# Dietary calcium or phosphorus deficiency impairs the bone development by regulating related calcium or phosphorus metabolic utilization parameters of broilers

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**ABSTRACT** An experiment was conducted to investigate the effect of dietary calcium (**Ca**) or phosphorus (**P**) deficiency on bone development and related Ca or P metabolic utilization parameters of broilers. A total of 504 one-day-old Arbor Acres male broilers were randomly assigned to 1 of 4 treatments with 7 replicates of 18 birds per replicate in a completely randomized design. A 2 (Ca levels: 1.00 and 0.35%) × 2 (nonphytate P [**NPP**] levels: 0.45 and 0.23%) factorial arrangement of treatments was adopted in the 21-day trial. The 4 treatments were the Ca- and P-adequate diet (1.00% Ca + 0.45% NPP), the Ca-deficient diet (0.35% Ca + 0.45% NPP), the P-deficient diet (1.00% Ca + 0.23% NPP), and the Ca- and P-deficient diet (0.35% Ca + 0.23% NPP). The greatest impact on tibia bone mineral density, bone breaking strength, and ash content was in the P-deficient diets, especially in broilers fed with the Ca-adequate diet, whereas adequate P and reduced Ca reduced ($P < 0.05$) these parameters compared with adequate Ca and P, but not to the same level as P deficiency. Furthermore, dietary Ca or P deficiency, especially adequate Ca and P deficiency decreased ($P < 0.05$) serum P, 25-hydroxyvitamin $D_3$ (**25-OHD$_3$**) contents, and tibia ash Ca and P contents but increased ($P < 0.05$) the serum Ca content and tibia alkaline phosphatase (**ALP**) activity compared with adequate Ca and P. The results from this study indicated that the bone development and Ca or P metabolic utilization parameters of broilers were the most sensitive ones to dietary P deficiency, followed by dietary Ca deficiency or Ca and P deficiencies. Dietary P deficiency impaired the bone development by increasing serum Ca content and tibia ALP activity but decreasing serum P, 25-$OHD_3$ contents, and tibia ash Ca and P contents of broilers. Dietary Ca deficiency impaired bone development by increasing serum Ca content, tibia ALP activity, and tibia ash P content but decreasing serum P, 25-$OHD_3$ contents, and tibia ash Ca content of broilers.

**Key words:** calcium deficiency, phosphorus deficiency, bone development, metabolic utilization parameter, broiler

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## INTRODUCTION

As essential minerals, calcium (**Ca**) and phosphorus (**P**) play important and extensive roles in nucleic acid synthesis, energy metabolism, muscle contraction, enzyme activity, signal transduction, and bone mineralization (Li et al., 2017). Considering that these 2 minerals are major inorganic components of bone, the deficiency in Ca or P could result in leg weakness and, if severe enough, increase morbidity and mortality (Edwards, 2000; Venalainen et al., 2006). Over the past decades, constant improvements in genetic selection and nutrition have led to a fast growth rate in broilers. Unfortunately, early fast growth rate in broilers generally exacerbates skeletal diseases (such as rickets) associated with dietary inadequate supply or imbalances of Ca and P (Williams et al., 2000a, b; Dinev, 2012; Shao et al., 2019b).

Many studies have demonstrated that severe Ca or P deficiency could cause poor mineralization (Jiang et al., 2013; Valable et al., 2017). Bone development indices,



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such as bone mineral density (**BMD**), bone breaking strength (**BBS**), and bone ash content, are commonly used response indices for assessing bone mineralization of broilers (Onyango et al., 2003; Park et al., 2003; Kim et al., 2006). In addition, Ca and P concentrations in serum and bones can reflect the changes of Ca and P homeostasis, and their contents within a normal range are important for normal physiological function and optimal bone mineralization (Proszkowiec-Weglarz and Angel, 2013). As a Ca and P metabolic regulator, vitamin $D_3$ is involved in the absorption and utilization of Ca and P and thus required for normal growth and bone development in chickens (Christakos et al., 2011; Garcia et al., 2013; Shao et al., 2019c). Generally, 25-hydroxyvitamin $D_3$ (**25-OHD$_3$**) is the storable and stable form of vitamin $D_3$ in the circulation of chickens (Klasing, 1998). Alkaline phosphatase (**ALP**) is a hydrolase involved in the process of Ca and P deposition during bone mineralization and formation (Li et al., 2014), and its activity is directly related to the rate of bone mineralization in broilers (Tilgar et al., 2008; Shao et al., 2019a). However, it is still not clear whether the influence of dietary Ca or P deficiency on the bone development is related to the aforementioned Ca or P metabolic utilization parameters (Ca and P concentrations in serum and bone, serum 25-OHD$_3$, and ALP activities in serum and bone) of broilers. It was hypothesized that dietary Ca or P deficiency might impair the bone development of broilers by regulating related Ca or P metabolic utilization parameters. Therefore, the objective of the present study was to investigate the effect of dietary Ca or P deficiency on growth performance, bone development, and related Ca or P metabolic utilization parameters of broilers to test the aforementioned hypothesis.

