 mg, Organic Zinc 6.75 mg, Selenium 0.27 mg, Choline 0.4 g, Growth promoter (Zinc bacitracin) 30 mg, (narasin+nicarbazin) 0.1 g, Methionine 1.68 g. From 21 to 42 days of age - Vit. A 7,000 U.I., Vit. D3 3,000 U.I., Vit. E 25 U.I., Vit. K 0.98 mg, Vit. B1 1.78 mg, Vit. B2 9.6 mg, Vit. B6 3.5 mg, Vit. B12 10 µg, Folic Acid 0.57 mg, Biotin 0.16 mg, Niacin 34.5 mg, Calcium Pantothenate 9.8 mg, Copper 0.12 g, Cobalt 0.02 mg, Iodine 1.3 mg, Iron 0.05 g, Manganese 0.07 g, Zinc 0.09 mg, Organic Zinc 6.75 mg, Selenium 0.27 mg, Choline 0.6 g, Growth promoter (avilamycin) 7.5 mg, (monensin sodium) 0.1 g, Methionine 1.4 g.

### Bone composition, mineral density, and area

Bone mineral density (BMD,g/cm$^2$), area (cm$^2$), and mineral content (BMC,g), were determined in femora and tibia. Bone mineral density was measured in the proximal, distal, and diaphyseal region using dual-energyX-ray absorptiometry (DXA), calibrated by the manufacturer (DPX-Alpha, Lunar®), and a small animal software of DPX-Alpha, Lunar®. Clean bones were placed in an acrylic container with deionized water and scanned using a densitometer. The small animal software was used to select the region for subsequent densitometric analysis.

### Bone strength

The left femur and tibia were used for the mechanical bone strength tests (three-point bending and axial compression). The tests were conducted using an EMIC® (model DL 3000) universal testing machine (UMT). The load was applied at a rate of 5 mm/min with a force of 2000 N to determine the maximum permissible force (Fmax) of the bone, amount of deformation (bend) caused by the Fmax, and the determination of bone rigidity. The bones were fixed on two supports (two points), with the span adjusted according to the size of the smallest bone. Force was then applied at the bone's geometric mean point between the two supports (the middle third of the bone) and the equipment recorded the results. These variables express bone strength at the ends of the bone and the middle third. Bone rigidity was calculated, with the maximum force curve (N) x deformation (mm) of the elastic body phase (the straight part of the curve). These data were used to determine the linear equation (1 degree, y = a.x+b) that best fit the points. In this equation, the slope of the curve is shown by the constant a, which multiplied by the variable x, expresses the bone rigidity (slope of the curve in the elastic phase).

### Bone mineral composition

The right femur and tibia were used to determine bone calcium, phosphorus, and ash content. The analyses were performed at the Laboratory of Animal Technology, College of Agricultural and Veterinary Sciences, UNESP, Jaboticabal, SP, according to the methodologies described by Silva & Queiroz (2002), and expressed as a% of defatted dry matter.

### Statistical analysis

The data for all variables were checked for outliers and if they complied with normality assumptions of the studentized errors test (Cramer-von-Misses) and homogeneity of variances (Levene's test). After these tests, the data were subjected to analysis of variance using the General Linear Model (GLM) procedure of SAS® software (SAS Institute, 2002) and the Tukey test at 5% probability level.

## RESULTS

Hatchability was lower in the high-temperature groups (with and without vitamin C) than for in control group (control: 84.50a ± 1.12%, 39ºC: 74.70b ± 1.20%, 39ºC+vitamin C: 74.40b ± 9.24%, $p$<0.0189).

No significant effect (p<0.05) of the incubation treatments was observed on broiler weight gain or feed intake. However, it is possible that there is an effect, because these parameters were significantly affected (p<0.05) by the applied temperatures during

the rearing phase. The broilers reared under hot temperature showed lower weight gain (Figure 1A) and lower feed intake (Figure 1B) compared with those reared under thermoneutral and cold temperatures.

