Restriction of dietary non-phytate phosphorus on growth performance and expression of intestinal phosphate cotransporter genes in broilers

Aye Cho Tay-Zar ,[*] Pairat Srichana,[†] Muhammad Bilal Sadiq,[*] and Anil Kumar Anal[*,1]

[*]*Department of Food, Agriculture and BioResources, Asian Institute of Technology, Klong Luang 12120, Thailand; and* [†]*Feed Technology Department, Charoen Pokphand Group, Bangrak, Bangkok 10500, Thailand*

ABSTRACT Effects of dietary non-phytate phosphorus (nPP) restriction on growth and duodenal type IIb sodium-dependent phosphate cotransporter (*NaPi-IIb*) genes were observed. A total of 432 one-day old Cobb500 male broiler chickens in 36 cage pens were divided into 6 groups with each group containing 6 pens. Each group was treated with one of the diets containing 0.33, 0.37, 0.41, 0.45, 0.49, and 0.53% of nPP up to 14 D. During 15 to 31 D, birds were treated with one of the diets containing 0.23, 0.27, 0.31, 0.35, 0.39, and 0.43% of nPP. Level of Ca was kept the same across all treatments. Dietary nPP level influenced ($P < 0.001$) weight gain and feed intake in both growth phases, whereas effect on feed per gain ratio was seen only in the second phase. Toe ash, tibia ash, and tibia breaking strength responded to treatments ($P < 0.01$) at 14 D. Only tibia ash content was significantly improved ($P < 0.001$) at 31 D. Growth and bone parameters linearly improved with an increase in dietary nPP content ($P < 0.05$). Above dietary nPP 0.41% and 0.31% for first phase and second phase, respectively, no significant improvement was seen. Duodenal *NaPi-IIb* mRNA overexpressed with a decrease in dietary nPP in both phases ($P < 0.05$). Relative expression of *NaPi-IIb* in lowest nPP group were 2.2 folds higher in the first phase and 3.6 folds higher in the second phase compared to respective highest nPP groups of each phase. No significant change in *NaPi-IIb* expression was seen above 0.37% of dietary nPP for 14 D and 0.31% of dietary nPP for 31 D. Dietary requirements of nPP 0.41% for 0 to 14 D and 0.31% for 15 to 31 D were adequate for optimal growth and bone parameters. This study fills the gap in understanding of intestinal *NaPi-IIb* expression in response to dietary nPP restriction in broilers older than 21 D of age.

Key words: broiler, phosphorus, sodium-dependent phosphate cotransporter, gene expression, growth and bone characteristics

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INTRODUCTION

In the past decades, genetic selection in broiler strains improved a lot in growth rate and nutrient efficiency. As a result, optimum nutrient requirements for modern broiler strains need to be redefined (Rama Rao et al., 2006; Applegate and Angel, 2014). Phosphorus (**P**) and calcium (**Ca**) play important roles in bone development and mineralization in animals. Sufficient P uptake is vital for bone development, growth, and productivity. On the other hand, higher supply of P, which exceeds maximum retention requirement, is excreted through kidneys (Manangi and Coon, 2008) causing both economical loss and environmental pollution. Around 80% of inorganic phosphorus (**Pi**) in the body is stored in skeleton as hydroxyapatite and the remaining 20% is found in nucleic acids, phospholipids, and phosphorylated proteins (Veum, 2010). According to the National Research Council (NRC, 1994), broilers need 1.00% of total Ca and 0.45% of non-phytate phosphorus (**nPP**) for starter phase and 0.90% of total Ca and 0.35% of nPP for grower phase, respectively, in diets. Nevertheless, requirements for modern broiler strains might differ from NRC (1994) recommendations.

In poultry, Pi homeostasis is regulated mainly by the intestinal uptake, retention/release from skeleton, and regulated renal reabsorption (Sabbagh et al., 2011). Intestinal Pi uptake involves transcellular and paracellular pathways (Fuchs and Peterlik, 1979). Paracellular pathway is a passive transport system and mainly depends on the concentration gradients across the intestinal membrane, whereas transcellular pathway is regulated by hormones and metabolic factors (Fuchs and Peterlik, 1979). Transcellular active transport of Pi occurs throughout the intestinal tract and in renal tubules (Sabbagh et al., 2011). In this process, Pi transport in the intestine involves uptake of phosphorous by sodium (Na)-dependent cotransporters, translocation

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[1]Corresponding author: anilkumar@ait.asia

across the cell, and efflux at the basolateral membrane (Tenenhouse et al., 1998). The sodium-dependent cotransporters (NaPi-) actively regulate the intestinal Pi absorption and renal reabsorption in poultry (Forster et al., 2006; Yan et al., 2007; Han et al., 2009; Sabbagh et al., 2009; Li et al., 2017), whereas sodium-independent intestinal phosphate transport is unregulated (Danisi et al., 1980; Katai et al., 1999a,b). There are 3 types of NaPi- cotransporters: NaPi-I, NaPi-II, and NaPi-III. Among them, type II NaPi-cotransporter represents 84% of total NaPi- cotransporters (Miyamoto et al., 1997; Tenenhouse et al., 1998). Sodium-dependent phosphate cotransporters type IIa, IIb, and IIc dominate in both intestinal and renal epithelial cells of mammals and poultry (Custer et al., 1994; Hilfiker et al., 1998; Segawa et al., 2002) with type IIa hugely responsible for renal Pi reabsorption (Tenenhouse, 2005). In their review, Miyamoto et al. (2007) suggested that NaPi-IIc had minor influence on Pi transport in mammals. Studies revealed that NaPi-IIb is the most abundant NaPi- cotransporter in intestinal Pi absorption and accounts for over 90% of total sodium-dependent Pi transport, whereas NaPi-IIa is responsible for 70 to 80% of renal Pi reabsorption (Hilfiker et al., 1998; Tenenhouse et al., 1998; Sabbagh et al., 2009).

