

Dietary supplemental vitamin D₃ enhances phosphorus absorption and utilisation by regulating gene expression of related phosphate transporters in the small intestine of broilers

Yuxin Shao†, Qian Wen†, Shumin Zhang, Lin Lu, Liyang Zhang, Xiudong Liao* and Xugang Luo*
Mineral Nutrition Research Division, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, People's Republic of China

(Submitted 24 May 2018 – Final revision received 23 August 2018 – Accepted 23 August 2018 – First published online 29 October 2018)

Abstract

The present study was carried out to evaluate the effect of dietary supplemental vitamin D₃ (VD₃) on P absorption and utilisation as well as its related mechanisms in the small intestine of broilers. A total of 384 1-d-old Arbor Acres male broilers were assigned randomly into four treatments following a completely randomised design with a 2 (dietary non-phytate P (NPP) contents: 0.43 and 0.22%) × 2 (dietary VD₃ supplemental levels: 0 and 87.5 µg/kg) factorial arrangement. The experiment lasted for 22 d. The results showed that P contents in serum from the hepatic portal vein and tibia ash of broilers were higher ($P < 0.05$) for 0.43% NPP than for 0.22% NPP. The type IIb Na-dependent phosphate cotransporter (NaP-IIb) protein expressions in the duodenum and ileum were higher ($P < 0.05$) also for 0.43% NPP than 0.22% NPP. Supplementation of VD₃ enhanced ($P < 0.05$) tibia P retention rate and type III Na-dependent phosphate cotransporter (PiT)-1 protein expression in the duodenum of all broilers. Moreover, VD₃ supplementation decreased ($P < 0.002$) mortality and increased ($P < 0.02$) serum P content from the hepatic portal vein after 4 h of feeding, tibia ash content, tibia ash P content and protein expressions of NaP-IIb and PiT-1 in the jejunum of broilers fed diet with 0.22% NPP. Thus, dietary supplemental VD₃ promoted intestinal P absorption and bone P utilisation, and this effect might be associated with enhanced PiT-1 levels in the duodenum and PiT-1 and NaP-IIb levels in the jejunum respectively when dietary NPP is limiting.

Key words: Vitamin D₃; Phosphorus; Absorption; Utilisation; Sodium-dependent phosphate cotransporters; Broilers

As a necessary mineral element for human and animals, P has several essential functions in nutrient metabolism, bone mineralisation, nucleic acid synthesis and signal transduction⁽¹⁾. P deficiency can cause a reduced feed intake and abnormal skeletal development as well as a retarded growth performance and high mortality in chickens^(2,3). To meet the requirements of fast-growing chicks, adequate dietary non-phytate P (NPP) were added to diets⁽⁴⁾. P excess to requirement is excreted in the urine, leading not only to the economic cost but also to the environmental P pollution^(5,6).

Previous studies in human and rats have shown that vitamin D₃ (VD₃) plays a vital role in maintaining P homeostasis, improving intestinal P absorption and stimulating the differentiation and activation of osteoblasts^(7,8) as has been observed in chickens^(9,10). 25-Hydroxycholecalciferol (25(OH)-D₃) and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂-D₃) are two biological activated forms of VD₃. Many researchers found that VD₃ or 1,25(OH)₂-D₃ could increase bone mineral density and ash content, and reduce

leg abnormalities using chickens as a model^(11–13). Broiler chickens have the advantage of short feeding period and share significant genetic conservation with humans⁽¹⁴⁾. Therefore, broilers have served as an important model for investigating the mechanisms of dietary supplemental VD₃ in mediating the regulation of P absorption in the small intestine and P retention in the bone. The type IIb Na-dependent phosphate cotransporter (NaP-IIb) is considered to be responsible for regulating P transport and absorption^(15,16). Supplemental 1,25(OH)₂-D₃ enhanced P absorption by upregulation of *NaP-IIb* expression in the *in situ* ligated duodenal loop of broilers⁽¹⁷⁾ and in the jejunum of mice⁽¹⁸⁾. The type III Na-dependent phosphate cotransporters (PiT), PiT-1 and PiT-2 are reported to be associated with intestinal P absorption and renal P reabsorption in the rats⁽¹⁹⁾. The PiT mediate P transport by providing P for bone growth and mineralisation^(20,21). Moreover, dietary supplemental 1,25(OH)₂-D₃ or P deprivation could regulate the *PiT-1* and *PiT-2* expressions in the parathyroid⁽²²⁾ and intestine⁽²³⁾ of

Abbreviations: 1α-OH D₃, 1α-hydroxycholecalciferol; 1,25(OH)₂-D₃, 1,25-dihydroxyvitamin D₃; 25(OH)-D₃, 25-hydroxycholecalciferol; ADFI, average daily feed intake; ADG, average daily gain; F:G, feed:gain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NaP-IIb, type IIb Na-dependent phosphate cotransporter; NPP, non-phytate P; PiT, type III Na-dependent phosphate cotransporter; TPRR, tibia P retention rate; VD₃, vitamin D₃.

* **Corresponding authors:** X. Liao, email liaoxd56@163.com; X. Luo, fax +86 10 62810184, email wlysz@263.net

† These authors contributed equally to this work.

rats. Similar as the cotransporter NaP-IIb, the roles of PiT-1 and PiT-2 in P utilisation have not been studied well in the poultry. We hypothesised that dietary supplementation of VD₃ might increase P absorption in the small intestine and utilisation in bone of the fast-growth broiler chickens by regulating NaP-IIb, PiT-1 and PiT-2 expressions. Therefore, the objective of the present study was to test the above hypotheses by investigating the effect of dietary supplemental VD₃ on growth performance, serum P content from the hepatic portal vein, tibia ash content, tibia ash P content, tibia P retention rate (TPRR), mRNA and protein expressions of P transporters in the duodenum, jejunum and ileum of broilers.

Methods

Experimental design, animals and diets

All experimental procedures were approved by the Animal Management Committee (in charge of animal welfare issue) of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS-CAAS, Beijing, China) and performed in accordance with the Animals in Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines for reporting animal research⁽²⁴⁾. Ethical approval on animal survival was given by the animal ethics committee of IAS-CAAS. A completed ARRIVE guidelines checklist is included.

A completely randomised design involving in a 2 (dietary NPP contents) × 2 (VD₃ supplemental levels) factorial arrangement of treatments was used in this study. The two dietary NPP contents were a normal level of 0.45% and a P-deficient level of 0.23% (the analysed values were 0.43 and 0.22%, respectively). The two VD₃ supplemental levels were 0 and 87.5 µg/kg of the diet. A total of 384 1-d-old Arbor Acres commercial male broiler chicks (average body weight: 47.3 (SEM 0.32) g) were obtained from Huadu Broiler Breeding Corp. The birds were randomly divided into one of four treatments with eight replicate cages of twelve birds per cage for each treatment. In addition, forty-eight birds (four replicate cages of twelve broilers per cage) were assigned a group as the reference group and provided with *ad libitum* feed containing 0.43% NPP and supplemental 87.5 µg VD₃/kg. To ensure the equal amount of NPP intake in two levels of supplemental VD₃, the feed intake of the birds fed diets containing 0.43% NPP was restricted to 90% of that of the reference birds (the feeding amount was shown to be eaten up). The feed intake of the birds fed diets containing 0.22% NPP was restricted to 70% of that of the reference birds, because our preliminary experiment showed that the feed intake of the birds fed 0.22% NPP diets was reduced by 20% compared with that of the birds fed 0.43% NPP diets. The maize–soyabean-meal basal diet was formulated to meet or exceed National Research Council (NRC)⁽³⁾ requirements of 1–21 d-old broilers for all nutrients except for NPP (Table 1). All diets were fed in mash form.

All birds were housed in an electrically heated, thermostatically controlled room equipped with fibreglass feeders, waterers and stainless steel cages coated with plastics. The birds had free access to tap water, and the room was maintained on a 24 h constant white light (wavelength, 450–465 nm, which

Table 1. Ingredients and nutrient composition of basal diets for broilers from 1 to 21 d of age (as-fed basis)

Item	Dietary non-phytate P levels (%)	
	0.45	0.23
Ingredients (%)		
Maize	52.05	52.05
Soyabean meal	39.05	39.05
Soyabean oil	4.64	4.64
Dicalcium phosphate*	1.98	0.68
Limestone*	1.11	1.85
Sodium chloride*	0.30	0.30
DL-Met	0.33	0.33
Sands	0.09	0.65
Micronutrients†	0.35	0.35
Maize starch + VD ₃ ‡	0.10	0.10
Nutrient composition		
Metabolisable energy (MJ/kg)	12.53	12.53
Crude protein (%)*§	21.78	21.81
Lys (%)	1.16	1.16
Met (%)	0.62	0.62
Met + Cys (%)	0.92	0.92
Ca (%)*§	1.02	0.99
Total P (%)*§	0.70	0.48
Non-phytate P (%)*§	0.43	0.22

VD₃, vitamin D₃.

* Feed grade.

† Supplied per kg of diet: vitamin A, 4.5 mg; vitamin E, 24 mg; vitamin K, 3.5 mg; thiamin, 3.0 mg; riboflavin, 6 mg; vitamin B₆, 3.0 mg; pantothenic acid, 15 mg; cobalamin, 0.018 mg; niacin, 39 mg; biotin, 0.15 mg; folic acid, 1.5 mg and choline (choline chloride), 700 mg; Cu (CuSO₄·5H₂O), 8 mg; Zn (ZnSO₄·7H₂O), 60 mg; Fe (FeSO₄·7H₂O), 80 mg; Mn (MnSO₄·H₂O), 110 mg; Se (Na₂SeO₃), 0.15 mg; iodine (KI), 0.35 mg.

