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The Effect of Bone and Analytical Methods on the Assessment of Bone Mineralization Response to Dietary Phosphorus, Phytase, and Vitamin D in Nursery Pigs[1]

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Summary

Three hundred-fifty pigs (initially 26.2 ± 1.23 lb) were used to evaluate the effects of bone and analytical methods on the assessment of bone mineralization response to dietary P and vitamin D in nursery pigs. Pens of pigs (5 or 6 pigs/pen) were randomized to 6 dietary treatments in a randomized complete block design with 10 pens per treatment. Treatments were formulated to have varying levels of P, phytase, and vitamin D to provide differences in bone characteristics. After feeding diets for 28 d, eight pigs per treatment were euthanized for bone, blood, and urine analysis. The response to treatment for bone density and ash was dependent upon the bone analyzed (density × bone interaction, $P = 0.044$; non-defatted bone ash × bone interaction, $P = 0.060$; defatted bone ash × bone interaction, $P = 0.068$). Pigs fed 0.19% STTD P had decreased ($P < 0.05$) bone density and ash (non-defatted and defatted) for all bones compared to 0.44% STTD P, with 0.33% STTD P generally intermediate or similar to 0.44% STTD P. Pigs fed 0.44% STTD P with no vitamin D had greater ($P < 0.05$) non-defatted fibula ash compared to all treatments other than 0.44% STTD P with added HyD. Pigs fed the three diets with 0.44% STTD P had greater ($P < 0.05$) defatted 2nd rib ash compared to pigs fed 0.19% STTD P or 0.33% STTD P with no phytase. In summary, bone density and ash responses varied depending on the bone analyzed. Differences in bone density and ash in response to P and vitamin D were most apparent with fibulas and 2nd ribs. The difference between bone ash procedures was more apparent than the differences between treatments. For histopathology, 10th ribs were more sensitive than 2nd ribs or fibulas for detection of lesions.

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Introduction

In recent years, the occurrence of lameness in growing pigs has increased, leading to an increased incidence of removals and mortality. Lameness is defined as impaired movement or deviation from normal gait. There are many factors that can contribute to lameness, including infectious disease, genetic and conformational deficiencies, impaired physiological development of articular surfaces within joints, and skeletal mineralization. Metabolic bone disease is a common cause of lameness in swine production and is often caused by inappropriate levels of essential vitamins or minerals.

Appropriate mineral supply is essential for the bone structure to become sufficiently strong and to ensure optimal bone mass accumulation during growth. Currently, it is a common practice to feed slightly higher Ca and P levels in swine diets than recommended by NRC.[6] Multiple experiments have showed improved growth performance and bone mineralization when feeding levels of P greater than NRC[6] recommendations when expressed on a dietary percentage basis.

An important component of lameness evaluations is the histopathological examination of tissues including articular surfaces, synovium, and growth plates. While these tissues can provide an indication of a variety of pathological processes, such as metabolic bone disease induced by vitamin D deficiency,[7] they do not always result in a definitive diagnosis. Ancillary diagnostic tests that can be used in a workup of clinical lameness include measures of bone mineralization such as bone ash, and serum concentrations of Ca, P, and vitamin D.

Serum 25(OH)D has been the standard for determination of vitamin D status within swine; however, there has been recent speculation that the activated form, $1,25(OH)_2D$, may provide additional benefit in assessing vitamin D status.[8] There is limited information regarding serum $1,25(OH)_2D$ levels in swine under a variety of feeding conditions.

Bone ash is an established method for measuring bone mineralization. However, questions remain whether defatted or non-defatted bone ash is the more accurate method. Wensley et al.[9] compared defatted and non-defatted processing methods to determine their effects on the ability to detect treatment differences. The authors observed that either non-defatted or defatted bone processing methods can be used to determine the bone ash weight and percentage bone ash to assess bone mineralization in nursery pigs although bone ash percentage is greater for the defatted method compared to the non-defatted method.

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[6] National Research Council. 2012. Nutrient Requirements of Swine: Eleventh Revised Edition. Washington, DC: The National Academies Press. https://doi.org/10.17226/13298.

[7] Madson, D. M., S. M. Ensley, P. C. Gauger, K. J. Schwartz, G. W. Stevenson, V. L. Cooper, B. H. Janke, E. R. Burrough, J. P. Goff, and R. L. Horst. Rickets: case series and diagnostic review of hypovitaminosis D in swine. J. Vet. Diagn. Invest. 24(6):1137-1144. doi:10.1177/1040638712461487.