Poultry NaPi-IIb encode for solute carrier family 34 member 2 (SLC34A2) (Werner and Kinne, 2001; Yan et al., 2007). In chicken intestine, highest expression of *NaPi-IIb* mRNA was found in the duodenum, followed by jejunum and ileum (Liu et al., 2006; Yan et al., 2007; Han et al., 2009, 2018). Dietary levels of Pi hugely influence the expression of *NaPi-IIb* in small intestine, where *NaPi-IIb* genes get overexpressed during the Pi-deficient conditions (Yan et al., 2007; Han et al., 2009; Nie et al., 2013; Li et al., 2017). Few studies have been conducted to evaluate the effects of Pi content *NaPi-IIb* expression and growth performance in broilers and layers (Driver et al., 2006; Rama Rao et al., 2006; Li et al., 2012; Nie et al., 2013; Hamdi et al., 2015). However, understanding of *NaPi-IIb* in response to different dietary nPP levels without changing Ca levels in both starter and grower phases is lacking, especially for broilers older than 21 D. Since both Ca levels and Ca to nPP ratio effects on broilers' P metabolism, it would give a clear picture on dietary response to nPP alone if Ca level is kept at standard while varying dietary nPP levels. Moreover, varying dietary nPP or Ca alone might address the precise role of *NaPi-II* in Ca and P homeostasis. The present study aimed to find out the role of intestinal *NaPi-IIb* in response to dietary nPP inclusions without changing Ca levels and suitable dietary nPP levels that could benefit to optimal growth and bone performance at 14 and 31 D of age.

MATERIALS AND METHODS

This experiment was conducted in accordance with the principles and guidelines presented in the Guide for the Care and Use of Agricultural Animals in Research and Teaching (National Research Council, 2010). The experimental design and procedures involved were approved by the Institutional Animal Care and Use Committee.

Table 1. Treatment groups with different levels of dietary nPP %.

	Dietary nPP levels (%)					
Age (days)	T1	T2	T3	T4	T5	T6
0 to 14	0.33	0.37	0.41	0.45	0.49	0.53
15 to 31	0.23	0.27	0.31	0.35	0.39	0.43

nPP = non-phytate phosphorus.

Animal and Diets

The male Cobb500 broilers (1 day old; n = 432) with average initial body weight of 47 g were housed in cage pens and randomly assigned to 6 dietary treatments. Each treatment consisted of 6 replicate pens with 12 birds in each pen. Each treatment group received diets for starter phase (0 to14 D) and grower phase (15 to 31 D). Birds were raised inside the cages under controlled environment for 31 D with ab libitum of feed and water. Housing and management were according to the standard guidelines. Briefly, birds were housed under tunnel ventilation system with maximum stocking density of 40 kg/m^2. Temperature was gradually reduced from 36 to 25°C during first 14 D of age and maintained at 25°C till the end of experiment. Relative humidity was maintained between 40 and 70% throughout the experiment. During first 7 D, light intensity of 30 lux was provided for 23 h per day and gradually reduced to 18 h per day by 14 D of age. From 14 D onwards, lighting period was kept 18 h per day at the intensity of 20 lux. Basal feed was formulated to meet minimum nutrient requirements by National Research Council (NRC, 1994) except for nPP. Six experimental diets for each phase with different nPP levels were prepared in mash form and fed to the animals. During 0 to 14 D, each treatment group of birds was fed with one of the diets containing dietary nPP % of 0.33, 0.37, 0.41, 0.45, 0.49, and 0.53%. During 15 to 31 D, birds were treated with one of the diets containing 0.23, 0.27, 0.31, 0.35, 0.39, and 0.43% of nPP. Treatment groups with respective dietary nPP % for starter and grower phases are described in Table 1. Ca in all treatments was maintained at 1.00% for starter phase and 0.90% for grower phase. Diet composition and calculated nutritional values are presented in Tables 2 and 3. Prepared diets were sent to the CP Bangna Central Laboratory (Charoen Pokphand Group), Bangkok, and confirmed that the analyzed nutrient values were in accordance with calculated values.

Table 2. Diet composition (%) and calculated nutrient values (0 to14 D).

	nPP (%)					
	T1	T2	T3	T4	T5	T6
Items	0.33	0.37	0.41	0.45	0.49	0.53
Ingredients (%)						
Corn	57.06	56.95	56.85	56.75	56.65	56.55
Soybean meal	35.60	35.60	35.60	35.60	35.60	35.60
Crude palm oil	3.20	3.20	3.20	3.20	3.20	3.20
Mono calcium phosphate	0.82	1.00	1.18	1.35	1.53	1.71
Limestone	1.91	1.83	1.76	1.68	1.60	1.53
Salt	0.41	0.41	0.41	0.41	0.41	0.41
Sodium bicarbonate	0.10	0.10	0.10	0.10	0.10	0.10
DL-methionine	0.30	0.30	0.30	0.30	0.30	0.30
L-lysine	0.19	0.19	0.19	0.19	0.19	0.19
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10
Premixes[1]	0.32	0.32	0.32	0.32	0.32	0.32
Total	100.00	100.00	100.00	100.00	100.00	100.00
Calculated values (%)						
ME (kcal/kg)	3,032	3,028	3,025	3,022	3,018	3,015
Crude protein	21.54	21.53	21.52	21.51	21.50	21.50
Crude fat	5.79	5.79	5.78	5.78	5.77	5.77
Crude fiber	2.39	2.39	2.39	2.39	2.39	2.38
Ca	1.00	1.00	1.00	1.00	1.00	1.00
Total phosphorus	0.60	0.64	0.68	0.72	0.76	0.80
nPP	0.33	0.37	0.41	0.45	0.49	0.53
Ca/nPP	3.03	2.71	2.44	2.22	2.04	1.89
Sodium	0.20	0.20	0.20	0.20	0.20	0.20
Chloride	0.30	0.30	0.30	0.30	0.30	0.30
Lysine	1.33	1.33	1.33	1.33	1.33	1.33
Methionine	0.63	0.63	0.63	0.63	0.63	0.63
Methionine + Cystine	0.97	0.97	0.97	0.97	0.97	0.97
Threonine	0.84	0.84	0.84	0.84	0.84	0.84
Tryptophan	0.27	0.27	0.27	0.27	0.27	0.27
Analyzed values (%)						
Ca	1.02	1.00	1.00	1.02	1.04	1.03
Total phosphorus	0.56	0.6	0.63	0.66	0.72	0.76

[1] Per kg of diet: copper, 8 mg; iodine, 0.35 mg; iron, 80 mg; manganese, 60 mg; selenium, 0.15 mg; zinc, 40 mg; vitamin A, 1,500 IU; vitamin D_3, 200 ICU; vitamin E, 10 IU; vitamin K 0.50 mg; vitamin B_{12}, 0.01 mg; riboflavin 3.6 mg; choline, 1,000 mg; thiamin, 1.8 mg; biotin, 0.15 mg.

Ca = calcium.

nPP = non-phytate phosphorus.

Growth Performance

Feed intake and live body weight were recorded at the end of each phase (14 and 31 D). Feed per gain (amount of feed consumed/ live weight gain) was calculated for each phase.

