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RESEARCH ARTICLE

Challenges of using tibia bone ash and toe ash as biomarkers of phosphorus nutrition for meat chickens

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Abstract

Two experiments were conducted to determine the suitability of tibia bone ash and toe ash as biomarkers of phosphorus (P) status of broilers from day 1 to 21 (Experiment 1) and from day 21 to 49 (Experiment 2). Experimental diets, including sorghum, soybean meal and other minor ingredients, were formulated to meet all nutrient requirements according to the nutrition specification recommended by Aviagen Inc (2022) except for calcium (Ca) and P. The diets contained the same concentrations of Ca of 10.0 and 9.0 g/kg diet for starter and grower/finisher, respectively and graded levels of non-phytate P (NPP) from 2.5 to 5.5 g/kg diet for starter; from 2.0 to 5.0 g/kg diet for grower/finisher in increment of 1.0 g/kg. Each diet was fed to one of five replicate pens (10 chicks per pen), starter from day 1 to 14; grower from day 15 to 28 and finisher from day 29 to 49. Body weight and feed intake were recorded weekly and feed conversion ratios (FCR) calculated. Ash content of tibia bones and toes were determined at the completion of both experiments. The results indicated that the NPP requirement of meat chickens was lower than the NRC (1994) recommendations and values currently used by the industry. Phytase supplementation significantly ($P < 0.05$) increased body weight of birds fed diets containing the lowest NPP concentrations (2.5/2.0 and 3.5/3.0 g/kg), but had no effect when the NPP levels were 4.5/4.0 and 5.5/5.0 g/kg. Growth performance and FCR are recommended as the primary parameters for evaluating the P requirement of meat chickens. Tibia bone and toe ash contents were poorly correlated with growth performance and were therefore not good biomarkers of dietary P status, especially in finisher birds. Only one Ca concentration was used in the current study and further research on P nutrition with different concentrations of Ca with or without supplemental phytase is warranted.

Keywords

calcium – phosphorus – tibia bone – toe – broilers – biomarker

1 Introduction

Phosphorus (P) is an essential element and the third most costly dietary component in poultry production (Li *et al.*, 2016). Cereal grains and their by-products, the main ingredients for monogastric animals, contain P as phytate, which represents up to 70% of the total P in poultry diets. Poultry cannot efficiently utilise P in this form as birds lack sufficient endogenous phytase to hydrolyse phytate (Li *et al.*, 2016; Nelson, 1967). The necessity of supplementing poultry diets with P, especially those of plant origin, has been recognised for over

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a century (Kaupp, 1918; Kennard, 1920). However, it is important that diets are not over supplemented with P due to cost, pollution from excretion and difficulties of maintaining the supply to the feed industry due to finite reserves of this essential nutrient (Cordel *et al.*, 2009). Thus, it is important to know the P requirement of broilers. This has been the subject of numerous investigations, but the requirement for this nutrient has not been established with any certainty (David *et al.*, 2022; Li *et al.*, 2016, 2017). This is due in part to published results including many variables, such as broiler strain, age, feed ingredients, phytase supplementation and different Ca and P sources. Moreover, interpretation of published data is further complicated by the different assessment criteria, including body weight gain, feed conversion ratio (FCR), tibia bone breaking strength, tibia bone ash, or toe ash that are used to determine requirements.

In many publications, the P requirements of meat chickens or broilers (David *et al.*, 2022; Dhandu and Angel, 2003; Leske and Coon, 2002; Li *et al.*, 2016; Persia and Saylor, 2006; Waldroup, 1999a,b, 2000; Yan and Waldroup, 2006; Yan *et al.*, 2001) are considerably lower than the NRC (1994) recommendations that were based on research from 40 years ago (Li *et al.*, 2017; Waldroup, 1999b). During this period, the genetics of the broiler have changed with a greater emphasis skeletal development and health in breeding programs (Kapell *et al.*, 2012). Moreover, the widespread addition of microbial phytase to poultry diets has reignited the debate on P requirements. Phytase provides an opportunity to reduce inorganic P supplementation of broiler diets due to increased hydrolysis and improved utilisation of phytate P and reduced environment pollution by excretion of undigested P (Dersjant-Li *et al.*, 2022; Selle and Ravindran, 2007). Moreover, this raises the question of the most appropriate criteria or biomarkers to determine the inorganic P or non-phytate P (NPP) status of diets.

Phosphorus plays an important role in bone mineralisation and, for that reason, ash content of the tibia or toes is used to assess P status. However, there are discrepancies in the literature when these biomarkers are used (Ravindran *et al.*, 1995). This may be related to the age of the bird as the rate of bone development and mineralisation changes as birds develop (Gulizia *et al.*, 2022; Talaty *et al.*, 2009). Hence, the objectives of this study were to examine the suitability of tibia bone ash and toe ash as criteria for determining P status of broilers fed typical Australian broiler diets with or without phytase supplementation. The influence of broiler age on the suitability of these biomarkers was determined at day 21 (Experiment 1) and day 49 (Experiment 2).

2 Materials and methods

All experimental procedures were approved by the Animal Care and Ethics Committee of the University of Queensland and complied with the Australia Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013).

Birds and housing

Ross 308, one-day-old, male, broilers chicks were purchased from a commercial hatchery (Baiada, Tamworth, NSW, Australia). Upon arrival, chicks were placed in floor pens (3.0 × 1.1 m) containing wood shavings, with the brooding temperatures and a lighting regimen recommended by the breeding company (Aviagen Inc, 2014). Prior to each experiment, birds were weighted individually and allocated to a pen containing 10 chicks by stratified randomisation, so that each group of chicks within an experiment had the same initial mean and range of live weights. Chicks commenced Experiment 1 at one-day-old, and Experiment 2, on day 21 post-hatch. The chicks in Experiments 2 were fed a commercial starter diet from day 1 to 14 and a commercial grower diet from day 15 to 21. For the duration of both experiments, birds were monitored twice daily.

