

Effects of available phosphorus source and concentration on performance and expression of sodium phosphate type IIb cotransporter, vitamin D-1 α -hydroxylase, and vitamin D-24-hydroxylase mRNA in broiler chicks

I. I. Omara,^{*,†} C. T. Mou,^{*} M. E. Persia,^{*} and E. A. Wong^{*,1}

^{*}Department of Animal and Poultry Sciences, Virginia Tech, Blacksburg, VA 24060, USA; and [†]Animal Production Department, Faculty of Agriculture, Cairo University, Giza 12613, Egypt

ABSTRACT This experiment was conducted to examine the effect of 2 phosphorus (P) sources on broiler performance to day 14. The P bioavailability was estimated using bird performance and tibia ash measurements, whereas P digestibility, intestinal P transporter, kidney vitamin D-1 α -hydroxylase, and vitamin D-24-hydroxylase mRNA abundances were also determined. Slope regression analysis was used to determine the bioavailability of dicalcium phosphate (**Dical P**) and nanocalcium phosphate (**Nano P**) with dietary available P (**AvP**) set to 0.20% P (control) using AvP from the major ingredients and Dical P. The experimental treatments were achieved by supplementation with either Dical P or Nano P to generate 0.24, 0.28, 0.32, and 0.36% AvP. A total of 648-day-old unsexed broiler chicks were divided into 72 birds per treatment (8 replicate cages of 9 birds). Slope regression analysis showed positive linear relationships between BW, feed intake (**FI**), tibia ash weight (**TAW**), and tibia ash percentage (**TAP**) with dietary Dical P and Nano P levels. Comparisons between regression

slopes for Dical P and Nano P fed birds were not significantly different for BW, feed intake, tibia ash weight, and tibia ash percentage, indicating similar P bioavailability from Dical P and Nano P. There were interactions between P source and AvP for feed efficiency (**FE**) and apparent ileal P digestibility (**AIPD**). Dicalcium phosphate had greater FE than Nano P at 0.28% AvP and greater AIPD than Nano P at 0.24% AvP. The addition of AvP from Dical P and Nano P resulted in reduced sodium phosphate cotransporter mRNA abundance in the duodenum in a dose-dependent response. In the kidney, vitamin D-1 α -hydroxylase mRNA abundance was greater at 0.36% Nano P compared with control, but there was no difference with Dical P. There was no difference in vitamin D-24-hydroxylase mRNA abundance between control and supplementation with Nano P or Dical P. In conclusion, Nano P and Dical P had the same bioavailability but had different effects on gene expression.

Key words: dicalcium phosphate, nanophosphate, phosphate transporter, vitamin D hydroxylase

2020 Poultry Science 99:1822–1831

<https://doi.org/10.1016/j.psj.2019.12.035>

INTRODUCTION

In poultry, phosphorus (P) is an essential nutrient in metabolic processes and a component of nucleic acids, membrane phospholipids, and bones (Nie et al., 2013). Much effort has been made to increase P absorption in poultry to improve metabolic performance and reduce

the environmental impact of phosphate (Pi) release from manure (Ahmadi and Rodehutschord, 2012; Létourneau-Montminy et al., 2013).

Dietary P is absorbed in the small intestine by both passive and active transport processes. Active transport of intestinal Pi is mainly mediated by sodium-dependent Pi transporters that belong to the Solute Carrier 34 (SLC34) family (Murer et al., 2001; Forster et al., 2013). Intestinal Pi absorption and renal Pi resorption are regulated by type II NaPi cotransporters (**NaPi-II**), which are further subdivided into NaPi-IIa (SLC34A1) and NaPi-IIb (SLC34A2) (Hilfiker et al., 1998; Werner and Kinne, 2001). The NaPi-IIb cotransporter is considered to be the major Na-Pi cotransporter that is expressed in the brush border membrane of the small

© 2019 The Authors. Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Received August 19, 2019.

Accepted December 10, 2019.

¹Corresponding author: ewong@vt.edu

intestinal epithelium but is also expressed in the lung and secretory tissues (Hilfiker et al., 1998; Feild et al., 1999). In chickens, NaPi cotransporter mRNA abundance was greatest in the duodenum, intermediate in the jejunum, and lowest in the ileum (Huber et al., 2015; Li et al., 2018).

A number of factors regulate expression of Pi transporters. Dietary P is a main modulator of Pi transporter expression (Sabbagh et al., 2011; Huber et al., 2015). Broilers and laying hens fed diets restricted (0.20/0.25% vs. 0.40/0.55% of diet) in nonphytate phosphorus (NPP) increased NaPi-IIb mRNA abundance in all segments of the small intestine (Yan et al., 2007; Nie et al., 2013). The expression of NaPi-IIb mRNA in the duodenum during 1 to 21 D of age decreased linearly ($P < 0.002$) as dietary NPP levels increased from 0.10 to 0.50% (Liu et al., 2017). This effect was dependent upon both the magnitude and length of dietary Pi deprivation (Capuano et al., 2005; Radanovic et al., 2005). Vitamin D is also part of the control mechanism for Ca and P absorption, because it can influence NaPi-IIb cotransporter gene transcription (Christakos et al., 2014; Han et al., 2018). In the kidney, 2 enzymes regulate vitamin D levels. Vitamin D-1 α -hydroxylase catalyzes the conversion of 25-hydroxy vitamin D to the hormonally active 1 α , 25 dihydroxy vitamin D, while vitamin D-24-hydroxylase converts the active form of vitamin D into inactive calcitroic acid (Christakos et al., 2010; Shanmugasundaram and Selvaraj, 2012).

Supplementation of minerals as nanoparticles, which are defined as particles less than 100 nm, increases the surface area, which potentially could increase absorption and utilization leading to higher bioavailability and a reduction in the quantity of supplements needed for broiler production (Weiss et al., 2006; Nikonov et al., 2011; Al-Beitawi et al., 2017; Sabry et al., 2018). The P digestibility depends on solubility of the limestone, particle size of the Ca source, in addition to total Ca concentration in the broiler diet (Kim et al., 2018). The bioavailability and effects of nanocalcium phosphate (Nano P) compared with dicalcium phosphate (Dical P) on broiler performance have been studied. Vijayakumar and Balakrishnan (2014) reported that the bioavailability of calcium phosphate nanoparticles was 200% when compared with Dical P. Hassan et al. (2016) similarly showed that a diet containing Nano P at only 25% of NPP levels was equivalent to 100% Dical P and resulted in a 50% reduction in excreted Ca and P. These findings may have great potential in the poultry industry especially in feed management by minimizing mineral loss in fecal material, which is both an economic and environmental issue. The growing concerns with regard to the potential contribution of P in poultry excreta on eutrophication of surface waters has led to increasing pressure to limit excess P in poultry rations, thus reducing fecal output of P and the resulting environmental contamination (Waldroup, 1999). Although nanoparticles are showing promise in animal agriculture, it is important that the safety of nanomaterials to both the animals and the environment, such as soil biota, is carefully considered (Mishra et al., 2017; Sabry et al., 2018).