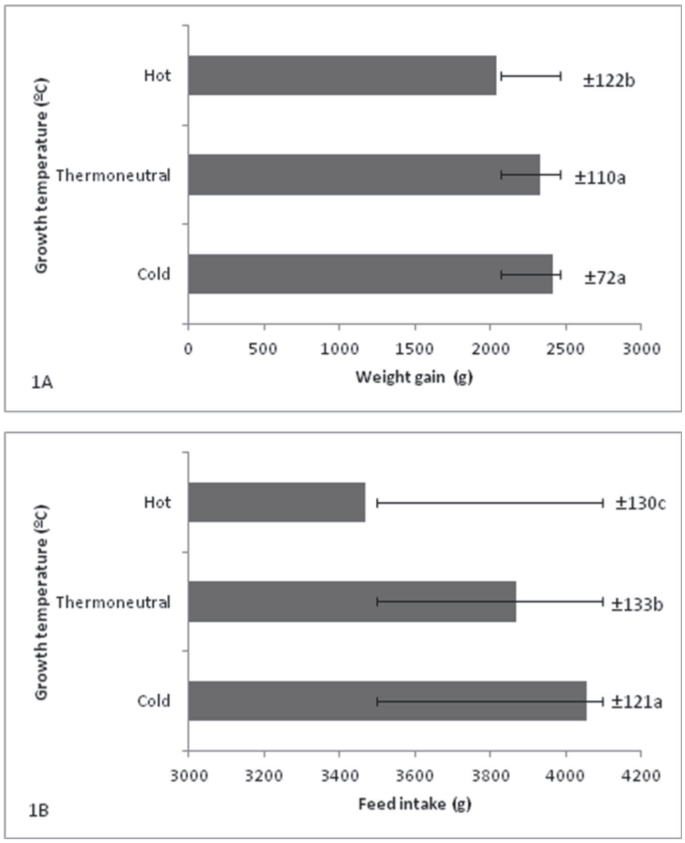


**Figure 1** – Weight gain of the broilers at 42 days of age (A) and feed intake from 1-42 days (B) according to the rearing temperature. a-c: means followed by different letters differ significantly by Tukey test at 5% probability.

The statistical analysis showed a significant effect ($p<0.05$) between the growth phase of rearing temperatures on femur width and bird weight, with the lowest values observed in birds reared under the hot temperature. Tibia length and weight were also significant different ($p<0.05$) among treatments, with lower values found in the birds reared under hot temperatures (Table 3). There was no significant effect ($p>0.05$) of any incubation treatments on bone length, width, or weight (Table 3).

Bone mineral composition, density, and area were measured for both in the femur and in the tibia. Bone area and mineral content were significantly lower ($p<0.05$) in reared under the hot temperature (Tables 4 and 6).

Femur mineral density was significantly lower ($p<0.05$) in birds reared under hot temperature (Table 4). In addition, for femur mineral density, a significant interaction ($p<0.5$) was found between the incubation treatments and rearing temperatures. Lower femur mineral densities were determined in chickens reared under the hot temperature and hatched from eggs incubated at 39°C, regardless of AA injection (Table 5). However, the broilers reared under thermoneutral temperature (37.5°C) presented the lowest mineral density.

Relative to the tibia, the lowest diaphyseal mineral density value was observed in the chickens reared under hot temperature (Table 6). Again, there was significant interaction between the incubation treatments and the rearing temperatures for the mineral density of the proximal tibia and distal epiphysis ($p<0.05$). Lower mineral density was observed in the proximal epiphysis of chickens hatched from eggs injected with AA and incubated at 39°C, then subsequently reared both

**Table 3 –** Effect of incubation treatments and rearing temperatures on femur and tibia length, width and weight of broilers at 42 days of age.