‡ VD₃ was added to diets by replacing an equal weight of maize starch.

§ These values were determined based on triplicate assays; and other nutrient levels were calculated values.

would not allow endogenous vitamin D synthesis) schedule throughout the entire experiment. The experiment lasted for 22 d. Mortalities and health status were observed visually and recorded daily throughout the experiment. Body weight and feed intake of birds were measured and corrected for mortality to calculate the average daily gain (ADG), average daily feed intake (ADFI), the ratio of feed:gain (F:G) and mortality of broilers from 1 to 21 d of age.

Sample collections and preparations

After being fed for 2 or 4 h, two birds close to the cage average body weight were selected from each replicate cage at 21 d of age, respectively. Chicks were anaesthetised by injections of sodium pentobarbital (20 mg/kg body weight) through the wing vein. Then the blood was collected aseptically from the hepatic portal vein under anaesthetic, and the serum was obtained after centrifuging at 3000 g for 10 min at 4°C and stored at –20°C for analysis of P content. After the blood sampling, the birds fed for 4 h were killed by cervical dislocation. The duodenum (about 10 cm distal to the pylorus), jejunum (about 10 cm preceding the Meckel's diverticulum) and ileum (about 10 cm preceding the ileocaecal junction) were separated⁽²⁵⁾. The subsection of each part of the small intestine was flushed gently with 0.1% diethyl pyrocarbonate and the mucosa samples were scraped

with an ice-cold slide and snap-frozen in liquid N₂ for analyses of mRNA and protein expressions, respectively. Right tibia was peeled and frozen at -20°C for analyses of the contents of bone ash and bone ash P. Besides, after fasting for 6 or 10 h, another two birds close to the cage average body weight were selected from each replicate cage at 22 d of age, respectively. The serum from the hepatic portal blood, the mucosa from the intestine and the bone from right tibia were collected following the same methods described as above. To reduce individual biological variation, the samples with equal weight from two birds in each replicate cage were pooled into one sample for analyses.

Tibia ash content

All soft tissues of the collected tibia were stripped and then the tibia were dried in an oven at 105°C for 24 h and defatted with fresh diethyl ether for 48 h, and then dried at 105°C for 12 h. The dried defatted bones were weighted and finally ashed in a muffle furnace at 550°C for 16 h. After that, the tibia ash content was expressed relative to a dry defatted tibia weight.

Measurements of crude protein, calcium, total phosphorus and phytate phosphorus

The crude protein contents in feed ingredients and diet samples were analysed with the Kjeldahl method⁽²⁶⁾ and Ca contents were determined by an inductively coupled plasma spectroscopy (model IRIS Intrepid II; Thermo Jarrell Ash)^(27,28). Total P contents in feed ingredients, diet samples and tibia ash were measured colorimetrically using the vanadate-molybdate method⁽²⁹⁾. Validations of the Ca and total P analyses were conducted concurrently using bovine liver powder (GBW (E) 080193; National Institute of Standards and Technology) as a standard reference material. The actual Ca and P recovery rates for bovine liver powder were both determined to be about 99% in the present study. Dietary phytate P contents were determined according to the ferric precipitation method as described previously^(30,31). Dietary NPP = dietary total P - dietary phytate P. Serum P contents were determined with molybdenum-blue method according to Goldenberg & Fernandez⁽³²⁾.

Calculation of the tibia phosphorus retention rate

The TPRR was calculated according to the following equation:

$$\text{TPRR}(\%) = 2 \times (\text{right tibia ash weight (g/bird)} \\ \times \text{right tibia ash P content (\%)}) \\ / (\text{total dietary P intake (g/bird)}) \times 100\%.$$

RNA extraction, RT and real-time quantitative PCR

Total RNA was isolated using TRIzol reagent (catalogue no. 15596018; Life Technologies) and reversely transcribed into complementary DNA (cDNA) according to the manufacturer's instructions. The concentration of total RNA was estimated by

measuring its optical density at 260 and 280 nm with a spectrophotometer (ND-100; NanoDrop Technologies), and the purity was determined in agarose gels stained with ethidium bromide. In all, 500 ng of total RNA was reversely transcribed into cDNA using PrimeScript™ RT Master Mix (catalogue no. RR047A; TaKaRa Bio Inc.) according to the manufacturer's instructions. cDNA was used as templates for real-time quantitative PCR amplification using SYBR green master mix (catalogue no. 4367659; Life Technologies) in the ABI 7500 Real-Time PCR machine following the manufacturer's guidelines. The gene-specific primers for *NaP-IIb*, *PiT-1*, *PiT-2*, *β-actin* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used as follows: forward CTGGATGCACTCCCTAGAGC, reverse TTATC TTTGGCACCTCCTG for *NaP-IIb* (NM_204474.1); forward GCT CGTGGCTTCGTTCTTG, reverse GGACCAITTTGACGCCTTTCT for *PiT-1* (XM_015297502.1); forward GCAGCAGATACATCAACTC, reverse ATTTCCACTCCACCCTC for *PiT-2*; forward GAG AAATTG TGCGTGACATCA, reverse CCTGAACCTCTCATTGCCA for *β-actin* (NM_205528.1); forward CTTTGGCATTGTGGAGGGTC, reverse ACGCTGGGATGATGTTCTGG for *GAPDH* (K01458.1). Internal reference genes, both *β-actin* and *GAPDH*, were constant across the dietary treatment groups, and thus their geometric mean could be used to normalise the expression of the targeted gene. Relative gene expression was calculated using the 2^{-ΔΔC_t} method⁽³³⁾. All the samples were analysed in triplicates.

Western blotting assay

Frozen intestinal mucosal samples (40 mg) were homogenised in 0.5 ml of ice-cold radio immunoprecipitation assay (RIPA) lysis buffer (catalogue no. P0013B; Beyotime Institute of Biotechnology) supplemented with 5 μl of protease inhibitor (catalogue no. B14001; BioTool). The homogenate was centrifuged at 10 000 g for 10 min at 4°C, and the supernatant was collected for total protein determination using a bicinchoninic acid Protein Assay kit (catalogue no. 23225; Pierce). The extracted protein (20 μg) was subjected to electrophoresis on a 10% SDS-PAGE gel, and then electrotransferred onto the polyvinylidene fluoride membranes (catalogue no. IPVH00010; Merck-Millipore). After the transfer, membranes were blocked for 1 h at room temperature in a blocking buffer with 5% non-fat milk, and then incubated overnight at 4°C with the following primary antibodies: NaP-IIb (catalogue no. sc-160605; Santa Cruz Biotechnology), PiT-1 (catalogue no. sc-98814; Santa Cruz Biotechnology), PiT-2 (catalogue no. ab64412; Abcam), GAPDH (catalogue no. HX1828; Huaxingbio) and *β-actin* (catalogue no. HX1827; Huaxingbio). After washing, membranes were incubated with the secondary antibody of goat anti-rabbit (catalogue no. HX2030; Huaxingbio) or goat anti-mouse (catalogue no. HX2032; Huaxingbio) for 1 h at room temperature. The signals were recorded with an Image Quant LAS 4000 scanner (GE Healthcare Life Sciences) by enhanced chemiluminescence using a Super Signal West Pico Trail Kit (catalogue no. 34077; Pierce). Data were presented as the ratio of NaP-IIb or PiT-1 protein band intensity to GAPDH protein band intensity, and the ratio of PiT-2 protein band intensity to *β-actin* protein band intensity.



Statistical analyses

The calculation of sample size was performed using software PASS 13 (NCSS LLC). In our previous study⁽¹⁷⁾, the means of duodenal *NaP-IIIb* mRNA expression levels for control and supplemental VD₃ (30 pmol/l) treatments were 0.77 and 1.20 with a pooled SE of 0.20. Thus, four replicates per treatment are needed to achieve 80% power using a two-sided two-sample *t* test at a significance level of 0.05. Our proposed sample size in this experiment was eight replicates per treatment and should provide an additional power for the multiple comparisons as reported previously⁽³⁴⁾. All data (except for mRNA expressions of the P cotransporters in different small intestinal segments and multiple correlations) were analysed by two-way ANOVA using the general linear model procedure of SAS (version 9.2; SAS Institute Inc.). The model was comprised of the main effects (dietary NPP level and supplemental VD₃ level) and interactions between the main effects. The data from mRNA expressions of the P cotransporters in different small intestinal segments were analysed by one-way ANOVA. Multiple correlations between P absorption, utilisation or bone ash and all of the transporter protein levels were analysed using Pearson procedure⁽³⁵⁾. The replicate cage of twelve chicks for growth performance or two chicks for other indices served as the experimental unit. Data from mortality were transformed to arcsine for analysis. Differences

among means were tested by the least significant difference method, and the statistical significance was set at $P \leq 0.05$.

Results

Growth performance and mortality

Dietary NPP level affected ($P < 0.0001$) ADG and ADFI of broilers from 1 to 21 d of age, but did not affect ($P > 0.13$) F:G (Table 2). Dietary supplemental VD₃ level did not affect ($P > 0.32$) ADG, ADFI and F:G. However, there was an interaction ($P < 0.002$) between NPP level and VD₃ level on the mortality. Compared with the 0.22% NPP, the 0.43% NPP increased ($P < 0.0001$) ADG and ADFI. When the broilers were fed diets containing the 0.43% NPP level, VD₃ supplementation had no effect ($P > 0.83$) on mortality of broilers. At dietary 0.22% NPP level, however, VD₃ supplementation decreased ($P < 0.002$) mortality of broilers.