Bone Characteristics

At the end of the first phase (14 D), half of the birds from each replicate were sacrificed by bleeding. The rest of the birds were sacrificed at the end of the experiment (31 D). From all birds, tibia and middle toes were collected. Tibias were de-flashed followed by removing the patella. All tibias and toes were kept for 24 h at 25°C and oven-dried at 105°C for 24 h. To determine bone ash percentage, left tibia and toes were burnt in muffle furnace at 600°C for 6 h to quantify the ash content. Right tibia bones were subjected to bone breaking strength test using Instron Materials tester (Instron 5965, Instron Corp., Canton, MA) with automated materials test system software version 8.0 (Shim et al., 2012). Briefly, each tibia was placed on 2 fulcrum which were 6 cm apart. Bending force was applied at a speed of 5 mm/min at the center of 2 fulcrum points, and tibia breaking strength was obtained from the load-deformation curve generated by the computerized software.

Tissue Samples

At the end of each phase (day 14 and 31), one bird from each replicate with close resemblance to the average body weight of the treatment was sacrificed. Lumen of the duodenum was flashed with sterile cold normal saline to clean the digesta. Duodenal mucosa was scrapped and immediately kept into cryovials filled with RNAlater (Sigma-Aldrich, St. Louis, MO). Cryovials with samples were incubated overnight at 4°C and subsequently kept at –80°C for RNA extraction.

Real-Time PCR

Total RNAs from samples were collected using RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Purity and concentration of extracted RNA were checked by

Table 3. Diet composition (%) and calculated nutrient values (15 to 31 D).

	nPP (%)					
	T1	T2	T3	T4	T5	T6
Items	0.23	0.27	0.31	0.35	0.39	0.43
Ingredients (%)						
Corn	65.49	65.39	65.28	65.18	65.08	64.97
Soybean meal	28.30	28.30	28.30	28.30	28.30	28.30
Crude palm oil	2.50	2.50	2.50	2.50	2.50	2.50
Mono calcium phosphate	0.42	0.59	0.77	0.95	1.13	1.31
Limestone	1.86	1.78	1.71	1.63	1.56	1.48
Salt	0.33	0.33	0.33	0.33	0.33	0.33
Sodium bicarbonate	0.10	0.10	0.10	0.10	0.10	0.10
DL-Methionine	0.31	0.31	0.31	0.31	0.31	0.31
L-Lysine	0.26	0.26	0.26	0.26	0.26	0.26
L-Threonine	0.01	0.01	0.01	0.01	0.01	0.01
Choline chloride	0.10	0.10	0.10	0.10	0.10	0.10
Premixes[1]	0.32	0.32	0.32	0.32	0.32	0.32
Total	100.00	100.00	100.00	100.00	100.00	100.00
Calculated values (%)						
ME (kcal/kg)	3,081	3,077	3,074	3,071	3,067	3,064
Crude protein	18.84	18.83	18.83	18.82	18.81	18.80
Crude fat	5.32	5.32	5.32	5.31	5.31	5.30
Crude fiber	2.31	2.30	2.30	2.30	2.30	2.30
Ca	0.90	0.90	0.90	0.90	0.90	0.90
Total phosphorus	0.49	0.53	0.57	0.61	0.65	0.69
nPP	0.23	0.27	0.31	0.35	0.39	0.43
Ca/nPP	3.92	3.34	2.90	2.57	2.31	2.09
Sodium	0.17	0.17	0.17	0.17	0.17	0.17
Chloride	0.27	0.27	0.27	0.27	0.27	0.27
Lysine	1.20	1.20	1.20	1.20	1.20	1.20
Methionine	0.61	0.61	0.61	0.61	0.61	0.61
Methionine + Cystine	0.91	0.91	0.91	0.91	0.91	0.91
Threonine	0.74	0.74	0.74	0.74	0.74	0.74
Tryptophan	0.23	0.23	0.23	0.23	0.23	0.23
Analyzed values (%)						
Ca	0.89	0.91	0.89	0.90	0.91	0.88
Total phosphorus	0.42	0.44	0.49	0.52	0.59	0.63

[1] Per kg of diet: copper, 8 mg; iodine, 0.35 mg; iron, 80 mg; manganese, 60 mg; selenium, 0.15 mg; zinc, 40 mg; vitamin A, 1,500 IU; vitamin D_3, 200 ICU; vitamin E, 10 IU; vitamin K 0.50 mg; vitamin B_{12}, 0.01 mg; riboflavin 3.6 mg; choline, 1,000 mg; thiamin, 1.8 mg; biotin, 0.15 mg.
Ca = calcium.
nPP = non-phytate phosphorus.

Table 4. Polymerase chain reaction (PCR) primer sequences and product sizes (Yan et al., 2007).

Name		Sequence (5′ - 3′)	Product size (bps)	Accession number
NaPi-IIb	Forward	CTGGATGCACTCCCTAGAGC	126	NM_204474.2
	Reverse	TTATCTTTGGCACCCTCCTG		
β-actin	Forward	GAGAAATTGTGCGTGACATCA	152	NM_205518.1
	Reverse	CCTGAACCTCTCATTGCCA		

NaPi-IIb = Sodium dependent phosphate cotransporter type IIb.
β-actin = beta actin.

spectrophotometric method using Nanodrop (Thermo Fisher, Waltham, MA). Additionally, integrity of RNA was visualized by QIAxcel capillary gel electrophoresis (Qiagen). Complementary DNA was synthesized from 1 μg of RNA using Omniscript reverse transcription kit (Qiagen) following the manufacturer's instructions. Real-time PCR for *NaPi-IIb* was performed using *β-actin* gene as endogenous control. Primer sequences (Yan et al., 2007) and predicted size of PCR amplicons are described in Table 4. The efficiencies of both primers were determined by serial dilutions and used in relative gene expression calculations. Real-time PCR was performed on Roche Lightcycler96 (Roche, Basel, Switzerland) using QuantiFast SYBR Green PCR Kit (Qiagen) followed by cycling protocol as: 95°C for 5 min; 95°C for 30 s; 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s; 72°C for 5 min. Fluorescence measurements were collected at every cycle during the extension step (72°C). Additionally, melting curve analysis of each reaction was also performed to determine

Table 5. Growth performance of broilers from 0 to 31 D of age.