Diets and experimental design

All experimental diets were formulated with sorghum, soybean meal, canola meal and meat and bone meal according to Ross nutrition specifications (Aviagen Inc, 2022) to meet all nutrient requirements, except for Ca and P. The experimental diets contained the same levels of Ca, 10.0 and 9.0 g/kg diet for starter and grower/finisher, respectively. Dietary non-phytate P (NPP) concentrations were graded from 2.5 to 5.5 g/kg diet for starter (Table 1) and from 2.0 to 5.0 g/kg diet for grower/finisher (Tables 2 and 3) in increments of 1.0 g/kg. This wide range of NPP concentrations covered a potential P deficiency status at the lowest level, to the value recommended by NRC (1994). The Ca and P concentrations in the respective diets were achieved by adjusting inclusion levels of meat and bone meat, monodicalcium phosphate (MDCP) and limestone.

Both experiments had a factorial design and the experimental diets were prepared either with or without phytase (Feedzyme XP 1000 FTU/g, Danisco Animal

TABLE 1 Composition (g/kg) of starter diet (Experiment 1)[1]

Ingredient	Dietary NPP (g/kg)			
	2.5	3.5	4.5	5.5
Sorghum	523.3	529.1	524.4	519.8
Soybean meal	350.0	330.0	330.0	330.0
Canola meal	30.0	33.0	33.0	33.0
Meat and bone meal	28.0	46.0	47.0	47.0
Soy oil	37.4	34.7	36.4	38.0
Lysine	2.2	2.3	2.3	2.3
DL Methionine	3.6	3.7	3.7	3.7
L-Threonine	1.0	1.0	1.0	1.0
Limestone	14.5	9.5	7.5	5.5
MDCP		1.0	5.0	10.0
Salt	2.4	2.3	2.3	2.3
Sodium bicarbonate	2.1	1.9	1.9	1.9
Vitamin and mineral premix[2]	5.0	5.0	5.0	5.0
Choline chloride	0.5	0.5	0.5	0.5
Total	1000	1000	1000	1000
Nutrient calculation (g/kg)				
AME (MJ/kg)	12.7	12.7	12.7	12.7
Protein	239.8	241.0	241.1	240.7
Ca	10.0	10.0	10.0	10.0
Total P	5.6	6.7	7.5	8.6

[1] NPP = non- phytate phosphorus; MDCP = monodicalcium phosphate; AME = apparent metabolisable energy.
[2] Each kg of premix contained the following: vitamin A, 4.8 MIU.; vitamin D_3, 0.96 MIU.; vitamin E, 10 g; vitamin K_3; 0.8 g; riboflavin (vitamin B_2), 5 g; pyridoxine (vitamin B_6), 2.4 g; cyanocobalamin (vitamin B_{12}), 6 mg; biotin, 0.06 g; niacin, 24 g; thiamine (vitamin B_1); 0.8 g; pantothenic acid, 6 g; folic acid, 0.6 g; magnesium (Mg), 40 g; manganese (Mn), 40 g; zinc, 32 g; iron, 32 g; copper, 4 g; iodine, 0.2 g; cobalt, 0.04 g; selenium, 0.04 g; molybdenum, 0.04 g; Endox, 50 g and Liquid Paraffin white, 10 g.

TABLE 2 Composition (g/kg) of grower diet (Experiments 1 and 2)[1]

Ingredient	Dietary NPP (g/kg)			
	2.0	3.0	4.0	5.0
Sorghum	576.2	581.3	576.7	570.1
Soybean meal	285.0	279.0	279.0	280.0
Canola meal	38.0	30.0	30.0	30.0
Meat and bone meal	20.0	35.0	35.0	36.0
Soy oil	50.0	47.8	49.4	51.0
Lysine	2.2	2.1	2.1	2.1
DL Methionine	3.3	3.3	3.3	3.3
L-Threonine	0.9	0.9	0.9	0.9
Limestone	14.5	10.0	8.0	6.0
MDCP		1.0	6.0	11.0
Salt	2.5	2.4	2.4	2.4

[1] NPP = non- phytate phosphorus; MDCP = monodicalcium phosphate; AME = apparent metabolisable energy.

TABLE 2 Composition (g/kg) of grower diet (Experiments 1 and 2) (*cont.*)

Ingredient	Dietary NPP (g/kg)			
Sodium bicarbonate	1.8	1.6	1.6	1.6
Vitamin and mineral premix[2]	5.0	5.0	5.0	5.0
Choline chloride	0.6	0.6	0.6	0.6
Total	1000	1000	1000	1000
Nutrient calculation (g/kg)				
AME (MJ/kg)	13.2	13.2	13.2	13.2
Protein	211.9	214.2	213.8	214.2
Ca	9.0	9.0	9.0	9.0
Total P	5.1	5.9	7.0	8.0

[2] Each kg of premix contained the following: vitamin A, 4.8 MIU.; vitamin D_3, 0.96 MIU.; vitamin E, 10 g; vitamin K_3; 0.8 g; riboflavin (vitamin B_2), 5 g; pyridoxine (vitamin B_6), 2.4 g; cyanocobalamin (vitamin B_{12}), 6 mg; biotin, 0.06 g; niacin, 24 g; thiamine (vitamin B_1); 0.8 g; pantothenic acid, 6 g; folic acid, 0.6 g; magnesium (Mg), 40 g; manganese (Mn), 40 g; zinc, 32 g; iron, 32 g; copper, 4 g; iodine, 0.2 g; cobalt, 0.04 g; selenium, 0.04 g; molybdenum, 0.04 g; Endox, 50 g and Liquid Paraffin white, 10 g.

TABLE 3 Composition (g/kg) of finisher diet (Experiment 2)[1]

Ingredient	Dietary NPP (g/kg)			
	2.0	3.0	4.0	5.0
Sorghum	627.7	624.6	620.0	613.4
Soybean meal	237.7	234.7	234.7	235.7
Canola meal	30.0	30.0	30.0	30.0
Meat and bone meal	22.0	27.0	28.0	29.0
Soy oil	50.7	51.3	52.9	54.5
Lysine	2.1	2.0	2.0	2.0
DL Methionine	3.0	3.0	3.0	3.0
L-Threonine	0.9	0.8	0.8	0.8
Limestone	14.6	11.7	9.6	7.3
MDCP	0.0	3.6	8.2	13.0
Salt	2.4	2.3	2.3	2.3
Sodium bicarbonate	2.0	1.9	1.9	1.9
Vitamin and mineral premix[2]	5.0	5.0	5.0	5.0
Choline chloride	0.8	0.8	0.8	0.8
Total	1000	1000	1000	1000
Nutrient calculation (g/kg)				
AME (MJ/kg)	13.4	13.4	13.4	13.4
Protein	192.1	192.7	192.8	193.2
Ca	9.0	9.0	9.0	9.0
Total P	4.9	5.9	6.9	7.9