Serum phosphorus content from the hepatic portal vein

The serum P contents of the broilers fed for 2 h at 21 d of age or fasting for either 6 or 10 h at 22 d of age were affected ($P < 0.0001$) by dietary NPP level, but not ($P > 0.22$) by sup-

Table 2. Effects of dietary non-phytate phosphorus (NPP) and supplemental vitamin D₃ (VD₃) levels on growth performance and mortality of broilers from 1 to 21 d of age (Mean values with their standard errors)

Dietary NPP levels (%)*...	0.22†		0.43†		SEM	NPP levels‡		VD ₃ levels‡		P		
	0	87.5	0	87.5		0.22	0.43	0	87.5	NPP levels	VD ₃ levels	NPP × VD ₃
Added VD ₃ levels (µg/kg)...	0	87.5	0	87.5		0.22	0.43	0	87.5			
ADG (g/d)	25.6	25.9	33.2	33.7	0.38	25.7 ^b	33.4 ^a	29.4	29.8	<0.0001	0.3299	0.8055
ADFI (g/d)	36.5	37.4	46.9	46.7	0.66	36.9 ^b	46.9 ^a	41.7	42.0	<0.0001	0.6039	0.4863
F:G (g/g)	1.43	1.45	1.42	1.39	0.022	1.44	1.40	1.42	1.42	0.1359	0.8438	0.3441
Mortality (%)	19.8 ^a	2.08 ^b	1.04 ^b	0.00 ^b	0.045	10.9	0.52	10.4	1.04	<0.0001	<0.0001	0.0013

ADG, average daily gain; ADFI, average daily feed intake; F:G, feed:gain.
^{a,b} Mean values with unlike superscript letters within the same row are significantly different ($P < 0.01$).
 * The levels were determined based on triplicate assays.
 † Data represent the mean values of eight replicates of twelve birds each ($n 8$).
 ‡ Data represent the mean values of sixteen replicates of twelve birds each ($n 16$).

Table 3. Effects of dietary non-phytate phosphorus (NPP) and supplemental vitamin D₃ (VD₃) levels on phosphorus contents in serum from the hepatic portal vein of broilers at 21 or 22 d of age (Mean values with their standard errors)

Dietary NPP levels (%)*...		0.22†		0.43†		SEM	NPP levels‡		VD ₃ levels‡		P		
		0	87.5	0	87.5		0.22	0.43	0	87.5	NPP levels	VD ₃ levels	NPP × VD ₃
Added VD ₃ levels (µg/kg)...		0	87.5	0	87.5		0.22	0.43	0	87.5			
P contents in serum (mmol/l)	After 2 h of feeding§	0.96	1.00	2.14	2.02	0.108	0.98 ^b	2.08 ^a	1.55	1.51	<0.0001	0.2693	0.1348
	After 4 h of feeding§	0.85 ^c	1.34 ^b	2.35 ^a	2.24 ^a	0.120	1.09	2.30	1.60	1.79	<0.0001	0.0610	0.0013
	After 6 h of fasting	0.87	0.95	1.98	2.06	0.105	0.91 ^b	2.02 ^a	1.43	1.51	<0.0001	0.2285	0.9006
	After 10 h of fasting	1.03	0.95	1.87	1.84	0.085	0.99 ^b	1.86 ^a	1.45	1.36	<0.0001	0.3570	0.6765

^{a,b,c} Mean values with unlike superscript letters within the same row are significantly different ($P < 0.005$).
 * The levels were determined based on triplicate assays.
 † Data represent the mean values of eight replicates of two birds each ($n 8$).
 ‡ Data represent the mean values of sixteen replicates of two birds each ($n 16$).
 § Data were from the hepatic portal vein of broilers at 21 d of age.
 || Data were from the hepatic portal vein of broilers at 22 d of age.

Table 4. Effects of dietary non-phytate phosphorus (NPP) and supplemental vitamin D₃ (VD₃) levels on tibia ash, ash phosphorus and phosphorus retention rate of broilers at 21 d of age (Mean values with their standard errors)

Dietary NPP levels (%)*...	0.22†		0.43†		SEM	NPP levels‡		VD ₃ levels‡		P		
	0	87.5	0	87.5		0.22	0.43	0	87.5	NPP levels	VD ₃ levels	NPP × VD ₃
Added VD ₃ levels (µg/kg)...	0	87.5	0	87.5		0.22	0.43	0	87.5			
Tibia ash (%)§	39.5 ^c	44.6 ^b	48.7 ^a	49.2 ^a	0.93	42.1	46.9	44.1	46.9	<0.0001	0.0053	0.0192
Tibia ash P (%)	16.9 ^c	17.2 ^b	17.6 ^a	17.5 ^a	0.06	17.0	17.6	17.2	17.3	<0.0001	0.3154	0.0014
Tibia P retention rate (%)	5.36	6.15	4.67	4.96	0.153	5.76 ^a	4.82 ^b	5.02 ^b	5.55 ^a	0.001	0.0340	0.3060

a,b,c Mean values with unlike superscript letters within the same row are significantly different ($P \leq 0.05$).

* The levels were determined based on triplicate assays.

† Data represent the mean values of eight replicates of two birds each ($n = 8$).

‡ Data represent the mean values of sixteen replicates of two birds each ($n = 16$).

§ Data are expressed on a dry and defatted weight basis of tibia.

Table 5. Effects of dietary non-phytate phosphorus (NPP) and supplemental vitamin D₃ (VD₃) levels on type IIb sodium-dependent phosphate cotransporter (*NaP-IIb*) mRNA expression levels in small intestinal segments of broilers at 21 d of age* (Mean values with their standard errors)

Dietary NPP levels (%)†...	0.22‡		0.43‡		SEM	NPP levels§		VD ₃ levels§		P		
	0	87.5	0	87.5		0.22	0.43	0	87.5	NPP levels	VD ₃ levels	NPP × VD ₃
Duodenum	1.28 ^A	1.34 ^A	0.86 ^A	0.72 ^A	0.093	1.31 ^a	0.79 ^b	1.07	1.03	<0.0001	0.6878	0.2890
Jejunum	0.26 ^B	0.28 ^B	0.20 ^B	0.13 ^B	0.023	0.27 ^a	0.16 ^b	0.23	0.21	<0.0001	0.3107	0.0608
Ileum	0.069 ^{c,C}	0.277 ^{a,B}	0.130 ^{b,B}	0.108 ^{b,c,B}	0.0190	0.173	0.119	0.099	0.193	0.0074	<0.0001	<0.0001
SEM	0.059	0.055	0.068	0.041								
P	<0.0001	<0.0001	<0.0001	<0.0001								

RQ, relative quantity.

a,b,c Mean values with unlike superscript letters within the same row are significantly different ($P < 0.0001$).

A,B,C Mean values with unlike superscript letters within the same column are significantly different ($P < 0.0001$).

* *NaP-IIb* mRNA abundance was calculated as the RQ of *NaP-IIb* mRNA to the geometric mean of β -actin and *GAPDH* mRNA, and $RQ = 2^{-\Delta\Delta C_t}$ (C_t = threshold cycle).

† The levels were determined based on triplicate assays.

‡ Data represent the mean values of eight replicates of two birds each ($n = 8$).

§ Data represent the mean values of sixteen replicates of two birds each ($n = 16$).

plemental VD₃ level (Table 3). The interaction between NPP level and VD₃ level was significant ($P < 0.002$) for serum P content of the broilers fed for 4 h at 21 d of age. Compared with the 0.22% NPP, the 0.43% NPP increased ($P < 0.001$) serum P contents of the broilers fed for 2 h at 21 d of age or fasting for either 6 or 10 h at 22 d of age. When broilers were fed diets containing the 0.43% NPP level, supplementation of VD₃ had no effect ($P > 0.36$) on serum P content of the broilers fed diets for 4 h. However, when the diets contained the 0.22% NPP, the addition of VD₃ increased ($P < 0.0005$) serum P content of the broilers fed diets for 4 h. Thus, the right tibia and the mucosa of small intestinal segments of broilers fed for 4 h at 21 d of age were used for the further analyses in this study.

Tibia ash, ash phosphorus and phosphorus retention rate

Both dietary NPP level and supplemental VD₃ level affected ($P < 0.05$) the TPRR of broilers at 21 d of age (Table 4). The interactions between NPP level and VD₃ level were substantial ($P < 0.02$) for tibia ash and ash P. Compared with the 0.22% NPP, the 0.43% NPP decreased ($P = 0.001$) TPRR. In contrast, dietary VD₃ supplementation increased ($P = 0.034$) TPRR. When broilers were fed the diets containing the 0.43% NPP level, no differences ($P > 0.70$) in tibia ash and ash P were observed in the broilers fed diets with or without addition of VD₃. However, when broilers

were fed the diet containing the 0.22% NPP, the addition of VD₃ increased ($P < 0.004$) tibia ash and ash P.

Type IIb sodium-dependent phosphate cotransporter and type III sodium-dependent phosphate cotransporter-1, 2 mRNA expressions in the duodenum, jejunum and ileum

The *NaP-IIb* mRNA expressions in the duodenum and jejunum of broilers at 21 d of age were affected by ($P < 0.008$) dietary NPP level, but not ($P > 0.31$) by supplemental VD₃ level (Table 5). The interaction between NPP level and VD₃ level was significant ($P < 0.0001$) for the *NaP-IIb* mRNA expression in the ileum. Compared with the 0.22% NPP, the 0.43% NPP led to a decline ($P < 0.001$) in the *NaP-IIb* mRNA expressions in the duodenum and jejunum. When broilers were fed the diet containing the 0.43% NPP level, VD₃ supplementation did not affect ($P > 0.40$) the *NaP-IIb* mRNA expression in the ileum. At dietary 0.22% NPP level, however, VD₃ supplementation increased ($P < 0.001$) the *NaP-IIb* mRNA expression in the ileum. In addition, the *NaP-IIb* mRNA expressions were higher ($P < 0.001$) in the duodenum than in the jejunum and ileum of all broilers. No difference ($P > 0.25$) was detected between the jejunum and ileum for broilers in other three groups except for the broilers fed the diet containing the 0.22% NPP level with no addition of VD₃, which had greater ($P < 0.05$) *NaP-IIb* mRNA expressions in the jejunum than in the ileum.