| | Femur | | | Tíbia | | |
|---|---|---|---|---|---|---|
| | Length (mm) | Width (mm) | Weight (g) | Length (mm) | Width (mm) | Weight (g) |
| Treatment (T) | | | | | | |
| 37.5° C | 68.53±3.46 | 7.40±0.91 | 6.82±0.94 | 91.73±4.59 | 5.87±0.83 | 9.15±1.27 |
| 39°C | 67.13±2.77 | 7.20±0.68 | 6.81±1.21 | 92.29±4.94 | 6.00±0.68 | 8.70±1.74 |
| Ascorbic Acid 39° C | 69.47±2.42 | 7.80±0.68 | 6.90±1.03 | 94.14±3.61 | 6.14±0.86 | 9.62±1.61 |
| Temperature (TP) | | | | | | |
| Cold | 68.67±3.15 | 7.60±0.83ab | 7.16±0.97a | 92.07±4.77ab | 6.20±0.94 | 9.58±1.42a |
| Thermoneutral | 69.00±2.39 | 7.73±0.70a | 7.39±0.79a | 94.86±4.26a | 6.14±0.66 | 10.02±1.11a |
| Hot | 67.47±3.38 | 7.07±0.70b | 5.99±0.76b | 91.21±3.62b | 5.64±0.63 | 7.81±1.21b |
| Probability | | | | | | |
| T | 0.0915 | 0.0946 | 0.9637 | 0.2076 | 0.6456 | 0.2215 |
| TP | 0.3112 | 0.0459 | 0.0002 | 0.0424 | 0.1527 | <0.0001 |
| T x TP | 0.2418 | 0.8760 | 0.7029 | 0.2218 | 0.9564 | 0.2220 |
| CV (%) | 4.16 | 9.98 | 12.96 | 4.42 | 13.50 | 13.17 |

CV: Variation coefficient. a-b: Means followed by different letters in columns differ significantly by Tukey test at 5% probability.

**Table 4 –** Effect of incubation treatments and rearing temperatures on mineral composition, area, total mineral density of the proximal epiphysis, diaphysis and distal epiphysis of the femur of broilers at 42 days of age.

| | Femur | | | | | |
|---|---|---|---|---|---|---|
| | Mineral composition (g) | Area ($cm^2$) | Mineral density | Proximal epiphysis** | Diaphysis** | Distal epiphysis[1]** |
| | | | ($g/cm^2$) | | | |
| Treatments (T) | | | | | | |
| 37.5ºC | 0.121±0.11 | 6.17±0.62 | 0.019±0.02 | 0.044±0.03 | 0.007±0.01 | 0.005 (0.409) ±0.01 |
| 39ºC | 0.175±0.13 | 6.06±0.64 | 0.028±0.02 | 0.052±0.03 | 0.028±0.03 | 0.003 (0.408) ±0.00 |
| Ascorbic acid-39ºC | 0.202±0.18 | 6.38±0.51 | 0.030±0.02 | 0.048±0.03 | 0.032±0.03 | 0.000 (0.406) ±0.00 |
| Temperatures (TP) | | | | | | |
| Cold | 0.164±0.11ab | 6.37±0.51a | 0.025±0.02ab | 0.041±0.02 | 0.021±0.02 | 0.005 (0.409) ±0.01 |
| Thermoneutral | 0.251±0.17a | 6.48±0.55a | 0.038±0.02a | 0.059±0.03 | 0.040±0.03 | 0.003 (0.407) ±0.00 |
| Hot | 0.082±0.08b | 5.77±0.49b | 0.014±0.01b | 0.043±0.03 | 0.006±0.01 | 0.002 (0.407) ±0.00 |
| Probability | | | | | | |
| T | 0.1661 | 0.2535 | 0.1581 | 0.7396 | 0.0008 | 0.1118 |
| TP | 0.0013 | 0.0015 | 0.0016 | 0.1739 | <0.0001 | 0.3905 |
| T x TP | 0.0635 | 0.8386 | 0.0841 | 0.3945 | 0.0048 | 0.4214 |
| CV (%) | 69.83 | 8.50 | 65.48 | 59.03 | 73.03 | 1.08 |

CV: variation coefficient. a-b: means followed by different letters in columns differ significantly by Tukey test at 5% probability. [1] comparison from log-transformed data (values in parentheses). ** considering an area of 0.231 $cm^2$.