The objective of the current experiment was to determine the relative bioavailability of dietary Nano P compared with Dical P and the effects of various concentrations of these two P sources on performance and mRNA abundance of duodenal NaPi-IIb and kidney hydroxylases when fed to broiler chickens from 1 to 14 D of age.

MATERIALS AND METHODS

Birds and Management

All animal procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee. A total of 648, day-old unsexed Cobb 500 broiler chicks were obtained from a commercial hatchery in Virginia. Chicks were individually weighed and allocated to 72 cages (9 treatments of 8 replicate groups of 9 chicks) in electrically heated battery brooders (377 cm²/bird), so that bird weights were similar across all pens. Experimental diets were randomly assigned to cages of chicks. Over the 14 D experimental period, the temperature was maintained at 34°C from day 1 to 3 posthatch, 31°C from day 4 to 7, and was progressively reduced to 27°C by day 14. The light cycle was set to 24 h of light for day 0 and 23 h of light for day 1. Lighting was adjusted to 18 h of light from day 2 to 14. The chicks were provided *ad libitum* access to water and mash feed.

Diets

Nine experimental mash starter diets (Table 1) were formulated based on maize and soybean meal and were formulated to meet nutrient requirements of chickens according to the recommended allowances of the breed (Cobb 500 broiler performance and nutrition supplement guide, 2015) with the exception of available phosphorus (AvP). Two main sources of P were used to design the experimental treatments: Dical P and Nano P. Calcium phosphate nanopowder (American Elements Co., Los Angeles, CA) contained 98.5% calcium phosphate (39.90% Ca and 18.50% P) with a Ca:P ratio of 2.15:1.00. The particle size ranged from 500 to 1,000 μ m for Dical P and <100 nm for Nano P. Experimental diets were formulated to contain 0.20, 0.24, 0.28, 0.32, and 0.36% AvP by adding Dical P or Nano P (Table 1). All diets were formulated to contain the same metabolizable energy (3,035 kcal ME/kg), crude protein (21.51%), and Ca (0.90%). Diets contained 0.25% titanium dioxide as an indigestible marker.

Animal Performance

Bird BW (g/chick) were averaged by cage for statistical analysis. Feed offered was measured on day of hatch (doh) and day 14, while feed refusal was measured on day 7 and day 14. Feed offered and feed refusal were then used to determine feed intake (FI, g/pen) between doh and day 14. Feed intake was calculated for each cage by [(initial feed pan

Table 1. Dietary ingredients (%) and chemical composition of diets fed to broiler chicks with various concentration of dicalcium phosphate (Dical P) or nanocalcium phosphate (Nano P) from day 1 to 14.

Ingredients	Control ¹	Dical P				Nano P ²			
	0.20%	0.24%	0.28%	0.32%	0.36%	0.24%	0.28%	0.32%	0.36%
Yellow corn	63.36	63.20	63.03	62.86	62.69	63.55	63.57	63.59	63.61
Soybean meal (48% CP)	28.08	28.11	28.14	28.17	28.20	28.05	28.04	28.04	28.04
Poultry bi-product meal	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Soy oil	0.71	0.77	0.82	0.88	0.93	0.65	0.64	0.64	0.63
Limestone	1.71	1.57	1.44	1.30	1.16	1.40	1.18	0.95	0.72
Dical P	0.19	0.41	0.63	0.85	1.07	-	-	-	-
Nano P	-	-	-	-	-	0.40	0.62	0.83	1.05
Salt	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42
DL-methionine	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28
Lysine-HCl	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23
Threonine	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
Choline chloride (60%)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin-mineral premix ³	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63	0.63
Titanium oxide (TiO ₂)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Nutrient composition									
Crude protein, %	21.51	21.51	21.51	21.51	21.51	21.51	21.51	21.51	21.51
ME, kcal/kg	3,035	3,035	3,035	3,035	3,035	3,035	3,035	3,035	3,035
Calcium, %	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90
Total phosphorus (TP), %	0.46	0.50	0.55	0.59	0.64	0.50	0.54	0.58	0.62
Available phosphorus, %	0.20	0.24	0.28	0.32	0.36	0.24	0.28	0.32	0.36

¹The low available P diet was generated by the addition of dicalcium phosphate to the basal diet before supplementation with either dicalcium phosphate or nanoparticle hydroxyapatite, which were added at 0.04% increments to generate experimental diets.

²Nanocalcium phosphate—nanoparticle hydroxyapatite product: hydroxyapatite calcium phosphorus (Ca₁₀(PO₄)₆(OH)₂), Form: Dry nanoparticle powder.

³Provided per kilogram of diet: cobalt, 30 ppm; copper, 4.75 ppm; iodine, 1.18 ppm; iron, 59.40 ppm; manganese, 75.50 ppm; zinc, 57.20 ppm; vitamin A, 7749 IU; vitamin D₃, 2596 IU; vitamin E, 1.94 IU; vitamin B₁₂, 0.01 mg; menadione, 1.36 mg; riboflavin, 4.84 mg; D-pantothenic acid, 7.13 mg; niacin, 233 mg; choline, 448.4 mg.

and feed weight + feed added over duration of experiment) – feed pan weight and remaining feed]. Total cage BW gain (final BW at day 14 – initial BW at doh) and FI were used to calculate feed efficiency (FE, g gain per g feed intake). Mortality was recorded daily, and any dead birds were weighed for inclusion into the FE calculation to adjust FE for mortality. Feed efficiency was calculated as total pen weight gain plus mortality weight gain divided by pen feed intake.

Apparent Ileal Phosphorus Digestibility

On day 14, all remaining birds were euthanized by cervical dislocation. The small intestine was immediately removed, and the ileal contents of each bird between Meckel's diverticulum and the ileal-cecal junction were collected by gentle manipulation for determination of apparent ileal phosphorus digestibility (AIPD). Owing to the small amount of intestinal contents, all samples within a replicate cage were pooled together for analysis resulting in 9 replicate samples per treatment as outlined by Douglas et al. (2003). Ileal contents were dried at 55°C for 72 h and then ground using a mill grinder to pass through a 0.50 mm sieve and stored frozen at –20°C until laboratory analysis. Once ground, both feed and ileal samples were analyzed to determine P and Ti concentrations. Feed samples were analyzed in quadruplet, while ileal samples were analyzed in duplicate. One g of feed or 0.5 g of dried ileal digesta was weighed into a 125 mL Erlenmeyer