Table 6. Effects of dietary non-phytate phosphorus (NPP) and supplemental vitamin D₃ (VD₃) levels on type III sodium-dependent phosphate cotransporter 1 (*PiT-1*) mRNA expression levels in small intestinal segments of broilers at 21 d of age* (Mean values with their standard errors)

Dietary NPP levels (%)†...	0.22‡		0.43‡		SEM	NPP levels§		VD ₃ levels§		P		
	0	87.5	0	87.5		0.22	0.43	0	87.5	NPP levels	VD ₃ levels	NPP × VD ₃
Added VD ₃ levels (µg/kg)...	0	87.5	0	87.5		0.22	0.43	0	87.5			
Duodenum	1.53 ^{a,A}	0.93 ^{b,A}	0.82 ^{b,A}	0.95 ^{b,A}	0.152	1.23	0.88	1.17	0.94	0.0319	0.1292	0.0242
Jejunum	0.58 ^{a,B}	0.43 ^{b,B}	0.40 ^{b,B}	0.50 ^{a,b,B}	0.051	0.51	0.45	0.49	0.47	0.2524	0.5915	0.0250
Ileum	0.83 ^B	0.78 ^A	0.82 ^A	0.85 ^A	0.091	0.81	0.84	0.83	0.82	0.7293	0.9015	0.6592
SEM	0.144	0.089	0.061	0.114								
P	0.0004	0.0023	<0.0001	0.0267								

RQ, relative quantity.
^{a,b} Mean values with unlike superscript letters within the same row are significantly different ($P < 0.05$).
^{A,B} Mean values with unlike superscript letters within the same column are significantly different ($P < 0.05$).
* *PiT-1* mRNA abundance was calculated as the RQ of *PiT-1* mRNA to the geometric mean of β -actin and *GAPDH* mRNA, and $RQ = 2^{-\Delta\Delta C_t}$ (C_t = threshold cycle).
† The levels were determined based on triplicate assays.
‡ Data represent the mean values of eight replicates of two birds each ($n = 8$).
§ Data represent the mean values of sixteen replicates of two birds each ($n = 16$).

Table 7. Effects of dietary non-phytate phosphorus (NPP) and supplemental vitamin D₃ (VD₃) level on type III sodium-dependent phosphate cotransporter 2 (*PiT-2*) mRNA expression levels in small intestinal segments of broilers at 21 d of age* (Mean values with their standard errors)

Dietary NPP levels (%)†...	0.22‡		0.43‡		SEM	NPP levels§		VD ₃ levels§		P		
	0	87.5	0	87.5		0.22	0.43	0	87.5	NPP levels	VD ₃ levels	NPP × VD ₃
Added VD ₃ levels (µg/kg)...	0	87.5	0	87.5		0.22	0.43	0	87.5			
Duodenum	0.83 ^B	0.85 ^B	1.15	1.03 ^B	0.089	0.84 ^b	1.09 ^a	0.99	0.94	0.0089	0.5988	0.4440
Jejunum	1.25 ^A	1.43 ^A	1.30	1.74 ^A	0.130	1.34	1.52	1.28 ^b	1.58 ^a	0.1805	0.0272	0.3479
Ileum	1.28 ^A	1.53 ^A	1.52	1.48 ^A	0.152	1.41	1.50	1.40	1.50	0.5454	0.5155	0.3721
SEM	0.106	0.087	0.154	0.146								
P	0.0119	<0.0001	0.2555	0.0093								

RQ, relative quantity.
^{a,b} Mean values with unlike superscript letters within the same row are significantly different ($P < 0.05$).
^{A,B} Mean values with unlike superscript letters within the same column are significantly different ($P < 0.05$).
* *PiT-2* mRNA abundance was calculated as the RQ of *PiT-2* mRNA to the geometric mean of β -actin and *GAPDH* mRNA, and $RQ = 2^{-\Delta\Delta C_t}$ (C_t = threshold cycle).
† The levels were determined based on triplicate assays.
‡ Data represent the mean values of eight replicates of two birds each ($n = 8$).
§ Data represent the mean values of sixteen replicates of two birds each ($n = 16$).

Dietary NPP level, supplemental VD₃ level and their interaction had no effect ($P > 0.6$) on the *PiT-1* mRNA expression in the ileum of broilers at 21 d of age (Table 6). However, the NPP level and VD₃ level had interactive effects ($P < 0.03$) on the *PiT-1* mRNA expressions in the duodenum and jejunum. When broilers were fed the diet containing the 0.43% NPP level, dietary supplemental VD₃ level did not affect ($P > 0.20$) the *PiT-1* mRNA expressions in the duodenum and jejunum. However, when broilers were fed the diet containing the 0.22% NPP level, the addition of VD₃ reduced ($P < 0.05$) the *PiT-1* mRNA expressions in the duodenum and jejunum. In addition, the *PiT-1* mRNA expressions were higher ($P < 0.04$) in the duodenum and ileum than in the jejunum of all broilers. However, no difference ($P > 0.25$) was detected between the duodenum and ileum for broilers in other three groups except for the broilers fed the diet containing the 0.22% NPP level with no addition of VD₃, which had greater ($P < 0.05$) *PiT-1* mRNA expression in the duodenum than in the ileum.

Dietary NPP level affected ($P < 0.009$) the *PiT-2* mRNA expression in the duodenum, but had no effects ($P > 0.18$) on the *PiT-2* mRNA expressions in the jejunum and ileum of broilers at 21 d of age (Table 7). Dietary supplemental VD₃ level affected ($P < 0.03$) the *PiT-2* mRNA expression in the jejunum, but had no

effects ($P > 0.51$) on the *PiT-2* mRNA expressions in the duodenum and ileum. No interactions ($P > 0.34$) were observed between NPP level and VD₃ level for the *PiT-2* mRNA expressions in the small intestinal segments. Compared with the 0.22% NPP, the 0.43% NPP increased ($P < 0.01$) the *PiT-2* mRNA level in the duodenum. Dietary supplemental VD₃ increased ($P < 0.03$) the *PiT-2* mRNA expression in the jejunum. In addition, the *PiT-2* mRNA expressions were higher ($P < 0.05$) in the jejunum and ileum than in the duodenum, but no difference ($P > 0.24$) was detected between the jejunum and ileum.

Type IIb sodium-dependent phosphate cotransporter and type III sodium-dependent phosphate cotransporter-1, 2 protein expressions in the duodenum, jejunum and ileum

The NaP-IIb protein expressions in the duodenum and ileum of broilers at 21 d of age were affected ($P < 0.01$) by dietary NPP level, but not ($P > 0.03$) by supplemental VD₃ level (Fig. 1(A) and (C)). There was an interaction ($P = 0.002$) observed between NPP and VD₃ levels on the NaP-IIb protein expression in the jejunum. Compared with the 0.22% NPP level, the 0.43%