[8] Hurst, E. A., N. Z. Homer, and R. J. Mellanby. 2020. Vitamin D metabolism and profiling in veterinary species. Metabolites. 10:371. doi: 10.3390/metabo10090371.

[9] Wensley, M. R., C. M. Vier, J. T. Gebhardt, M. D. Tokach, J. C. Woodworth, R. D. Goodband, and J. M. DeRouchey. 2020. Technical note: assessment of two methods for estimating bone ash in pigs. J. Anim. Sci. 98:1-8. doi: 10.1093/jas/skaa251.

Urine samples can be collected and analyzed to evaluate the mineralization status. This sampling technique is non-invasive and can be used while the animal is alive. Urinary Ca and P can be measured, and when put in a ratio to creatinine to standardize for potential differences in urine volume, has been shown to be a promising indicator of status.[10] On their own, the presented assays are limited in their ability to diagnose metabolic bone disease and identify the cause. However, when evaluated collectively, the assays can result in a diagnosis with an identified cause that can lead to intervention. Therefore, the objective of this study was to evaluate the effect of different bone and analytical methods on the assessment of bone mineralization responses to dietary P, phytase, and vitamin D in nursery pigs.

Procedures

The Kansas State University Institutional Animal Care and Use Committee approved the protocol used in this experiment. This experiment was conducted at the Kansas State University Swine Teaching and Research Center in Manhattan, KS. Each pen (4 × 4 ft) was equipped with a 4-hole, dry self-feeder, and a nipple waterer to provide *ad libitum* access to feed and water.

Animals and diets

A total of 350 pigs (DNA 241 × 600; initially 26.2 ± 1.23 lb) were used in a 28-d trial. Pigs were weaned at approximately 21 d of age and placed in pens of 5 pigs based on initial weight and gender. Common phase 1 and 2 diets were fed for 24 d after weaning. Dietary vitamin D, calcium, and phosphorus levels during phases 1 and 2 were at or above the NRC recommendations and resulted in mean serum levels of 14.6 ng/mL of 25-hydroxyvitamin D_3, 10.5 mg/dl of Ca, and 9.3 mg/dl of P at d 24 post-weaning. On d 24 after weaning, which was considered d 0 of the trial, pens of pigs were randomly allotted to 1 of 6 dietary treatments with 10 replications per treatment. The dietary treatments were: 1) 0.19% STTD P (deficient); 2) 0.33% STTD P (NRC requirement) using monocalcium phosphate; 3) 0.33% STTD P including 0.14% release from phytase; 4) 0.44% STTD P (industry level) using monocalcium phosphate, phytase, no vitamin D; 5) diet 4 with vitamin D (1,653 IU/kg); and 6) diet 5 with additional 2,000 IU/kg 25(OH)D_3 (HyD) (DSM Nutritional Products, Parsippany, NJ). All diets were manufactured in meal form at Hubbard Feeds in Beloit, KS (Table 1). Treatment 1 had an STTD P level of 0.19%, which was 57% of the NRC requirement for pigs of this weight range. Treatments 2 and 3 had an STTD P level of 0.33%, which was 100% of the NRC requirement for this weight range. Treatments 4, 5, and 6 had an STTD P level of 0.44%, which was approximately 133% of the NRC requirement for this weight range. For treatments 1 and 2, STTD P levels were met by only using monocalcium phosphate. All other treatments had 2,000 FYT/kg of phytase included in the diet to meet the desired STTD P levels, with an assumed STTD P release of 0.14%. Vitamin D_3 was included in the diet for treatments 1, 2, 3, 5, and 6 via the same vitamin premix used during the common phase 1 and 2 periods to provide 1,653 IU/kg. Treatment 4 had a special vitamin premix that had no vitamin D. Treatment 6 had 1,653 IU/kg of vitamin D_3 from the vitamin premix and an additional 2,000 IU/kg of vitamin D_3 from HyD. All diets were formulated to an analyzed Ca:analyzed P ratio of 1.20:1. Experimental diets were fed for 28 d.

[10] Hagemoser, W. A., J. P. Goff, T. P. Sanderson, and J. S. Haynes. 2000. Osteopenic disease in growing pigs: diagnostic methods using serum and urine calcium and phosphorus values, parathormone assay, and bone analysis. J. Vet. Diagn. Invest. 12:525-534. doi:10.1177/104063780001200606.