flask. Twenty mL of sulfuric acid and 2 mL of nitric acid were then added to each flask. Samples were boiled for 5 min, then allowed to cool completely. Another 2.5 mL of nitric acid were added to each flask and boiled for an additional 20 min before being cooled completely to form a clear colored solution free of precipitates. The last step was repeated with 1.0 mL of nitric acid if the solution was not colorless and not free of precipitates. The digested sample was then standardized using a 100 mL volumetric flask by addition of sample and deionized water to exactly 100 mL. The standardized samples were then poured through circular 90 mm filter paper, and 50 mL samples were retained in 50 mL conical tubes for final analysis (Boguhn et al., 2009). Samples were sent to the Virginia Tech Soil Testing Laboratory for an inductively coupled plasma atomic emission spectroscopy analysis (ICP) to determine P and Ti concentrations. The ICP measures characteristic atomic emission spectra. Samples were introduced into a nebulizer to create an aerosol, which in turn were introduced into argon plasma. In the plasma, at approximately 8,000 K, molecules were almost completely dissociated, and the resulting ions emitted absorbed energy as a characteristic wavelength of light. The light intensity emitted at each wavelength was proportional to the amount of the element that was excited. A calibration curve was created so that the unknown samples could be measured against known standards. Determined values were represented as mg/L (Martin et al., 1994). Total digesta and feed P and Ti values were determined by

multiplying the weight of the original sample by the dilution factor and the final P and Ti concentrations in solution. The following equation was used to determine AIPD:

$$\text{AIPD} = P_{\text{diet}} - (P_{\text{ileum}}^* (\text{Ti}_{\text{diet}} / \text{Ti}_{\text{ileum}})) / P_{\text{diet}}^* 100$$

where (P_{diet}) is the phosphorus determined concentration in the diet; (P_{ileum}) is the concentration of phosphorus in the ileal digesta; (Ti_{diet}) is the determined concentration of titanium in the diet; and (Ti_{ileum}) is the determined concentration of titanium in the ileal digesta.

Tissue Sampling

All birds euthanized for digesta collection on day 14 also had the right leg collected to determine tibia ash weight (**TAW**) and tibia ash percentage (**TAP**). Tibias from the right leg were removed, packaged in labeled plastic bags, and frozen at -20°C . Collected legs were autoclaved at 121°C at 18 psi for 30 min, and all adhering tissue was removed including both tibia cartilage caps. Bones were then dried in a convection oven at 100°C for 24 h (Persia et al., 2003). The dried bones were weighed before they were dry-ashed for 24 h at 600°C in a muffle furnace. The bones from all 9 birds per replicate pen were pooled to generate a single sample for analysis ($n = 8$). The remaining ash was weighed to determine tibia ash, expressed as total ash g per tibia and as a percentage of dry bone. Six euthanized birds per treatment were randomly selected for collecting intestinal samples. The duodenal segment (from distal of the gizzard to 1 cm distal of the bile duct) was isolated immediately from the gastrointestinal tract (Rodehutscord et al., 2012). The gut lumen was opened at the mesenteric border and gently rinsed with ice-cold phosphate buffered saline (1%) to remove gut contents. Intestinal tissues were scraped with a glass microscope slide to collect the mucosa from the underlying muscle layer from a 5 cm section in the center of the duodenum, frozen in liquid nitrogen, and stored at -80°C . Kidney samples were also collected from these birds. Kidney tissue was minced and then placed immediately into RNAlater solution (RNAlater, Ambion, Austin, TX) and stored at -80°C until RNA extraction.

RNA Extraction, Reverse Transcription, and Real-Time qPCR

Total RNA was extracted from the intestine and kidney samples ($n = 6$ per treatment) with Trizol reagent and Direct-zol RNA MiniPrep kit (Zymo Research Inc., Irvine, CA) according to the manufacturer's instructions. The concentration and purity of the RNA were determined by measuring the absorbance at 260 and 280 nm. After extraction, total RNA was reverse transcribed into cDNA by using 2.0 μL of 10xRT Buffer, 0.8 μL of 25xNTP Mix, 2.0 μL of 10xRT Random Primers, 1.0 μL of MultiScribe Reverse transcriptase (Applied Biosystems, Foster City, CA), 10 μL of RNA

(0.2 $\mu\text{g}/\text{mL}$), and 4.2 μL of RNase-free water. The mRNA abundances of duodenal NaPi-IIb, kidney vitamin D-1 α -hydroxylase, and vitamin D-24-hydroxylase mRNA, and the reference gene β -actin were determined by real-time quantitative PCR, using the primers listed in Table 2. A 10 μL reaction was prepared containing 5 μL of fast SYBR Green Master mix (Applied Biosystems), 1.0 μL of forward primer (5 μM), 1.0 μL of reverse primer (5 μM), 1.0 μL of cDNA (diluted 1:30), and 2.0 μL of RNase-free water. The PCR reaction was conducted in a 7,500 Fast Real-Time PCR instrument (Applied Biosystems). The amplification protocol used was 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Each sample was amplified in triplicate and amplification efficiency was determined by standard curves to ensure equal efficiency between target genes and the reference gene. Fold change was calculated using the $2^{-\Delta\Delta\text{CT}}$ method of Schmittgen and Livak (2008).

Statistical Analysis

Data were analyzed using PROC GLM, version 9.2 (SAS Institute, 2004). For BW, FI, TAW, and TAP data, a slope regression line for various concentrations of P from Dical P and Nano P was calculated. The analysis of covariance test was used to compare the slope regression lines for Dical P and Nano P (Onyiah, 2008). For FE and AIPD data, a factorial analysis (2 P sources and 4 AvP levels) was used. For gene expression data, one-way ANOVA was used to compare the various concentrations of P from Dical P and Nano P treatments vs. control. When significant differences were noted, Tukey's Honestly Significant Difference test was used to separate means, and significance was accepted at $P \leq 0.05$.

RESULTS AND DISCUSSION

This experiment investigated the relative bioavailability of dietary Nano P compared with Dical P, and the effects of various concentrations of these 2 P sources on performance and mRNA abundance of intestinal NaPi-IIb and kidney hydroxylases when fed to broiler chickens from day 1 to 14. Regression analysis performed for both BW (Figure 1A) and FI (Figure 1B) for Dical P and Nano P fed at various concentrations showed significant positive linear responses to supplemental P from both sources. For both BW ($P = 0.75$) and FI ($P = 0.71$), there were no differences between the slopes for Dical P and Nano P. The response for FE (Figure 2A) showed a P source \times P level interaction. At 0.28% AvP, FE for Nano P was lower than Dical P; whereas at all other AvP levels, there was no difference between Nano P and Dical P.

The increased BW gain and FI with increased dietary AvP in the current experiment were similar to Jiang et al. (2013) who reported that increasing concentrations of NPP (0.25, 0.35, and 0.45%) in starter broiler diets increased ($P \leq 0.01$) both BW (560.5, 662.1, and

Table 2. Primers used for qPCR.

Genes ¹	GenBank accession no	Primer sequences (Forward/Reverse)
β-actin	NM_205518.1	5'-GTCCACCGCAAATGCTTCTAA-3'/ 5'-TGCGCATTATGGGTTTTGTT-3'
NaPi-IIb	NM_204474.1	5'-GGAAGCATTGCTGCGGATT-3'/ 5'-GCACCCTCCTGTTCTGCATT-3'
Vitamin D-1α-hydroxylase	XM_422077.2	5'-ACTTTGGTCCCCAGTTTGTGT-3'/ 5'-CGGAGCACTTGAGCAACCATA-3'
Vitamin D-24-hydroxylase	AF019142.1	5'-GCAGCGCTGGAGATCAA-3'/ 5'-AGCCCTCGTCGCGATAGTC-3'

¹NaPi-IIb, sodium phosphate cotransporter IIb.