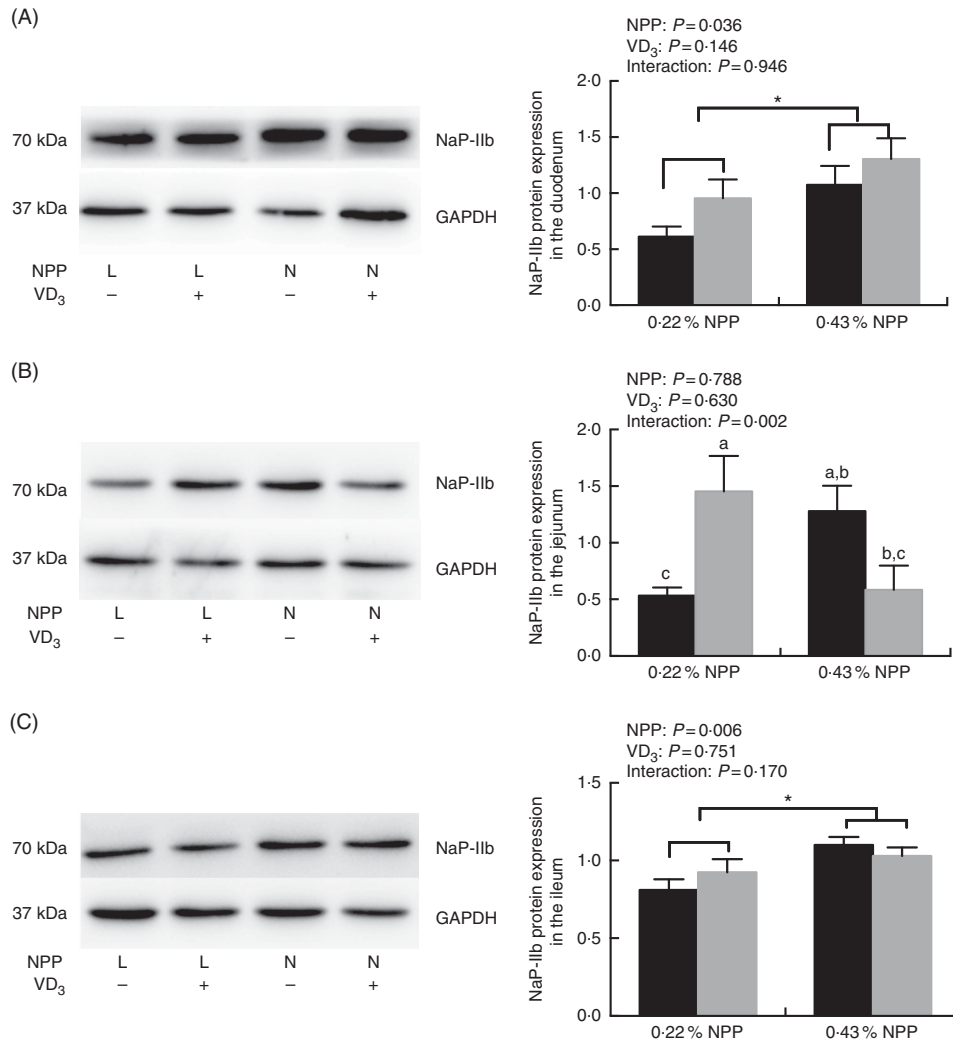


Fig. 1. Effects of dietary non-phytate phosphorus (NPP) and supplemental vitamin D₃ (VD₃) levels on type IIb sodium-dependent phosphate cotransporter (NaP-IIb) protein levels in small intestinal segments of broilers at 21 d of age. (A) The protein concentrations of NaP-IIb in the duodenum. (B) The protein concentrations of NaP-IIb in the jejunum. (C) The protein concentrations of NaP-IIb in the ileum. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Values are mean (n 8), with their standard errors represented by vertical bars. ^{a,b,c} Based on the two-way ANOVA analyses, mean values with unlike letters were significantly different at $P < 0.05$ between single bars (n 8) as determined by their interaction. * Mean values were significantly different at $P < 0.05$ between group bars (n 16) as determined by a main effect of dietary NPP levels. L, low level of NPP (0.22%); N, normal level of NPP (0.43%). –, No addition of VD₃; +, dietary supplemental 87.5 μ g VD₃/kg. ■, 0 μ g/kg VD₃; □, 87.5 μ g/kg VD₃.

NPP level increased ($P < 0.001$) the NaP-IIb protein expressions in the duodenum and ileum (Fig. 1(A) and (C)). Dietary supplemental VD₃ level did not affect ($P = 0.0521$) the NaP-IIb protein expression in the jejunum when broilers were fed the diet containing the 0.43% NPP level. However, the supplementation of VD₃ increased ($P = 0.0076$) the NaP-IIb protein expression in the jejunum when broilers were fed the diet containing the 0.22% NPP level (Fig. 1(B)).

Dietary NPP level affected ($P = 0.034$) the PiT-1 protein expression in the ileum (Fig. 2(C)), but had no effect ($P > 0.25$) on the PiT-1 protein expression in the duodenum of broilers at 21 d of age (Fig. 2(A) and (C)). Dietary supplemental VD₃ affected ($P = 0.01$) the PiT-1 protein expression in the duodenum, but had no effect ($P > 0.95$) on the PiT-1 protein expression in the ileum (Fig. 2(A) and (C)). There was an interaction ($P = 0.01$) between NPP and VD₃ levels on the PiT-1 protein

expression in the jejunum. Compared with the diet with 0.22% NPP level, the 0.43% NPP level decreased ($P < 0.001$) the PiT-1 protein expression in the ileum (Fig. 2(C)). Dietary VD₃ supplementation increased ($P = 0.01$) the PiT-1 protein expression in the duodenum. When broilers were fed the diet containing the 0.43% NPP level, dietary supplemental VD₃ did not affect ($P = 0.117$) the PiT-1 protein expression in the jejunum. At dietary 0.22% NPP level, however, the PiT-1 protein expression in the jejunum was greater ($P = 0.03$) in broilers fed diets with supplemental VD₃ than without supplemental VD₃ (Fig. 2(B)).

Dietary NPP level affected ($P < 0.05$) the PiT-2 protein expressions in the jejunum and ileum (Fig. 3(b) and (c)), but had no effect ($P = 0.734$) on the PiT-2 protein expression in the duodenum of broilers at 21 d of age (Fig. 3(a)). Dietary supplemental VD₃ level did not affect ($P > 0.45$) the PiT-2 protein expressions in all small intestinal segments (Fig. 3(a)–(c)). No

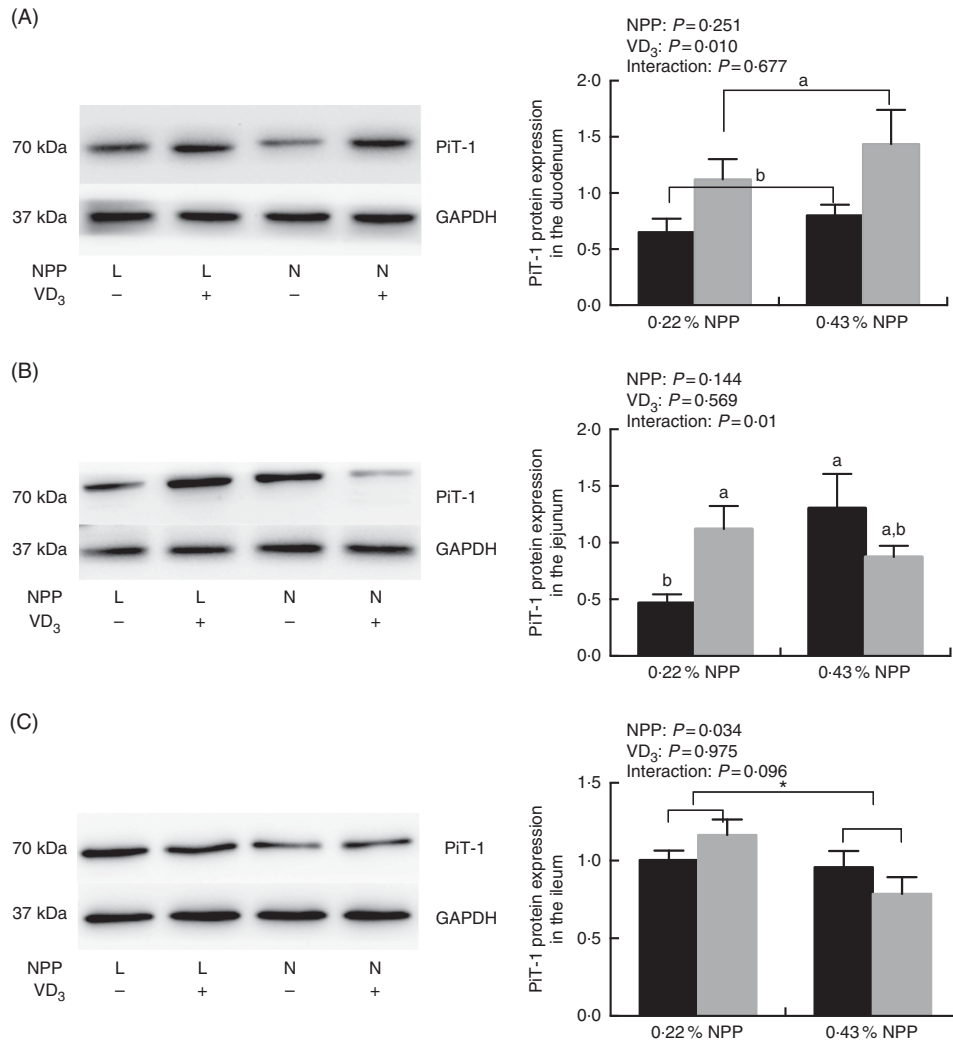


Fig. 2. Effects of dietary non-phytate phosphorus (NPP) and supplemental vitamin D₃ (VD₃) levels on type III sodium-dependent phosphate cotransporter 1 (PiT-1) protein levels in small intestinal segments of broilers at 21 d of age. (A) The protein concentrations of PiT-1 in the duodenum. (B) The protein concentrations of PiT-1 in the jejunum. (C) The protein concentrations of PiT-1 in the ileum. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Values are mean (n 8), with their standard errors represented by vertical bars. ^{a,b} Based on the two-way ANOVA analyses, mean values with unlike letters were significantly different at $P < 0.05$ between single bars (n 8) as determined by their interaction or between group bars (n 16) as determined by a main effect of supplemental VD₃ levels. * Mean values were significantly different at $P \leq 0.05$ between group bars (n 16) as determined by a main effect of dietary NPP levels. L, low level of NPP (0.22%); N, normal level of NPP (0.43%). –, No addition of VD₃; +, dietary supplemental 87.5 μ g VD₃/kg. ■, 0 μ g/kg VD₃; □, 87.5 μ g/kg VD₃.

interactions ($P > 0.45$) were observed between NPP level and VD₃ level for the PiT-2 protein expression in all small intestinal segments. Compared to diets with the 0.22% NPP level, the 0.43% NPP level decreased ($P < 0.05$) the PiT-2 protein expression in the jejunum and ileum (Fig. 3(b) and (c)).

Multiple correlations between phosphorus absorption, utilisation or bone ash and all of the transporter protein levels in the duodenum, jejunum and ileum

The correlation coefficients between the serum P, the tibia ash P or tibia ash and the protein levels of transporters (NaP-IIb, PiT-1 and PiT-2) in three small intestinal segments of broilers fed 0.22% NPP diets were shown in Table 8. The serum P content exhibited positive correlations with the PiT-1 protein

expressions in the duodenum (r 0.519, $P=0.04$) and jejunum (r 0.741, $P=0.004$), but had no correlations ($P > 0.05$) with other transporters. The tibia ash P was positively correlated with NaP-IIb protein expression (r 0.675, $P=0.016$) and PiT-1 protein expression (r 0.553, $P=0.05$) in the jejunum. In addition, no correlations ($P > 0.10$) were observed between tibia ash and all of the transporters.