Pens of pigs were weighed, and feed disappearance was measured every 7 d to determine ADG, ADFI, and F/G. On d 28, 8 pigs per treatment were euthanized and used for the analysis of bones, blood, and urine. The right and left metacarpal, fibula, 2nd rib, and 10th rib were collected from each pig for a total of 8 bones per pig. All bones were analyzed using dual-energy X-ray absorptiometry (DEXA) scans, bone density, breaking strength, bone ash, and bone Ca and P were determined. Histologic evaluation of hematoxylin and eosin (H&E stained) sections of 2nd rib, 10th rib, and fibula was performed by three blinded diagnostic pathologists. Hematoxylin stains the cell nuclei a purplish blue and eosin stains the extracellular matrix and cytoplasm pink, with other structures taking on different shades, hues, and combinations of these colors. Bones were scored for lesions of failure of endochondral ossification of the physis and microscopic fractures (infractions). Medullary trabecular and cortical bone thickness was measured. Ten mL of blood was collected to measure serum chemistry, and 10 mL of urine was collected directly from the bladder to measure Ca, P, and creatinine.

Statistical analysis

Data were analyzed as a randomized complete block design for one-way ANOVA using the lmer function from the lme4 package in R version 3.5.1 (2018-07-2) with pen considered the experimental unit, body weight as a blocking factor, and treatment as a fixed effect. Linear and quadratic effects between treatments were measured based on STTD P % in the diet. A Tukey multiple comparison adjustment was used when appropriate. Differences between treatments were considered significant at $P \leq 0.05$ and marginally significant at $0.05 < P \leq 0.10$.

Results and Discussion

Growth performance

On d 28, pigs fed 0.19% STTD P had reduced final body weight ($P < 0.001$) compared to pigs fed 0.33% and 0.44% STTD P, with no differences observed between the 2 higher P treatments ($P > 0.10$; Table 2). Overall, pigs fed 0.19% STTD P had decreased ADG compared to pigs fed 0.33% and 0.44 % STTD P ($P < 0.001$). Pigs fed 0.44% STTD P had increased ADFI ($P < 0.05$) compared to pigs fed 0.19% STTD P, with pigs fed 0.33% STTD P intermediate. Pigs fed 0.33% STTD P with phytase had improved F/G compared to pigs fed 0.19% STTD P and 0.44% STTD P and HyD ($P < 0.05$), with pigs fed 0.33% STTD P with no phytase, 0.44% STTD P with no vitamin D, and 0.44% STTD P with 1,653 IU/kg of vitamin D intermediate.

Serum analysis

For serum Ca, there was a tendency for a difference between treatments, but no significant mean separation was observed ($P = 0.096$). For serum P, pigs fed 0.19% P had lower levels compared to pigs fed 0.33% and 0.44% STTD P ($P < 0.05$). Pigs fed no vitamin D in the diet had the lowest ($P < 0.05$) circulating 25-hydroxyvitamin D_3, and pigs fed HyD in the diet had the greatest ($P < 0.05$), and pigs fed only 1,653 IU/kg of vitamin D_3 intermediate. The compound 25-hydroxyvitamin D_3 is the precursor to the active form of vitamin D and undergoes a bioconversion process within the kidney to form the active metabolite, 1,25-dihydroxyvitamin D_3. Pigs fed no vitamin D in the diet had the lowest circulating level of 1,25-dihydroxyvitamin D_3 but pigs fed 0.19% STTD P had the highest ($P < 0.05$). Pigs fed 1,653 IU/kg of vitamin D with 0.33% STTD P, 0.44% STTD P, and added HyD were intermediate. Pigs fed P deficient diets had

increased Ca:creatinine levels ($P < 0.001$) in the urine compared to pigs fed diets with industry levels of P, with pigs fed NRC P with no phytase being intermediate. For P:creatinine levels, pigs fed no vitamin D, and excess vitamin D in the diet from HyD had increased P levels in the urine ($P = 0.004$) compared to pigs fed the deficient P levels, with all other treatments being intermediate.

Bone analysis

The response to treatment for bone density and ash was dependent upon the bone that was analyzed (density × bone interaction, $P = 0.044$; non-defatted bone ash × bone interaction, $P = 0.060$; defatted bone ash × bone interaction, $P = 0.068$; Table 4). Pigs fed 0.19% STTD P had decreased ($P < 0.05$) bone density and ash (non-defatted and defatted) for all bones compared to 0.44% STTD P, with 0.33% STTD P generally intermediate or similar to 0.44% STTD P. Pigs fed 0.44% STTD P with no vitamin D had the greatest ($P < 0.05$) non-defatted fibula ash compared to all treatments other than 0.44% STTD P with added HyD. Pigs fed the three diets with 0.44% STTD P had greater ($P < 0.05$) defatted 2nd rib ash compared to pigs fed 0.19% STTD P or 0.33% STTD P with no phytase.