670.5 g) and FI (52.88, 61.93, and 62.38 g/d), respectively. These responses are expected as dietary concentrations of AvP or NPP were generally below the requirement of starter chicks for performance allowing for generation of a linear slope response to estimate bioavailability (NRC, 1994; Ammerman et al., 1995). The negative impact of P deficiency on BW gain, FI, and FE was in birds fed the 0.20% NPP diet (574 g/bird, 821 g/bird, and 698g:kg, respectively) compared with those fed 0.40% NPP diet (798 g/bird, 984 g/bird, and 813 g:kg, respectively) from days 6 to 22 (Babatunde et al., 2019). The similar slopes between the Dical P and Nano P fed chicks, and the differences in growth response between previous reports and the current data suggest that there is minimal to no difference in P bioavailability between Dical P and Nano P supplementation. Our result is in contrast to those reported by Hassan et al. (2016) and Vijayakumar and Balakrishnan (2014) who reported that Nano P had increased bioavailability compared with Dical P. The difference in results could be because of the method of analysis and levels of P used. In our study, we used slope regression of BW, FI, TAW, and TAP to assess bioavailability, while Hassan et al. (2016) used BW, FI, and FCR, and Vijayakumar and Balakrishnan (2014) used BW and tibial bone ash, calcium, and phosphorus content to assess bioavailability. In addition, in our study, total P ranged from 0.46 to 0.64%; whereas, Hassan et al. (2016) used NPP levels from 0.21 to 0.45%.

Regression analysis was performed for both TAW (Figure 1C) and TAP (Figure 1D) for Dical P and Nano P fed at various concentrations, resulting in significant positive linear responses to supplemental P from both sources. For both TAW ($P = 0.40$) and TAP ($P = 0.83$), there were no differences between the slopes generated for Dical P and Nano P responses. The slope regression analysis generated slope value responses that were similar to those of the BW gain and FI, providing further support that Dical P and Nano P had similar bioavailability. It is of note that the fit of the regression lines was more consistent with bone mineral analysis in comparison to direct performance responses.

These results were not surprising and were in agreement with previous reports (Powell et al., 2011; Shastak et al., 2012; Li et al., 2015; Gautier et al., 2018). Broiler birds that received a low-NPP diet (0.20%) showed reduced TAP (33.7%; $P < 0.01$)

compared with birds fed the 0.40% NPP diet (48.4%) from days 6 to 22 (Babatunde et al., 2019). This is because of the importance of P and Ca as components of hydroxyapatite in the bone and because 80% of P found in the body is located in the bone. Thus, an insufficient dietary P level in low P diets led to adverse effects on bone formation and mineralization except in the presence of endogenous enzymes such as phytase (Olukosi et al., 2013).

Apparent ileal P digestibility % (Figure 2B) showed a P source \times P level interaction. At 0.24% AvP, AIPD for Dical P was greater than Nano P; whereas, at all other levels of AvP (0.28–0.36%), AIPD was not different between Dical P and Nano P. Furthermore, AIPD at 0.24% AvP from Dical P was greater than AIPD at all other levels of AvP regardless of P source, with the exception of 0.28% Dical P. These data are in contrast to Hamdi et al. (2015), where increasing NPP concentrations of 0.30, 0.35, 0.40, and 0.45% increased AIPD 76.8, 81.3, 82.3, and 86.1%, respectively ($P \leq 0.01$). These differences demonstrate that mineral digestion is a complex process that depends on a number of physiological variables, including but not limited to transporter abundance and vitamin D metabolism. Babatunde et al. (2019) showed that there was a decrease ($P < 0.01$) in AIPD in birds fed a 0.20% NPP diet as compared with birds fed a 0.40% NPP diet from days 6 to 22. Tibia ash may reflect a cumulative process and may not react as quickly to changes in dietary P as observed in the ileal digestibility of P.

The Ca:P ratio can affect P digestibility. Liu et al. (2013) reported greater digestibility when birds were fed a diet with a Ca:total P ratio of 0.8; whereas, there was no difference in P digestibility for Ca:total P ratios between 1.2 and 2.0. Because the Ca:total P ratios ranged from 1.4 to 2.0 in our study, we did not expect any differences. As expected, Nano P showed no difference in AIPD for 0.24 to 0.36% AvP; however, AIPD for Dical P at 0.24% AvP was greater than that at 0.32 and 0.36% AvP.

In the duodenal mucosa, mRNA abundance of the NaPi-IIb transporter was greatest in the 0.20% AvP fed birds compared with all other concentrations of P for both Dical P and Nano P (Figure 3A). There was no difference, however, in mRNA abundance between 0.24, 0.28, 0.32, and 0.36% P for both Dical P and Nano P. Consistent with our results, Li et al. (2016) showed no difference in NaPi-IIb mRNA abundance