[1] NPP = non-phytate phosphorus; MDCP = monodicalcium phosphate; AME = apparent metabolisable energy.
[2] Each kg of premix contained the following: vitamin A, 4.8 MIU.; vitamin D_3, 0.96 MIU.; vitamin E, 10 g; vitamin K_3; 0.8 g; riboflavin (vitamin B_2), 5 g; pyridoxine (vitamin B_6), 2.4 g; cyanocobalamin (vitamin B_{12}), 6 mg; biotin, 0.06 g; niacin, 24 g; thiamine (vitamin B_1); 0.8 g; pantothenic acid, 6 g; folic acid, 0.6 g; magnesium (Mg), 40 g; manganese (Mn), 40 g; zinc, 32 g; iron, 32 g; copper, 4 g; iodine, 0.2 g; cobalt, 0.04 g; selenium, 0.04 g; molybdenum, 0.04 g; Endox, 50 g and Liquid Paraffin white, 10 g.

Nutrition) inclusion at 500 FTU/kg, where FTU refers to phytase units and is defined as the amount of enzyme required to hydrolyse 1 μmol inorganic P per minute from 0.0051 mol/l sodium phytate at pH 5.5 and a temperature of 37 °C (AOAC, 2000). Each experimental diet was fed to five replicate pens: starter diet from day 1 to 14 in Experiment 1; grower diet from day 15 to 21 in Experiment 1 and from day 21 to 28 in Experiment 2; finisher diet from day 29 to 49 in Experiment 2.

Measurements and sample collections

Feed intake and body weight were measured weekly and FCR was calculated accordingly. Gait scores (scores 0 to 5; 0 = normal, 5 = Complete lameness) of all birds in each pen were assessed independently by three people according to the system of Garner *et al.* (2002) from day 13 to 15 in Experiment 1 and day 43 to 45 in Experiment 2. The average of three scores was used for statistical analysis. At the end of each experiment, two birds per replicate were euthanised by an intracardial injection of pentobarbitone sodium. Both tibia bones and left middle toes were collected and all soft tissues removed and the left tibia bone and toe were frozen. The head of the right tibia bone was sliced longitudinally with a scalpel and examined for the incidence and severity of tibia dyschondroplasia (TD). The size of the area with an abnormal mass of cartilage was scored (scores 0 to 5; 0 = normal, 5 = extensive deposition of abnormal cartilage) according to Edwards and Veltmann (1983).

Analysis of tibia bone ash and toe ash content

The left tibia bones were autoclaved at 121 °C and 16 psi for 15 (Experiment 1) and 30 (Experiment 2) min, respectively, to facilitate manual removal of soft tissue. After cleaning, the left tibia bones were weighed into pre-cleaned, -ashed and -weighed crucibles, dried at 105 °C in an oven for 48 h to a constant weight. Crucibles with dried tibia bones were then ashed at 550 °C for 24 h (AOAC, 1990). Left middle toes were cleaned, and dried, and then ashed as described for tibia bones. Tibia bone and toe ash contents were expressed as percentages of dry tibia bone and toe weight, respectively.

Statistical analysis

General linear model and Minitab program (version 16.0, Minitab Pty Ltd., Sydney NSW, Australia) were used to analyse all the data according to Steele *et al.* (1997). Main factors were dietary NPP level and phytase supplementation. TD scores were transformed using a square root (TD + 0.5) to improve the distribution of the residuals (Zar, 1984) before statistical analysis was conducted. The significant difference level was $P < 0.05$.

3 Results

In both experiments birds appeared clinically normal for the duration of the studies.

Experiment 1

The growth performance of the birds in this study is shown in Table 4. Birds fed on the diet containing the lowest NPP concentration (2.5/2.0 g/kg) without phytase supplementation had significantly ($P < 0.05$) lower body weight and feed intake than those fed on diets containing higher NPP levels at day 21. Phytase supplementation significantly ($P < 0.05$) increased body weight and feed intake of birds fed the lowest NPP diet numerically the body weight of the birds fed NPP of 3.5/3.0 g/kg without phytase supplementation was 12% lower at day 21 than the average of birds fed on higher NPP levels and those with phytase supplementation. The FCR was significantly affected by dietary NPP level with 5.3% improvement achieved in birds fed on the two higher NPP levels than those on the lowest NPP level. Birds fed low NPP of 2.5/2.0 g/kg without phytase supplementation had significantly ($P < 0.05$) higher FCR than those on the high NPP diets (4.5/4.0 and 5.5/5.0 g/kg) without phytase supplementation from day 1 to 21 (Table 4).

Gait score was higher for birds consuming the low NPP diet (2.5/2.0 g/kg) without phytase than those fed on the other diets ($P < 0.05$; Table 5). Phytase supplementation significantly ($P < 0.001$) reduced the gait score of birds fed the low NPP diet (2.5/2.0 g/kg). There were no significant differences in TD scores among the treatments ($P > 0.05$).

Tibia bone ash and toe ash contents were lower ($P < 0.05$) in birds fed the low NPP diets (2.5/2.0 and 3.5/3.0 g/kg) without phytase. Phytase supplementation increased tibia bone ash and toe ash contents markedly at the lower levels of NPP (2.5/2.0 g/kg); whereas at higher levels of NPP (4.5/4.0 g/kg) close to the requirement for maximal tibia bone ash, the addition of phytase showed little or no benefit (Table 5). The relationship between the tibia bone ash and toe ash contents ($R^2 = 0.78$, $P < 0.01$ without phytase; $R^2 = 0.04$, $P = 0.04$ with phytase) are shown in Figure 1.

As dietary NPP levels increased, tibia bone and toe ash contents improved. However, this increase appears to plateau when dietary NPP levels increased above 4.0 g/kg (Figures 2 and 3).