Discussion

The requirement of NPP for fast-growing broilers from 1 to 21 d of age was 0.45% of feed as recommended by the NRC⁽³⁾. However, it is reported that the restriction of dietary NPP ($< 0.45\%$) in diets could result in a lower growth performance in broilers and laying hens^(2,3,36–38). In the present study, the

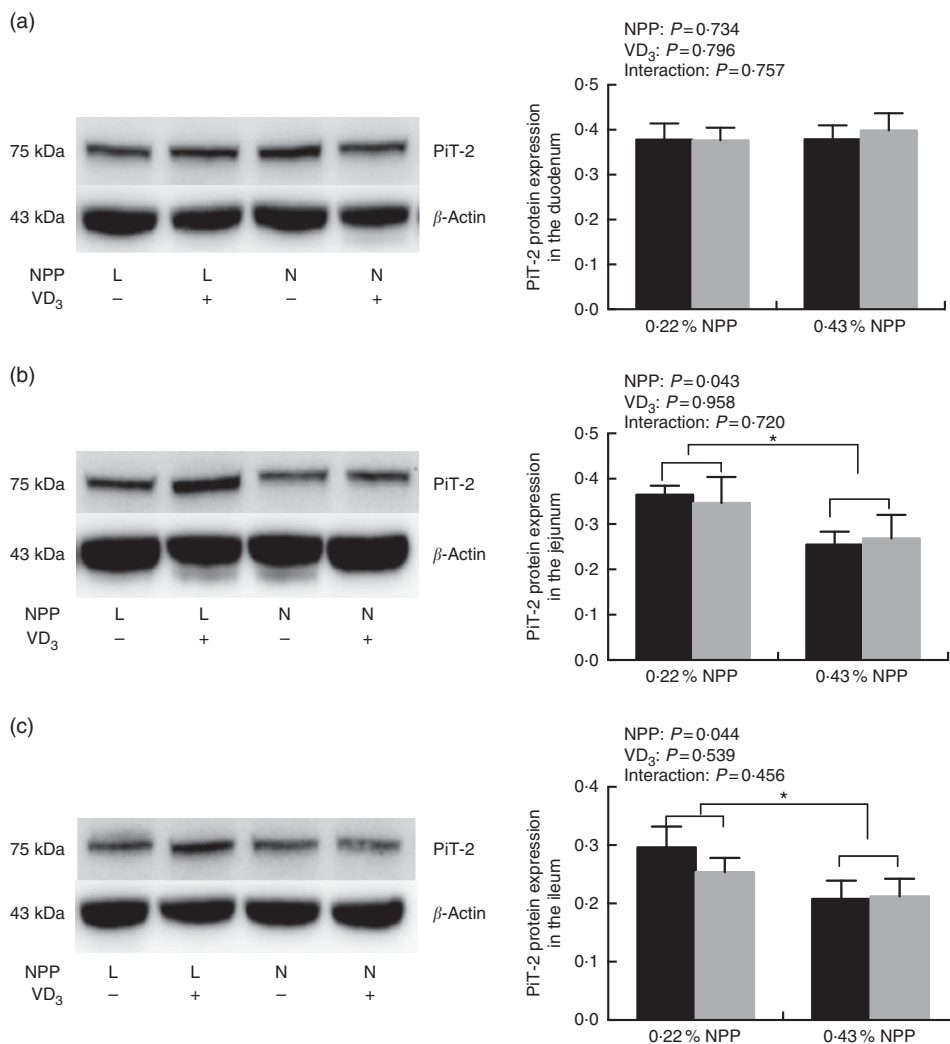


Fig. 3. Effects of dietary non-phytate phosphorus (NPP) and supplemental vitamin D₃ (VD₃) levels on type III sodium-dependent phosphate cotransporter 2 (PiT-2) protein levels in small intestinal segments of broilers at 21 d of age. (a) The protein concentrations of PiT-2 in the duodenum. (b) The protein concentrations of PiT-2 in the jejunum. (c) The protein concentrations of PiT-2 in the ileum. β -Actin was used as a loading control. Values are mean (n 8), with their standard errors represented by vertical bars. * Based on the two-way ANOVA analyses, mean values were significantly different at $P < 0.05$ between group bars (n 16) as determined by a main effect of dietary NPP levels. L, low level of NPP (0.22%); N, normal level of NPP (0.43%). –, No addition of VD₃; +, dietary supplemental 87.5 μ g VD₃/kg. ■, 0 μ g/kg VD₃; □, 87.5 μ g/kg VD₃.

Table 8. Correlations between phosphorus absorption, utilisation or bone ash and all of the transporter protein levels of in small intestinal segments of broilers fed 0.22% non-phytate phosphorus (NPP) diets†

Traits		Serum P	Tibia ash P	Tibia ash
Duodenum	NaP-IIb	0.006	0.095	0.378
	PiT-1	0.519*	-0.001	0.371
	PiT-2	0.285	0.176	0.092
Jejunum	NaP-IIb	0.377	0.675*	0.263
	PiT-1	0.741**	0.553*	0.308
	PiT-2	0.262	-0.179	0.370
Ileum	NaP-IIb	0.382	0.214	0.253
	PiT-1	0.51	0.309	0.086
	PiT-2	0.067	-0.348	-0.148

NaP-IIb, type IIb Na-dependent phosphate cotransporter; PiT-1, type III Na-dependent phosphate cotransporter 1; PiT-2, type III Na-dependent phosphate cotransporter 2.

* $P < 0.05$, ** $P < 0.01$.

† Data represent the correlation coefficients (r) between P absorption, utilisation or bone ash and all of the transporter protein levels in small intestinal segments of broilers fed 0.22% NPP diets with or without supplemental VD₃ (n 16).

reductions in ADG and ADFI were observed in the broilers fed diets containing 0.22% NPP level, which confirmed the results of previous studies. In addition, the high mortality of the broilers fed P-deficient diets without supplemental VD₃ could be caused by the extreme differences in the Ca:P ratio (4.5 Ca:NPP) of the low P diets. Supplemental VD₃ did not influence the growth performance of broilers in despite of the dietary NPP level, which was inconsistent with the previous observation⁽¹²⁾. One possible explanation is that the increased P absorption and utilisation by VD₃ might be not sufficient enough to increase the gain of broilers. In addition, the growth rate (gain) is generally not a very sensitive index in reflecting dietary P absorption and utilisation since it is affected by many other factors except for P, such as the feed intake and so on. In the present study, broilers were restrictedly fed the same amount of feed intake especially under the low-P diets with or without VD₃ supplementation, which restricted the gain of broilers. Furthermore, a previous study has also shown that 1,25(OH)₂-D₃ or

1 α -hydroxycholecalciferol (1 α -OH D₃) increased the contents of bone ash and plasma P and decreased the incidence of P rickets, but had no effect on the body weight of broilers fed diets containing a low level of NPP⁽³⁹⁾.

Dietary P absorbed from the small intestine is transported to liver via the hepatic portal vein and distributed finally into all organs for the body utilisation⁽⁴⁰⁾. Therefore, the serum P content from the hepatic portal vein would reflect the dietary P level, and could be used to evaluate the P absorption in the small intestine of broilers. In the present study, the broilers fed the diets containing the 0.43% NPP level had a higher serum P content than those fed the P-deficient diet, indicating that the absorption of P in the small intestine increased with the dietary NPP levels. This is consistent with the results reported in previous studies in which the concentration of serum inorganic P responded very quickly to the dietary NPP levels from 0.24 to 0.40%^(41,42). In addition, we found that the serum P content was enhanced indeed by VD₃ addition in the broilers fed diets containing 0.22% NPP, demonstrating that supplementation of VD₃ in a NPP deficient diet is capable of improving the P absorption of broilers. The improvement of P absorption could be associated with the enhanced P transport from the intestinal brush border membrane to intracellular stimulated by supplementation of 1,25(OH)₂-D₃^(43,44). Furthermore, an increase in serum P concentration was observed in the broilers fed the diet with supplementation of 1 α -OH D₃^(39,45). However, we found that the feed intake was higher in the broilers fed diets with supplementation of 1 α -OH D₃ than with no addition of 1 α -OH D₃ in their studies^(39,45), and the P intake could be different due to the variable feed intake. Therefore, it may be inaccurate to evaluate the effect of VD₃ or 1 α -OH D₃ on P transport and absorption based on the serum P content from birds that are not pair fed. To get rid of this shortcoming, the broilers fed the diets with or without supplementation of VD₃ were offered an equal amount of P intake in our current study. The results showed that addition of VD₃ in the diets had no influence on serum P content in the broilers fed for 2 h or fasting for 6 and 10 h, suggesting that the effect of VD₃ on serum P content from the hepatic portal blood was associated with the status of digestive physiology. The digestion and absorption of P in the small intestine could not be completed in broilers fed for 2 h⁽⁴⁶⁾, while the blood P might be cleared partly and controlled within a narrow physiological range through feedback mechanisms in broilers fasting more than 6 h with no P intake⁽⁴⁷⁾.