## MATERIAL AND METHODS

All experimental procedures were approved by the Animal Management Committee (in charge of animal welfare issue) of Institute of Animal Science, Chinese Academy of Agricultural Sciences (Beijing, China) and performed in accordance with the guidelines. Ethical approval on animal survival was given by the Animal Ethics Committee of Institute of Animal Science, Chinese Academy of Agricultural Sciences.

### Experimental Design and Treatments

A completely randomized design involving a 2 (dietary Ca levels) × 2 (dietary nonphytate P [**NPP**] levels) factorial arrangement of treatments was used in this experiment. The 2 dietary Ca levels were a normal Ca level of 1.00% and a low Ca level of 0.35%. The 2 dietary NPP levels were a normal NPP level of 0.45% and a low NPP level of 0.23%. Thus, there were a total of 4 dietary treatments, including the Ca- and P-adequate diet (1.00% Ca + 0.45% NPP), the Ca-deficient diet (0.35% Ca + 0.45% NPP), the P-deficient diet (1.00% Ca + 0.23% NPP), and the Ca- and P-deficient diet (0.35% Ca + 0.23% NPP).

### Birds and Diets

A total of 504 one-day-old Arbor Acres male broiler chicks (Huadu Broiler Breeding Corp., Hebei, PR China) were randomly allotted by body weight to 1 of 4 treatments with 7 replicate cages of 18 chicks per replicate cage for each treatment. The birds were housed in an electrically heated, thermostatically controlled room with fiberglass feeder (150 cm, length) and stainless-steel cages (160 × 90 × 75 cm, length × width × height) coated with plastic, and maintained on a continuous 18-h light and 6-h of dark schedule for 21 D. Environmental temperature was maintained at 35°C for the first 3 D, after which the temperature was gradually reduced by 3°C per week until it reached 24°C and then was maintained at this temperature until the end of the experiment. The light intensity of 20 lux was used during the entire experimental period. Feed and tap water were available ad libitum, and the tap water contained no detectable Ca or P. The basal corn–soybean meal diet (Table 1) was formulated to meet or exceed the nutrient requirements (NRC, 1994) for broilers, except for Ca and P. Dietary treatments included the basal diets supplemented with Ca and P in the form of $CaHPO_4 \cdot 2H_2O$, $Ca(H_2PO_4)_2 \cdot H_2O$, and limestone. Mortality was recorded daily, and chick weight

**Table 1.** Composition and nutrient levels of experimental diets (as-fed basis).

| | Ca levels, % | | | |
|---|---|---|---|---|
| | 1.00 | | 0.35 | |
| | NPP levels, % | | | |
| Items | 0.45 | 0.23 | 0.45 | 0.23 |
| Ingredients, % | | | | |
| Corn | 53.42 | 53.42 | 53.42 | 53.42 |
| Soybean meal | 38.10 | 38.10 | 38.10 | 38.10 |
| Soybean oil | 4.42 | 4.42 | 4.42 | 4.42 |
| NaCl[1] | 0.30 | 0.30 | 0.30 | 0.30 |
| $CaHPO_4$[1] | 1.82 | 0.50 | - | 0.50 |
| $Ca(H_2PO_4)_2$[1] | - | - | 1.36 | - |
| Limestone[1] | 1.32 | 2.00 | - | 0.23 |
| DL-Methionine[1] | 0.30 | 0.30 | 0.30 | 0.30 |
| Premix[2] | 0.32 | 0.32 | 0.32 | 0.32 |
| Sand[3] | - | 0.64 | 1.79 | 2.41 |
| Nutrient composition, % | | | | |
| Metabolizable energy, MJ/kg | 12.6 | 12.6 | 12.6 | 12.6 |
| Crude protein[4] | 22.42 | 22.11 | 22.17 | 22.07 |
| Lysine | 1.12 | 1.12 | 1.12 | 1.12 |
| Methionine | 0.61 | 0.61 | 0.61 | 0.61 |
| Methionine + Cysteine | 0.90 | 0.90 | 0.90 | 0.90 |
| Ca[4] | 0.95 | 0.97 | 0.36 | 0.34 |
| Total P[4] | 0.69 | 0.48 | 0.71 | 0.46 |
| Non-phytate P (NPP)[4] | 0.41 | 0.27 | 0.43 | 0.24 |

[1]Feed grade.