TABLE 4 Body weight at day 21 (g/bird), feed intake (g/bird/day) and feed conversion ratio (FCR) (g feed/g gain) of the birds from day 1 to 21 post-hatch in Experiment 1[1,2]

(g/kg)	Phytase[3]	Body weight (g/bird)	Feed intake (g/bird/d)	FCR (g feed/g gain)
NPP				
2.5/2.0		719[b]	42.7[b]	1.38[a]
3.5/3.0		806[a]	48.2[a]	1.33[ab]
4.5/4.0		875[a]	49.9[a]	1.31[b]
5.5/5.0		854[a]	49.7[a]	1.31[b]
Phytase				
	–	784[b]	46.0[b]	1.34
	+	843[a]	49.2[a]	1.33
NPP × phytase				
2.5/2.0	–	619[b]	38.4[b]	1.42[a]
	+	819[a]	47.0[a]	1.33[ab]
3.5/3.0	–	762[a]	45.8[a]	1.34[ab]
	+	850[a]	50.6[a]	1.32[ab]
4.5/4.0	–	880[a]	49.8[a]	1.30[b]
	+	871[a]	50.1[a]	1.33[ab]
5.5/5.0	–	875[a]	50.2[a]	1.29[b]
	+	833[a]	49.2[a]	1.32[ab]
P-value				
NPP		<0.001	<0.001	0.018
Phytase		0.004	0.001	0.515
NPP × Phytase		<0.001	0.002	0.056
Pooled SEM		27.2	1.24	0.024

1 NPP (g/kg): non-phytate phosphorus; 2.5, 3.5, 4.5 and 5.5 g/kg for starter diet fed to birds from day 1 to 14 post-hatch; 2.0, 3.0, 4.0 and 5.0 g/kg for grower diet fed to birds from day 15 to 21 post-hatch.
2 SEM = standard error of mean. Means in comparisons with the different superscripts ([a,b]) differ ($P < 0.05$).
3 (+/–) with or without phytase supplementation of 500 FTU/kg.

TABLE 5 Gait score, tibia dyschondroplasia (TD) score, tibia bone and toe ash contents (%DM) in Experiment 1[1,2]

(g/kg)	Phytase[3]	Gait[4] score	TD[5] score	Tibia bone ash (%DM)	Toe ash (%DM)
NPP					
2.5/2.0		1.87[a]	0.71	44.1[c]	12.3[b]
3.5/3.0		1.10[b]	0.81	47.8[b]	13.7[a]
4.5/4.0		1.14[b]	0.95	50.1[a]	14.3[a]
5.5/5.0		1.05[b]	0.86	50.4[a]	14.5[a]
Phytase					
	–	1.49[a]	0.80	46.2[b]	12.9[b]
	+	1.09[b]	0.86	50.0[a]	14.5[a]

1 NPP: non-phytate phosphorus; 2.5, 3.5, 4.5 and 5.5 g/kg for starter diet fed to birds from day 1 to 14 post-hatch; 2.0, 3.0, 4.0 and 5.0 g/kg for grower diet fed to birds from day 15 to 21 post-hatch.
2 SEM: standard error of mean. Means in comparisons with the different superscripts ([a,b,c]) differ ($P < 0.05$).
3 (+/–) with or without phytase supplementation of 500 FTU/kg.
4 Gait score: Gait scores 0 to 5.
5 TD score was transformed by square root (TD score + 0.5); TD score 0 to 5 (0 = normal, 5 = extensive deposition of abnormal cartilage).

TABLE 5 Gait score, tibia dyschondroplasia (TD) score, tibia bone and toe ash contents (%DM) in Experiment 1 (*cont.*)

(g/kg)	Phytase	Gait score	TD score	Tibia bone ash (%DM)	Toe ash (%DM)
NPP × phytase					
2.5/2.0	–	2.65[a]	0.71	38.7[c]	10.6[c]
	+	1.08[b]	0.71	49.6[a]	13.9[a]
3.5/3.0	–	1.18[b]	0.76	45.5[b]	12.4[b]
	+	1.03[b]	0.87	50.1[a]	15.0[a]
4.5/4.0	–	1.10[b]	0.82	49.7[a]	14.1[a]
	+	1.17[b]	1.07	50.5[a]	14.6[a]
5.5/5.0	–	1.03[b]	0.93	50.8[a]	14.4[a]
	+	1.06[b]	0.79	49.9[a]	14.5[a]
P-value					
NPP		< 0.001	0.21	< 0.001	< 0.001
Phytase		< 0.001	0.478	< 0.001	< 0.001
NPP × Phytase		< 0.001	0.390	< 0.001	< 0.001
Pooled SEM		0.062	0.114	0.75	0.32