	nPP (%)									
	T1	T2	T3	T4	T5	T6				
0 to 14 D	0.33	0.37	0.41	0.45	0.49	0.53				
15 to 31 D	0.23	0.27	0.31	0.35	0.39	0.43	SEM	*P*-value		
								nPP	Linear	Quadratic
At 14 D										
Weight gain (g)	429^{c}	434^{c}	469^{b}	482^{a,b}	494^{a}	493^{a}	0.03	< 0.001	< 0.001	0.018
Feed intake (g)	477^{b}	481^{b}	512^{a}	523^{a}	535^{a}	534^{a}	0.03	< 0.001	< 0.001	0.136
Feed/gain	1.111	1.108	1.091	1.084	1.084	1.082	0.02	0.130	-	-
Depletion (%)	4.17	0.00	1.34	2.78	0.00	2.78	0.88	0.230	-	-
At 31 D										
Weight gain (g)	1,480^{b}	1,516^{b}	1,709^{a}	1,725^{a}	1,778^{a}	1,769^{a}	0.02	< 0.001	< 0.001	0.013
Feed intake (g)	2,203^{b}	2,235^{b}	2,454^{a}	2,475^{a}	2,526^{a}	2,490^{a}	0.03	< 0.001	< 0.001	0.007
Feed/gain	1.491^{a}	1.474^{a,b}	1.436$^{a–c}$	1.436$^{a–c}$	1.421^{b,c}	1.409^{c}	0.01	< 0.001	< 0.001	0.243
Depletion (%)	8.33	0.00	2.78	2.78	0.00	2.78	0.88	0.060	-	-

[a-c]Within comparisons, means in a row with no common superscript differs significantly ($P < 0.05$).

the specificity of each amplification. Each gene was amplified in duplicate. Relative expression of *NaPi-IIb* was evaluated by delta CT ($2^{-\Delta\Delta CT}$) method using *β-actin* as an internal control gene (Pfaffl, 2001).

Statistical Analysis

Each pen served as an experimental unit. All data were analyzed by 1-way ANOVA using GLM procedure followed by Tukey's HSD test in SAS 9.0 (SAS Inst. Inc., Cary, NC). A value of $P < 0.05$ was considered statistically significant. Variables numerically significant by dietary nPP levels were analyzed by orthogonal polynomial contrasts to determine linear and quadratic trends. A P value of less than 0.05 was considered significant.

RESULTS

Growth Performance

Growth performances of the birds are summarized in Table 5. At 14 D, effects of dietary nPP levels on body weight gain and feed intake were mathematically significant ($P < 0.001$). Feed per gain ratio and depletion were not affected by dietary nPP levels. Weight gain and feed intake reached plateau when dietary nPP were 0.45% and 0. 41%, respectively. Both linear ($P < 0.001$) and quadratic responses ($P < 0.05$) were observed in weight gain, whereas linear response ($P < 0.001$) was found in feed intake. At 31 D, weight gain, feed intake, and feed per gain ratio were significantly affected ($P < 0.001$) by dietary nPP levels. Both weight gain and feed intake reached to plateau at 0.31% of dietary nPP. Above 0.27% of dietary nPP, feed per gain ratio was not improved by an increase in dietary nPP levels. Overall (0 to 31 D), the increase in dietary nPP levels improved weight gain, feed intake, and feed per gain ratio ($P < 0.001$). Dietary nPP levels of 0.41% for starter phase (0 to 14 D) and 0.31% for grower phase (15 to 31 D) appeared to be adequate. An increase of dietary nPP beyond that level did not improve the growth parameters significantly. Nevertheless, all 3 parameters linearly improved ($P < 0.001$) with the increase in dietary nPP %, whereas weight gain and feed intake responded in quadratic trend as well ($P < 0.05$). There was no interaction between depletion rates and dietary nPP levels in both phases.

Bone Characteristics

Influences of dietary nPP levels on tibia and toe ash, and tibia breaking strength are summarized in Table 6. At day 14, dietary nPP levels influenced toe ash content, tibia ash content, and tibia breaking strength ($P < 0.01$). Toe ash content did not respond to changes in dietary nPP levels above 0.45%. However, tibia ash content and tibia breaking strength reached to plateau at 0.37% of dietary nPP. There were linear responses for all 3 parameters ($P < 0.01$) and quadratic responses for toe ash and tibia breaking strength ($P < 0.01$). At 31 D of age, only tibia ash content was influenced by dietary nPP levels ($P < 0.001$). Above 0.31% of dietary nPP, tibia ash did not respond to increase in dietary nPP contents. There were both linear and quadratic responses of tibia ash to dietary nPP levels ($P < 0.001$).

Expression of NaPi-IIb

Relative expression of *NaPi-IIb* with reference to *β-actin* is described in Figures 1 and 2. In both phases, the group treated with highest level of dietary nPP (0.5 3% for 0 to14 D and 0.43% for 15 to 31 D) was assumed as calibrator and compared with other groups within the same period. At 14 D, relative expression of *NaPi-IIb* significantly increased with a reduction in dietary nPP ($P = 0.033$). Below 0.49% of dietary nPP content, expression of *NaPi-IIb* did not respond to the reduction in dietary nPP content. Nevertheless, expression of *NaPi-IIb* linearly increased ($P = 0.001$) with a decrease in dietary nPP levels. Similarly, relative expression of *NaPi-IIb* was influenced by dietary nPP levels

Table 6. Toe ash, tibia ash, and tibia breaking strength at 14 and 31 D of age.

	nPP (%)									
	T1	T2	T3	T4	T5	T6				
0 to 14 D	0.33	0.37	0.41	0.45	0.49	0.53				
15 to 31 D	0.23	0.27	0.31	0.35	0.39	0.43	SEM	*P*-value		
								nPP	Linear	Quadratic
At 14 D										
Toe ash (%)	9.32[d]	10.68[c]	11.83[b]	12.18[a,b]	12.48[a,b]	12.54[a]	0.22	< 0.001	< 0.001	< 0.001
Tibia ash (%)	36.15[b]	38.75[a,b]	39.44[a,b]	40.92[a]	40.88[a]	41.31[a]	0.47	0.002	< 0.001	0.067
Tibia strength (kgf/mm)[1]	7.40[b]	7.54[a,b]	8.66[a]	8.70[a]	8.73[a]	8.77[a]	0.13	0.001	0.005	0.006
At 31 D										
Toe ash (%)	13.36	14.08	14.28	14.24	14.97	15.47	0.66	0.782	-	-
Tibia ash (%)	37.64[c]	40.80[b,c]	44.24[a,b]	45.47[a]	44.74[a]	44.61[a]	0.57	< 0.001	< 0.001	< 0.001
Tibia strength (kgf/mm)	17.83	18.91	19.29	19.50	19.58	19.72	0.30	0.520	-	-

[1]Kilogram force per millimeter.
[a–d]Within comparisons, means in a row with no common superscript differs significantly ($P < 0.05$).

at 31 D of age ($P < 0.001$). Compared to the birds fed with highest nPP content (T6), expression of *NaPi-IIb* in birds fed with lowest nPP group (T1) was 2.20 folds higher at 14 D and 3.58 folds higher at 31 D. There were both linear and quadratic trends ($P < 0.001$) of *NaPi-IIb* expression in response to changes in dietary nPP levels.