[2]Provided per kilogram of diets: 15,000 IU vitamin A (all-retinol acetate); 4,500 IU cholecalciferol; 24 IU vitamin E (all-rac-α-tocopherol acetate); 3 mg vitamin K (menadione sodium bisulfate); 3 mg thiamin (thiamin mononitrate); 9.6 mg riboflavin; 3 mg vitamin $B_6$; 0.018 mg vitamin $B_{12}$; 15 mg pantothenic acid calcium; 39 mg niacin; 1.5 mg folic acid; 0.15 mg biotin; 700 mg choline (choline chloride); 8 mg Cu ($CuSO_4 \cdot 5H_2O$); 110 mg Mn ($MnSO_4 \cdot H_2O$); 40 mg Fe ($FeSO_4 \cdot H_2O$); 60 mg Zn ($ZnSO_4 \cdot H_2O$); 0.35 mg I (KI); 0.15 mg Se ($Na_2SeO_3$); 50 mg chlortetracycline.

[3]Washed building sand without Ca and P, which was used to adjust amounts of $CaHPO_4$, $Ca(H_2PO_4)_2$ and limestone.

[4]Analyzed values. Each value based on triplicate determinations. The others were calculated values.

and feed intake per cage were measured at 21 D of age to calculate average daily gain, average daily feed intake, and feed-to-gain ratio (F/G) from 1 to 21 D of age.

### Sample Collections and Preparations

Samples of the diets and tap water were collected for analyses of Ca, P, or dietary crude protein contents. At 7, 14, and 21 D of age, 4, 3, and 2 birds close to the average weight of the replicate were selected for collection of blood and tibias, respectively. Blood samples of 5 mL were collected from the wing vein in 10-mL tubes without an anticoagulant and immediately centrifuged for 10 min at 3,000 × *g* at 4°C for analyses of serum Ca, P, 25-$OHD_3$ contents, and ALP activity. And then, the selected birds from each replicate cage were subsequently stunned using an electrical stunner (40 V: alternating current, 400 Hz for 5 s) and immediately exsanguinated. The right and left tibias were freed from adhering tissue, sealed in plastics bags, and stored at −20°C until further analysis. The right tibias were used for determining the BMD, BBS, and percentages of ash, Ca, and P. The left tibias were used to analyze the ALP activity. To reduce individual biological variation, the samples from the selected birds in each replicate cage were pooled into one sample in equal ratios before analysis.

### Determination of the Serum Ca, P, 25-$OHD_3$ Contents, and ALP Activity

Serum was thawed and analyzed for the Ca content using a microplate reader with Ca assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Serum P contents were determined by the molybdenum blue method (Goldenberg and Fernandez, 1966). The 25-$OHD_3$ level in serum was determined by the method of ELISA with the assay kits (Nanjing Jiancheng Bioengineering Institute). The left tibias were grinded, homogenized, and then sonicated and centrifuged at 1,000 × *g* for 10 min at 4°C to harvest the supernatants for ALP activity analysis. The ALP activities in serum and the tibia were measured using a microplate reader with ALP assay kits (Nanjing Jiancheng Bioengineering Institute).

### Determination of the Tibia BMD, BBS, and Ash Content

The frozen tibiae were thawed at room temperature for 2 h and then stripped of all soft tissues. The tibia BMD (Liu et al., 2017; Jing et al., 2018) was determined by dual-energy X-ray absorptiometry. The tibia BBS was determined by a 3-point bending test (HDP/3PB Texture Analyzers, West Sussex, UK). The tibia bone of the broilers on days 7, 14, or 21 was put on a fulcrum point with 2.0, 2.5, or 3.0 cm apart, respectively. The loading point was located in the midpoint of fulcrum points. The breaking force was determined by the shear test at a speed of 5 mm/min with a 50 kg loading cell until fracture occurred (Crenshaw et al., 1981; Shim et al., 2012; Sadeq et al., 2018). The ultimate tibia breaking force was directly obtained from the loaded-deformation curve recorded by a computerized monitor (Jiang et al., 2016; Liu et al., 2017). The tibia bone was dried using an oven at 105°C for 24 h and then defatted with fresh diethyl ether for 48 h. The fat-free, dried bone was finally ashed using a muffle furnace at 550°C for 16 h. The tibia ash content was expressed on a dried and defatted weight basis of tibia.

### Determination of the Dietary and Tibia Ca and P Contents

Concentrations of Ca in diets and tibia ash were determined by inductively coupled plasma spectroscopy (Model IRIS Intrepid II; Thermo Jarrell Ash, Waltham, MA) as described by Li et al. (2011). Total P concentrations in diets and tibia ash were determined by the spectrophotometric method (Procedure 3.4.11; AOAC, 2000). Diets were analyzed for phytate P as per the ferric precipitation method (Rutherfurd et al., 2004; Leytem et al., 2008).