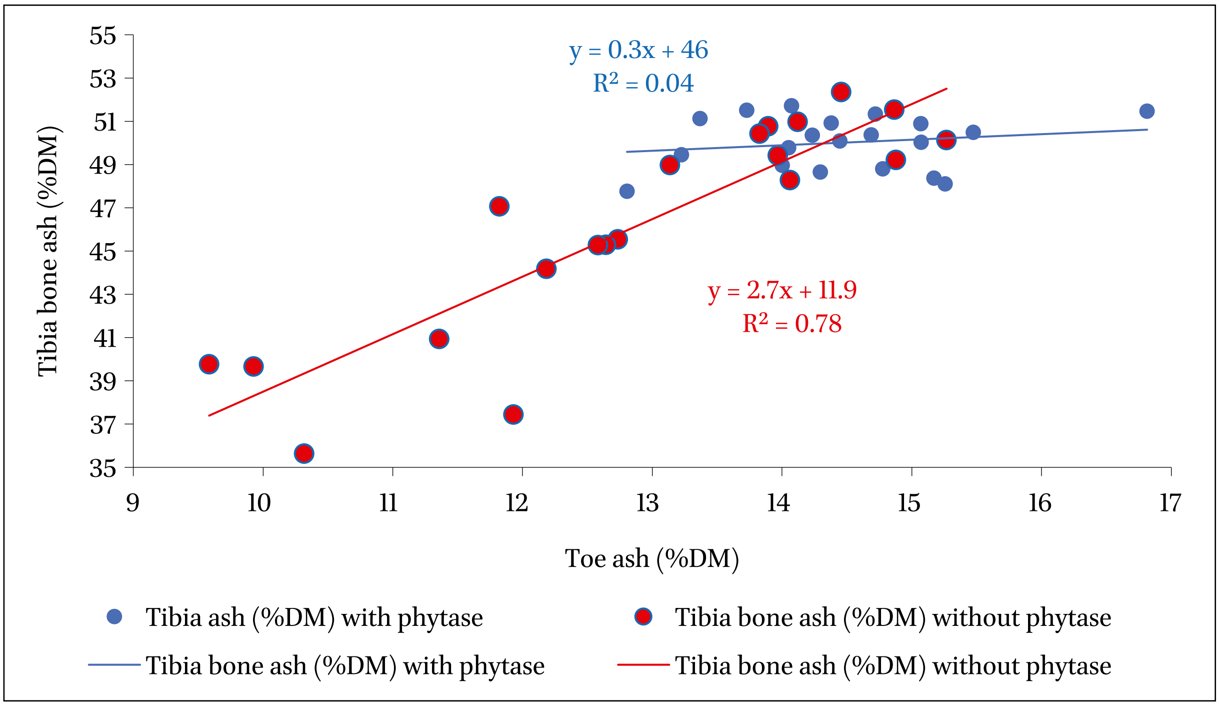

FIGURE 1 Correlation scatter plot of percentage tibia bone ash and toe ash contents of birds fed diets with or without phytase supplementation at day 21; Experiment 1

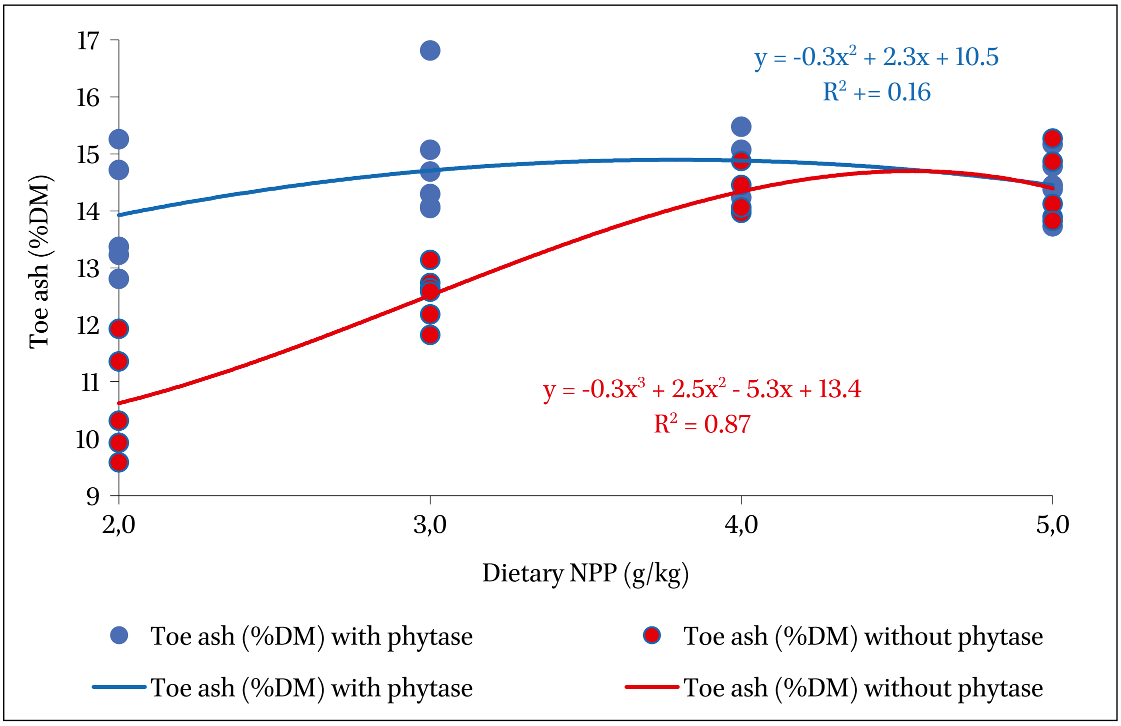

FIGURE 2 Correlation scatter plot of dietary NPP concentration and percentage toe ash of the birds fed diets with or without phytase supplementation at day 21; Experiment 1