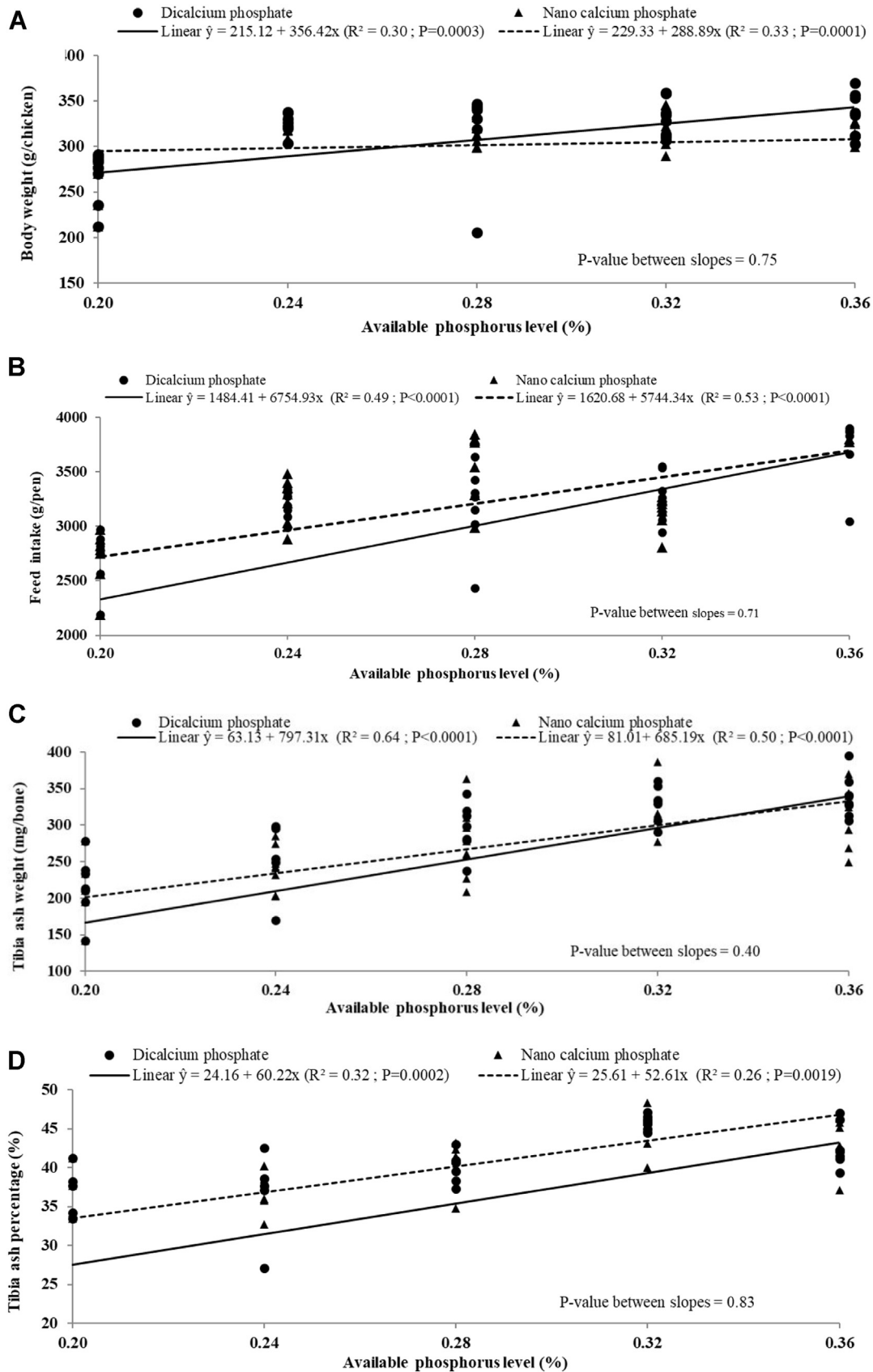


Figure 1. Body weight (A), feed intake (B), tibia ash weight (C), and tibia ash percentage (D) of chicks fed diets containing 0.20, 0.24, 0.28, 0.32, and 0.36% available phosphorus from either dicalcium phosphate (circles and solid lines) or nanocalcium phosphate (triangles and dashed lines) from 0 to 14 D. Slope regression analysis was performed to determine differences in response slopes between the 2 sources of P for body weight, feed intake, tibia ash weight, and tibia ash percentage.

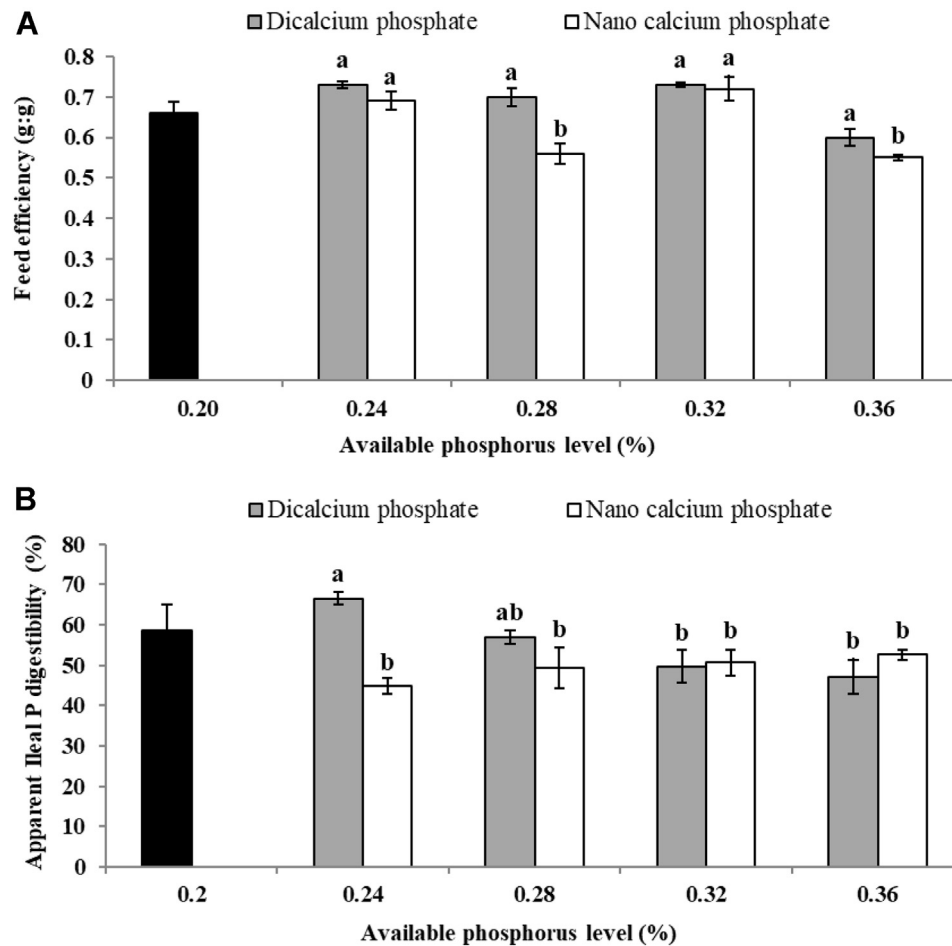


Figure 2. Feed efficiency (A) and apparent ileal phosphorus digestibility (B) of chicks fed diets containing 0.20 (control), 0.24, 0.28, 0.32, and 0.36% available phosphorus from either dicalcium phosphate or nanocalcium phosphate from 0 to 14 D. A 2×4 factorial (2 P sources and 4 available P levels) analysis was performed without the 0.20% P treatment. Bars (means \pm SE) without common superscript letters (a, b) for feed efficiency and apparent ileal digestibility are different ($P \leq 0.05$).

when broilers were fed both low and high nutrient density diets supplemented with 0.25 to 0.4% NPP. The inverse relationship between NaPi-IIb mRNA abundance and dietary P levels has been reported previously for both broilers and layers. Yan et al. (2007) were the first to clone and functionally characterize chicken NaPi-IIb. They found that NaPi-IIb mRNA was expressed greatest in the duodenum followed by the jejunum and ileum and that a reduction of P in the diets of Ross 308 broilers from hatch to day 4 post-hatch led to an induction of NaPi-IIb mRNA abundance. Nie et al. (2013) also showed a similar decrease in duodenal NaPi-IIb mRNA abundance, when Dwarf pink shell laying hens were fed increasing levels of NPP from 28 to 41 wk of age.

The NaPi-IIb gene expression is regulated by dietary P and crude protein levels in broiler chickens (Xue et al., 2016; Liu et al., 2017). Using Western blots, Huber et al. (2015) detected 2 isoforms of NaPi-IIb protein of 105 kDa and 90 kDa in the jejunum but only the 90 kDa protein in the ileum. Supplementation of the diet with monocalcium phosphate did not affect expression of either NaPi-IIb isoform.