### Statistical Analyses

Data from the present study were subjected to two-way ANOVA using the general linear model procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). The model included the main effects of dietary Ca level, dietary NPP level, and their interaction. The replicate cage was the experimental unit. Differences among means were tested by the least significant difference method, and the statistical significance was set at $P < 0.05$.

## RESULTS

### Dietary Ca and NPP Contents

The analyzed Ca and NPP contents in experimental diets are presented in Table 1. Analyzed values generally agree with calculated values.

### Growth Performance and Mortality

The broilers fed with the P-deficient diet had the lowest ($P < 0.05$) average daily feed intake and average daily gain, as well as the highest ($P < 0.05$) feed-to-gain ratio and mortality as compared with those fed with the Ca- and P-adequate diet, Ca-deficient diet, or Ca- and P-deficient diet. The detailed data about the growth performance and mortality of broilers have been published in our previous article (Shao et al., 2019b).

### Tibia BMD, BBS, and Ash Content

The interaction between Ca and the NPP level affected ($P < 0.05$) BMD, BBS, and ash content in tibiae on days 7, 14, and 21 (Table 2). The broilers fed with the Ca- and P-adequate diet had higher ($P < 0.05$) BMD,

BBS, and ash content in tibia on days 7, 14, and 21 than those fed with the Ca- or P-deficient diets, and broilers fed with the P-deficient diet at adequate Ca had lower ($P < 0.05$) BMD, BBS, and ash content in tibia on days 7, 14, and 21 than those fed with the Ca- and P-deficient diet. All of the tibia development parameters of broilers were more sensitive to P deficiency, especially at adequate Ca and this was followed by the Ca- and P-deficient diet and then the Ca-deficient diet.

### Serum Ca, P, 25-OHD$_3$ Contents, and ALP Activity

Decreasing dietary Ca level decreased ($P < 0.05$) serum ALP on days 7, 14, and 21 as well as serum P content on day 14 (Table 3). The interaction between Ca and the NPP level had effects ($P < 0.05$) on serum Ca and 25-OHD$_3$ contents on days 7, 14, and 21 as well as serum P content on days 7 and 21 but had no effects ($P > 0.05$) on serum ALP activity on days 7, 14, and 21 as well as serum P content on day 14 (Table 3). In general, broilers fed with the Ca- and P-adequate diet had lower ($P < 0.05$) serum Ca when compared with broilers fed with the P-deficient diet. Serum Ca was similar ($P > 0.05$) in broilers fed with the Ca- and P-adequate diet and Ca- and P-deficient diet on days 14 and 21, whereas P deficiency in Ca-adequate diets resulted in an increase ($P < 0.05$) in serum Ca. Serum 25-OHD$_3$ was inversely related with serum Ca. Compared with broilers fed with the P-deficient diet or Ca-deficient diet, birds fed with the Ca- and P-deficient diet had increased ($P < 0.05$) serum P on days 7 and 21 but still had lower ($P < 0.05$) serum P than those fed with the Ca- and P-adequate diet on day 21. Overall, the broilers fed with the P-deficient diet had the highest serum Ca content and the lowest serum P and 25-OHD$_3$ contents compared with the broilers fed with the Ca-deficient, Ca- and P-deficient, or Ca- and P-adequate diets.

### Tibia Ash Ca, P Contents, and ALP Activity

Decreasing the dietary Ca level decreased ($P < 0.05$) tibia ALP activity on day 7 and tibia ash P content on day 14 but increased ($P < 0.05$) tibia ash P content on days 7 and 21 (Table 4). In addition, decreasing the dietary NPP level increased tibia ALP activity on day 7 but decreased tibia ash P content on days 7 and 14 (Table 4). The interaction between Ca and the NPP level affected ($P < 0.05$) tibia ALP activity on days 14 and 21, tibia ash Ca content on days 7, 14, and 21, as well as tibia ash P content on day 21 but did not affect ($P > 0.05$) tibia ALP activity on day 7 and tibia ash P content on days 7 and 14 (Table 4). The broilers fed with the Ca- and P-adequate diet had lower ($P < 0.05$) tibia ALP activity on day 14 and 21 than those fed with the Ca-deficient diet; however, the broilers fed with the P-deficient diet had higher ($P < 0.05$) tibia ALP activity on days 14 and 21 than those fed with the Ca- and P-deficient diet. No difference ($P > 0.05$) was observed in tibia ash Ca content on day 7 between the broilers fed with the P-deficient and Ca- and P-deficient diets; however, the broilers fed with the Ca-deficient diet had lower ($P < 0.05$) tibia ash Ca content on day 7 than those fed with the Ca- and P-adequate diet. Compared with the control broilers, the broilers fed with the P-deficient diet had lower ($P < 0.05$) tibia ash Ca content on days 14 and 21 as well as tibia ash P content on day 21; however, the broilers fed with the Ca- and P-deficient diet had higher ($P < 0.05$) tibia ash Ca and P contents on day 21 than those fed with the Ca-deficient diet. Overall, the broilers fed with the P-deficient diet had the highest tibia ALP activity and the lowest tibia ash P content compared with those fed with the Ca-deficient, Ca- and P-deficient, or control diets.

