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## Short Communication

# Anionic variation of diets on bone characteristics of broilers at 7 and 14 days of age

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**ABSTRACT** - The objective of this study was to evaluate the chemical composition and bone strength of the tibiotarsus of broilers at 7 and 14 days of age. Bone percentages of collagenous protein (CPr), non-collagenous protein (NCPr) and minerals (calcium, phosphorus, potassium and sodium) were determined. The experiment utilized 650 male Cobb chicks at one day of age, fed corn and soybean meal-based diets. The experimental design was of random blocks with five treatments, five replicates and 26 birds per experimental unit. The treatments consisted of a basal diet supplementated with $NH_4Cl$ to obtain five levels (-50, 0, 50, 100 and 150 mEq/kg) of electrolyte balance (EB). The levels of EB influenced the following characteristics: ash and potassium content; Ca:P ratio and the breaking strength (at 7 days); and phosphorus and potassium contents and breaking strength (at 14 days). At the starter phase of rearing, the supply of acidogenic diets affected the concentrations of potassium and phosphorus in the bone tissue, without altering the organic fraction.

Key Words: electrolyte balance, locomotor disorders, metabolic acid, tibiotarsus

## Introduction

The skeletal biology has been the subject of study for several years, mainly because of its influence on health and welfare of the animals, associated with the importance for the growth of broilers from strains selected for the food industry (Knott & Bailey, 1999).

Locomotion is the main part of the vital activities which, strictly speaking, should not be hindered by structural dysfunctions, with consequent discomfort and pain. Adequate bone mineralization is essential in poultry production because muscle development depends on a good bone support and is necessary for the proper functioning of the locomotor system (Schoulten et al., 2003).

The acid-base balance or electrolyte balance (EB) is defined as the difference between the main cations and anions in the diet. The acid-base balance is directly related to the electrolytes ingested by birds (Vieites et al., 2004). The primary role of electrolytes is to regulate the maintenance of body water and the ionic balance of the body (Ahmad & Sarwar, 2006). The EB can influence growth, appetite, bone development, response to heat stress and the metabolism of certain nutrients, such as amino acids, minerals and vitamins (Patience, 1990).

The optimal balance of minerals can improve animal performance and reduce leg problems in broilers (Oviedo Rondón et al., 2001). The incidence of tibial dyschondroplasia increases with excess of anion $Cl^-$ unbalanced with the cations $Na^+$ or $K^+$ (Leach & Nesheim, 1972).

The objective of this study was to evaluate the influence of low levels of dietary electrolyte balance on the concentration of collagenous protein and non-collagenous proteins, breaking strength, and the mineral composition of the tibiotarsi of broilers in the early stages of growth.

## Material and Methods

The chemical composition and bone strength of the tibiotarsi of broilers at 7 and 14 days of age were evaluated. Contents of collagenous protein (CPr), non-collagenous protein (NCPr) and minerals (calcium, phosphorus, potassium and sodium) were determined.

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The tibiotarsi evaluated in this study were obtained from an experiment conducted at the Poultry Section of Instituto Federal de Edução, Ciência e Tecnologia de Mato Grosso. Six hundred and fifty male Cobb chicks were used.

Birds were raised from 1 to 14 days of age in. Birds were housed in 25 pens of 3.2 × 1.46 m (4.67 $m^2$) within a facility with ceiling height of 3.0 m. A randomized block experimental design with five treatments (levels of electrolyte balance), five replications and 26 birds per experimental unit was used. Each block was composed of a replicate of each treatment. This distribution was chosen for greater uniformity in the plots in the experimental facility concerned to the position of the boxes.

During the experimental period, a program of continuous light (24 hours of daylight + artificial light) was used. The temperature and relative air humidity were measured with a maximum and minimum thermometer and a dry and wet bulb thermometer, respectively. Maximum and minimum temperatures of 30 ºC and 23 ºC, respectively, were recorded during the experimental period, with an average relative humidity of 67%.