DISCUSSION

In this study, experimental diets were prepared in practical approach by formulating diets for starter (0 to 14 D) and grower (15 to 31 D) phases. According to NRC (1994) guidelines and broiler breed recommendations, animals' nutrient requirements change with age and hence diets should be prepared in accordance with different requirements for each growth phase. It has been proved that dietary crude protein significantly influenced on P retention and intestinal *NaPi-IIb* expression (Ajuwon et al., 2016).

Dietary Ca and P have significant interactions on growth and bone performances in broilers. Imbalance in Ca and P ratio, whether one of them is deficient or excess, would adversely affect the homeostasis of the other that would subsequently lead to poor growth performance and bone mineralization (Shafey et al., 1990; Hurwitz et al., 1995). Nevertheless, it is suggested that broilers are highly adaptable to both Ca and P restriction throughout the growing phase (Bar et al., 2007). In the present study, dietary Ca content was kept the same across all treatments while varying nPP content resulting different Ca:nPP ratios in diet. Calcium content of 1.00% for 0 to 14 D and 0.90% for 15 to 31 D in this study met the recommended dose for the broilers (NRC, 1994).

In the current study, dietary nPP levels influenced growth parameters in both growing phases. Dietary nPP levels of 0.41 for 0 to14 D and 0.31 for 15 to 31 D were required for optimum growth performance (Table 5). Above those levels, growth performances were insignificant although linear improvement ($P < 0.001$) was seen with an increase in dietary nPP levels. This finding agrees with the recent studies in which broilers fed with corn-soy based diet containing different levels of nPP from 0.10 to 0.58% showed linear improvement in growth performance and daily weight gain reached plateau at 0.38% of dietary nPP content on 21 D of age (Waldroup et al., 2000; Liu et al., 2017). In the same study, Liu et al. (2017) found that feed per gain ratio did not improve further when dietary nPP level was 0.23% and above. Similar outcome was found in the present study in which there was no treatment effect on feed per gain ratio during first 14 D where dietary nPP levels ranged from 0.33 to 0.53% (Table 5). Nevertheless, feed per gain ratio was affected ($P < 0.001$) by dietary nPP levels from 15 to 31 D and an increase in dietary nPP content linearly improved the feed efficiency of the birds ($P < 0.001$). No significant interaction between depletion ratio and dietary nPP levels was also in agreement with the previous findings. High depletion was seen when birds were feed with dietary nPP of below 0.33% (Denbow et al., 1995; Liu et al., 2017).

In the present study, Ca:nPP ratio was decreased as dietary nPP contents were increased across the treatments. By increasing dietary nPP levels and consequently decreasing Ca:nPP ratio, growth parameters were linearly improved in both growth phases ($P < 0.05$). This suggests that the increase in dietary nPP improves growth performance while dietary Ca was adequate. In other words, high Ca:nPP ratio resulted in poor growth and bone performance compared to lower Ca:nPP ratio groups. This has been reported in previous studies (Rama Rao et al., 2006; Li et al., 2017; Liu et al., 2017). Improvement in broiler body weight gain, tibia ash, and tibia strength at the 42 D of age was seen with an increase in dietary nPP from 0.23 to 0.43% while keeping Ca at 0.90% (Rama Rao et al., 2006). The same authors reported in another study that the increase in dietary nPP from 0.25 to 0.40% improved body weight gain, feed per gain, and tibia ash content without changing feed Ca content (Rama Rao et al., 1999).

Bone ash contents and bone strengths are the good indicators to interpret the bone mineralization of Ca

and P. In the present study, fat was not extracted from the bones prior to ash analysis. Studies have confirmed that bone ash results were reliable and comparable regardless of fat extraction (Yan et al., 2005; Garcia and Dale, 2006). In their study, Liu et al. (2017) concluded that tibia ash, toe ash, and tibia breaking strength reached to plateau at dietary nPP level of 0.38% on 21 D of age. Optimum tibia ash content was found at dietary nPP levels of 0.35% in broiler at 21 D of age (Han et al., 2018). In the current study, tibia ash content reached to plateau at 0.37 and 0.31% for 14 and 31 D of age, respectively. Overall, dietary nPP levels of 0.41% for 0 to 14 D and 0.31% for 15 to 31 D were required for desirable tibia ash content. This finding further strengthens the assumption that an increase in dietary nPP contents with adequate dietary Ca improves bone characteristics in broilers. Toe ash and tibia breaking strength were significantly affected by dietary nPP levels only in the first phase.

Different studies on broiler intestinal *NaPi-IIb* expression related to Ca and nPP were reported at the broiler age between 0 to 21 D (Yan et al., 2007; Han et al., 2009; Li et al., 2012; Huber et al., 2015; Ajuwon et al., 2016; Liu et al., 2017). Data on *NaPi-IIb* expression in nPP restricted broilers older than 21 D are lacking. There were 2 reports that compared the expression of *NaPi-IIb* in broilers at different ages (Li et al., 2017; Han et al., 2018). However, those studies did not restrict dietary nPP levels. In this report, role of duodenal *NaPi-IIb* expression in response to dietary nPP restriction has been studied both at younger age (14 D) and older age (31 D). Intestinal *NaPi-IIb* is responsible for over 90% active Pi transport in mammals (Sabbagh et al., 2009) with the highest expression found in duodenum of the chickens (Yan et al., 2007; Han et al., 2009; Fang et al., 2012). In the present study, relative expression of *NaPi-IIb* in duodenal mucosal cells was analyzed by reverse transcriptase quantitative-PCR. In both growth phases, dietary nPP levels significantly influenced in relative expression of *NaPi-IIb* (Figures 1 and 2). Moreover, *NaPi-IIb* expression linearly increased with a reduction in dietary nPP ($P < 0.001$). During starter phase, *NaPi-IIb* in birds received lowest nPP diet (0.33%) expressed 2.20 folds higher than that of highest nPP group (0.53%). As dietary nPP inclusion increased, expression of *NaPi-IIb* consistently decreased. In grower phase, *NaPi-IIb* expressed 3.58 folds higher in lowest nPP group (0.23%) than in highest nPP group (0.43%). A similar linear increase in expression of *NaPi-IIb* in response to reduction in dietary nPP was observed in the recent studies in which broilers were fed with different levels of dietary nPP from 1 to 21 D of age (Liu et al., 2017; Han et al., 2018). Study in laying hens also showed the effect of dietary P restriction on *NaPi-IIb* expression (Nie et al., 2013). Contrary, no significant effect of dietary nPP content was observed in laying hen duodenal *NaPi-IIb* expression (Jing et al., 2018) and jejunal *NaPi-IIb* expression (Huber et al., 2006). Downregulation of *NaPi-IIb* in