Phytase supplementation, however, resulted in decreased jejunal expression of the 90 kDa NaPi-IIb protein. A comprehensive analysis of the effects of high and low Pi diets on not only NaPi-IIb mRNA and protein abundance but also Pi activity was conducted by Fang et al. (2012). In this study, Ross broilers fed a 0.6% total Pi diet, had maximal Pi uptake in the jejunum even though the greatest NaPi-IIb mRNA and protein abundance was in the duodenum. Furthermore, when 40-day-old broilers were fed low (0.2% Pi), normal (0.6% Pi), or high (1.0% Pi) diets for 7 D, there was increased NaPi-IIb mRNA and protein abundance and Pi uptake in the duodenum of the low Pi group, but no difference in the jejunum and ileum. Li et al. (2018) showed that diets fed to laying hens containing increasing levels of AvP (0.15–0.82%) caused an increase in NaPi-IIb protein but not NaPi-IIb mRNA in the duodenum and a decrease in NaPi-IIb mRNA but not NaPi-IIb protein in the ileum. These results demonstrate that regulation of NaPi-IIb mRNA and protein varies with intestinal segment and that Pi uptake and NaPi-IIb mRNA and protein levels are not always correlated.

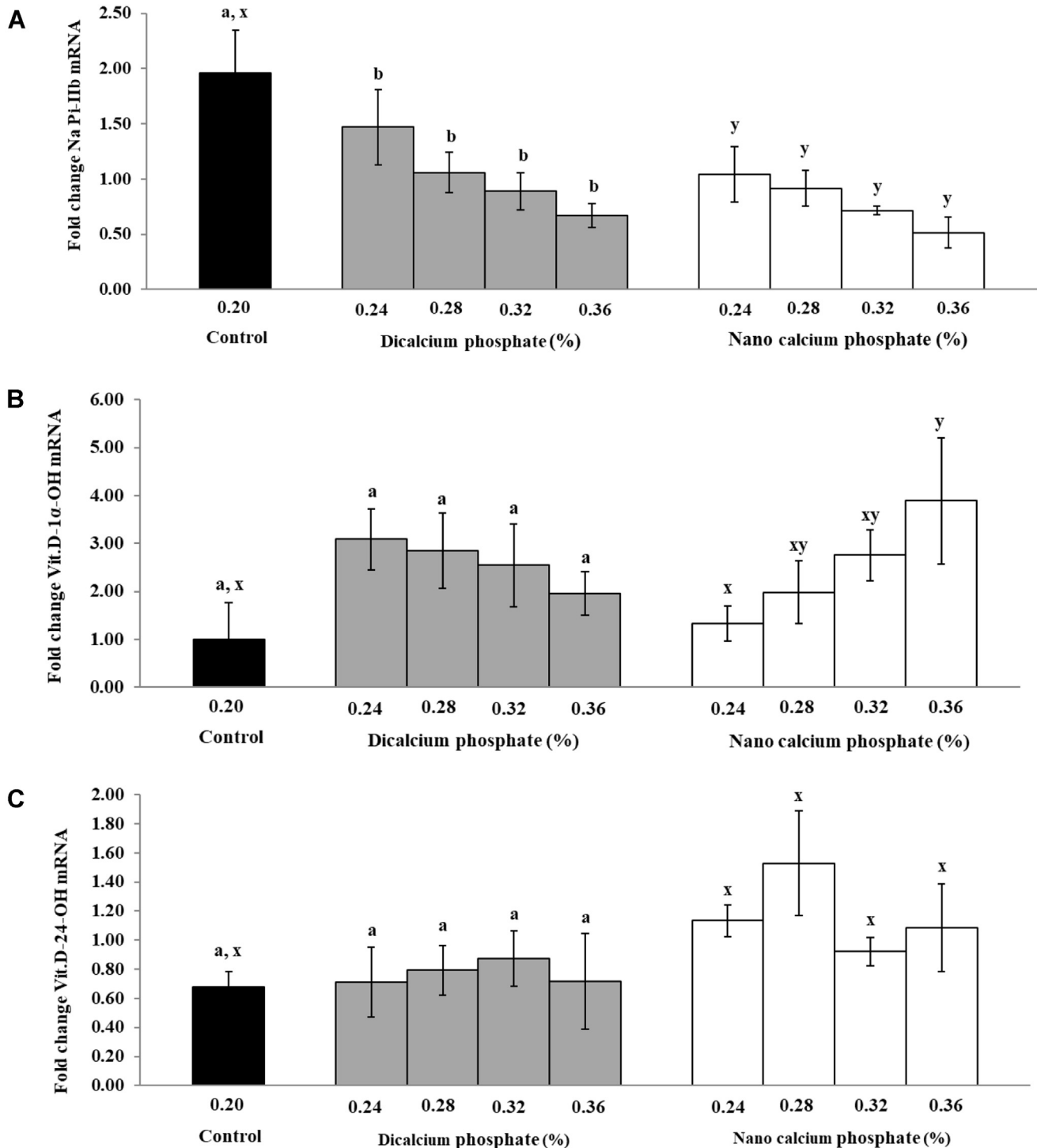


Figure 3. Expression of intestinal NaPi-IIb (A), kidney vitamin D-1 α -hydroxylase (B), and kidney vitamin D-24-hydroxylase (C) mRNA in chicks fed various levels of phosphorus in the diet. Chicks were fed diets containing 0.20 (control), 0.24, 0.28, 0.32, and 0.36% available phosphorus from dicalcium phosphate and nanocalcium phosphate sources from 0 to 14 D. The abundance of NaPi-IIb (sodium phosphate cotransporter IIb) mRNA in duodenal mucosa, Vit.D-1 α -OH (vitamin D-1 α -hydroxylase) mRNA in kidney and Vit.D-24-OH (vitamin D-24-hydroxylase) mRNA in kidney was determined by quantitative PCR. Analysis was performed separately for dicalcium phosphate (control plus 4 dicalcium phosphate levels) and nanocalcium phosphate (control plus 4 nanocalcium phosphate levels). Bars (means \pm SE) without common letters (a, b) are different for dicalcium phosphate ($P \leq 0.05$) and bars without common letters (x, y) are different for nanocalcium phosphate ($P \leq 0.05$).

In the kidney, there was an increase in mRNA abundance for vitamin D-1 α -hydroxylase at 0.36% Nano P compared with control (0.20%) (Figure 3B). There was no difference in vitamin D-1 α -hydroxylase mRNA abundance between 0.20% P and all other Dical P concentrations. For vitamin D-24-hydroxylase, there was no

difference in mRNA abundance between control (0.20%) and Dical P and Nano P levels (Figure 3C). The increase in vitamin D-1 α -hydroxylase mRNA in the current study was observed only at the highest level of Nano P and not with Dical P. This difference may be because of differences in solubility due to particle size

between Nano P (<100 nm) and Dical P (500–1,000 µm). The increase in vitamin D-1 α -hydroxylase and no change in vitamin D-24-hydroxylase could potentially lead to an increase in the concentration of active 1,25-dihydroxy vitamin D. When the vitamin D analog, 1 α -hydroxy-vitamin D3, was fed to chicks, it was rapidly metabolized to 1,25-dihydroxy vitamin D (Edelstein et al., 1978) and resulted in an increase in growth performance, plasma Pi concentration, and Na Pi-IIIb mRNA abundance (Han et al., 2009). Moreover, 25-OH vitamin D3 increased both vitamin D-1 α -hydroxylase and NaPi-IIIb mRNA abundance in broiler chickens from days 1 to 14, thereby promoting active Pi absorption in the duodenum of broilers (Han et al., 2018).