The basal diet consisted of corn and soybean meal to meet nutritional recommendations according to Rostagno at al. (2000) for the initial phase, comprising the period from 1 to 14 days, with electrolyte balance of 150 mEq/kg (Table 1). Animals were distributed uniformly in pens at one day of age and weighing 47 grams. Ammonium chloride ($NH_4CI$) was added to each basal diet, replacing the inert material in order to achieve five levels of EB (Table 2).

Values of EB were calculated through the formula suggested by Mongin (1981):

$$EB = (\%Na^+ \times 100/22.990 *) + (\% K^+ \times 100/39.102 *) – (\%Cl^- \times 100/35.453 *)$$

*(Equivalent gram of $Na^+$, $K^+$, or $Cl^-$ , respectively)

One bird at 7 and 14 days of age of each plot with the average weight of the experimental unit was sacrificed through cervical dislocation, and had the tibiotarsi of both legs removed and adherent tissues cleaned off. Bones were then identified and frozen at 5º C.

Laboratorial tests were conducted at the Laboratory of Animal Biochemistry, of Universidade Federal de Viçosa. To determine bone strength, left leg bones were thawed at room temperature and then sent to a bending test, which used a mechanical universal testing machine INSTRON-model 4204. All the bones were tested in the same position, with their ends laying on two supports appropriately separated according to their lengths, and a load was applied into the center (in the bone diaphysis region) at a constant speed of 10 mm/min.

After determination of bone strength by using the unit of force in Newton (N/mm), the tibiotarsi were cut lengthwise, removing the bone marrow with a flow of distilled and deionized water. After that, they were defatted with petroleum ether in Soxhlet for 12 hours to determine

Table 1 - Composition of experimental diets

| Ingredient | Starter diet (g/kg of natural matter) |
|---|---|
| Corn | 544.54 |
| Soybean meal | 361.79 |
| Soybean oil | 37.24 |
| Limestone | 9.77 |
| Dicalcium phosphate | 18.25 |
| DL-methionine 99% | 2.30 |
| L-lysine HCl 98% | 1.53 |
| Salt | 4.56 |
| Potassium carbonate | - |
| Ammonium chloride | 3.31 |
| Choline chloride 60% | 0.80 |
| Vitamin premix[1] | 1.00 |
| Mineral mix[2] | 0.50 |
| Virginiamycin[3] | 0.50 |
| Anticoccidial[4] | 0.50 |
| Antioxidant[5] | 0.10 |
| Washed sand (inert) | 13.31 |
| Total | 1,000.0 |
| Nutritional requirement | |
| Metabolizable energy, kcal/ kg | 3,000 |
| Crude protein, g/kg | 214.0 |
| Calcium, g/kg | 9.60 |
| Available phosphorus, g/kg | 4.50 |
| Sodium, g/kg | 2.22 |
| Potassium, g/kg | 8.29 |
| Chlorine, g/kg | 5.63 |
| Digestible arginine, g/kg | 13.36 |
| Glycine + serine, g/kg | 20.24 |
| Digestible methionine + cystine, g/kg | 8.07 |
| Digestible lysine, g/kg | 11.43 |
| Digestible threonine, g/kg | 7.17 |
| Digestible tryptophan, g/kg | 2.42 |
| Electrolyte balance, mEq/kg | 150 |

Supplementation of vitamin, minerals and additives (per kg of product):
[1] Vitamin A - 10,000,000 IU; vitamin D3 - 2,000,000 IU; vitamin E - 30,000 IU; vitamin B1 - 2.0 g; vitamin B6 - 4.0 g; pantothenic acid - 12.0g; biotin - 0.10 g; vitamin K3 - 3.0 g; folic acid - 1.0 g; nicotinic acid, 50.0g; vitamin B12 - 15,000 mcg, selenium - 0.25 g; excipient q.s. - 1.000 g.
[2] Roligomix (Roche). Guaranteed levels per kg of product: manganese 16.0 g; iron - 100.0 g; zinc - 100.0 g; copper - 20.0 g; cobalt - 2.0 g; iodine - 2.0 g; excipient q.s. - 1.000 g.
[3] Stafac® - 50%.
[4] Coxistac® (salinomycin) - 12%.
[5] Butylated hydroxytoluene.