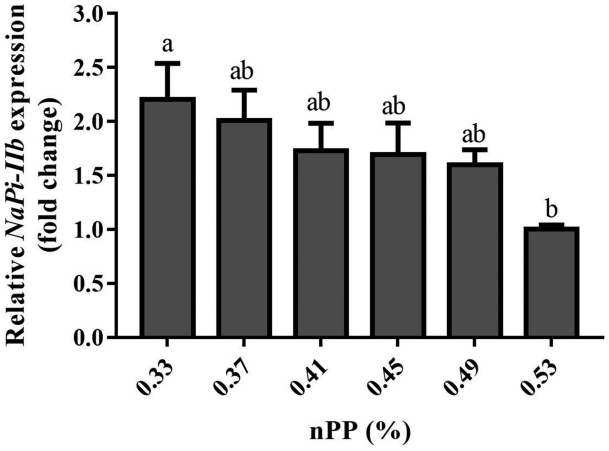

Figure 1. Relative quantitation of *NaPi-IIb* in broilers at 14 days of age in response to different (0.33-0.53%) dietary non-phytate phosphorus (nPP) levels. Mean fold changes in relative expression of *NaPi-IIb* compared to *β-actin* were calculated relative to the respective values at the nPP level of 0.53 (n = 6). Within comparisons, means in each bar with no common alphabet differs significantly ($P < 0.05$).

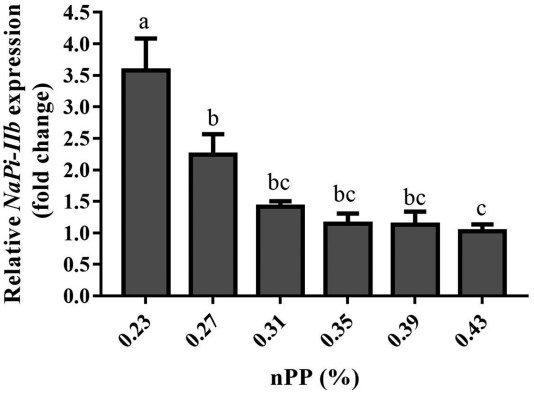

Figure 2. Relative quantitation of *NaPi-IIb* in broilers at 31 days of age in response to different dietary non-phytate phosphorus (nPP) levels. Mean fold changes in relative expression of *NaPi-IIb* compared to *β-actin* were calculated relative to the respective values at the nPP level of 0.43 (n = 6). Within comparisons, means in each bar with no common alphabet differs significantly ($P < 0.01$).

higher nPP groups was explained that the paracellular P transport pathway which transports intestinal P passively into blood predominates postprandial P transport pathway (Cross et al., 1990). Huge amount of phosphate in digesta after meal induces the Na-independent P transport and hence in high nPP diet groups, Na-dependent pathway might have less workload to transport P compared to low nPP diet groups (Cross et al., 1990). Our study clearly showed that regardless of Ca:nPP ratio, duodenal *NaPi-IIb* was upregulated in low nPP groups and the effect was linear ($P < 0.001$). The same response was also observed by Han et al. (2018), where Ca was included the same in all diets with different nPP contents. Moreover, a study in laying hens revealed that expression of *NaPi-IIb* linearly decreased with an increase in dietary nPP levels whereas Ca level was maintained the same for all diets (Nie et al., 2013). In their study, Li et al. (2012) found that it was dietary P that influences the intestinal *NaPi-IIb* expression and

Ca levels did not influence *NaPi-IIb* expression ($P = 0.77$). However, Li et al. (2012) claimed that imbalance of Ca in diets adversely affected expression of *NaPi-IIb* by impairing the relative absorption of nPP. The concentration of Ca does not effect on Na-dependent P transport, and hence Ca and P transport systems are assumed to work separately (Berner et al., 1976; Matsumoto et al., 1980; Murer and Hildmann, 1981). It is to note that, in our study, Ca was provided at standard does for all treatments. In pigs fed with low P diets, intestinal P uptake was independent of Ca concentration (Saddoris et al., 2010). Another study suggested that parathyroid gland could sense the changes in plasma P level, whereas Ca level was unchanged (Khoshniat et al., 2011). Broilers fed with low P diet (0.25%) from hatch to 4 D increased duodenal *NaPi-IIb* expression in 3 folds compared to control (P 0.50%), whereas Ca:P ratio was kept the same for each treatment (Ashwell and Angel, 2010). The authors concluded that broilers fed with low P diet from hatch to 4 D could handle P deficiency in the grower and finisher phases. In our study, broilers with low nPP diet (0.33%) from hatch to 14 D increased duodenal *NaPi-IIb* expression in 2.2 folds compared to diet with nPP level of 0.53%. Data from mice experiments suggested that increased expression of intestinal *NaPi-IIb* protected severe bone demineralization during Pi deficiency (Knöpfel et al., 2017). This might be true in the present study where tibia breaking strength was not numerically significant by the end of the second growth phase even though significant results were found in the first phase ($P < 0.01$). It was observed that a reduction of dietary nPP to 0.04% from the recommended dose (0.45 to 0.41% for phase 1 and 0.35 to 0.31% for phase 2) could be practiced without affecting growth performance and bone parameters adversely. Higher expression of *NaPi-IIb* in nPP-deficient diet groups might help in increasing Pi update from the small intestine.

In conclusion, body weight gain, feed efficiency, bone ash content, and bone strength were improved with the increase in dietary nPP at the adequate level of dietary Ca. Tibia ash content was found to be more sensitive than toe ash in response to dietary nPP in grower period (31 D). For optimum growth and bone characteristics, dietary nPP levels of 0.41% for 0 to 14 D and 0.31% for 15 to 31 D were required. These values were 8.9 and 11.4% lower than the NRC (1994) recommendations for starter and grower phase, respectively. Intestinal *NaPi-IIb* is a good candidate for dietary nPP levels in chickens. Expression of *NaPi-IIb* increased constantly with decrease in dietary nPP content in both growing periods. Intestinal *NaPi-IIb* works independently from Ca:nPP ratio when the Ca requirements of chickens were met. The gap in understanding of intestinal *NaPi-IIb* expression related to dietary nPP restriction in broilers older than 21 D of age was bridged in the present study. Role of *NaPi-IIb* in response to different Ca levels with fixed nPP in diets should be studied further.

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REFERENCES

Ajuwon, K. M., P. C. Xue, and O. Adeola. 2016. Phosphorus and nitrogen utilization responses of broiler chickens to dietary crude protein and phosphorus levels. Poult. Sci. 9511:2615–2623.