For bone ash content, there was no difference between treatments for the percentage of Ca and P in bone ash regardless of the level of P, phytase, and vitamin D in the diet ($P > 0.10$). Pigs fed 0.19% STTD P had reduced grams of P and Ca in the bone ash compared to pigs fed 0.33% and 0.44% STTD P ($P < 0.05$). This means the bone increases in mineralization when adequate levels of Ca and P are fed, but the ratio of Ca and P in the bone mineral remains unchanged, because the bone will only increase in size when adequate or excess levels of Ca and P are fed.

For histopathology, the 10th rib had more lesions of endochondral ossification and infraction than the 2nd rib or fibula ($P < 0.001$; Table 3). Pigs fed a P deficient diet had significantly higher scores indicating failure of endochondral ossification, more infractions, and thinner medullary trabecular bone compared to other treatment groups.

For defatted bone ash percent, the 2nd rib had the lowest, fibulas highest, and the 10th rib and metacarpal were intermediate ($P < 0.001$). For non-defatted bone ash percent, the bone ash percent changed by bone as the metacarpal was the lowest, then increased for the fibula, 2nd rib, and 10th rib, respectively ($P < 0.001$). For histopathology, the 10th rib had the highest physeal score, infraction score, fibrosis score, and lowest trabeculae bone thickness compared to the fibula and 2nd rib ($P < 0.001$).

In summary, bone density and ash responses varied depending on the bone analyzed. Differences in bone density and ash in response to P and vitamin D were most apparent with fibulas and 2nd ribs. The difference between bone ash procedures was more apparent than the differences between treatments. For histopathology, 10th ribs were more sensitive than 2nd ribs or fibulas for detection of lesions.

Table 1. Diet composition (as-fed basis)[1]

STTD P, %:	**0.19**	**0.33**	**0.33**	**0.44**	**0.44**	**0.44**
Vitamin D, IU/kg:	**1,653**	**1,653**	**1,653**	**0**	**1,653**	**1,653 + HyD**
Phytase, FYT/kg[3]:	**0**	**0**	**2,000**	**2,000**	**2,000**	**2,000**
Ingredients, %						
Corn	67.40	66.50	67.30	66.60	66.60	66.55
Soybean meal, 46.5% CP	29.65	29.65	29.65	29.65	29.65	29.65
Calcium carbonate	0.75	0.90	0.75	0.87	0.87	0.87
Monocalcium phosphate	0.15	0.90	0.15	0.75	0.75	0.75
Salt	0.60	0.60	0.60	0.60	0.60	0.60
Feed-grade amino acids	1.06	1.06	1.06	1.06	1.06	1.06
Trace mineral premix	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin premix[2]	0.25	0.25	0.25	---	0.25	0.25
Vitamin premix - no vitamin D	---	---	---	0.25	---	---
Phytase[3]	---	---	0.08	0.08	0.08	0.08
HyD[4]	---	---	---	---	---	0.04
Total	100	100	100	100	100	100
Calculated analysis, %						
SID Lys	1.30	1.30	1.30	1.30	1.30	1.30
NE, kcal/lb	1,115	1,104	1,114	1,105	1,105	1,105
Analyzed Ca	0.50	0.69	0.50	0.65	0.65	0.65
Analyzed P	0.42	0.58	0.42	0.55	0.55	0.55
STTD P, with phytase	0.19	0.33	0.33	0.44	0.44	0.44
Analyzed Ca:Analyzed P	1.20	1.20	1.20	1.20	1.20	1.20
Mineral analysis[5]						
Ca,%	0.66	0.50	0.64	0.78	0.55	0.67
P,%	0.41	0.39	0.43	0.52	0.44	0.49

[1]Diets were fed to pigs from approximately 26 to 65 lb BW.

[2]Vitamin premix contained 1,653 IU/kg of vitamin D_3 in the diet when the premix was included in the diet at 0.25%.

[3]Ronozyme HiPhos 2700 (DSM Nutritional Products, Parsippany, NJ) was included at 2,000 FYT/kg with an assumed release of 0.14 STTD P.

[4]HyD provided an additional 2,000 IU/kg 25(OH)D_3 to the diet.

[5]A representative sample of each diet was collected from each feeder at 3 different time points throughout the study. Samples were stored at -4°F until they were homogenized, subsampled, and submitted to the K-State Research and Extension Soil Test Laboratory (Manhattan, KS) for proximate analysis and Ca and P.