Table 2 - Treatments consisting of experimental diets (starter and growth) supplemented with $NH_4Cl$

| EB mEq/kg | Basal diet (kg) | Inert (g) | $NH_4Cl$ (g) | Total (kg) |
|---|---|---|---|---|
| - 50 | 98.669 | 255 | 1076 | 100 |
| 0 | 98.669 | 524 | 807 | 100 |
| 50 | 98.669 | 793 | 538 | 100 |
| 100 | 98.669 | 1062 | 269 | 100 |
| 150 | 98.669 | 1331 | 0 | 100 |

$NH_4Cl$ - molecular weight (AMU) = 53.45; 99.5% purity.
EB - electrolytic balance.

the concentrations of NCPr and CPr. Subsequently, tibiae were demineralized with a solution of disodium salt of EDTA (ethylenediaminetetraacetic acid tetracetic) 0.5 M and pH 8.2, according to the method proposed by Hauschka & Gallop (1977), for extraction of NCPr, whose molecular weights are less than 10 kilodalton. Oxalic acid was used at the end of the analytical procedures to be an indicator of complete bone demineralization.

The proteins were quantified in EDTA solution following the method of Bradford (1976), using bovine serum albumin as standard. After being degreased and demineralized, bones were washed with distilled and deionized water for the extraction of excess of EDTA, and then they were used to determine the level of CPr by the method of Robins (1995) to estimate total nitrogen. The CPr content was obtained by multiplying the nitrogen content by the 6.25 factor.

The right tibiotarsi were subsequently thawed and subjected to a temperature of 105 ºC for six hours and then defatted with hexane in a Soxhlet apparatus for four hours. After extraction of fat, the bones were dried once more in forced-ventilation oven at 105 ºC for 16 hours to obtain the dry and deffated weight. After, they were crushed and calcined in muffle furnace at 600 °C for two hours in order to determine ash, following the methodology of Silva & Queiroz (2002).

The ash was used to prepare mineral solution by being dissolved in hydrochloric acid solution in its purest form (1:1) following the methodology of Silva & Queiroz (2002) for concentrates. After obtaining this solution, calcium, potassium and sodium in the bones were obtained through the Optical Emission Spectrometry technique with Inductively Coupled Plasma source (ICP-OES) with the Perkin Elmer simultaneous spectrometer (Optima 3300 DV). The percentage of phosphorus in the tibiotarsi was determined by colorimetry, using a self-parametric analyzer device (Alizee) from Synermed®.

Percentages of NCPr and CPr were calculated based on the weight of dry bone and fat. The values of minerals were expressed as a percentage of ash weight, and the ratio between calcium and phosphorus was obtained by dividing the percentage of calcium by phosphorus in the ash.

Data were statistically analyzed on the program SAEG (System for Statistical Analysis and Genetics, version 7.1). The data were submitted to analysis of variance and regression according to methodology of Banzato & Kronka (1992).

## Results and Discussion

The results of the traits evaluated in broilers at seven days of age were influenced by the levels of EB for the following characteristics: ash and potassium, Ca:P ratio and resistance to breakage (Table 3).

The ash content in the tibiotarsi showed linear growth ($P<0.01$; Equation: $\hat{Y} = 33.1259 + 0.0214761X$; $r^2 = 0.97$) as the inclusion of $NH_4Cl^-$ in the diets was reduced, confirming the negative effect of the acidification of a diet under bone mineralization during this phase. Previous studies had already shown that the major locomotive disorder of young birds, Tibial Dicondroplasia, among other factors, is related to the dietary electrolyte balance (Mongin & Sauveur, 1977; Sauveur, 1984). Leeson & Summers (2001) reported that metabolic acidosis promoted by the diet of acidogenic products such as $NH_4Cl$ influenced negatively the normal development and maturation of the growth plate of the epiphyseal cartilage.

The potassium content showed linear effect ($P<0.05$; Equation: $\hat{Y} = 2.3878 + 0.00284204X$; $r^2 = 0.40$); however, the behavior of this mineral in this phase is not well-explained by the equation because it presented a low coefficient of determination, reflecting that not much of this trait can be explained by a decrease of studied EB levels.