Applegate, T. J., and R. Angel. 2014. Nutrient requirements of poultry publication: history and need for an update. J. Appl. Poult. Res. 233:567–575.

Ashwell, C. M., and R. Angel. 2010. Nutritional genomics: a practical approach by early life conditioning with dietary phosphorus. R. Bras. Zootec. 39 suppl spe:268–278.

Bar, A., D. Shinder, S. Yosefi, E. Vax, and I. Plavnik. 2007. Metabolism and requirements for calcium and phosphorus in the fast-growing chicken as affected by age. Br. J. Nutr. 891:51–60.

Berner, W., R. Kinne, and H. Murer. 1976. Phosphate transport into brush-border membrane vesicles isolated from rat small intestine. Biochem. J. 1603: 467–474.

Cross, H., H. Debiec, and M. Peterlik. 1990. Mechanism and regulation of intestinal phosphate absorption. Miner. Electrolyte. Metab. 162–3:115—124.

Custer, M., M. Lotscher, J. Biber, H. Murer, and B. Kaissling. 1994. Expression of Na-P(i) cotransport in rat kidney: localization by RT-PCR and immunohistochemistry. Am. J. Physiol. 2665 (Pt 2):F767–F774.

Danisi, G., J. P. Bonjour, and R. W. Straub. 1980. Regulation of Na-dependent phosphate influx across the mucosal border of duodenum by 1,25-dihydroxycholecalciferol. Pflugers Arch. 3883:227–232.

Denbow, D. M., V. Ravindran, E. T. Kornegay, Z. Yi, and R. M. Hulet. 1995. Improving phosphorus availability in soybean meal for broilers by supplemental phytase. Poult. Sci. 7411:1831–1842.

Driver, J. P., G. M. Pesti, R. I. Bakalli, and H. M. Edwards, Jr. 2006. The effect of feeding calcium- and phosphorus-deficient diets to broiler chickens during the starting and growing-finishing phases on carcass quality. Poult. Sci. 8511:1939–1946.

Fang, R., Z. Xiang, M. Cao, and J. He. 2012. Different phosphate transport in the duodenum and jejunum of chicken response to dietary phosphate adaptation. Asian Australas. J. Anim. Sci 2510:1457–1465.

Forster, I. C., L. Virkki, E. Bossi, H. Murer, and J. Biber. 2006. Electrogenic kinetics of a mammalian intestinal type IIb Na+/Pi cotransporter. J. Membr. Biol. 2123:177–190.

Fuchs, R., and M. Peterlik. 1979. Pathways of phosphate transport in chick jejunum. Pflugers Arch. 3813:217–222.

Garcia, A. R., and N. M. Dale. 2006. Foot ash as a means of quantifying bone mineralization in chicks. J. Appl. Poult. Res. 151:103–109.

Hamdi, M., S. López-Vergé, E. G. Manzanilla, A. C. Barroeta, and J. F. Pérez. 2015. Effect of different levels of calcium and phosphorus and their interaction on the performance of young broilers. Poult. Sci. 949:2144–2151.

Han, J. C., X. D. Yang, T. Zhang, H. Li, W. L. Li, Z. Y. Zhang, and J. H. Yao. 2009. Effects of 1alpha-hydroxycholecalciferol on growth performance, parameters of tibia and plasma, meat quality, and type IIb sodium phosphate cotransporter gene expression of one- to twenty-one-day-old broilers. Poult. Sci. 882:323–329.

Han, J. C., J. L. Zhang, N. Zhang, X. Yang, H. X. Qu, Y. Guo, C. X. Shi, and Y. F. Yan. 2018. Age, phosphorus, and 25-hydroxycholecalciferol regulate mRNA expression of vitamin D receptor and sodium-phosphate cotransporter in the small intestine of broiler chickens. Poult. Sci. 974:1199–1208.

Hilfiker, H., O. Hattenhauer, M. Traebert, I. Forster, H. Murer, and J. Biber. 1998. Characterization of a murine type II sodium-phosphate cotransporter expressed in mammalian small intestine. Proc. Natl. Acad. Sci. USA 9524:14564–14569.

Huber, K., R. Hempel, and M. Rodehutscord. 2006. Adaptation of epithelial sodium-dependent phosphate transport in jejunum and kidney of hens to variations in dietary phosphorus intake. Poult. Sci. 8511:1980–1986.

Huber, K., E. Zeller, and M. Rodehutscord. 2015. Modulation of small intestinal phosphate transporter by dietary supplements of mineral phosphorus and phytase in broilers. Poult. Sci. 945:1009–1017.

Hurwitz, S., I. Plavnik, A. Shapiro, E. Wax, H. Talpaz, and A. Bar. 1995. Calcium metabolism and requirements of chickens are affected by growth. J. Nutr. 12510:2679–2686.

Jing, M., S. Zhao, A. Rogiewicz, B. A. Slominski, and J. D. House. 2018. Assessment of the minimal available phosphorus needs of laying hens: Implications for phosphorus management strategies. Poult. Sci. 977:2400–2410.

Katai, K., K. Miyamoto, S. Kishida, H. Segawa, T. Nii, H. Tanaka, Y. Tani, H. Arai, S. Tatsumi, K. Morita, Y. Taketani, and E. Takeda. 1999a. Regulation of intestinal Na+-dependent phosphate cotransporters by a low-phosphate diet and 1,25-dihydroxyvitamin D3. Biochem. J. 343(Pt 3):705–712.

Katai, K., H. Tanaka, S. Tatsumi, Y. Fukunaga, K. Genjida, K. Morita, N. Kuboyama, T. Suzuki, T. Akiba, K. Miyamoto, and E. Takeda. 1999b. Nicotinamide inhibits sodium-dependent phosphate cotransport activity in rat small intestine. Nephrol. Dial. Transplant. 145:1195–1201.

Khoshniat, S., A. Bourgine, M. Julien, P. Weiss, J. Guicheux, and L. Beck. 2011. The emergence of phosphate as a specific signaling molecule in bone and other cell types in mammals. Cell. Mol. Life Sci. 682:205–218.

Knöpfel, T., E. M. Pastor-Arroyo, U. Schnitzbauer, D. V. Kratschmar, A. Odermatt, G. Pellegrini, N. Hernando, and C. A. Wagner. 2017. The intestinal phosphate transporter NaPi-IIb (Slc34a2) is required to protect bone during dietary phosphate restriction. Sci. Rep. 71:11018.

Li, J., J. Yuan, Y. Guo, Q. Sun, and X. Hu. 2012. The influence of dietary calcium and phosphorus imbalance on intestinal NaPi-IIb and calbindin mRNA expression and tibia parameters of broilers. Asian-Australas. J. Anim. Sci. 254:552–558.