**Table 5 –** Interactions between incubation treatments and rearing temperatures for the femoral diaphysis mineral density of broilers at 42 days of age.

| Diaphysis ($g/cm^2$) | Temperatures (ºC) | | | p |
|---|---|---|---|---|
| Treatments | Cold | Thermoneutral | Hot | |
| 37.5ºC | 0.011±0.01 | 0.008±0.01b | 0.002±0.01 | 0.7435 |
| 39ºC | 0.033±0.03A | 0.048±0.03Aa | 0.004±0.01B | 0.0007 |
| Ascorbic acid-39ºC | 0.016±0.00B | 0.065±0.02Aa | 0.011±0.01B | <0.0001 |
| p | 0.1135 | <0.0001 | 0.6856 | |

a-b; A-B: means followed by different letters in columns and rows, respectively, differ significantly by Tukey test at 5% probability.

**Table 6 –** Effect of incubation treatments and growth temperatures on mineral composition, area, total mineral density and on the tibial proximal epiphysis, diaphysis and distal epiphysis of the tibia of broilers at 42 days of age.

| | Tíbia | | | | | |
|---|---|---|---|---|---|---|
| | Mineral composition (g) | Area ($cm^2$) | Mineral density | Proximal epiphysis** | Diaphysis** | Distal epiphysis[1]** |
| | | | ($g/cm^2$) | | | |
| Treatments (T) | | | | | | |
| 37.5ºC | 0.218±0.18 | 8.92±0.84 | 0.024±0.02 | 0.021±0.02 | 0.042±0.03 | 0.011 (0.413) ±0.01 |
| 39ºC | 0.243±0.13 | 8.89±0.86 | 0.027±0.01 | 0.019±0.02 | 0.042±0.03 | 0.004 (0.408) ±0.01 |
| Ascorbic acid-39ºC | 0.267±0.20 | 9.50±1.00 | 0.027±0.02 | 0.037±0.04 | 0.053±0.04 | 0.011 (0.413) ±0.02 |
| Temperatures (TP) | | | | | | |
| Cold | 0.269±0.16ab | 9.23±0.94ab | 0.029±0.02 | 0.019±0.02 | 0.053±0.03ab | 0.010 (0.412) ±0.02 |
| Thermoneutral | 0.303±0.16a | 9.45±0.87a | 0.031±0.02 | 0.041±0.04 | 0.057±0.03a | 0.003 (0.407) ±0.00 |
| Hot | 0.152±0.15b | 8.58±0.77b | 0.17±0.02 | 0.016±0.02 | 0.027±0.03b | 0.012 (0.413) ±0.01 |
| Probability | | | | | | |
| T | 0.6232 | 0.0765 | 0.7532 | 0.0016 | 0.4754 | 0.5967 |
| TP | 0.0396 | 0.0161 | 0.0764 | 0.0001 | 0.0286 | 0.3498 |
| T x TP | 0.4736 | 0.5961 | 0.5779 | 0.0002 | 0.4629 | 0.0297 |
| CV (%) | 65.81 | 9.26 | 63.67 | 77.61 | 68.10 | 2.70 |

CV: variation coefficient. a-b: means followed by different letters in columns differ significantly by Tukey test at 5% probability. [1]comparison from log-transformed data (values in parentheses). **considering an area of 0.231 $cm^2$.

under hot and cold temperatures. This was also true for chickens from eggs not injected with AA and incubated both at 37.5°C and 39°C, but reared under thermoneutral temperature. For the distal epiphysis, the lowest values were observed in chickens from AA-injected eggs and incubated at 39°C, then reared under either thermoneutral or hot temperature (Table 7).

There was a significant effect ($p<0.05$) of rearing temperature on tibial calcium, phosphorus and ash percentages, with the lowest values determined in broilers reared under hot temperature. There was no influence of the treatments ($p>0.05$) on femur mineral composition (Table 8).