In conclusion, during the starter period, Dical P and Nano P sources significantly increased performance, TAW, and TAP of broiler chicks. In addition, there were no differences between Dical P and Nano P for BW, FI, TAW, and TAP of chicks, indicating that Dical P and Nano P had equivalent bioavailability. In the intestine, mRNA abundance of NaPi-IIIb mRNA decreased with increasing dietary Dical P and Nano P; whereas in the kidney, mRNA abundance of vitamin D-1 α -hydroxylase was increased by 0.36% Nano P, while vitamin D-24-hydroxylase mRNA abundance was unaffected by Dical P and Nano P. Although Dical P and Nano P showed the same bioavailability, they showed a differential effect on vitamin D-1 α -hydroxylase gene expression but not intestinal NaPi-IIIb gene expression.

ACKNOWLEDGMENTS

Funding for this research was provided in part by a grant from the USAID and Egyptian Ministry of Higher Education and Scientific Research (IO) and by the Virginia Agricultural Experiment Station and the Hatch Program of the National Institute of Food and Agriculture, U.S. Department of Agriculture. The authors would also like to express their appreciation to all members of the Persia and Wong labs, the VT farm crew, and the Faculty of Agriculture, Cairo University, Egypt, for their assistance and support.

REFERENCES

- Ahmadi, H., and M. Rodehutsord. 2012. A meta-analysis of responses to dietary nonphytate phosphorus and phytase in laying hens. *Poult. Sci.* 91:2072–2078.
- Al-Beitawi, N. A., M. M. Shaker, K. N. El-Shuraydeh, and J. Blaha. 2017. Effect of nanoclay minerals on growth performance, internal organs and blood biochemistry of broiler chickens compared to vaccines and antibiotics. *J. Appl. Anim. Res.* 45:543–549.
- Ammerman, C. B., D. H. Baker, and A. J. Lewis. 1995. *Bioavailability of Nutrients for Animals: Amino Acids, Minerals, and Vitamins*. Academic Press, San Diego, CA, USA, p. 441.
- Babatunde, O. O., A. J. Cowieson, J. W. Wilson, and O. Adeola. 2019. Influence of age and duration of feeding low-phosphorus diet on phytase efficacy in broiler chickens during the starter phase. *Poult. Sci.* 98:2588–2597.
- Boguhn, J., T. Baumgartel, A. Dieckmann, and M. Rodehutsord. 2009. Determination of titanium dioxide supplements in different matrices using two methods involving photometer and inductively coupled plasma optical emission spectrometer measurements. *Archiv. Anim. Nutr.* 4:337–342.
- Capuano, P., T. Radanovic, C. A. Wagner, D. Bacic, S. Kato, Y. Uchiyama, R. St. Arnaud, H. Murer, and J. Biber. 2005. Intestinal and renal adaptation to a low-Pi diet of type II NaPi cotransporters in vitamin D receptor- and 1 alpha OHase-deficient mice. *Am. J. Physiol. Cell Physiol.* 288:C429–C434.
- Christakos, S., D. V. Ajibade, P. Dhawan, A. J. Fechner, and L. J. Mady. 2010. Vitamin D: metabolism. *Endocrinol. Metab. Clin. North Am.* 39:243–253.
- Christakos, S., L. Lieben, R. Masuyama, and G. Carmeliet. 2014. Vitamin D endocrine system and the intestine. *Bonekey Rep.* 3:496. Article number 496.
- Cobb 500TM Broiler Performance & Nutrition Supplement. 2015. Accessed July 2015. <https://cobbstorage.blob.core.windows.net/guides/5a171aa0-6994-11e8-9f14-bdc382f8d47e>.
- Douglas, M. W., M. E. Persia, and C. M. Parson. 2003. Impact of galactose, lactose and Grobiotic-B70TM on growth performance and energy utilization when fed to broiler chicks. *Poult. Sci.* 82:1596–1601.
- Edelstein, S., D. Noff, D. Freeman, M. Sheves, and Y. Mazur. 1978. Synthesis of 1 α -hydroxy [7-³H] cholecalciferol and its metabolism in the chick. *Biochem. J.* 176:111–117.
- 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.* 25:1457–1465.
- Feild, J. A., L. Zhang, K. A. Brun, D. P. Brooks, and R. M. Edwards. 1999. Cloning and functional characterization of a sodium-dependent phosphate transporter expressed in human lung and small intestine. *Biochem. Biophys. Res. Commun.* 258:578–582.
- Forster, I. C., N. Hernando, J. Biber, and H. Murer. 2013. Phosphate transporters of the SLC20 and SLC34 families. *Mol. Asp. Med.* 34:386–395.
- Gautier, A. E., C. L. Walk, and R. N. Dilger. 2018. Effects of a high level of phytase on broiler performance, bone ash, phosphorus utilization, and phytate dephosphorylation to inositol. *Poult. Sci.* 97:211–218.
- Hamdi, M., D. Solà-Oriol, R. Davin, and J. F. Perez. 2015. Calcium sources and their interaction with the different levels of non-phytate phosphorus affect performance and bone mineralization in broiler chickens. *Poult. Sci.* 94:2136–2143.
- Han, J. C., X. D. Yang, T. Zhang, H. Li, W. L. Li, Z. Y. Zhang, and J. H. Yao. 2009. Effects of 1- α -hydroxycholecalciferol on growth performance, parameters of tibia and plasma, meat quality, and type IIb sodium phosphate co-transporter gene expression of one- to twenty-one-day-old broilers. *Poult. Sci.* 88:323–329.
- 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.* 97:1199–1208.
- Hassan, H. M. A., A. Samy, A. E. El-Sherbiny, M. A. Mohamed, and M. O. Abd-Elsamee. 2016. Application of nano-dicalcium phosphate in broiler nutrition: performance and excreted calcium and phosphorus. *As. J. Anim. Vet. Adv.* 11:477–483.
- 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 intestine. *Proc. Natl. Acad. Sci. USA.* 95:14564–14569.
- Huber, K., E. Zeller, and M. Rodehutsord. 2015. Modulation of small intestinal phosphate transporter by dietary supplements of mineral phosphorus and phytase in broilers. *Poult. Sci.* 94:1009–1017.
- Jiang, R. X., F. H. Luo, M. R. Qu, V. Bontempo, S. G. Wu, H. J. Zhang, H. Y. Yue, and G. H. Qi. 2013. Effects of non-phytate phosphorus levels and phytase sources on growth performance, serum biochemical and tibia parameters of broiler chickens. *Ital. J. Anim. Sci.* 12:375–380.
- Kim, S. W., W. Li, R. Angel, and M. Proszkowiec-Weglarz. 2018. Effects of limestone particle size and dietary Ca concentration on apparent P and Ca digestibility in the presence or absence of phytase. *Poult. Sci.* 97:4306–4314.
- Létourneau-Montminy, M. P., A. Narcy, P. Lescoat, J. F. Bernier, M. Magnin, C. Pomar, Y. Nys, D. Sauvart, and