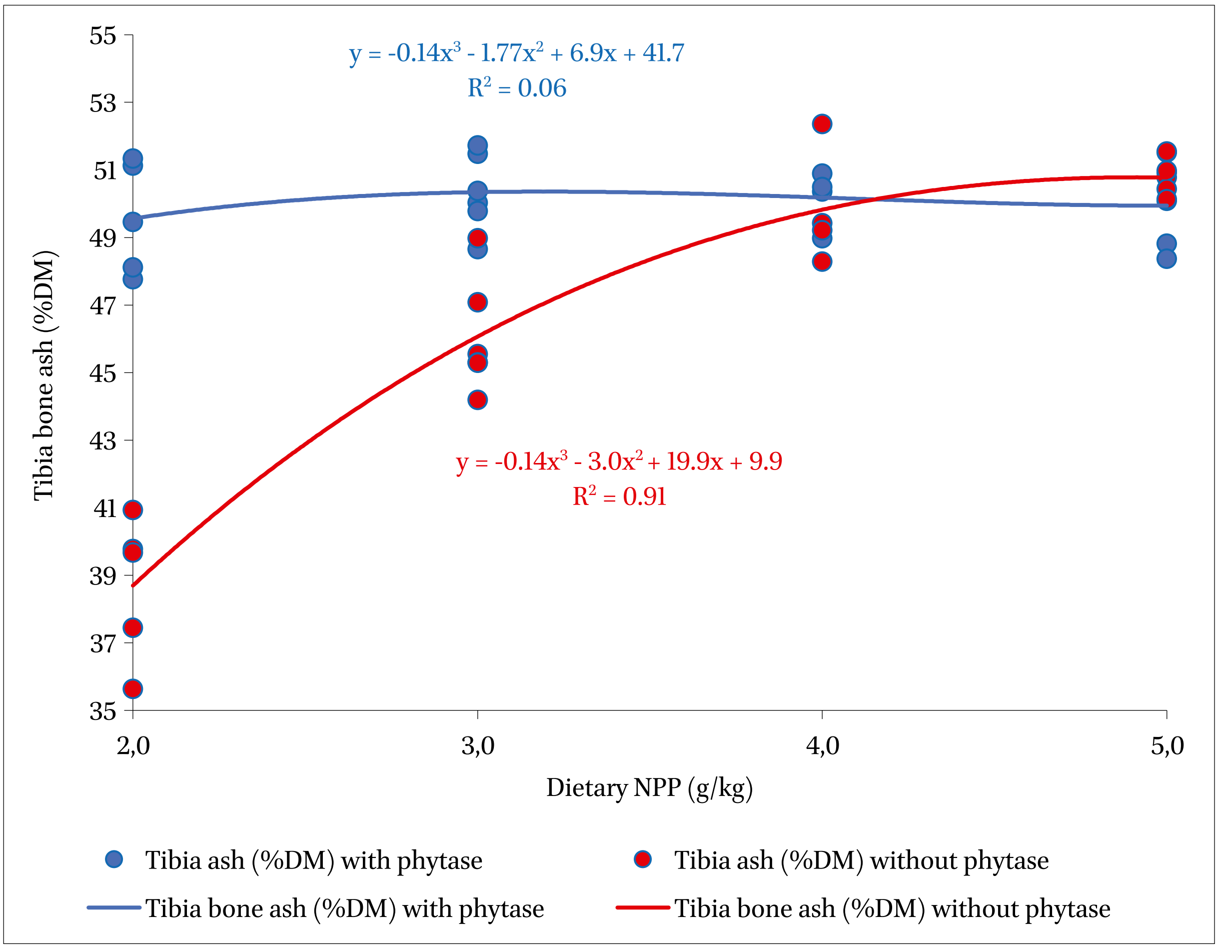

FIGURE 3 Correlation scatter plat of dietary NPP concentrations and percentage tibia bone ash content of birds fed diets with or without phytase supplementation at day 21; Experiment 1

Experiment 2

Body weight and FCR were significantly compromised by the lowest dietary NPP level (Table 6) but this was only when phytase was excluded from the diet. Phytase supplementation had significant beneficial effects on birds overall performance ($P < 0.05$). The body weights of the birds fed diets containing NPP of 2.0 g/kg without phytase supplementation were significantly lower ($P < 0.05$) than all the other treatments. However, for the birds fed on NPP of 3.0 g/kg without phytase supplementation, body weight was similar to those fed NPP of 2.0 g/kg ($P > 0.05$; Table 6). Phytase supplementation overcame this performance deficit.

Feed intake results were not affected by dietary NPP levels (Table 6). The combination of body weight and feed intake led to the FCR value of birds fed diets containing 2.0 g/kg NPP without supplemental phytase to be the highest among all the treatments (Table 6).

Dietary NPP levels and phytase supplementation had no effects on gait score, tibia bone ash and toe ash contents of birds in Experiment 2 (Table 7). Only one case of TD was detected within all the birds sampled on day 49, therefore, statistical analysis was not conducted. The poor relationship between tibia bone ash and toe ash contents in older birds (d 49) fed with and without phytase supplementation is shown in Figure 4.

The results of Experiment 2 indicated that phytase supplementation in a diet containing 2.0 g/kg of NPP met the requirement of broilers from day 21 to 49 of age. It was interesting to compare the outcomes of Experiments 1 and 2, and Tables 8 shows correlation coefficients between the various parameters, with and without phytase supplementation, in both experiments.

With no phytase supplementation, the relationship between tibia bone ash and body weight ($R^2 = 0.65$, $P < 0.01$), tibia bone ash and FCR ($R^2 = 0.35$, $P < 0.01$),

TABLE 6 Body weight (g/bird) on day 49, feed intake (g/bird/day) and feed conversion ratio (FCR) (g feed/g gain) from day 21 to 49 in Experiment 2[1,2]

(g/kg)	Phytase[3]	Body weight (g/b)	Feed intake (g/b/d)	FCR (g feed/g gain)
NPP				
2.0		4,087[b]	203.4	1.93[a]
3.0		4,171[ab]	205.6	1.91[ab]
4.0		4,234[a]	204.2	1.86[b]
5.0		4,189[ab]	207.6	1.89[ab]
Phytase				
	–	4,081[b]	202.7[b]	1.93[a]
	+	4,259[a]	207.8[a]	1.87[b]
NPP × phytase				
2.0	–	3,893[b]	199.1[b]	2.02[a]
	+	4,281[a]	207.8[ab]	1.84[b]
3.0	–	4,068[ab]	200.4[ab]	1.93[ab]
	+	4,273[a]	210.8[a]	1.90[b]
4.0	–	4,221[a]	204.2[ab]	1.85[b]
	+	4,247[a]	204.3[ab]	1.87[b]
5.0	–	4,143[a]	206.9[ab]	1.91[b]
	+	4,234[a]	208.3[ab]	1.86[b]
P-value				
NPP		0.052	0.345	0.012
Phytase		< 0.001	0.005	0.001
NPP × Phytase		0.007	0.094	0.001
Pooled SEM		51.3	2.4	0.023

[1] NPP (g/kg): non-phytate phosphorus; 2.5, 3.5, 4.5 and 5.5 g/kg for grower diet fed to birds from day 21 to 28 post-hatch; 2.0, 3.0, 4.0 and 5.0 g/kg for finisher diet fed to birds from day 29 to 49 post-hatch.

[2] SEM: standard error of mean. Means in comparisons with the different superscripts ([a,b]) differ ($P < 0.05$).

[3] (+/–) with or without phytase supplementation of 500 FTU/kg.

TABLE 7 Gait score, tibia bone ash and toe ash contents in Experiment 2[1]

(g/kg)	Phytase[2]	Gait score[3]	Tibia bone ash (%DM)	Toe ash (%DM)
NPP				
2.0		1.05	44.8	12.8
3.0		1.08	45.8	13.1
4.0		1.07	46.5	13.3
5.0		1.00	46.0	13.1
Phytase				
	–	1.09	45.9	13.2
	+	1.01	45.7	13.0
NPP × phytase				
2.0	–	1.10	43.9	12.7
	+	1.00	45.6	12.9
3.0	–	1.17	45.3	13.2
	+	1.00	46.4	13.0
4.0	–	1.10	47.4	13.6
	+	1.03	45.6	12.9
5.0	–	1.00	46.8	13.2
	+	1.00	45.2	13.1
P-value				
NPP		0.593	0.177	0.732
Phytase		0.073	0.751	0.376
NPP × Phytase		0.621	0.060	0.660
Pooled SEM		0.06	0.8	0.38

[1] NPP (g/kg): non-phytate phosphorus; 2.5, 3.5, 4.5 and 5.5 g/kg for grower diet fed to birds from day 21 to 28 post-hatch; 2.0, 3.0, 4.0 and 5.0 g/kg for finisher diet fed to birds from day 29 to 49 post-hatch. SEM: standard error of the mean.
[2] (+/–) with or without phytase supplementation of 500 FTU/kg.
[3] Gait score 0 to 5 (0 = normal, 5 = complete lameness).

or between toe ash and body weight ($R^2 = 0.71$, $P < 0.01$), toe ash and FCR ($R^2 = 0.53$, $P < 0.01$) were significant. Moreover, phytase supplementation reduced the correlations between these parameters (Table 8). After the finisher phase in Experiment 2, the correlation coefficients between tibia bone ash or toe ash contents with body weight and FCR (Table 8) were much lower than observed for Experiment 1, both without and with phytase supplementation.