## DISCUSSION

Fast-growing broilers are more susceptible to bone development abnormalities resulting from inadequate

**Table 2.** Effects of dietary Ca and NPP levels on tibia BMD, BBS, and ash content of broilers[1].

| Ca levels, % | 1.00 | | 0.35 | | | *P*-value |
|---|---|---|---|---|---|---|
| NPP levels, % | 0.45[2] | 0.23[2] | 0.45[2] | 0.23[2] | *SEM* | Ca × NPP |
| Day 7 | | | | | | |
| Tibia BMD, g/cm$^2$ | 0.094$^a$ | 0.043$^d$ | 0.072$^b$ | 0.067$^c$ | 0.001 | < 0.0001 |
| Tibia BBS, kg | 3.77$^a$ | 0.63$^d$ | 2.33$^b$ | 1.90$^c$ | 0.09 | < 0.0001 |
| Tibia ash content, % | 47.9$^a$ | 27.3$^c$ | 40.3$^b$ | 38.9$^b$ | 0.7 | < 0.0001 |
| Day 14 | | | | | | |
| Tibia BMD, g/cm$^2$ | 0.160$^a$ | 0.068$^d$ | 0.101$^b$ | 0.096$^c$ | 0.001 | < 0.0001 |
| Tibia BBS, kg | 10.5$^a$ | 1.19$^d$ | 4.99$^b$ | 3.55$^c$ | 0.28 | < 0.0001 |
| Tibia ash content, % | 50.4$^a$ | 30.1$^d$ | 40.5$^b$ | 39.1$^c$ | 0.5 | < 0.0001 |
| Day 21 | | | | | | |
| Tibia BMD, g/cm$^2$ | 0.201$^a$ | 0.098$^c$ | 0.124$^b$ | 0.130$^b$ | 0.004 | < 0.0001 |
| Tibia BBS, kg | 18.9$^a$ | 3.08$^c$ | 8.04$^b$ | 8.87$^b$ | 0.50 | < 0.0001 |
| Tibia ash content, % | 50.1$^a$ | 33.8$^c$ | 40.0$^b$ | 40.9$^b$ | 0.7 | < 0.0001 |

$^{a\text{-}d}$Means within a row lacking a common superscript differ ($P < 0.05$).

[1]Abbreviations:BMD, bone mineral density; BBS, bone breaking strength; NPP, nonphytate P.

[2]Data represented the means of 7 replicate cages (n = 7).

**Table 3.** Effects of dietary Ca and NPP levels on serum Ca, P, and 25-$OHD_3$ contents and ALP activity of broilers[1].