Li, J., J. Yuan, Z. Miao, and Y. Guo. 2017. Effects of age on intestinal phosphate transport and biochemical values of broiler chickens. Asian-Australas. J. Anim. Sci. 302:221–228.

Liu, S. B., X. D. Liao, L. Lu, S. F. Li, L. Wang, L. Y. Zhang, Y. Jiang, and X. G. Luo. 2017. Dietary non-phytate phosphorus requirement of broilers fed a conventional corn-soybean meal diet from 1 to 21 d of age. Poult. Sci. 961:151–159.

Liu, S., W. Tang, J. Zhou, J. R. Stubbs, Q. Luo, M. Pi, and L. D. Quarles. 2006. Fibroblast growth factor 23 is a counter-regulatory phosphaturic hormone for vitamin D. J. Am. Soc. Nephrol. 175:1305–1315.

Manangi, M. K., and C. N. Coon. 2008. Phytate phosphorus hydrolysis in broilers in response to dietary phytase, calcium, and phosphorus concentrations. Poult. Sci. 878:1577–1586.

Matsumoto, T., O. Fontaine, and H. Rasmussen. 1980. Effect of 1,25-dihydroxyvitamin D-3 on phosphate uptake into chick intestinal brush border membrane vesicles. Biochim. Biophys. Acta. 5991:13–23.

Miyamoto, K., M. Ito, S. Tatsumi, M. Kuwahata, and H. Segawa. 2007. New aspect of renal phosphate reabsorption: the type IIc sodium-dependent phosphate transporter. Am. J. Nephrol. 275:503–515.

Miyamoto, K., H. Segawa, K. Morita, T. Nii, S. Tatsumi, Y. Taketani, and E. Takeda. 1997. Relative contributions of Na+-dependent phosphate co-transporters to phosphate transport in mouse kidney: RNase H-mediated hybrid depletion analysis. Biochem. J. 327:735–739.

Murer, H., and B. Hildmann. 1981. Transcellular transport of calcium and inorganic phosphate in the small intestinal epithelium. Am. J. Physiol. 2406:G409–G416.

Nie, W., Y. Yang, J. Yuan, Z. Wang, and Y. Guo. 2013. Effect of dietary nonphytate phosphorus on laying performance and small intestinal epithelial phosphate transporter expression in Dwarf pink-shell laying hens. J. Anim. Sci. Biotechnol. 41:34.

National Research Council. 1994. Nutrient Requirements of Poult. 9th rev. ed. Natl. Acad. Press, Washington, DC.

National Research Council. 2010. Guide for the Care and Use of Laboratory Animals. 8th rev. ed. Natl. Acad. Press, Washington, DC.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic. Acids. Res. 299:45e.

Rama Rao, S. V, M. V. L. N. Raju, M. R. Reddy, and P. Pavani. 2006. Interaction between dietary calcium and non-phytate phosphorus levels on growth, bone mineralization and mineral excretion in commercial broilers. Anim. Feed Sci. Technol. 1311:135–150.

Rama Rao, S. V., V. Ramasubba Reddy, and V. Ravindra Reddy. 1999. Non-phytin phosphorus requirements of commercial broilers and White Leghorn layers. Anim. Feed Sci. Technol. 801:1–10.

Sabbagh, Y., H. Giral, Y. Caldas, M. Levi, and S. C. Schiavi. 2011. Intestinal phosphate transport. Adv. Chronic Kidney Dis. 182:85–90.

Sabbagh, Y., S. P. O'Brien, W. Song, J. H. Boulanger, A. Stockmann, C. Arbeeny, and S. C. Schiavi. 2009. Intestinal npt2b plays a major role in phosphate absorption and homeostasis. J. Am. Soc. Nephrol. 2011:2348–2358.

Saddoris, K. L., J. C. Fleet, and J. S. Radcliffe. 2010. Sodium-dependent phosphate uptake in the jejunum is post-transcriptionally regulated in pigs fed a low-phosphorus diet and is independent of dietary calcium concentration. J. Nutr. 1404:731–736.

Segawa, H., I. Kaneko, A. Takahashi, M. Kuwahata, M. Ito, I. Ohkido, S. Tatsumi, and K. Miyamoto. 2002. Growth-related renal type II Na/Pi cotransporter. J. Biol. Chem. 27722:19665–19672.

Shafey, T. M., M. W. McDonald, and R. A. Pym. 1990. Effects of dietary calcium, available phosphorus and vitamin D on growth rate, food utilisation, plasma and bone constituents and calcium and phosphorus retention of commercial broiler strains. Br. Poult. Sci. 313:587–602.

Shim, M. Y., A. B. Karnuah, A. D. Mitchell, N. B. Anthony, G. M. Pesti, and S. E. Aggrey. 2012. The effects of growth rate on leg morphology and tibia breaking strength, mineral density, mineral content, and bone ash in broilers. Poult. Sci. 918:1790–1795.

Tenenhouse, H. S. 2005. Regulation of phosphorus homeostasis by the type IIa na/phosphate cotransporter. Annu. Rev. Nutr. 25:197–214.

Tenenhouse, H. S., S. Roy, J. Martel, and C. Gauthier. 1998. Differential expression, abundance, and regulation of Na+-phosphate cotransporter genes in murine kidney. Am. J. Physiol. 2754 (Pt 2):F527–E534.

Veum, T. L. 2010. Phosphorus and calcium nutrition and metabolism. Pages 94–111 in Phosphorus and Calcium Utilization and Requirements in Farm Animals. M. S. S. V. Dorinha, and E. Kebreab, eds. Oxfordshire, UK, CAB International.

Waldroup, P. W., J. H. Kersey, E. A. Saleh, C. A. Fritts, F. Yan, H. L. Stilborn, J. R. C. Crum, and V. Raboy. 2000. Nonphytate phosphorus requirement and phosphorus excretion of broiler chicks fed diets composed of normal or high available phosphate corn with and without microbial phytase. Poult. Sci. 7910:1451–1459.

Werner, A., and R. K. Kinne. 2001. Evolution of the Na-P(i) cotransport systems. Am. J. Physiol. Regul. Integr. Comp. Physiol. 2802:R301–R312.

Yan, F., R. Angel, and C. M. Ashwell. 2007. Characterization of the chicken small intestine type IIb sodium phosphate cotransporter. Poult. Sci. 861:67–76.

Yan, F., C. A. Keen, K. Y. Zhang, and P. W. Waldroup. 2005. Comparison of methods to evaluate bone mineralization. J. Appl. Poult. Res. 143:492–498.