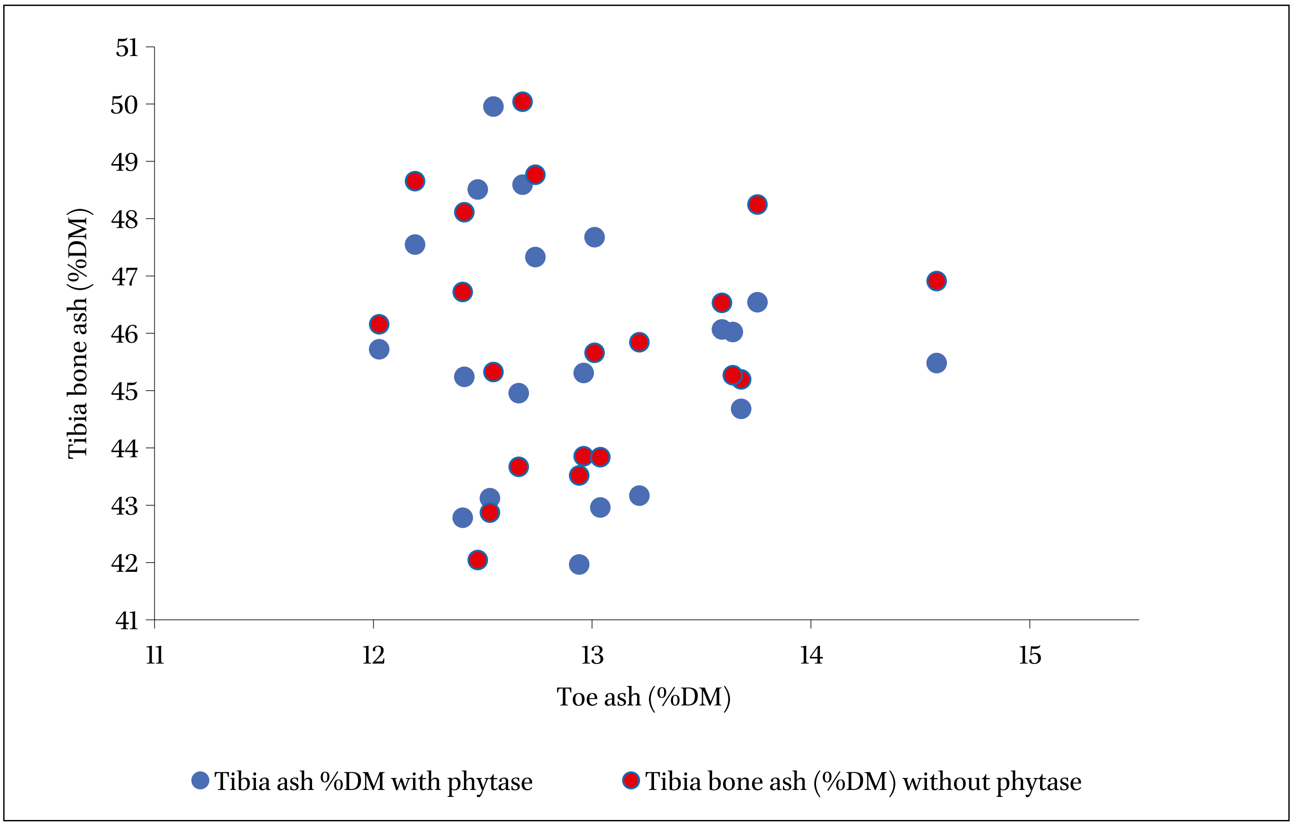

FIGURE 4 Correlation scatter plot of percentage tibia bone ash and toe ash of birds fed diets with or without phytase supplementation at day 49; Experiment 2

TABLE 8 Correlations (R^2) with *P*-values (in brackets) between percentage tibia bone ash or toe ash and body weight (g/bird) or feed conversion ratio (FCR) (g feed/g gain) in Experiments 1 and 2

	Tibia bone ash		Toe ash	
Experiment 1	−phytase[1]	+phytase[2]	−phytase	+phytase
Tibia bone ash	1	1	0.78 (< 0.001)	0.04 (0.44)
Toe ash	0.78 (< 0.001)	0.04 (0.44)	1	1
Body weight	0.65 (< 0.001)	0.0004 (0.93)	0.71 (< 0.001)	0.13 (0.12)
FCR	0.35 (0.004)	0.0004 (0.93)	0.53 (< 0.001)	0.19 (0.0497)
Experiment 2				
Tibia bone ash	1	1	0.08 (0.24)	0.015 (0.6134)
Toe ash	0.08 (0.24)	0.015 (0.6134)	1	1
Body weight	0.20 (0.049)	0.006 (0.76)	0.018 (0.58)	0.18 (0.06)
FCR	0.33 (0.007)	0.016 (0.60)	0.08 (0.24)	0.24 (0.03)

[1] (+/−) with or without phytase supplementation of 500 FTU/kg.