Table 2. Effect of STTD P, phytase, and vitamin D on growth performance, serum, and bone analysis of nursery pigs[1]

STTD P, %:	0.19	0.33	0.33	0.44	0.44	0.44		
Vitamin D, IU/kg:	1,653	1,653	1,653	0	1,653	1,653 + HyD		
Phytase FYT/kg:	No	No	2,000	2,000	2,000	2,000	SEM	*P*-value
Body weight, lb								
d 0	26.2	26.2	26.2	26.3	26.3	26.4	1.23	0.925
d 28	59.5^{a}	64.6^{b}	66.3^{b}	66.9^{b}	65.7^{b}	66.8^{b}	2.07	< 0.001
Overall								
ADG, lb[3]	1.19^{a}	1.37^{b}	1.43^{b}	1.45^{b}	1.41^{b}	1.44^{b}	0.035	< 0.001
ADFI, lb[4]	2.03^{a}	2.16^{ab}	2.18^{ab}	2.26^{b}	2.24^{b}	2.32^{b}	0.068	< 0.001
F/G[3]	1.70^{c}	1.57^{ab}	1.52^{a}	1.56^{ab}	1.59^{ab}	1.60^{b}	0.022	< 0.001
Serum[5]								
Ca, mg/dL	11.1	11.2	10.8	11.1	11.1	10.6	0.162	0.096
P, mg/dL	6.5^{a}	9.3^{b}	9.8^{b}	10.0^{b}	10.1^{b}	9.9^{b}	0.327	< 0.001
25-hydroxyvitamin D_3, ng/mL	10.9^{b}	14.1^{b}	12.2^{b}	4.1^{a}	13.5^{b}	35.6^{c}	1.47	< 0.001
1,25-dihydroxyvitamin D_3, pg/mL	588^{c}	418^{b}	387^{ab}	254^{a}	320^{ab}	318^{ab}	37.36	< 0.001
Urine								
Calcium:creatinine	0.80^{a}	0.68^{ab}	0.10^{c}	0.06^{c}	0.11^{bc}	0.04^{c}	0.138	< 0.001
Phosphorus:creatinine	0.06^{b}	0.21^{ab}	0.33^{ab}	0.78^{a}	0.29^{ab}	0.90^{a}	0.170	0.004
Defatted bone ash[6]								
Bone ash, g	0.90^{a}	1.31^{b}	1.44^{b}	1.65^{b}	1.49^{b}	1.62^{b}	0.093	< 0.001
Bone ash, %	54.4^{a}	57.5^{b}	59.0^{bc}	60.1^{c}	59.3^{bc}	60.0^{c}	0.514	0.006
Non-defatted bone ash[7]								
Bone ash, g	0.87^{a}	1.25^{ab}	1.24^{ab}	1.46^{b}	1.23^{ab}	1.42^{b}	0.092	< 0.001
Bone ash, %	42.4^{a}	46.7^{b}	46.4^{b}	49.5^{b}	48.0^{b}	48.1^{b}	0.724	< 0.001
Dual-energy X-ray absorptiometry								
Bone mineral density, g/cm^2	0.11^{a}	0.14^{b}	0.14^{b}	0.17^{c}	0.15^{bc}	0.16^{c}	0.004	< 0.001
Bone mineral content, g	0.85^{a}	1.16^{ab}	1.20^{b}	1.44^{b}	1.15^{ab}	1.41^{b}	0.080	< 0.001

continued

Table 2. Effect of STTD P, phytase, and vitamin D on growth performance, serum, and bone analysis of nursery pigs[1]

STTD P, %:	0.19	0.33	0.33	0.44	0.44	0.44		
Vitamin D, IU/kg:	1,653	1,653	1,653	0	1,653	1,653 + HyD		
Phytase FYT/kg:	No	No	2,000	2,000	2,000	2,000	SEM	*P*-value
Bone ash content[8]								
Ca, g	0.17[a]	0.27[b]	0.27[b]	0.32[b]	0.30[b]	0.33[b]	0.020	< 0.001
P, g	0.13[a]	0.21[b]	0.21[b]	0.25[b]	0.24[b]	0.26[b]	0.017	< 0.001
Ca, %	18.5	19.8	18.7	19.5	20.1	20.1	0.505	0.603
P, %	13.8	15.7	14.6	15.3	15.9	16.0	0.536	0.352
Histopathology[9]								
Physeal score	0.61[b]	0.12[a]	0.28[ab]	0.28[ab]	0.21[ab]	0.19[ab]	0.112	< 0.001
Infraction score	0.29[b]	0.00[a]	0.01[a]	0.07[a]	0.01[a]	0.03[a]	0.049	< 0.001
Fibrosis score	0.61	0.54	0.59	0.50	0.54	0.56	0.038	0.948
Trabeculae bone thickness, mm	28.6	29.0	30.3	31.4	31.7	31.0	1.851	0.232
Cortical bone thickness, mm	87.5	93.2	97.0	93.7	96.5	94.8	3.455	0.538

[abc]Means within a row with different superscripts differ ($P < 0.05$).