Tibia ash is one of the most sensitive criteria for evaluation of dietary P utilisation⁽⁴⁸⁾. Low dietary P level could decrease the bone ash content and bone strength, and increase the incidence of osteoporosis in layers⁽²⁾. In the present study, when broilers were fed the low NPP diets (0.22% NPP), tibia ash and tibia ash P increased with dietary supplementation of VD₃. These results demonstrated that VD₃ had a positive effect on P utilisation in bones of the broilers fed the P deficient diets. Consistent with our study, previous studies demonstrated that dietary 1 α -OH D₃ supplementation, as an analogue of VD₃, increased the contents of tibia ash and tibia ash P in broilers^(39,45,49–51). In addition, the TPRR was also enhanced by supplementation of VD₃, which further demonstrated that VD₃ indeed played an important role in promoting P retention and utilisation in broilers. However, low dietary P level

also resulted in greater tibia P retention, which may be due to greater efficiency of use of P by these birds through more efficient utilisation of phytate P which is known to occur in low P diets⁽⁵²⁾.

The NaP-IIb is a well-known protein that plays a pivotal role in P transport through apical membrane of the small intestinal epithelial cells^(11,53,54). Radanovic *et al.*⁽⁵⁵⁾ concluded that low P intake was an important stimulating factor for NaP-IIb expression. It was very interesting that the low level of NPP caused an up-regulation of *NaP-IIb* mRNA expressions in the duodenum and jejunum but down-regulation of NaP-IIb protein expressions in the duodenum and ileum. The above differences between mRNA expression and protein expression may be a function of the time of sample collection. The selected time for analyses of tibia and mucosa assays was based on the time (after 4 h of feeding) at which differences were detected in serum P levels. However, there may be a delayed response for protein expression. Therefore, if protein expression were measured at 6 h, the same differences would be expected. In addition, a post-transcriptional regulatory mechanisms including glycosylation or ubiquitination may be also involved in the NaP-IIb protein expression induced by P deficiency in this study⁽⁵⁶⁾, but it needs to be investigated further. Hu *et al.*⁽²⁵⁾ reported that a higher dietary NPP level promoted intestinal P absorption by increasing NaP-IIb protein expression, which was consistent with our results. Besides, dietary supplemental VD₃ enhanced NaP-IIb protein expression in the jejunum of the broilers fed the low NPP diets, suggesting that the increased absorption and utilisation of P induced by supplemental VD₃ might be partially associated with the enhanced NaP-IIb protein expression. Similarly, it is reported that VD₃ could increase the NaP-IIb protein abundance in the upper small intestine of mouse^(18,57).

In addition to the NaP-IIb, PiT-1 and PiT-2 are shown to serve as Na⁺-dependent P cotransporters in several tissues^(58–60). However, the regulatory effect of VD₃ on intestinal PiT-1 and PiT-2 expressions has not been studied extensively in animals. In the current study, dietary supplemental VD₃ decreased the *PiT-1* mRNA level by 34%, but increased the PiT-1 protein level by 138% in the jejunum of broilers fed the low NPP diets. Tatsumi *et al.*⁽²²⁾ reported that the administration of 1,25(OH)₂-D₃ inhibited *PiT-1* mRNA expression in the jejunum of vitamin D-deficient rats, but the PiT-1 protein abundance was not determined in their study. Given the potential for translation rate, post-translational modification, compartmentalization, etc. of the protein, mRNA levels are not always correlated with protein levels⁽⁶¹⁾. Since the mRNA is eventually translated into functional proteins, the protein level may be more indicative of the functioning of the transporters than mRNA level. Therefore, the upregulation of PiT-1 protein expression by supplemental VD₃ may partially enhance P absorption and utilisation. In addition, it is reported that the PiT-2 is likely to be involved in intestinal P influx by targeting to the basolateral membrane in the jejunal epithelial cells of rats⁽¹⁸⁾. However, we did not observe an increase in PiT-2 protein expression in all small intestinal segments, suggesting that the PiT-2 might be unlikely to be involved in intestinal P absorption of broilers.

It is likely that one or two of the above transporters may be involved in the enhanced P absorption and utilisation by VD₃ supplementation in broilers fed a low NPP diet. The analyses of



multiple correlations showed the relative contribution of each transporter and each section of the gut towards the P absorption and utilisation. It is suggested that the PiT-1 in the jejunum could be the most important transporter for P absorption and utilisation, while the NaP-IIb transporter in the jejunum and the PiT-1 in the duodenum might be just partially involved in P absorption and utilisation. Besides, the distribution of the above transporters in each section of the small intestine was different in the present study. The *NaP-IIb* mRNA abundance was greater in the duodenum than in the jejunum and ileum, which was in accordance with the results published previously^(25,62). However, the higher *PiT-2* abundances were observed in the jejunum and ileum than in the duodenum. Because the activity of these transporters is highly dependent on luminal pH⁽⁶³⁾, the variations in mRNA abundances of these transporters observed in different segments of the small intestine may be associated with the status of digestive physiology in each segment such as luminal pH. To explore the functional mechanism of the transporters in P absorption and utilisation, further studies appeared to be needed using the molecular techniques such as the method of gene silencing.

In conclusion, dietary supplemental VD₃ decreased the mortality of P-deficient broilers and increased P absorption in the intestine, as well as promoted P utilisation in bone via upregulating protein expressions of PiT-1 in the duodenum and NaP-IIb and PiT-1 in the jejunum from the P-deficient broilers. Overall, this study would be of important theoretical and practical significances for providing a nutritional strategy and mechanisms to promote P absorption and utilisation for animals, and reduce the dietary inorganic P addition and environmental pollution caused by P emissions in broiler production.

Acknowledgements

The authors would highly appreciate Dr Lin Xi in the North Carolina State University for assisting with the English polishing of this manuscript.

The present study was supported by the Key Program of the National Natural Science Foundation of China (project no. 31630073; Beijing, P. R. China), the National Nonprofit Institute Research Grant (2016ywf-yb-8; Beijing, P. R. China), the Special Funds of CAAS for Distinguished Scientists (Beijing, P. R. China), the Basic Research Program of Science and Technology (project no. 2014FY111000; Beijing, P. R. China), the National Natural Science Foundation of China (project no. 31472116; Beijing, P. R. China), the National Key R&D Program of China (project no. 2017YFD0502200; Beijing, P. R. China), the China Agriculture Research System (project no. CARS-41; Beijing, P. R. China), and the Agricultural Science and Technology Innovation Program (ASTIP-IAS08; Beijing, P. R. China).

The authors' contributions are as follows: X. L. designed the experiment; Y. S. drafted the manuscript; X. Lu. and X. Li. participated in writing and editing the manuscript; Q. W. and S. Z. conducted most of the experiments and analysed the data; L. Z. and L. L. performed the P analysis; X. Lu. and X. Li. had the primary responsibility for the final content. All authors have read and approved the final version of the manuscript.

The authors have no financial or personal conflicts of interests to declare.

References

- Berndt T & Kumar R (2009) Novel mechanisms in the regulation of phosphorus homeostasis. *Physiology (Bethesda)* **24**, 17–25.
- Webster AB (2004) Welfare implications of avian osteoporosis. *Poult Sci* **83**, 184–192.
- Long PH, Lee SR, Rowland GN, *et al.* (1984) Experimental rickets in broilers: gross, microscopic, and radiographic lesions. II. Calcium deficiency. *Avian Dis* **28**, 921–932.
- National Research Council (1994) *Nutrient Requirements of Poultry*, 9th ed. Washington, DC: National Academies Press.
- Liu SB, Li SF, Lu L, *et al.* (2012) Estimation of standardized phosphorus retention for corn, soybean meal, and corn–soybean meal diet in broilers. *Poult Sci* **91**, 1879–1885.
- Liu SB, Xie JJ, Lu L, *et al.* (2013) Estimation of standardized phosphorus retention for inorganic phosphate sources in broilers. *J Anim Sci* **91**, 3766–3771.
- Chen TC, Castillo L, Korycka-Dahl M, *et al.* (1974) Role of vitamin D metabolites in phosphate transport of rat intestine. *J Nutr* **104**, 1056–1060.
- Holick MF, Binkley NC, Bischoff-Ferrari HA, *et al.* (2011) Evaluation, treatment, and prevention of vitamin D deficiency: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab* **96**, 1911–1930.
- Peterlik M & Wasserman RH (1978) Effect of vitamin D on transepithelial phosphate transport in chick intestine. *Am J Physiol* **234**, 379–388.
- Mohammed A, Gibney MJ & Taylor TG (1991) The effects of dietary levels of inorganic phosphorus, calcium and cholecalciferol on the digestibility of phytate-P by the chick. *Br J Nutr* **66**, 251–259.
- Edwards HM Jr (1993) Dietary 1,25-dihydroxycholecalciferol supplementation increases natural phytate phosphorus utilization in chickens. *J Nutr* **123**, 567–577.
- Fritts CA & Waldroup PW (2003) Effect of source and level of vitamin D on live performance and bone development in growing broilers. *J Appl Poultry Res* **12**, 25–45.
- Rama Rao SV, Mvln R & Reddy MR (2007) Performance of broiler chicks fed high levels of cholecalciferol in diets containing sub-optimal levels of calcium and non-phytate phosphorus. *Anim Feed Sci Technol* **134**, 77–88.
- Furlong RF (2005) Insights into vertebrate evolution from the chicken genome sequence. *Genome Biol* **6**, 207.
- Werner A & Kinne RK (2001) Evolution of the Na–P(i) cotransport systems. *Am J Physiol Regul Integr Comp Physiol* **280**, 301–312.
- Murer H, Forster I & Biber J (2004) The sodium phosphate cotransporter family SLC34. *Pflugers Arch* **447**, 763–767.
- Liao XD, Suo HQ, Lu L, *et al.* (2017) Effects of sodium, 1,25-dihydroxyvitamin D₃ and parathyroid hormone fragment on inorganic P absorption and type IIB sodium–phosphate cotransporter expression in ligated duodenal loops of broilers. *Poult Sci* **96**, 2344–2350.
- Marks J, Srani SK, Biber J, *et al.* (2006) Intestinal phosphate absorption and the effect of vitamin D: a comparison of rats with mice. *Exp Physiol* **91**, 531–537.
- Collins JF, Bai L & Ghishan FK (2004) The SLC20 family of proteins: dual functions as sodium–phosphate cotransporters and viral receptors. *Pflugers Arch* **447**, 647–652.
- Zoidis E, Ghirlanda-Keller C, Gosteli-Peter M, *et al.* (2004) Regulation of phosphate (Pi) transport and NaPi-III