- C. Jondreville. 2013. Meta-analysis of phosphorus utilisation by broilers receiving corn soyabean meal diets: influence of dietary calcium and microbial phytase. *Animal* 4:1844–1853.
- Li, J., J. Yuan, Z. Miao, Z. Song, Y. Yang, W. Tian, and Y. Guo. 2016. Effect of dietary nutrient density on small intestinal phosphate transport and bone mineralization of broilers during the growing period. *PLoS One* 11:e0153859.
- Li, P., R. Wang, H. Jiao, X. Wang, J. Zhao, and H. Lin. 2018. Effects of dietary phosphorus level on the expression of calcium and phosphorus transporters in laying hens. *Front. Physiol.* 9:627.
- Li, W., R. Angel, S. W. Kim, E. Jiménez-Moreno, M. Proszkowiec-Weglarz, and P. W. Plumstead. 2015. Impact of response criteria (tibia ash weight vs. percent) on phytase relative non phytate phosphorus equivalence. *Poult. Sci.* 94:2228–2234.
- Liu, J. B., D. W. Chen, and O. Adeola. 2013. Phosphorus digestibility response of broiler chickens to dietary calcium-to-phosphorus ratios. *Poult. Sci.* 92:1572–1578.
- 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.* 96:151–159.
- Martin, T. D., and C. A. Brockhoff. J. T. Creed and EMMC Methods Work Group. 1994. Method 200.7 Determination of Metals and Trace Elements in Water and Wastes by Inductively Coupled Plasma-Atomic Emission Spectrometry, Revision 4.4. Environmental Monitoring Systems Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, OH.
- Mishra, S., C. Keswani, P. C. Abhilash, L. F. Fraceto, and H. B. Singh. 2017. Integrated approach of agri-nanotechnology: challenges and future trends. *Front. Plant Sci.* 8:471.
- Murer, H., N. Hernando, L. Forster, and J. Biber. 2001. Molecular mechanisms in proximal tubular and small intestinal phosphate reabsorption (Plenary Lecture). *Mol. Membr. Biol.* 18:3–11.
- National Research Council. 1994. Nutrient Requirements of Poultry, 9th rev. ed. Natl. Acad. Press, Washington, DC.
- 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.* 4:34–40.
- Nikonov, I. N., Yu. G. Folmanis, G. E. Folmanis, L. V. Kovalenko, G. Y. Laptsev, I. A. Egorov, V. I. Fisinin, and I. G. Tananaev. 2011. Iron nanoparticles as a food additive for poultry. *Dokl. Biol. Sci.* 440:328–331.
- Olukosi, O. A., C. Kong, F. Fru-Nji, K. M. Ajuwon, and O. Adeola. 2013. Assessment of a bacterial 6-phytase in the diets of broiler chickens. *Poult. Sci.* 92:2101–2108.
- Onyiah, C. L. 2008. Design and Analysis of Experiments: Classical and Regression Approaches with SAS. Statistics: Textbooks and Monographs. St. Cloud State University, Minnesota, U.S.A., p 817.
- Persia, M. E., P. L. Utterback, P. E. Biggs, K. W. Koelkebeck, and C. M. Parsons. 2003. Interrelationship between environmental temperature and dietary nonphytate phosphorus in laying hens. *Poult. Sci.* 82:1763–1768.
- Powell, S., T. D. Bidner, and L. L. Southern. 2011. The effects of feeding various levels of nonphytate phosphorus in the starter phase on broiler growth performance and bone characteristics in subsequent phases. *J. Appl. Poult. Res.* 20:181–189.
- Radanovic, T., C. A. Wagner, H. Murer, and J. Biber. 2005. Regulation of intestinal phosphate transport I. Segmental expression and adaptation to low-Pi diet of the type IIb Na⁺-Pi cotransporter in mouse small intestine. *Am. J. Physiol. Gastrointest. Liver Physiol.* 288:G496–G500.
- Rodehutsord, M., A. Dieckmann, M. Witzig, and Y. Shastak. 2012. A note on sampling digesta from the ileum of broilers in phosphorus digestibility studies. *Poult. Sci.* 91:965–971.
- Sabbagh, Y., H. Giral, Y. Caldas, M. Levi, and S. C. Schiavi. 2011. Intestinal phosphate transport. *Adv. Chronic Kidney Dis.* 18:85–90.
- Sabry, M. I., K. W. McMillan, and C. M. Sabliov. 2018. Nanotechnology considerations for poultry and livestock production systems-A review. *Ann. Anim. Sci.* 18:319–334.
- SAS Institute. 2004. SAS User's Guide: Statistics, Version. 9, 1 ed. SAS Inst. Inc., Cary, NC.
- Schmittgen, T. D., and K. J. Livak. 2008. Analyzing real-time PCR data by the comparative C_T method. *Nat. Protoc* 3:1101–1108.
- Shanmugasundaram, R., and R. K. Selvaraj. 2012. Vitamin D-1 α -hydroxylase and vitamin D-24-hydroxylase mRNA studies in chickens. *Poult. Sci.* 91:1819–1824.
- Shastak, Y., M. Witzig, K. Hartung, W. Bessei, and M. Rodehutsord. 2012. Comparison and evaluation of bone measurements for the assessment of mineral phosphorus sources in broilers. *Poult. Sci.* 91:2210–2220.
- Vijayakumar, M. P., and V. Balakrishnan. 2014. Evaluating the bioavailability of calcium phosphate nanoparticles as mineral supplement in broiler chicken. *Indian J. Sci. Tech.* 7:1475–1480.
- Waldroup, P. W. 1999. Nutritional approaches to reducing phosphorus excretion by poultry. *Poult. Sci.* 78:683–691.
- Weiss, J., P. Takhistov, and J. McClements. 2006. Functional materials in food nanotechnology. *J. Food Sci.* 71:107–116.
- Werner, A., and R. K. H. Kinne. 2001. Evolution of the Na-Pi cotransporter systems. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 280:R301–R312.
- Xue, P. C., K. M. Ajuwon, and O. Adeola. 2016. Phosphorus and nitrogen utilization responses of broiler chickens to dietary crude protein and phosphorus levels. *Poult. Sci.* 95:2615–2623.
- Yan, F., R. Angel, and C. M. Ashwell. 2007. Characterization of the chicken small intestine type IIb sodium phosphate cotransporter. *Poult. Sci.* 86:67–76.