| Ca levels, % | 1.00 | | 0.35 | | | Ca level[3], % | | | NPP level[3], % | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NPP levels, % | 0.45[2] | 0.23[2] | 0.45[2] | 0.23[2] | *SEM* | 1.00 | 0.35 | *SEM* | 0.45 | 0.23 | *SEM* | Ca | NPP | Ca × NPP |
| Day 7 | | | | | | | | | | | | | | |
| Serum Ca, mmol/L | 1.79$^{c}$ | 2.19$^{a}$ | 2.06$^{b}$ | 1.94$^{b}$ | 0.04 | 1.99 | 2.00 | 0.03 | 1.93 | 2.07 | 0.03 | 0.79 | 0.003 | < 0.0001 |
| Serum P, mmol/L | 1.90$^{a}$ | 0.96$^{c}$ | 1.41$^{b}$ | 1.89$^{a}$ | 0.08 | 1.43 | 1.65 | 0.05 | 1.66 | 1.42 | 0.05 | 0.009 | 0.006 | < 0.0001 |
| Serum ALP, U/L | 207 | 291 | 149 | 183 | 30 | 249$^{a}$ | 166$^{b}$ | 21.5 | 178 | 237 | 21.5 | 0.012 | 0.06 | 0.42 |
| Serum 25-$OHD_3$, ng/mL | 24.4$^{a}$ | 18.4$^{c}$ | 23.5$^{a}$ | 20.6$^{b}$ | 0.7 | 21.4 | 22.1 | 0.49 | 24.0 | 19.5 | 0.49 | 0.31 | < 0.0001 | 0.03 |
| Day 14 | | | | | | | | | | | | | | |
| Serum Ca, mmol/L | 2.03$^{b}$ | 2.58$^{a}$ | 2.29$^{a,b}$ | 2.17$^{b}$ | 0.11 | 2.31 | 2.23 | 0.08 | 2.16 | 2.38 | 0.08 | 0.49 | 0.07 | 0.006 |
| Serum P, mmol/L | 1.78 | 1.83 | 1.34 | 1.44 | 0.08 | 1.80$^{a}$ | 1.39$^{b}$ | 0.06 | 1.56 | 1.64 | 0.06 | < 0.0001 | 0.38 | 0.76 |
| Serum ALP, U/L | 186 | 227 | 106 | 92 | 15.0 | 206$^{a}$ | 99.2$^{b}$ | 10.6 | 146 | 159 | 10.6 | < 0.0001 | 0.38 | 0.08 |
| Serum 25-$OHD_3$, ng/mL | 34.1$^{a}$ | 29.4$^{c}$ | 30.9$^{b,c}$ | 32.1$^{a,b}$ | 0.77 | 31.7 | 31.5 | 0.54 | 32.5 | 30.7 | 0.54 | 0.73 | 0.03 | 0.0008 |
| Day 21 | | | | | | | | | | | | | | |
| Serum Ca, mmol/L | 1.78$^{c}$ | 2.26$^{a}$ | 2.01$^{b}$ | 1.86$^{c}$ | 0.03 | 2.04 | 1.94 | 0.02 | 1.91 | 2.06 | 0.02 | 0.009 | < 0.0001 | < 0.0001 |
| Serum P, mmol/L | 1.90$^{a}$ | 1.22$^{c}$ | 1.30$^{c}$ | 1.62$^{b}$ | 0.04 | 1.56 | 1.45 | 0.03 | 1.6 | 1.4 | 0.03 | 0.03 | 0.0005 | < 0.0001 |
| Serum ALP, U/L | 148 | 192 | 69 | 54 | 25 | 170$^{a}$ | 61.6$^{b}$ | 17.5 | 108 | 123 | 17.5 | 0.0002 | 0.56 | 0.25 |
| Serum 25-$OHD_3$, ng/mL | 33.4$^{a,b}$ | 29.4$^{b}$ | 32.4$^{a,b}$ | 35.7$^{a}$ | 1.4 | 31.4 | 34.0 | 1.01 | 32.9 | 32.5 | 1.01 | 0.08 | 0.78 | 0.017 |

[a–c]Means within a row lacking a common superscript differ ($P < 0.05$).
[1]Abbreviations: 25-$OHD_3$, 25-hydroxyvitamin $D_3$; ALP, alkaline phosphatase; NPP, nonphytate P.
[2]Data represented the means of 7 replicate cages (n = 7).
[3]Data represented the means of 12 replicate cages (n = 14).

mineral supply, especially Ca or P deficiency (Williams et al., 2000b). Moreover, dietary Ca or P deficiency might first change Ca or P metabolic utilization parameters and then influence bone development of broilers. The results from the present study supported our hypothesis, indicating that dietary P deficiency impaired the bone development by increasing serum Ca content and tibia ALP activity but decreasing serum P, 25-$OHD_3$ contents, and tibia ash Ca and P contents of broilers. Dietary Ca deficiency impaired bone development by increasing serum Ca content, tibia ALP activity, and tibia ash P content but decreasing serum P, 25-$OHD_3$ contents, and tibia ash Ca content of broilers. The aforementioned findings provided scientific experimental bases for monitoring and regulating Ca and P homeostasis of broilers.

Many studies demonstrated that the bone development parameters, such as tibia BMD, BBS, and ash content of broilers, were susceptible to dietary Ca or P level (Williams et al., 2000a; Rama Rao et al., 2003; Liu et al., 2017). Results from the present study showed that the Ca- or P-deficient diets negatively affected the tibia BMD, BBS, and ash content of broilers. Similar results were observed in the previous study of Liu et al. (2017), who found that broilers fed with 0.23% NPP diet had lower tibia BMD, BBS, and ash content than the control broilers. Rama Rao et al. (2003) also found that a decrease of Ca and NPP levels in the broiler diet had a negative effect on tibia BBS and ash content. Venalainen et al. (2006) reported that tibia ash content decreased quadratically with dietary NPP level decreased but had no effect on BBS of broilers. However, Huyghebaert (1996) found that both tibia ash content and BBS diminished when the dietary NPP level decreased from 0.45 to 0.30%. In addition, the results from the current study demonstrated that tibia BMD, BBS, and ash content of broilers showed stronger responses to P deficiency as compared with Ca deficiency or Ca and P deficiencies. Similar results in rats showed that the influence coefficient of P on femoral ash was six-fold larger than that of Ca (Shapiro and Heaney, 2003). These aforementioned results indicated that the animals are more sensitive to insufficient P than inadequate Ca for bone mineralization.