- transporter (Pit-1) mRNA in rat osteoblasts. *J Endocrinol* **181**, 531–540.
21. Suzuki A, Ghayor C, Guicheux J, *et al.* (2006) Enhanced expression of the inorganic phosphate transporter Pit-1 is involved in BMP-2-induced matrix mineralization in osteoblast-like cells. *J Bone Miner Res* **21**, 674–683.
 22. Tatsumi S, Segawa H, Morita K, *et al.* (1998) Molecular cloning and hormonal regulation of PiT-1, a sodium-dependent phosphate cotransporter from rat parathyroid glands. *Endocrinology* **139**, 1692–1699.
 23. Katai K, Miyamoto K, Kishida S, *et al.* (1999) Regulation of intestinal Na⁺-dependent phosphate co-transporters by a low-phosphate diet and 1,25-dihydroxyvitamin D₃. *Biochem J* **343**, 705–712.
 24. Kilkenny C, Browne WJ, Cuthill IC, *et al.* (2010) Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *Plos Biol* **8**, e1000412.
 25. Hu YX, Liao XD, Wen Q, *et al.* (2018) Phosphorus absorption and gene expressions of related transporters in the small intestine of broilers. *Br J Nutr* **119**, 1346–1354.
 26. Thiex NJ, Manson H, Andersson S, *et al.* (2002) Determination of crude protein in animal feed, forage, grain, and oilseeds by using block digestion with a copper catalyst and steam distillation into boric acid: collaborative study. *J AOAC Int* **85**, 309–317.
 27. Li S, Lin Y, Lu L, *et al.* (2011) An estimation of the manganese requirement for broilers from 1 to 21 d of age. *Biol Trace Elem Res* **143**, 939–948.
 28. Huang YL, Lu L, Xie JJ, *et al.* (2013) Relative bioavailabilities of organic zinc sources with different chelation strengths for broilers fed diets with low or high phytate content. *Anim Feed Sci Technol* **179**, 144–148.
 29. Davies JL, Andrews GS, Miller R, *et al.* (1973) Comparison of the stannous chloride and vanadate methods for estimation of serum inorganic phosphorus by use of the “SMA12-60”. *Clin Chem* **19**, 411–414.
 30. Rutherford SM, Chung TK, Morel PC, *et al.* (2004) Effect of microbial phytase on ileal digestibility of phytate phosphorus, total phosphorus, and amino acids in a low-phosphorus diet for broilers. *Poult Sci* **83**, 61–68.
 31. Leytem AB, Kwanyuen P & Thacker P (2008) Nutrient excretion, phosphorus characterization, and phosphorus solubility in excreta from broiler chicks fed diets containing graded levels of wheat distillers grains with solubles. *Poult Sci* **87**, 2505–2511.
 32. Goldenberg H & Fernandez A (1966) Simplified method for the estimation of inorganic phosphorus in body fluids. *Clin Chem* **12**, 871–882.
 33. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T) (–Delta Delta C) method. *Methods* **25**, 402–408.
 34. Chow S-C, Wang H & Shao J (2007) *Sample Size Calculations in Clinical Research*, 2nd ed. Boca Raton, FL: Chapman & Hall/CRC Press.
 35. Steel RGD, Torrie JH & Dickey DA (1997) *Principles and Procedures of Statistics: A Biometrical Approach*, 3rd ed. New York: McGraw Hill.
 36. Li J, Yuan J, Guo Y, *et al.* (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* **25**, 552–558.
 37. Nie W, Yang Y, Yuan J, *et al.* (2013) Effect of dietary non-phytate phosphorus on laying performance and small intestinal epithelial phosphate transporter expression in Dwarf pink-shell laying hens. *J Anim Sci Biotechnol* **4**, 34.
 38. Hamdi M, Lopez-Verge S, Manzanilla EG, *et al.* (2015) Effect of different levels of calcium and phosphorus and their interaction on the performance of young broilers. *Poult Sci* **94**, 2144–2151.
 39. Edwards HM Jr (2002) Studies on the efficacy of cholecalciferol and derivatives for stimulating phytate utilization in broilers. *Poult Sci* **81**, 1026–1031.
 40. Chun S, Bamba T, Suyama T, *et al.* (2016) A high phosphorus diet affects lipid metabolism in rat liver: a DNA microarray analysis. *PLOS ONE* **11**, e0155386.
 41. Gardiner EE (1962) The relationship between dietary phosphorus level and the level of plasma inorganic phosphorus of chicks. *Poult Sci* **41**, 1156–1163.
 42. Abudabos AM (2012) Optimal dietary phosphorus for broiler performance, bone integrity and reduction of phosphorus excretion. *Asian J Anim Vet Adv* **7**, 288–298.
 43. Matsumoto T, Fontaine O & Rasmussen H (1980) Effect of 1,25-dihydroxyvitamin D-3 on phosphate uptake into chick intestinal brush border membrane vesicles. *Biochim Biophys Acta* **599**, 13–23.
 44. Lee DB, Walling MW & Corry DB (1986) Phosphorus transport across rat jejunum: influence of sodium, pH, and 1,25-(OH)₂D₃. *Am J Physiol* **250**, G369–G373.
 45. Han JC, Yang XD, Zhang T, *et al.* (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* **88**, 323–329.
 46. Birger S (2017) Function of the digestive system. *J Appl Poult Res* **23**, 306–314.
 47. Veum TL (2010) Phosphorus and calcium nutrition and metabolism. In *Phosphorus and Calcium Utilization and Requirements in Farm Animals*, pp. 94–111 [DMSS Vitti and E Kebreab, editors]. Oxfordshire: CAB International.
 48. Nelson TS (1967) The utilization of phytate phosphorus by poultry – a review. *Poult Sci* **46**, 862–871.
 49. Biehl RR & Baker DH (1997) 1Alpha-hydroxycholecalciferol does not increase the specific activity of intestinal phytase but does improve phosphorus utilization in both cecectomized and sham-operated chicks fed cholecalciferol-adequate diets. *J Nutr* **127**, 2054–2059.
 50. Biehl RR & Baker DH (1997) Utilization of phytate and non-phytate phosphorus in chicks as affected by source and amount of vitamin D₃. *J Anim Sci* **75**, 2986–2993.
 51. Snow JL, Baker DH & Parsons CM (2004) Phytase, citric acid, and 1alpha-hydroxycholecalciferol improve phytate phosphorus utilization in chicks fed a corn–soybean meal diet. *Poult Sci* **83**, 1187–1192.
 52. Onyango EM, Asem EK & Adeola O (2006) Dietary cholecalciferol and phosphorus influence intestinal mucosa phytase activity in broiler chicks. *Br Poult Sci* **47**, 632–639.
 53. Hilfiker H, Hattenhauer O, Traebert M, *et al.* (1998) Characterization of a murine type II sodium–phosphate cotransporter expressed in mammalian small intestine. *Proc Natl Acad Sci U S A* **95**, 14564–14569.
 54. Liu SB, Hu YX, Liao XD, *et al.* (2016) Kinetics of phosphorus absorption in ligated small intestinal segments of broilers. *J Anim Sci* **94**, 3312–3320.
 55. Radanovic T, Wagner CA, Murer H, *et al.* (2005) Regulation of intestinal phosphate transport. I. Segmental expression and adaptation to low-P(i) diet of the type IIb Na(+)-P(i) cotransporter in mouse small intestine. *Am J Physiol Gastrointest Liver Physiol* **288**, 496–500.
 56. Saddoris KL, Fleet JC & Radcliffe JS (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* **140**, 731–736.
57. Hattenhauer O, Traebert M, Murer H, *et al.* (1999) Regulation of small intestinal Na–Pi type IIb cotransporter by dietary phosphate intake. *Am J Physiol Gastrointest Liver Physiol* **277**, G756–G762.
 58. Biber J, Custer M, Magagnin S, *et al.* (1996) Renal Na/Pi-cotransporters. *Kidney Int* **49**, 981–985.
 59. Boyer CJ, Baines AD, Beaulieu E, *et al.* (1998) Immunodetection of a type III sodium-dependent phosphate cotransporter in tissues and OK cells. *Biochim Biophys Acta* **1368**, 73–83.
 60. Custer M, Spindler B, Verrey F, *et al.* (1997) Identification of a new gene product (diphor-1) regulated by dietary phosphate. *Am J Physiol* **273**, 801–806.
 61. Liu Y, Beyer A & Aebersold R (2016) On the dependency of cellular protein levels on mRNA abundance. *Cell* **165**, 535–550.
 62. Yan F, Angel R & Ashwell CM (2007) Characterization of the chicken small intestine type IIb sodium phosphate cotransporter. *Poult Sci* **86**, 67–76.
 63. Bai L, Collins JF & Ghishan FK (2000) Cloning and characterization of a type III Na-dependent phosphate cotransporter from mouse intestine. *Am J Physiol Cell Physiol* **279**, 1135–1143.