Table 3 - Effect of levels of electrolyte balance on bone characteristics of birds at seven days of age

| Characteristics | Eletrolytic balance (mEq/kg) | | | | | CV (%) | Effect | |
|---|---|---|---|---|---|---|---|---|
| | -50 | 0 | 50 | 100 | 150 | | Linear | Quadratic |
| Ash (mg/g) | 319.6 | 335.4 | 337.7 | 352.8 | 364.5 | 5.30 | 0.001 | 0.121 |
| Calcium (mg/g) | 265.9 | 240 | 237 | 235.1 | 259.6 | 16.80 | 0.089 | 0.163 |
| Phosphorus (mg/g) | 261 | 221.3 | 240.6 | 257.1 | 246.1 | 14.13 | 0.180 | 0.885 |
| Potassium (mg/g) | 21.8 | 27.1 | 21.1 | 28.1 | 28.4 | 14.41 | 0.014 | 0.231 |
| Sodium (mg/g) | 6.0 | 1.3 | 5.7 | 4.9 | 4.0 | 31.41 | 0.073 | 0.326 |
| Calcium:phosphorus ratio | 1.02 | 1.08 | 0.98 | 0.91 | 1.05 | 5.69 | 0.070 | 0.021 |
| Breaking strength (N/mm) | 3.10 | 3.46 | 2.75 | 2.50 | 2.28 | 26.66 | 0.023 | 0.247 |
| Collagenous protein (mg/g) | 416 | 468.1 | 498.2 | 404.3 | 450.3 | 21.04 | 0.115 | 0.748 |
| Non-collagenous protein (mg/g) | 7.8 | 8.0 | 6.2 | 9.2 | 7.8 | 16.95 | 0.428 | 0.427 |

CV - coefficient of variation.

Contrarily to expectations, there was a decrease in the maximum load supported by the bones as the diets were getting higher levels of electrolytic balance ($P<0.05$; Equation: $\hat{Y} = 3.093 – 0.005356X$; $r^2 = 0.79$). Currey (2003) reported that the mechanical property of bone is determined by the interaction between mineral content, water and the property and the amount of organic matrix, especially of type I collagen.

Quadratic effect ($P<0.05$; Equation: $\hat{Y} = 1.07097 – 0.00107939X + 0.00000743852X^2$; $R^2 = 0.88$) was found for the Ca:P ratio in the bones, in which the level of EB was estimated to be 73 mEq/kg, corresponding to lower Ca:P ratio (1.03). In the formation of hydroxyapatite bone crystal, this ratio is within the range of 2:1. It is clear that this ratio varies according to age, and is influenced by nutritional factors. Williams et al. (2000) showed that this ratio varies according to the age of broilers and among genetic strains. The authors found a Ca:P ratio ranging from 1.8:1 to 3.9:1 in birds at 18 days of age, concluding that the type of bone, quantity, distribution and the ratio between collagen and mineral is more important for the locomotor system than the exact shape of the bone crystal.

Significant levels of EB were found for the percentage of phosphorus and potassium in in the ash of birds at 14 days of age, as well as breaking strength (Table 4).

The content of phosphorus showed negative linear relationship ($P<0.05$; Equation: $\hat{Y} = 21.7132 – 0.00711288X$; $r^2 = 69$) as the diets were acidified, and the lower level of EB corresponded to the greater percentage of this element in the bones. According to the description of the ash content in birds of up to seven days of age (Table 4), anionic diets reduce bone mineralization, which may affect calcium and phosphorus. However, the literature is not well-versed on the influence of diet on the deposition of mineral in the skeleton of birds.

Tardin (1995) reports that deficiency of phosphorus in the diets results in hypophosphatemic raquitism, causing the accumulation of hypertrophied chondrocytes, which, according to Murakami (2000), characterizes tibial dyschondroplasia. On the other hand, Riddell & Pass (1987) concluded that excess of phosphorus in diets causes thickening in the area of bone growth in broiler chickens at two weeks of age, which may lead to the development of tibial dyschondroplasia. The levels of phosphorus used in this study met the requirements for maximum growth of high-performance broiler chickens. Therefore, one can assume that anionic diets increase the percentage of phosphorus in the bones.