The Ca and P levels in serum and bone can reflect the nutritional status of Ca and P in broilers. Insufficient intake of one or both minerals, when the deficiency of one of them interferes with homeostasis of second one, results in retarded growth rate and poor bone mineralization (Shafey et al., 1990; Hurwitz et al., 1995). In our present study, broilers fed with the Ca-deficient diet with adequate P or the P-deficient diet with adequate Ca had severely lower serum P level and higher serum Ca level compared with the control broilers. The depressed serum P level may be a result of reduction of the P available for absorption in the gut through the formation of a flocculent precipitate of Ca phosphate when the ratio of Ca and NPP is disproportionate in the diet (Driver et al., 2005). Subsequently, a low serum P concentration leads to the activation of osteoclasts that, in turn, leads to increased bone resorption for maintaining

**Table 4.** Effects of dietary Ca and NPP levels on tibia ALP activity and ash Ca and P contents of broilers[1].

| Ca levels, % | 1.00 | | 0.35 | | | Ca level[3], % | | | NPP level[3], % | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NPP levels, % | 0.45[2] | 0.23[2] | 0.45[2] | 0.23[2] | *SEM* | 1.00 | 0.35 | *SEM* | 0.45 | 0.23 | *SEM* | Ca | NPP | Ca × NPP |
| Day 7 | | | | | | | | | | | | | | |
| Tibia ALP, U/g protein | 595 | 793 | 399 | 595 | 44 | 694[a] | 497[b] | 32 | 497[b] | 694[a] | 32 | 0.0002 | 0.0002 | 0.99 |
| Tibia ash Ca content, % | 38.4[a] | 33.7[b,c] | 34.4[b] | 33.0[c] | 0.3 | 36.0 | 33.7 | 0.2 | 36.5 | 33.4 | 0.2 | < 0.0001 | < 0.0001 | < 0.0001 |
| Tibia ash P content, % | 16.6 | 15.6 | 17.4 | 16.6 | 0.24 | 16.1[b] | 17.0[a] | 0.2 | 17.0[a] | 16.1[b] | 0.2 | 0.001 | 0.001 | 0.94 |
| Day 14 | | | | | | | | | | | | | | |
| Tibia ALP, U/g protein | 352[c] | 661[a] | 458[b] | 466[b] | 34 | 507 | 462 | 24 | 405 | 564 | 24 | 0.20 | < 0.0001 | 0.0002 |
| Tibia ash Ca content, % | 35.3[a] | 34.0[b] | 31.8[c] | 31.8[c] | 0.2 | 34.7 | 31.8 | 0.1 | 33.6 | 33.0 | 0.1 | < 0.0001 | 0.0002 | 0.001 |
| Tibia ash P content, % | 16.7 | 16.2 | 16.1 | 15.8 | 0.1 | 16.5[a] | 15.9[b] | 0.1 | 16.4[a] | 16.0[b] | 0.1 | 0.0002 | 0.007 | 0.46 |
| Day 21 | | | | | | | | | | | | | | |
| Tibia ALP, U/g protein | 254[c] | 508[a] | 491[a,b] | 426[b] | 26 | 459 | 381 | 18.6 | 373 | 467 | 19 | 0.007 | 0.002 | < 0.0001 |
| Tibia ash Ca content, % | 36.2[a] | 33.7[b] | 31.1[d] | 32.5[c] | 0.2 | 35.1 | 31.8 | 0.1 | 33.7 | 33.0 | 0.1 | < 0.0001 | 0.001 | < 0.0001 |
| Tibia ash P content, % | 16.7[c] | 16.2[d] | 17.4[b] | 17.6[a] | 0.1 | 16.4 | 17.5 | 0.1 | 17.0 | 16.9 | 0.1 | < 0.0001 | 0.38 | 0.0008 |

[a–d]Means within a row lacking a common superscript differ ($P < 0.05$).
[1]Abbreviations: ALP, alkaline phosphatase; NPP, nonphytate P.
[2]Data represented the means of 7 replicate cages (n = 7).
[3]Data represented the means of 12 replicate cages (n = 14).

a normal serum P level and simultaneously increasing serum Ca level (Proszkowiec-Weglarz and Angel, 2013). Therefore, the severely depressed tibia ash Ca content might be due to the increased bone resorption for maintaining serum Ca homeostasis, which could partly explain the elevated serum Ca level. These findings suggested that dietary Ca or P deficiency might impair the bone development by disturbing Ca and P homoeostasis of broilers. However, the tibia ash Ca and P contents seemed to change little over time, possibly because the tibia ash Ca and P contents are relative values and less affected by age of birds than their absolute values (Shastak et al., 2012; Shao et al., 2019a).