Significant effects of the evaluated treatments ($p<0.05$) on femur maximum strength and deformation were observed. Lower values were found in the birds derived from AA-injected eggs and incubated at 39°C and reared under hot temperatures (Table 9). Significant effects ($p<0.05$) were also found on tibial maximum strength, with the lowest values determined in chickens reared under hot temperature (Table 10). There were no effects of the treatments ($p>0.05$) for on femur and tibia rigidity.

## DISCUSSION

In the present study, chickens reared under the cold temperature presented higher weight gain and feed intake. This result is consistent with the results observed by Oliveira *et al.* (2006), and appears to be related to body temperature maintenance. However, the hot rearing temperature impaired bird performance, as shown by the lower feed intake and weight gain of the broilers reared under the hot temperature. Ain

**Table 7 –** Interactions between incubation treatments and rearing temperatures for the mineral density of the tibial proximal epiphysis and distal epiphysis of broilers at 42 days of age.

| Proximal epiphysis (g/cm²) | Temperatures (°C) | | | p |
|---|---|---|---|---|
| Treatments | Cold | Thermoneutral | Hot | |
| 37.5°C | 0.026±0.03 | 0.019±0.02b | 0.017±0.02 | 0.7668 |
| 39°C | 0.014±0.01 | 0.028±0.02b | 0.013±0.01 | 0.4291 |
| Ascorbic acid-39°C | 0.018±0.02B | 0.102±0.03Aa | 0.017±0.02B | <0.0001 |
| p | 0.6275 | <0.0001 | 0.9387 | |
| Distal epiphysis[1] (g/cm²) | Temperatures (°C) | | | p |
| Treatments | Cold | Thermoneutral | Hot | |
| 37.5°C | 0.000 (0.405)±0.00b | 0.008 (0.411)±0.00 | 0.024 (0.421)±0.02 | 0.0974 |
| 39°C | 0.002 (0.407)±0.00b | 0.000 (0.405)±0.00 | 0.012 (0.413)±0.01 | 0.5579 |
| Ascorbic acid-39°C | 0.028 (0.424) ±0.03Aa | 0.001 (0.406)±0.00B | 0.001 (0.406)±0.00B | 0.0264 |
| p | 0.0223 | 0.7523 | 0.1323 | |

a-b; A-B: means followed by different letters in columns and rows, respectively, differ significantly by Tukey test at 5% probability. [1] comparison from log-transformed data (values in parentheses).

**Table 8 –** Effect of incubation treatments and rearing temperatures on femur and tibia calcium, phosphorus and ash contents of broilers at 42 days of age.

| | Femur | | | Tibia | | |
|---|---|---|---|---|---|---|
| | Calcium | Phosphorus | Ash | Calcium | Phosphorus | Ash |
| Treatments (T) | (%) based on defatted DM | | | | | |
| 37.5°C | 14.91±1.20 | 7.37±0.61 | 40.51±3.43 | 15.13±0.94 | 7.49±0.45 | 41.93±2.61 |
| 39°C | 15.18±1.37 | 7.65±0.76 | 41.11±2.58 | 15.33±0.76 | 7.61±0.53 | 42.78±2.98 |
| Ascorbic acid-39°C | 15.09±1.15 | 7.66±0.52 | 41.90±3.36 | 14.88±0.99 | 7.53±0.57 | 42.46±3.28 |
| Temperatures (TP) | | | | | | |
| Cold | 15.02±1.30 | 7.59±0.66 | 41.66±3.20 | 15.57±0.98a | 7.73±0.52a | 43.53±2.72a |
| Thermoneutral | 15.42±1.08 | 7.72±0.53 | 41.96±3.75 | 15.28±0.73a | 7.62±0.43ab | 43.16±2.26a |
| Hot | 14.73±1.25 | 7.37±0.69 | 39.94±2.67 | 14.45±0.58b | 7.24±0.46b | 40.38±2.84b |
| Probability | | | | | | |
| T | 0.8312 | 0.3858 | 0.5040 | 0.1848 | 0.7775 | 0.5984 |
| TP | 0.3214 | 0.3205 | 0.1931 | 0.0018 | 0.0241 | 0.0073 |
| T x TP | 0.4525 | 0.5225 | 0.6141 | 0.6001 | 0.2002 | 0.4521 |
| CV (%) | 8.24 | 8.42 | 7.81 | 5.15 | 6.18 | 6.27 |

CV: variation coeffecient. a-b: means followed by different letters in columns differ significantly by Tukey test at 5% probability.