[1]A total of 350 pigs were used in a 28-d nursery trial with 5 pigs per pen and 10 pens per treatment. Pigs were placed on experimental diets 24 days post-weaning (~19 d of age).

[2]BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; F/G = feed efficiency.

[3]STTD P, quadratic, $P < 0.05$.

[4]STTD P, linear, $P < 0.05$.

[5]Serum Ca and P were measured at the Iowa State University College of Veterinary Medicine Veterinary Diagnostic Laboratory (Ames, IA) as a part of the large animal complete profile. The vitamin D serum analysis was conducted at Heartland Assays (Ames, IA).

[6]Eight pigs per treatment were euthanized and the left and right metacarpal, fibula, 2nd rib, and 10th rib were collected to determine bone ash weight and percentage bone ash. All bones were cleaned of tissue and then placed in Soxhlet extractors containing petroleum ether for 7 days as a means of removing water and fat. Bones were then dried at 221°F for 7 days and then ashed in a muffle furnace at 1,112°F for 24 h.

[7]Eight pigs per treatment were euthanized and the left and right metacarpal, fibula, 2nd rib, and 10th rib were collected to determine bone ash weight and percentage bone ash. All bones were cleaned of tissue and then dried at 221°F for 7 days and then ashed in a muffle furnace at 1,112°F for 24 h.

[8]After bone ash was completed, the ash was digested and sent to the K-State Research and Extension Soil Testing Laboratory (Manhattan, KS) for analysis of Ca and P via ICP machine.

[9]The left fibula, 2nd rib, and 10th rib were taken to Iowa State University VDL for analysis of histopathology. Each bone was scored on a scale of 0 to 3 on the severity of fractures on the physis of each growth plate.

Table 3. Effect of STTD P, phytase, and vitamin D on bone analysis of nursery pigs[1]

	Metacarpal	Fibula	2nd rib	10th rib	SEM	*P*-value
Defatted bone ash[2]						
Bone ash, g	1.70[c]	1.25[b]	0.92[a]	1.74[c]	0.048	< 0.001
Bone ash, %	58.3[b]	61.3[c]	55.7[a]	58.3[b]	0.325	< 0.001
Non-defatted bone ash[3]						
Bone ash, g	1.89[d]	1.06[b]	0.70[a]	1.34[c]	0.046	< 0.001
Bone ash, %	37.8[a]	48.0[b]	50.0[c]	51.5[d]	0.416	< 0.001
Dual-energy X-ray absorptiometry						
Bone mineral density, g/cm^2	0.22[d]	0.12[b]	0.09[a]	0.16[c]	0.004	< 0.001
Bone mineral content, g	2.41[d]	0.92[b]	0.14[a]	1.34[c]	0.047	< 0.001
Bone ash content[4]						
Ca, g	0.34[c]	0.25[b]	0.17[a]	0.35[c]	0.012	< 0.001
P, g	0.27[c]	0.19[b]	0.13[a]	0.27[c]	0.010	< 0.001
Ca, %	20.0[b]	19.6[ab]	18.3[a]	19.9[b]	0.403	0.025
P, %	15.6[ab]	15.4[ab]	14.2[a]	15.7[b]	0.423	0.047
Histopathology[5]						
Physeal score	---	0.12[a]	0.16[a]	0.56[b]	0.071	< 0.001
Infraction score	---	0.01[a]	0.02[a]	0.18[b]	0.033	< 0.001
Fibrosis score	---	0.44[a]	0.57[b]	0.66[c]	0.027	0.083
Trabeculae bone thickness	---	38.7[c]	28.8[b]	23.5[a]	1.09	< 0.001
Cortical bone thickness	---	97.1	94.8	89.4	2.22	0.898

[abc]Means within a row with different superscripts differ ($P < 0.05$).

[1]A total of 350 pigs were used in a 28-d nursery trial with 5 pigs per pen and 10 pens per treatment. Pigs were placed on experimental diets 24 days post-weaning (~19 d of age).