A variety of factors, such as 25-$OHD_3$ and ALP, could regulate the Ca and P metabolic utilization and homeostasis in serum and bone of animals. As a metabolite of vitamin $D_3$, 25-$OHD_3$ is involved in the absorption and utilization of Ca and P and thus required for proper bone and eggshell mineralization in chickens. In the present study, dietary Ca deficiency in P-adequate diets or P-deficiency in Ca-adequate diets decreased serum 25-$OHD_3$ content, suggesting that dietary Ca or P deficiency might directly weaken the 25-hydroxylation of vitamin $D_3$ with a decreased serum 25-$OHD_3$ level (Berlin and Bjorkhem, 1988). Low serum 25-$OHD_3$ level has been associated with an increased risk of fracture, osteoporosis, and rickets in humans (Raghuramulu and Reddy, 1980; Bahlous et al., 2009; Ohta et al., 2014). The study in children showed that serum 25-$OHD_3$ level was positively associated with BMD, and low 25-$OHD_3$ level could result in low BMD (Fu et al., 2016). Therefore, dietary Ca or P deficiency might impair the bone development by depressing serum 25-$OHD_3$ level of broilers. It is reported that Ca and P absorption is stimulated as part of adaptive mechanism to Ca or P restriction (Rousseau et al., 2016). Therefore, the present study showed that the serum 25-$OHD_3$ increased as age of broilers when fed with Ca- or P-deficient diets, which might be related to adaptive mechanism to enhance Ca or P absorption by increasing 25-$OHD_3$. In addition, as a bone formation–related enzyme, the ALP was involved in the process of Ca and P deposition of bone, and its activity is usually elevated when bone formation rates increased (Tilgar et al., 2008). In the present study, we found that the ALP activity decreased as birds age, suggesting that bone formation rate might progressively decrease with age of broilers. However, an increase in ALP activity is also usually associated with poor bone mineralization (Sarac and Saygili, 2007). As per our results, inadequate supply of Ca or P was associated with an increase in tibia ALP activity. One explanation for this phenomenon is that inadequate supply of Ca or P decreased the serum P level and the P available for bone mineralization, thereby leading to activation of ALP (Haraikawa et al., 2012; Christmann et al., 2016). Liu et al. (2017) also found that serum ALP activity of broilers was linearly increased as the dietary NPP level decreased. Similar results in humans showed that bone ALP activity was negatively correlated with intake of Ca and P (Haraikawa et al., 2012). Therefore, the

previous results indicated that dietary Ca or P deficiency might impair the bone development by elevating tibia ALP activity of broilers.

Skeletal health could be adversely influenced by diets with an imbalance of the Ca: NPP ratio. The results from our recent study showed that the growth performance and rickets incidence of broilers were the most sensitive ones to dietary P deficiency, followed by dietary Ca deficiency or Ca and P deficiencies (Shao et al., 2019b). Furthermore, the results from the present study showed that the P-deficient diet had greater influence on bone development and Ca or P metabolic utilization parameters than the Ca-deficient diet or Ca- and P-deficient diet. Bradbury et al. (2014) also reported similar findings that the broilers fed with the P-deficient diet had lower tibia ash content than those fed with the Ca-deficient diet. The previous study showed that the effect of the P-deficient diet was exacerbated with the increasing concentration of Ca in the diet (Driver et al., 2005). However, dietary Ca was shown to have a modest influence on tibia ash content at high NPP concentration (Bradbury et al., 2014). The possibility is that broilers might be more willing to over consume NPP to reach a Ca target than over consuming Ca to reach an NPP intake in case with dietary Ca or P deficiency (Bradbury et al., 2014; Wilkinson et al., 2014). It is well known that the Ca: NPP ratio in poultry diets plays an important role in both Ca and P absorption and utilization, especially if the diet has minimal inclusion levels of both minerals (Li et al., 2000). In our present study, the Ca- and P-deficient diet contained a low concentration of NPP (0.23%) but the concentration of Ca was also low (0.35%) and therefore more balanced than the P-deficient diet. These aforementioned studies indicated that the ratio of Ca: NPP was more important for skeletal health of broilers than absolute concentrations of either mineral (Driver et al., 2005; Bradbury et al., 2014).

In conclusion, the results from the present study indicated that the bone development and Ca or P metabolic utilization parameters of broilers were the most sensitive ones to dietary P deficiency, followed by dietary Ca deficiency or Ca and P deficiencies. Dietary Ca or P deficiency impaired the bone development by regulating related Ca or P metabolic utilization parameters of broilers.

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