**Table 9 –** Effect of the incubation treatments and rearing temperatures on the femoral bone resistance of broilers at 42 days of age.

| | Femur | | |
|---|---|---|---|
| | Maximum force (N) | Deformation at maximum force (mm) | Rigidity (N/mm) |
| Treatments (T) | | | |
| 37.5°C | 141.85±27.70 | 2.57±0.40ab | 84.99±28.11 |
| 39°C | 158.28±38.84 | 2.95±0.65a | 80.50±29.14 |
| Ascorbic acid-39°C | 156.38±37.35 | 2.42±0.78b | 82.14±27.80 |
| Temperatures (TP) | | | |
| Cold | 156.97±29.56a | 2.87±0.71 | 81.40±30.17 |
| Thermoneutral | 170.33±38.13a | 2.58±0.78 | 91.71±26.38 |
| Hot | 129.21±23.94b | 2.49±0.39 | 74.52±25.64 |
| Probability | | | |
| T | 0.2700 | 0.0432 | 0.9008 |
| TP | 0.0020 | 0.1842 | 0.2333 |
| T x TP | 0.1726 | 0.0652 | 0.1854 |
| CV (%) | 19.64 | 21.71 | 32.97 |

CV: variation coeffecient. a-b: means followed by different letters in columns differ significantly by Tukey test at 5% probability.

Baziz *et al.* (1996) and Geraert et al. (1996) obtained similar results in their studies on the effect of heat stress during broiler growth. Furthermore, none of the incubation treatments prevented or minimized the effects of heat stress during rearing, indicating that egg incubation at 39°C, independently of ascorbic acid (AA) injection, did not produce an effective epigenetic heat adaptation in broilers.

Locomotion disorders hinder broilers' ability to access feed and water. Birds with locomotion disorders can suffer starvation and dehydration. Furthermore, according to Almeida Paz (2008b), starvation and dehydration, in addition of impairing performance, may hamper intestinal broiler development, leading to further performance losses. Therefore, broiler tibia and femur development and their study are of paramount importance.

The tibia has been used in the evaluation of bone metabolic disorders, but the development in this bone is centripetal, with maturation from the gradient distal (end) to the proximal limb bones. Therefore, the femur is more important because, according to Kwakkel *et al.* (1998), the mineralization rate and other bone development aspects occur more slowly in the femur than in the tibia. This is because the femur is closer to the center of the body and therefore, it reflect more clearly the relation g between fast weight gain and leg problems (Barbosa *et al.,* 2010).

Bone characteristics of the chickens were negatively influenced by the hot rearing temperature. These birds presented lower femur width, tibia and femur weight, tibia length, femur and tibia mineral content and area, and tibia shaft values. These results are consistent with the findings of Bruno *et al.* (2000), who reported that

**Table 10 –** Effects of incubation treatments and rearing temperatures on tibial bone resistance of broilers at 42 days of age.

| | Tibia | | |
|---|---|---|---|
| | Maximum force (N) | Deformation at maximum force (mm) | Rigidity (N/mm) |
| Treatments (T) | | | |
| 37.5°C | 198.79±59.37 | 2.33±0.28 | 113.42±55.48 |
| 39°C | 177.19±47.28 | 2.38±0.30 | 104.30±31.53 |
| Ascorbic acid-39°C | 177.20±61.29 | 2.52±0.29 | 96.99±26.37 |
| Temperatures (TP) | | | |
| Cold | 201.42±47.65a | 2.44±0.28 | 119.48±39.59 |
| Thermoneutral | 207.01±50.07a | 2.50±0.20 | 109.23±45.93 |
| Hot | 144.56±50.92b | 2.27±0.35 | 88.57±28.47 |
| Probability | | | |
| T | 0.3290 | 0.1371 | 0.5473 |
| TP | 0.0011 | 0.0711 | 0.0925 |
| T x TP | 0.0546 | 0.7397 | 0.1698 |
| CV (%) | 24.81 | 11.68 | |

CV: variation coeffecient. a-b: means followed by different letters in columns differ significantly by Tukey test at 5% probability.

broilers reared under heat stress conditions showed a decrease in tibia and femur length and width.