[2]One pig per pen (8 pigs per treatment) were euthanized and the left and right metacarpal, fibula, 2nd rib, and 10th rib were collected to determine bone ash weight and percentage bone ash. All bones were cleaned of tissue and then placed in Soxhlet extractors containing petroleum ether for 7 days as a means of removing water and fat. Bones were then dried at 221°F for 7 days and then ashed in a muffle furnace at 1,112°F for 24 h.

[3]One pig per pen (8 pens per treatment) were euthanized and the left and right metacarpal, fibula, 2nd rib, and 10th rib were collected to determine bone ash weight and percentage bone ash. All bones were cleaned of tissue and then dried at 221°F for 7 days and then ashed in a muffle furnace at 1,112°F for 24 h.

[4]After bone ash was completed, the ash was digested and sent to the K-State Research and Extension Soil Testing Laboratory (Manhattan, KS) for analysis of Ca and P via ICP machine.

[5]The left fibula, 2nd rib, and 10th rib were taken to Iowa State VDL for analysis of histopathology. Each bone was scored on a scale of 0 to 3 on the severity of fractures on the physis of each growth plate.

Table 4. Interactive effects of STTD P, phytase, and vitamin D on bone analysis

STTD P, %:	0.19	0.33	0.33	0.44	0.44	0.44	
Vitamin D, IU/kg:	1,653	1,653	1,653	0	1,653	1,653 + HyD	
Phytase FYT/kg[1]:	No	No	2,000	2,000	2,000	2,000	SEM
Bone density, g/mL[2]							
Metacarpal	1.13[a]	1.17[ab]	1.17[ab]	1.20[b]	1.19[b]	1.19[b]	0.014
Fibula	1.19[a]	1.26[b]	1.25[b]	1.33[c]	1.33[c]	1.29[bc]	
2nd rib	1.15[a]	1.22[b]	1.26[bc]	1.28[c]	1.27[c]	1.26[bc]	
10th rib	1.19[a]	1.26[b]	1.27[b]	1.31[b]	1.31[b]	1.30[b]	
Non-de-fat bone ash, %[3]							
Metacarpal	33.9[a]	38.8[b]	36.8[b]	39.0[b]	39.1[b]	39.1[b]	1.02
Fibula	45.2[a]	47.0[ab]	47.4[ab]	51.6[c]	47.3[ab]	49.5[bc]	
2nd rib	44.2[a]	49.6[b]	49.9[b]	53.0[b]	51.9[b]	51.4[b]	
10th rib	46.2[a]	51.3[b]	51.6[b]	54.3[b]	53.7[b]	52.2[b]	
Non-de-fat bone ash, g[4]							
Metacarpal	1.38	2.01	1.84	2.04	1.90	2.11	0.113
Fibula	0.84	1.04	1.07	1.25	0.93	1.19	
2nd rib	0.42	0.66	0.70	0.92	0.67	0.84	
10th rib	0.84	1.30	1.34	1.61	1.42	1.55	
Defatted bone ash, %[5]							
Metacarpal	54.0[a]	58.5[b]	58.3[b]	59.7[b]	59.1[b]	60.0[b]	0.72
Fibula	57.5[a]	61.3[b]	61.9[b]	62.7[b]	61.8[b]	62.6[b]	
2nd rib	50.6[a]	54.1[b]	57.1[bc]	57.9[c]	57.8[c]	58.4[c]	
10th rib	55.7[a]	57.7[ab]	58.7[b]	59.9[b]	58.7[b]	58.9[b]	
Defatted bone ash, g[6]							
Metacarpal	1.18[a]	1.56[ab]	1.80[bc]	1.87[bc]	1.75[bc]	2.05[c]	0.117
Fibula	0.86[a]	1.22[ab]	1.30[ab]	1.37[b]	1.35[b]	1.37[b]	
2nd rib	0.49[a]	0.85[ab]	0.86[ab]	1.29[b]	0.96[ab]	1.07[b]	
10th rib	1.04[a]	1.65[b]	1.79[b]	2.10[b]	1.88[b]	2.00[b]	
Histopathology physeal score[7]							
Fibula	0.17	0.04	0.17	0.08	0.12	0.17	0.173
2nd rib	0.33	0.08	0.33	0.12	0.04	0.04	
10th rib	1.33[b]	0.25[a]	0.33[a]	0.62[a]	0.48[a]	0.38[a]	