4 Discussion

Phosphorus is essential for skeletal development and bone mineralisation. The dietary requirements of P in the modern broiler would be expected to change as the growth rate of the bird has increased. In addition, the availability of P in poultry diets has increased following routine supplementation with phytase (Selle and Ravindran, 2007). This was demonstrated in this study, where 2.5 g/kg of NPP in the starter (day 1–14), and 2.0 g/kg of NPP in the grower/finisher (day 15–49) supported normal growth performance with dietary inclusion of phytase. In other words, the lowest level of NPP is deficient and the addition of phytase corrects the deficiency. Moreover, these NPP levels were much lower than the breeder recommendations of 0.5–0.42 g/kg for birds from day 0 to 24 and 0.34–0.36 g/kg for various ages after day 24 post-hatch (Aviagen Inc., 2022). However, supplementation of phytase to diets containing NPP levels above 4.5 and 4.0 g/kg for starter and grower/finisher diets, respectively, showed no benefit to bird growth performance. It should be noted that in this study all the experimental diets contained the same concentration of Ca (10.0 g/kg for starter and 9.0 g/kg for grower/finisher). The Ca to P ratio is important in poultry diets (Li *et al.*, 2017; Shafey, 1993) and it is possible that these dietary Ca concentrations in combination with lower dietary NPP caused the detrimental effects on broiler performance noted in this study, as has been documented previously (Keshavarz, 1986, 1999; Li *et al.*, 2017; Yang *et al.*, 2013).

The body pool of P is concentrated in bone and assessment of bone health is an important criterion when assessing P requirements. In this instance three aspects of bone health were examined; TD incidence, gait score, and bone ash content. The lowest NPP concentration resulted in impaired gait scores in the starter phase, as noted by others (Xu *et al.*, 2021) but this was not seen in the finisher phase. There was no effect on the incidence of TD, which is thought to be induced by imbalanced Ca to P ratios (Li *et al.*, 2012). However, there was no evidence of a relationship between dietary Ca and P balance and the incidence of TD in this or other studies (Edwards and Veltmann, 1983; Praul *et al.*, 2000). Therefore, TD score is not a suitable criterion to evaluate the P status of birds.

Tibia bone ash has been widely used as the most meaningful response criteria for assessing P status, determining biological availability of P in feedstuffs (Dersjant-Li *et al.*, 2022; Gillis *et al.*, 1954; Ketels and De Groote, 1988; Potchanacom and Potter, 1987; Potter, 1988; Potter *et al.*, 1995) and the P requirement of birds (David *et al.*, 2022; Malloy *et al.*, 2017; Moran and Todd, 1994; NRC, 1994; Persia and Saylor, 2006; Yan *et al.*, 2001, 2003). Moreover, Rama Rao *et al.* (2006) reported that the tibia bone ash content was maximal when the dietary Ca to NPP ratio was maintained at 2:1, irrespective of the concentrations of these minerals in the diet. Nevertheless, toe ash content, which is more convenient to measure, has been found to provide a similar bone mineralisation assessment to tibia bone ash (Fritz *et al.*, 1969; Grau and Zweigart, 1953; Malloy *et al.*, 2017; Ravindran *et al.*, 1995; Yoshida and Hoshii, 1977) in response to dietary P concentrations; reflecting changes in its net utilisation.

In the current study there was a relatively high correlation between tibia bone ash and toe ash in birds at day 21 (Figure 1) but this was not evident at day 49 (Figure 4). This discrepancy was evident in other studies (Malloy *et al.*, 2017; Ravindran *et al.*, 1995; Scholey and Burton, 2017) and may reflect the age at which different skeletal components mineralise. Bone mineral density of commercial male and female meat chickens has been shown to peak at day 28 (Talaty *et al.*, 2009). While Barreiro *et al.* (2009) found that bone ash and mineral percentage were higher at day 22 than day 42, which reflected the higher mineral requirement at this age. Moreover, although relationships between tibia bone ash or toe ash and body weight or FCR were stronger in younger birds than in older birds; neither was robust enough to be used as a criterion to evaluate the P requirement of broilers.

In general, as dietary NPP levels increased the tibia bone ash and toe ash contents increased in a curvilinear manner in Experiment 1 (Figures 2 and 3) but was not apparent in Experiment 2. However, the increase noted in Experiment 1 appeared to peak or plateau when dietary NPP concentration increased to 4.0 g/kg. This non-linear relationship raises a number of questions with regard to interpretation. Which dietary NPP concentration and tibia bone ash or toe ash content are most meaningful in regard to P retention and growth performance, and over what period of the production cycle do these relationships remain valid. Angel *et al.* (2006) fed various levels of NPP to birds and found differences in P and NPP intakes, bone ash weights and density measurements, but did not observe differences in carcass yield or in the incidence of bruised or broken legs and wings at processing. Therefore, using tibia bone/toe parameters to determine P requirement is challenging as it appears that the maximum is different depending on bird production parameters. Waldroup *et al.* (2000) suggested that meeting the P requirements for maximum tibia bone ash and body weight gain would provide sufficient P to maximise FCR, which is somewhat more variable than tibia bone ash and body weight gain. In relative terms, the amount of P required to maximise various criteria was in the following order: bone calcification > weight gain > FCR > mortality (Waldroup, 1999a). It appears that P requirement for tibia bone ash is much higher than for weight gain and FCR (Persia and Saylor, 2006; Waldroup, 1999a, 2000; Yan *et al.*, 2001). However, maximal tibia bone ash may be required to sustain optimal production of meat chickens. Ravindran *et al.* (1995) found that body weight gain and toe ash percentage were equally or a more sensitive criteria for assessment of P availability than tibia bone ash. They found that tibia bone specific gravity, tibia, toe bone and metatarsal shear force were of limited value as response criteria in P availability assays due to large variations and failure to detect statistically significant effects. Blood P and alkaline phosphatase have also been ruled out as suitable criteria since neither are not very sensitive nor show a linear response to the amount of P absorbed (Guéguen, 1994).

5 Conclusions

The results demonstrated that dietary concentration of NPP can be reduced substantially in broiler diets relative to current industry practice provided the diets are supplemented with phytase. Although tibia and toe ash increased with dietary P concentration, the relationships between tibia bone ash or toe ash content and body weight and FCR were not strong enough to be used as criteria or biomarkers to evaluate the NPP status of diets or P requirement of broilers. The minimal concentrations tested in this study were 2.5/2.0 NPP g/kg for starter and grower/finisher diets with only one level of Ca of 10.0/9.0 for starter and grower/finisher diets.

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Conflict of interest

WLB is a member of the Editorial Board of JAAN but was not involved in the review and editorial process for this paper. The authors have no further conflicts of interest to declare.

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