The environmental conditions of broiler houses can trigger locomotion problems (Nääs, 2008). This is due to the fact that during heat stress, body electrolyte balance is compromised, affecting bone development and contributing for the increase in the incidence of leg problems in young broiler chickens (Patience, 1990). According to Simons *et al.* (1987), birds under metabolic acidosis have a higher incidence of leg problems than birds in metabolic balance. However, the exact mechanism of the influence of the acid-base balance on bone calcification is not fully elucidated. Sauver & Mongin (1974) suggest that during metabolic acidosis the hydroxylation of vitamin $D_3$ into its biologically active form, 1.25-dihydroxycholecalciferol, is reduced, and may influence bone mineralization. Vitamin $D_3$ helps stimulating intestinal calcium absorption, thereby increasing bone osteoclast recruitment, promoting protein synthesis in the osteoblasts, in addition of being involved in bone matrix mineralization (Fernandes, 2005).

According to Bushinky & Sessler (1992), respiratory alkalosis causes greater complexation of calcium by blood proteins, consequently reducing the free ionized calcium available for bone deposition. However, t rearing temperature did not influence blood ionized calcium levels, as reported in another article on the present experiment (Sgavioli *et al.,* Submitted). According to Vargas *et al.* (2004), calcium and phosphorus are the most essential minerals for bone formation, since 98% of the calcium and 80% of the phosphorus in the body are found in the bones. Free ionized calcium reduction may impair bone calcification process, favoring an increase in the area of the hypertrophic zone, whereas chondrocytes remain pre-hypertrophic when there is poor calcification, resulting in an accumulation of cartilaginous, non-calcified masses in this area. This is a characteristic of tibial chondrodysplasia.

Rearing temperature negatively influenced tibia calcium, phosphorus, and ash contents. Sahin *et al.* (2006) demonstrated that Japanese quails (*Coturnix japonica coturnux*) exposed to cyclic heat stress (34°C, from 9:00 am to 5:00 pm, from the 10th to 42nd day of age) also presented low tibial calcium, phosphorus, magnesium and manganese concentrations due to an increased excretion of these minerals, impairing bone mineralization.

Biomechanical parameters are direct indicators of bone quality and include by bone density (Barreiro *et al.,* 2011) and bone maximum flexibility strength (Reis *et al.,* 2011). Heat-stressed birds develop metabolic acidosis, which first stimulates mineral dissolution and then the cell-mediated bone resorption due to the increased calcium excretion by the kidneys (Riond, 2001; Oliveira *et al.,* 2010). This calcium loss results in reduced bone mineralization and may affect mechanical quality of the bones. Bone calcium content is also associated with bone fragility. Bone maximum strength was reduced in the broilers submitted to hot temperature, indicating lower tibia and femur strength to impact.

Thorp & Waddington (1997), studying the relationships between bone pathologies and ash and mineral content of the long bones of35-day-old broiler chickens, observed that bones with lower calcium concentration presented more fractures during processing, evidencing that these bones have lower maximum force. Crespo *et al.* (2002) also found a higher incidence of femur fractures in adult turkeys with low bone calcium content. The relative amounts and properties of calcium, also known as the organic matrix, determine the mechanical strength.

## CONCLUSION

The *in-ovo* injection of ascorbic acid of eggs incubated at high temperature (39ºC) did not alleviate the effects of heat stress on during rearing. Broilers reared under high environmental temperature (heat treatement) presented lower weight gain and feed intake, and lower rates of bone development compared with those reared under cold and thermoneutral temperatures, independently of incubation temperatures or *in-ovo* AA injection.

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