continued

Table 4. Interactive effects of STTD P, phytase, and vitamin D on bone analysis

STTD P, %:	0.19	0.33	0.33	0.44	0.44	0.44	
Vitamin D, IU/kg:	1,653	1,653	1,653	0	1,653	1,653 + HyD	
Phytase FYT/kg[1]:	No	No	2,000	2,000	2,000	2,000	SEM
Dual-energy X-ray absorptiometry							
Bone mineral content, g[8]							
Metacarpal	1.99	2.23	2.49	2.69	2.31	2.75	0.115
Fibula	0.61	0.97	0.87	1.17	0.82	1.08	
2nd rib	0.05	0.08	0.13	0.20	0.09	0.27	
10th rib	0.78	1.37	1.30	1.70	1.36	1.55	
Bone mineral density, g/cm[9]							
Metacarpal	0.18	0.21	0.22	0.25	0.24	0.24	0.007
Fibula	0.09	0.11	0.11	0.14	0.12	0.13	
2nd rib	0.05	0.07	0.09	0.10	0.09	0.11	
10th rib	0.11	0.16	0.16	0.19	0.16	0.17	

continued

Table 4. Interactive effects of STTD P, phytase, and vitamin D on bone analysis

STTD P, %:	0.19	0.33	0.33	0.44	0.44	0.44	
Vitamin D, IU/kg:	1,653	1,653	1,653	0	1,653	1,653 + HyD	
Phytase FYT/kg[1]:	No	No	2,000	2,000	2,000	2,000	SEM
Bone ash content							
Phosphorus, g[10]							
Metacarpal	0.19	0.27	0.28	0.28	0.27	0.34	0.025
Fibula	0.12	0.20	0.18	0.21	0.22	0.23	
2nd rib	0.06	0.12	0.12	0.19	0.14	0.17	
10th rib	0.14	0.27	0.28	0.33	0.32	0.30	
Calcium, g[11]							
Metacarpal	0.24	0.32	0.35	0.36	0.35	0.42	0.028
Fibula	0.16	0.25	0.22	0.27	0.28	0.29	
2nd rib	0.08	0.16	0.15	0.24	0.17	0.21	
10th rib	0.19	0.34	0.35	0.41	0.39	0.39	
Phosphorus, %[12]							
Metacarpal	15.8	16.2	15.3	14.7	15.3	16.4	1.10
Fibula	13.9	16.4	14.0	15.5	16.1	16.4	
2nd rib	117	13.8	13.6	15.3	14.9	15.7	
10th rib	13.9	16.4	15.4	15.9	17.2	15.5	
Calcium, %[13]							
Metacarpal	20.4	20.5	19.5	19.1	19.8	20.6	1.045
Fibula	18.7	20.7	17.8	19.9	204	20.4	
2nd rib	16.2	17.6	18.0	19.4	19.0	19.6	
10th rib	18.6	205	19.6	19.7	21.1	19.6	

[abc]Means within a row with different superscripts differ ($P < 0.05$).

[1]Ronozyme HiPhos 2700 (DSM Nutritional Products, Parsippany, NJ) was included at 2,000 FYT/kg with an assumed release of 0.14 STTD P.

[2]Bone density was measured on each bone based on Archimedes principle. Bone × treatment, $P = 0.044$.

[3]Eight pigs per treatment were euthanized and the left and right metacarpal, fibula, 2nd rib, and 10th rib were collected to determine bone ash weight and percentage bone ash. All bones were cleaned of tissue and then dried at 221°F for 7 days and then ashed in a muffle furnace at 1,112°F for 24 h. Bone × treatment, $P = 0.060$.

[4]Bone × treatment, $P = 0.131$.

[5]Eight pigs per treatment were euthanized and the left and right metacarpal, fibula, 2nd rib, and 10th rib were collected to determine bone ash weight and percentage bone ash. All bones were cleaned of tissue and then placed in Soxhlet extractors containing petroleum ether for 7 days as a means of removing water and fat. Bones were then dried at 221°F for 7 days, and then ashed in a muffle furnace at 1,112°F for 24 h. Bone × treatment, $P = 0.068$.

[6]Bone × treatment, $P = 0.063$

[7]The left fibula, 2nd rib, and 10th rib were taken to Iowa State VDL for analysis of histopathology. Each bone was scored on a scale of 0 to 3 on the severity of fractures on the physis of each growth plate. Bone × treatment, $P = 0.077$.

[8]Bone × treatment, $P = 0.045$.

[9]Bone × treatment, $P = 0.132$.

[10]Bone × treatment, $P = 0.421$.

[11]Bone × treatment, $P = 0.283$.

[12]Bone × treatment, $P = 0.790$.

[13]Bone × treatment, $P = 0.707$.