In relation to potassium concentration, quadratic effect ($P<0.05$; Equation: $\hat{Y} = 2.51427+0.000714042X – 0.0000260361X^2$; $R^2 = 0.61$) was found, in which the maximum deposition of bone mineral (2.52%) corresponded to EB of 14 mEq kg. According to Patience (1990), acid-base disorders affect the metabolism of potassium. This author observed that in acidosis, there is translocation of potassium from the cell to the extracellular fluid, whereas in alkalosis, the effect is opposite. Because there is proton inflow to the cells in this condition, the output of potassium is necessary for the maintenance of their electrical neutrality.

It is clearly demonstrated that at both ages, the behavior of potassium against the alteration of electrolyte balance is related to increased chlorine levels in the diet. It is very likely that the bone tissue and other tissues will store or eliminate more potassium in function of the chloride concentration in the body. Austic, cited by Leeson & Summers (2001), showed that the variation in the Cl:K ratio in the diet negatively influences the severity of the imbalance of arginine:lysine, which hinders the growth of birds. More studies are needed to set the correct concentration of potassium in the tibiotarsus of broilers at different ages and different nutritional conditions.

Unlike birds at seven days of age, breaking strength increased as the levels of dietary electrolyte balance

Table 4 - Effect of the levels of electrolyte balance on bone characteristics of birds at 14 days of age

| Characteristics | Eletrolytic balance levels (mEq/kg) | | | | | CV (%) | Effect | |
|---|---|---|---|---|---|---|---|---|
| | -50 | 0 | 50 | 100 | 150 | | Linear | Quadratic |
| Ash (mg/g) | 385 | 392.7 | 415.7 | 370.3 | 417.8 | 4.91 | 0.137 | 0.121 |
| Calcium (mg/g) | 266.5 | 275.4 | 266.6 | 258.8 | 226.1 | 9.78 | 0.150 | 0.069 |
| Phosphorus (mg/g) | 223.5 | 214.2 | 209.9 | 215 | 205.3 | 4.88 | 0.028 | 0.233 |
| Potassium (mg/g) | 24.8 | 24.2 | 23.8 | 25.6 | 19.3 | 9.56 | 0.058 | 0.027 |
| Sodium (mg/g) | 6.4 | 7.9 | 4.4 | 5.4 | 6.8 | 28.92 | 0.417 | 0.182 |
| Calcium:phosphorus ratio | 1.19 | 1.28 | 1.35 | 1.270 | 1.10 | 7.99 | 0.297 | 0.083 |
| Breaking strength (N/mm) | 7.78 | 8.79 | 10.17 | 13.68 | 11.95 | 16.87 | 0.001 | 0.272 |
| Collagenous protein (mg/g) | 341.4 | 397.6 | 385.8 | 316.6 | 356 | 9.31 | 0.289 | 0.123 |
| Non-collagenous protein (mg/g) | 7.8 | 7.5 | 6.5 | 6.8 | 6.8 | 15.76 | 0.101 | 0.298 |

CV - coefficient of variation.

became higher: Linear effect, $P<0.01$; Equation: $\hat{Y} = 9.153+0.026452X$; $r^2 = 0.77$). The physical characteristics and strength are well used by nutritionists to evaluate bone mineralization (Crenshaw et al., 1981). Rath et al. (2000) stated that many factors that can influence bone strength, such as: anti-nutrients and toxins, growth and age, gender, nutrition, physical load, endocrinology, genetics and disease. Because of the divergence of results for this variable at different ages, some hypotheses can be remarked: first, in these stages, the bone tissue is going through major changes due to higher rates of mineralization (Angel, 2007), so it should not present standard behavior until it reaches the maximum mineralization. Secondly, the resistance was modulated by physical load, since, in the performance assay (Vieites et al., 2010), the weight of broilers at 14 days decreased linearly as the electrolyte balance of the diet was reduced.

## Conclusions

Anionic diets influence the concentration of potassium and phosphorus in the bones, but do not change the organic fraction. Further studies are needed to correlate the mineral composition of bones with the organizational structure and its implications with the mechanical characteristics of this tissue.

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